Entrapped in Cage (EiC) Scaffolds of 3D-Printed Polycaprolactone and Porous Silk Fibroin for Meniscus Tissue Engineering

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Abstract

The meniscus has critical functions in the knee joint kinematics and homeostasis. Injuries of the meniscus are frequent, and the lack of a functional meniscus between the femur and tibial plateau can cause articular cartilage degeneration leading to osteoarthritis development and progression. Regeneration of meniscus tissue has outstanding challenges to be addressed. In the current study, novel Entrapped in Cage (EiC) scaffolds of 3D-printed polycaprolactone (PCL) and porous silk fibroin were proposed for meniscus tissue engineering. As confirmed by micro-structural analysis the entrapment of silk fibroin was successful, and all scaffolds had excellent interconnectivity ($\geq 99\%$). The EiC scaffolds had more favorable microstructure compared with the PCL cage scaffolds by improving the pore size while keeping the interconnectivity almost the same. When compared with the PCL cage, the entrapment of porous silk fibroin into the PCL cage decreased the high compressive modulus in a favorable matter in the wet state thanks to the silk fibroin's high swelling properties. The *in vitro* studies with human stem cells or meniscocytes seeded constructs, demonstrated that the EiC scaffolds had superior cell adhesion, metabolic activity, and proliferation compared to the PCL cage scaffolds. Upon subcutaneous implantation of scaffolds in nude mice, all groups were free of adverse incidents, and mildly invaded by inflammatory cells with neovascularization, while the EiC scaffolds showed better tissue infiltration. The results of this work indicated that the EiC scaffolds of PCL and silk fibroin are favorable for meniscus tissue engineering, and the findings are encouraging for further studies using a larger animal model.

Keywords: Silk fibroin, Polycaprolactone, Meniscocytes, Human adipose-derived stem cells, Meniscus, Tissue engineering, Scaffold

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1 **1. Introduction**

2 The menisci are fundamental tissues involved in the homeostasis of a healthy knee. The
3 meniscus lesions are the most frequent lesions in the knee, which are partially unsolved [1, 2].
4 The prognosis and clinical intervention are associated with the characteristics and location of the
5 lesion in the meniscus (*e.g.*, vascular or avascular region), the conditions of the knee and the
6 patient [2-5]. Therefore, different surgical interventions are available, *e.g.*, meniscectomy,
7 suturing, use of allografts, or scaffold implantation, each with their indications and contra-
8 indications [2, 6-9]. Lack of proper chondroprotective function of meniscus inhibits healthy
9 articulation, affects the patient's daily life with pain and loss of proper knee function, and can
10 lead to osteoarthritis with time. Total knee arthroplasty can be an option for end-stage
11 osteoarthritic knees. However, even when the arthroplasty procedures are successful, a
12 significant number of patients experiences post-operative pain that is challenging to manage
13 [10].

14 Tissue engineering strategies are emerging as a reliable solution to repair or regenerate
15 diseased/damaged tissues as it involves the use of cells, scaffolds and bioactive agents, alone or
16 in combination, and in this way, they have potential to contribute to the clinical management of
17 meniscus lesions with indications and contraindications [1, 2, 11, 12]. Given the biomechanical
18 functions of the menisci [13], the use of scaffolds in meniscus tissue engineering strategies
19 seems to be indispensable. Numerous scaffolds from different biomaterials have been studied for
20 meniscus tissue engineering including but not limited to collagen [14, 15], silk [16-18],
21 polycaprolactone (PCL) [19-21], bacterial cellulose [22, 23], hyaluronic acid/PCL [24, 25],
22 polyvinyl alcohol/chitosan [26], silk/polyvinyl alcohol [27]. In addition to the required *in vitro*
23 biological outcomes, the appropriate surface smoothness and mechanical flexibility, as well as

1 the suturability/fixation of the scaffold are indispensable requirements that need to be met.
2 Suturability and patient-specificity of the meniscal implants are two of the main requirements
3 which are for the dimensional fit of the implant, fixation of the implant, and avoidance of the
4 post-operative extrusion of the implant [2, 28-30].

5 In this work, we manufactured novel Entrapped in Cage (EiC) scaffolds of 3D-printed
6 PCL and porous silk fibroin. Three different scaffolds were produced: PCL cage alone or
7 combined either with 8 or 16 wt.% silk fibroin. We hypothesized that such a design of the EiC
8 scaffolds could enhance the overall performance of the constructs by combining the PCL's
9 mechanical strength and silk fibroin's favorable biological features. To test this hypothesis, we
10 characterized the scaffolds, and performed *in vitro* and *in vivo* assessments of cellular constructs.
11 Scaffolds' the micro-structure and mechanical evaluation were performed by means of micro-
12 computed tomography (micro-CT) analysis, scanning electron microscopy (SEM) imaging, and
13 compression testing. The water uptake properties of the scaffolds were assessed by means of
14 using a gravimetric method up to 7 days. Human meniscocytes or human Hoffa's fat pad-derived
15 stem cells were seeded onto the scaffolds, and the *in vitro* studies with the constructs were
16 carried out for up to 21 days. Human meniscocytes are the primary cells of the native meniscus,
17 and Hoffa's fat pad is an adipose body located in the knee close to the meniscus, and it is an
18 intracapsular extrasynovial source of stem cells. Cell adhesion and migration, viability, and
19 proliferation were assessed by SEM, live/dead study, luminescent cell viability, and DNA
20 quantification, respectively. The constructs' *in vivo* biological performance, tissue infiltration,
21 inflammation, neovascularization were evaluated using micro-CT, histology, and semi-
22 quantitative evaluation after a 4-week subcutaneous implantation using a nude mice model.

2. Materials and methods

2.1. Materials and reagents

Silk cocoons of the mulberry silkworm, *Bombyx mori*, were purchased from the Portuguese Association of Parents and Friends of Mentally Disabled Citizens (APPACDM, Castelo Branco, Portugal). Commercial grade sodium chloride particles were purchased from a local store in Portugal. The other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless indicated otherwise.

2.2. Scaffold design and production

In this study, three different scaffolds were produced: PCL cage alone or combined either with 8 or 16 wt.% silk fibroin. The cages from PCL (average M_n 45,000) were 3D-printed with a 4th Generation 3D-Bioplotter (EnvisionTec GmbH, Germany) layer by layer through a 22G metallic needle at 110°C under the dispensing pressure of 5.5 bars [28, 31]. Each layer had parallel strands that were 1.2 mm apart from each other, and the 3D cubic cage had the pattern with layer-wise alternating strand directions of 90° and 0°. The aqueous-derived silk fibroin solutions [32] were prepared the silk cocoons of the mulberry silkworm, *Bombyx mori*, with the following main steps: cleaning, degumming, dissolving, filtering, dialysis, and concentration. The cocoons were first cut, separated from the worm, and cleaned from macroscopic impurities. Clean cocoons were cut further into small pieces for the degumming process in which the sericine, glue-like protein, is eliminated. The 5 g of cocoons were boiled in 2 L of distilled water with 0.02 M sodium carbonate for 1 h, then boiled in only distilled water for 0.5 h, and washed several times with distilled water, and the obtained silk fibroin was air-dried inside a flow cabinet for 48 h. A 9.3 M BrLi solution was prepared with distilled water to dissolve silk fibroin

at 70°C for 1 h with the ratio of 5 mL:1 g, BrLi solution : silk fibroin. After the solution reached to room temperature, it was purified with 5 µm filters, and dialyzed in distilled water for 48 h using a benzoylated dialysis tubing (MWCO: 2 kDa) with 4 water changes per day. After the dialysis, the solution was concentrated using poly(ethylene glycol), and the solution stored at 4°C until further use. The final concentration was determined by measuring the dry weight of the silk fibroin solution after being at 70°C for 24 h.

Silk fibroin solution was used at two concentrations either 8 or 16 wt.%. PCL cages were immersed in silk fibroin solution for 0.5 h, and then placed in a silicon mold that is filled with silk fibroin solution followed by the gradual addition of granular sodium chloride with the particle size range of 250-500 µm, and kept inside a flow cabinet for 72 h to dry. Dry samples were salt-leached by being placed in distilled water under agitation for 3 d with 5 times of daily water change. The side surfaces of the cage structure and the excess silk on the top and bottom of the cage were cut with a surgical blade in wet state to obtain 5 mm³ structures. The wet scaffolds were dried inside a flow cabinet for 48 h, and the scaffolds alone were sterilized by ethylene oxide with 3M Steri-Vac Sterilizer/Aerator - 5XL equipment (3M, USA).

2.3. Characterizations of scaffolds

2.3.1. Micro-computed tomography analysis

Scaffolds (n=3) were scanned with a high-resolution micro-CT system (SkyScan 1272, Bruker, Belgium) for the micro-structural analysis [33, 34]. The source voltage and current were set to 50 kV and 200 µA, respectively. 2D X-ray projections were acquired without any filter over a sample rotation range of 360° with a rotation step of 0.45° with 10 µm of pixel size. The 2D cross-sectional images were obtained by reconstruction of the projections. The 3D models

1 were built from the 2D images, and the micro-structural analysis performed by using the
2 manufacturer's software.

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10 2.3.2. Scanning electron microscopy analysis

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12 The surface and cross-sectional morphology of the scaffolds were analyzed by means of
13 performing SEM analyses (JEOL JSM-6010 LV, Tokyo, Japan). First, the scaffolds were sputter-
14 coated with gold (Cressington Sputter Coater 108A, Watford, UK) and their surfaces were
15 imaged. Then, the samples were cut on the transverse plane into half, and the cross-sectional
16 surfaces were sputter-coated with gold, and observed. The images were taken at 40x and 150x
17 magnifications.

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30 The water uptake behaviors of the scaffolds were assessed through a gravimetric method.
31 The scaffolds (n=3) were weighed in dry condition (M_d), and wet condition at each time point
32 (M_w) (24 h, 72, and 168 h) after being soaked in PBS solution and kept at 37°C in an incubator
33 (BE500, Memmert, Germany). The water uptake percentage was calculated using the equation:
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Water uptake % = $(M_w - M_d) / M_d \times 100$.

19 2.3.4. Compression testing

20 The mechanical properties of the scaffolds (n=6) were assessed through unconfined
21 compression testing using a universal mechanical testing machine (Instron 5543, USA) with the
22 crosshead speed set at 1 mm/min. The scaffolds were tested in both dry and wet conditions after
23 being soaked in PBS solution and kept 24 h at 37°C in an incubator (BE500, Memmert,
24 Germany). The compressive modulus was obtained from the slope of the linear region of the

1 stress-strain curve. The compressive stress was calculated by dividing the load by the initial
2 cross-sectional area. The strain was calculated by dividing the absolute value of the change in the
3 height of the scaffold to the original height of the scaffold.

4 5 **2.4. *In vitro* cell studies**

6 **2.4.1. Tissue harvest**

7 The human tissues were processed after being collected in a clinical situation in which
8 the tissues have already been removed for surgical treatment as described elsewhere [13, 35]. In
9 brief, the harvesting of the tissues was performed in aseptic conditions with cold blade. After
10 harvesting, the tissues were immediately placed in sterile containers with 75 mL of sterile
11 Dulbecco's Phosphate Buffered Saline (dPBS) (Life Technologies, USA) with 1.0% of an
12 antibiotic-antimycotic mixture (Invitrogen, USA) containing 10,000 U/mL penicillin G sodium,
13 10,000 µg/mL streptomycin sulphate and 25 µg/mL amphotericin B as Fungizone antimycotic in
14 0.85% saline [35]. The tissues were kept refrigerated and processed within 1 d for cell isolation.
15 The donors gave their informed written consent, and the Local Ethics Committee approved
16 processing protocols for the human tissue collection. The ages of the donors ranged between 29
17 and 63 years. All samples were anonymized. The tissues were intact without macroscopic signs
18 of degeneration. There was no intervention in the normal course of treatment.

19 20 **2.4.2. Cell isolation and culturing**

21 Human adipose-derived stem cells (hASCs) were enzymatically isolated from the Hoffa's
22 fat pad. Briefly, the tissue washed twice with dPBS, and any the non-fat tissue were cut with a
23 sterile surgical blade and discarded. The fat tissue was cut further into small pieces and digested

1 in the mixture of collagenase solution: α -MEM medium 1:1 (v/v) with the final volume of 15 mL
2 at 37°C in a water bath for 1 h with agitation. The collagenase solution had collagenase type II at
3 the concentration of 0.15% (w/v) in dPBS. α -MEM medium (Invitrogen, USA) contained 10%
4 fetal bovine serum (Gibco, USA) and 1% antibiotics/ antimycotic (Invitrogen, USA). The
5 digestion mixture was passed through a 100 μ m cell strainer (BD Biosciences, USA) to eliminate
6 anything that is not digested, and centrifuged at 800 g for 10 min (5810R, Eppendorf, Germany).
7 The supernatants were discarded; the cell pellet was re-suspended in 15 mL of dPBS and
8 centrifuged at 300 g for 10 min. The supernatants were discarded, the cell pellet re-suspended in
9 10 mL of α -MEM medium, passed through a 100 μ m cell strainer (BD Biosciences, USA) to
10 avoid agglomeration, and plated and cultured in a CO₂ incubator (MCO-18AIC (UV), Sanyo,
11 Japan) at 37°C and 5% CO₂ by changing the culture medium first after the first at 24 h, then
12 every 2-3 d.

13 Isolation of primary human meniscocytes was similar to that of hASCs, all the fat tissue
14 and vascularized tissue were cut and discarded. The remaining meniscus tissue was cut with a
15 surgical blade into small pieces, and washed twice with dPBS, digested for 8 h, and passed
16 through a strainer and centrifuged at 300 g for 7 min (5810R, Eppendorf, Germany). The
17 supernatants were discarded and the pellet re-suspended in medium and cultured in a CO₂
18 incubator (MCO-18AIC (UV), Sanyo, Japan). After 48 h, the media was collected from the flask,
19 and the flask was washed with dPBS which was added to the collected media and centrifuged
20 300 g for 5 min. The obtained pellet was re-suspended in the media that was freshly introduced
21 to the flask, cultured until reaching confluency in an incubator (MCO-18AIC (UV), Sanyo,
22 Japan) by changing the culture medium first at 48 h, then every 2-3 d after washing with dPBS.
23 When confluent, the cells were trypsinized with TrypLE Express (ThermoFisher Scientific,

USA) after washing twice with dPBS, centrifuged for 5 min and re-plated. Meniscocytes and hASCs were cryopreserved respectively at P4 and P3 in liquid nitrogen until seeding, 10^6 cells/mL of 90% fetal bovine serum (Gibco, USA) and 10% dimethyl sulfoxide (VWR International, USA).

2.4.3 Cell seeding

The three types of scaffolds were hydrated in culture medium (α -MEM medium (Invitrogen, USA) contained 10% fetal bovine serum (Gibco, USA) and 1% antibiotics/antimycotic (Invitrogen, USA)) overnight prior to cell seeding; the cryopreserved cells were defrosted, centrifuged, and counted with a cell counting chamber using the trypan blue exclusion method (Trypan blue solution, 0.4%, VWR International, USA). The cells were seeded onto scaffolds at a density of 1×10^5 /scaffold that were placed in multi-well suspension culture plates (Greiner Bio-One GmbH, Austria). After incubation in the CO₂ incubator for 3 h, the culture medium was added to each well. Every 2-3 d the scaffolds were transferred to new well-plates with new culture medium and cultured up to 21 d. Three independent experiments were performed using three samples per group per time point.

2.4.4. Cell adhesion and migration

The cell adhesion and migration were assessed by means of carrying out SEM analyses (JEOL JSM-6010 LV, Tokyo, Japan). At each time point, the scaffolds were rinsed with PBS, and fixed with 10% formalin solution (ThermoFisher Scientific, USA). The fixed samples were dehydrated in a series of aqueous ethanol solutions (30, 50, 70, 90 and 100% v/v), twice at each

concentration for 20 min, then dried inside a flow cabinet for 48 h. Dry scaffolds were sputter-coated with gold (Cressington Sputter Coater 108A, Watford, UK) before SEM observation.

2.4.5. Live/Dead study

Cell viability at the scaffolds was confirmed at each time point with live/dead staining, calcein-acetoxymethyl (Life Technologies, USA) and propidium iodide (Life Technologies, USA) staining. Samples were washed twice with dPBS, and incubated in 1 mg/mL calcein-acetoxymethyl and 5 mg/mL propidium iodide in the dark, then washed with dPBS and observed under fluorescent illumination using Axio Imager Z1 microscope (Zeiss, Germany).

2.4.6. Luminescent cell viability assay

An adenosine triphosphate (ATP) bioluminescence assay was performed using the CellTiter-Glo luminescent cell viability assay (Promega, USA) based on the manufacturer's protocol to assess the presence of metabolically active cells. The quantity of ATP was detected through luminescence intensity reading [36] using a microplate reader (Synergy HT Multi-Detection Microplate Reader, BioTek, USA). Three independent experiments were performed using three samples per group per time point.

2.4.7. Proliferation study

The proliferation of cells in the cell-scaffold constructs was analyzed by quantification of the total double-strand DNA (dsDNA), after seeding and culturing for 1 and 21 d. The quantifications were performed in triplicates for each time point three samples per group. At certain time points, the cell culture media was removed from the well with the sample, and the samples were washed twice with dPBS, and placed in a vial with 1 mL ultrapure water, and kept

1 in a water bath at 37°C for 1 h before being stored at -80°C until next step. The samples were
2 thawed to room temperature and sonicated for 30 min in an ultrasonic water bath. The dsDNA
3 quantification kit (Quant-iT PicoGreen dsDNA Assay Kit, Life Technologies, USA) was used as
4 instructed in the manufacturer's assay protocol. The lambda DNA standard of the kit was used to
5 obtain the standard curve. The fluorescence intensity was read using a microplate reader
6 (Synergy HT Multi-Detection Microplate Reader, BioTek, USA) at excitation and emission at
7 485 nm and 528 nm, respectively. Three independent experiments were performed using three
8 samples per group per time point.

2.4.8. Immunofluorescence staining for filamentous actin (F-actin)

11 Cytoskeleton of the cells was observed by phalloidin staining of F-actin filaments.
12 Briefly, the samples were fixed with 10% formalin (ThermoFisher Scientific, USA), washed
13 with PBS, and permeabilized for 5 min with 0.1% v/v Triton X-100 in PBS. Then, the samples
14 were incubated in Phalloidin-Tetramethylrhodamine B isothiocyanate (Phalloidin-TRITC)
15 (1:80) for 1 h at room temperature. The nuclei were counterstained with 1:5000 of the stock of
16 4,6-Diamidino-2-phenylindole, dilactate solution (DAPI, 1mg/mL, Biotium). The samples were
17 observed with an inverted confocal microscope (TCS SP8, Leica, Germany).

2.5. *In vivo* study

20 The scaffold manufacture and sterilization, and cell isolation, culturing, and
21 cryopreservation were the same as aforementioned. The scaffolds were hydrated in culture media
22 overnight before cell seeding onto scaffolds at a density of 1×10^6 /scaffold. After 1 h of
23 incubation in the CO₂ incubator post-seeding, the culture medium was added to the suspension

1 culture wells (Greiner Bio-One GmbH, Austria). Every 2-3 d the scaffolds were transferred to
2 new well-plates with new culture medium and cultured 7 d before implantation.

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10 2.5.1. Subcutaneous implantation

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12 The *in vivo* biological response to the scaffolds and constructs were evaluated through
13 subcutaneous implantation in seven 5-week-old male nude mice (CrI:NU(NCr)-*Foxn1*^{nu}, Charles
14 River, France). The animal experimentation complies with the 3Rs (Replacement, Reduction,
15 and Refinement) [37-39] and approved by the Ethical Committee, and the Portuguese National
16 Authority for Animal Health, Direção Geral de Alimentação e Veterinária (DGAV). Animals
17 were anesthetized with intraperitoneal injection of a mixture of ketamine 75 mg/Kg (Imalgene
18 1000, 100 mg/mL, Merial, France) and medetomidine 1 mg/Kg (Domitor 1 mg/mL, Orion,
19 Finland). After the confirmation of anesthesia/ analgesia and disinfection of the dorsal skin of the
20 animals, two short incisions were performed on the dorsal midline (cranial and caudal) of the
21 animals. Through each incision, one pocket on each side of the dorsa was made by blunt
22 dissection (4 pockets/animal), and the cell-seeded scaffolds and control scaffolds without cells
23 were implanted (1 scaffold/pocket). The incisions were sutured, the animals were observed and
24 maintained on the heated platform for recovery, and upon the recovery from anesthesia, the
25 animals were maintained in their normal housing conditions. The mice were checked on a daily
26 basis and allowed food and drink *ad libitum* throughout the experiment. The animals were
27 euthanized by CO₂ inhalation 4 weeks after the implantation for implant retrieval for
28 characterization. Three samples per scaffold type and condition were implanted.

2.5.2. Micro-CT analysis of the explants

One explant from each sample group was randomly pre-selected before explant collection to be visualized using a micro-CT system (SkyScan 1272, Bruker, Belgium). The explants were maintained in dPBS with 1.0% of an antibiotic-antimycotic solution, and scanned without delay with micro-CT as described above, with an additional step of covering of samples with Parafilm M wrapping film to avoid drying after fixation on the sample holder of the equipment. The 3D images of the explants were built from the 2D reconstructed images by using the manufacturer's software.

2.5.3. Hematoxylin and eosin (H&E) staining

The samples (n=3) were first fixed with 10% formalin (ThermoFisher Scientific, USA) then placed in histological cassettes and processed in a spin tissue processor (STP120-2, Microm, ThermoFisher Scientific, USA) following standard protocols. After embedded in paraffin, the histological sections were obtained from the histoblocks using a rotary microtome (HM355S, Microm, ThermoFisher Scientific, USA), mounted onto histological slides. The sections were deparaffinized and stained with H&E using an automatic stainer (HMS740, Microm, ThermoFisher Scientific, USA). The stained sections were analyzed with a Leica DM750 microscope.

2.5.4. Masson's trichrome staining

The histological sections were prepared as described in the previous section, and Masson's trichrome staining was performed using a staining kit (Bio-Optica, Italy) as described in the manufacturer's protocol. Briefly, reacting the sections with sequentially with Weigert's iron hematoxylin, picric acid alcoholic solution, ponceau acid fuchsin according to Mallory,

phosphomolybdic acid solution, and Masson aniline blue; all provided with the kit. After the staining, the sections were analyzed with a Leica DM750 microscope.

2.5.5. Semi-quantitative evaluation of the explants

The explants were evaluated through a semi-quantitative scoring by three independent evaluators using H&E stained histological images (n=6). After the images were anonymized, and analysis was performed using the QuPath software [40] that is a software for quantitative pathology installed on a computer with the 64-bit operating system of Windows 10. Each independent evaluator marked the areas with inflammation on the images using the brush and wand tools of the software. To determine the percent of the areas with inflammation, firstly all markings within an image were merged into a single area to avoid counting a particular area more than once, then the percentage was calculated using the entire area of the image. Regarding the tissue infiltration and scaffold fragmentation scaffolds were scored according to Table 1.

2.6. Statistical analysis

Statistical analyses were performed using the SPSS® (IBM® SPSS® Statistics version 24.0, IBM, USA). Values are presented as “mean (standard error of the mean)”, M (SE). The error bars indicate the standard error of the mean where present. The statistical differences between the means of the quantitative results of three scaffold groups were investigated using a one-way analysis of variance (ANOVA) tests. For each ANOVA test, the null hypothesis (H₀) is that the means of the examined parameter are equal for each scaffold type, while the alternative hypothesis is that at least one of the scaffold group’s mean is different from others.

$$H_0: \text{Mean}_{\text{PCL cage}} = \text{Mean}_{\text{PCL cage} + 8\% \text{ silk fibroin}} = \text{Mean}_{\text{PCL cage} + 16\% \text{ silk fibroin}}$$

Acceptance or rejection of the H_0 is based on the absence or presence of the statistically significant differences. The statistical significance level was set at 0.05. Upon rejection of H_0 , the F -ratio (F) with the significance was provided, and the underlying difference between the scaffolds was investigated by the Tukey post hoc test with equal variances. Upon unequal variances, the Welch's ANOVA was used, and the Welch's F (F_w) was obtained that is the corrected F -ratio, and accordingly, the underlying differences were investigated by Games-Howell post hoc test. The quantified differences between the scaffolds were indicated as difference's "mean (the 95% confidence intervals, lower bound of the mean difference-upper bound of the mean difference)", M (95% CI, LB - UB). The absence of outliers was checked with boxplots, Levene's tests were used to confirm the equality of variances, and Shapiro-Wilk's tests were used to confirm the presence of normal distributions [33, 35]. The general linear model analysis provided the η^2 values as the effect size, and the Cohen's effect size benchmarks [41, 42] were used for the effect size classes' determination [35]. The η^2 values of 0.01, 0.06, and 0.14 correspond to small, medium, and large effect size classes, respectively.

3. Results and Discussion

3.1. Physicochemical characterization of scaffolds

In this study we demonstrated the production of the EiC scaffolds for meniscus tissue engineering that were produced from 3D-printed PCL and porous regenerated silk fibroin by combining 3D-printing and salt-leaching, and the results of *in vitro* and *in vivo* studies after seeding with hASCs or human meniscocytes. PCL [43-45] and silk fibroin [46-49] are appealing biomaterials used in tissue engineering. Herein, the EiC scaffolds were designed in a way to take advantage of the PCL's mechanical strength and convenience for 3D-printing owing to its

1 thermoplasticity, and silk fibroin's favorable biological features for cell culture [50, 51] by
2 entrapping silk fibroin inside the PCL cage. Fig. 1 illustrates the design and the architecture of
3 the EiC scaffolds that are shown by the cross-sectional 2D and 3D micro-CT images. Fig. 2
4 shows entrapment of silk fibroin in the 3D-printed PCL cage on the SEM images from the cross-
5 section and the surface of the scaffolds. In an orthotopic application of meniscal scaffolds
6 anatomically correct shape and size is of critical importance for the performance of the implant
7 [2]. In our previous studies, we manufactured the first patient-specific 3D-printed meniscal
8 scaffolds from PCL using the patients' volumetric knee magnetic resonance imaging datasets
9 [28] and quantified the 3D cellular density of human menisci in a segmental and regional manner
10 for first time in the literature [35]. Accordingly, the strategy that was developed and evaluated in
11 the current study, has a high capacity for advancement towards tissue engineering constructs that
12 emulate the native human meniscus cellularity, size and shape which would be needed also for
13 large animal models such as sheep or goat with clinically relevant meniscal defect models [2].
14 Although in this study the EiC scaffolds were seeded with Hoffa's fat pad-derived ASCs and
15 meniscocytes, the EiC scaffold system described herein can also be beneficial for other orthopedic
16 tissues such as bone and cartilage where micro-structure and mechanical properties can be
17 tailored by modulating the 3D-printed cage structure and the concentration of aqueous-derived
18 silk fibroin solution, and also where the patient-specific scaffold manufacture is useful to treat
19 certain orthopedic lesions.

20 The micro-structural parameters (Table 2), *i.e.*, mean porosity, mean pore size, mean wall
21 thickness, and interconnectivity were quantified with micro-CT. The mean porosity of the
22 scaffolds were 61.1 (1.7)%, 54.6 (1.0)%, and 50.0 (2.2)% respectively for the PCL cage, PCL
23 cage + 8% silk fibroin, and the PCL cage + 16% silk fibroin scaffolds. The scaffolds were

1 statistically significantly different from each other in mean porosity $F(2, 6) = 11.4$, $p = 0.009$ (η^2
2 $= 0.792$) with a difference of 11.4% (95% CI, 4.0 - 18.8), $p = 0.008$ between the PCL cage and
3 the PCL cage + 16% silk fibroin scaffolds. The scaffolds' mean pore size were 679.1 (25.3) μm ,
4 278.7 (15.5) μm , and 287.2 (25.4) μm respectively for PCL cage, PCL cage + 8% silk fibroin,
5 and PCL cage + 16% silk fibroin scaffolds. The scaffolds were statistically significantly different
6 from each other in mean pore size $F(2, 6) = 103.0$, $p < 0.0005$ ($\eta^2 = 0.972$) with the differences of
7 (i) 400.4 μm (95% CI, 302.6 - 498.1), $p < 0.0005$ between the PCL cage and the PCL cage + 8%
8 silk fibroin scaffolds, and (ii) 391.8 μm (95% CI, 294.1 - 489.6), $p < 0.0005$ between the PCL
9 cage and PCL cage + 16% silk fibroin scaffolds. The mean wall thickness of the scaffolds were
10 390.2 (4.6) μm , 387.7 (21.8) μm , and 427.0 (38.4) μm respectively for the PCL cage, PCL cage
11 + 8% silk fibroin, and the PCL cage + 16% silk fibroin scaffolds. The pore size and wall
12 thickness distributions are provided in Figs. 3 A-B. The interconnectivity values were 99.8
13 (0.0)%, 99.0 (0.1)%, and 98.6 (0.4)% respectively for the PCL cage, PCL cage + 8% silk fibroin,
14 and PCL cage + 16% silk fibroin scaffolds. The scaffolds were not statistically significantly
15 different from each other in mean wall thickness and interconnectivity. The inclusion of 16% silk
16 fibroin to PCL cage scaffold caused a significant decrease of 11.4 % in the mean porosity.
17 Nevertheless, the mean porosity of the scaffolds seems to be lower than the generally desired
18 porosity from scaffolds, although silk fibroin itself is highly porous. This is due to the fact that
19 thick PCL structures occupy a considerable amount of space. We previously reported the
20 porosity of the 16% silk fibroin scaffolds to be around 80% [33]. With the presence of silk
21 fibroin, the mean pore size becomes more favorable when compared with PCL cage scaffolds.
22 The microstructural characteristics of the scaffolds such as porosity, pore size, and
23 interconnectivity can affect the cellular functions and thus the performance of the scaffolds [34].

Highly interconnected pores facilitate tissue infiltration and vascularization, and all the scaffolds had excellent interconnectivity (98.6% - 99.8%). While scaffolds with silk fibroin did not have significantly higher compression modulus as compared to that of PCL cage scaffolds in the dry state, there were significant differences in the wet state thanks to water uptake kinetics of the silk fibroin. Fast water uptake is a favorable feature for implantation. The PCL fibers were compact while silk fibroin was in porous form. This led to significant differences in the water uptake behavior in the first 24 h.

The compression tests were performed on both dry and wet scaffolds (Table 2). The compression modulus in dry state values were 53.0 (6.6) MPa, 57.6 (2.0) MPa, and 57.8 (6.3) MPa respectively for PCL cage, PCL cage + 8% silk fibroin, and PCL cage + 16% silk fibroin scaffolds, and there were no statistically significant differences. The compression modulus in wet state values were 61.6 (0.9) MPa, 49.5 (8.4) MPa, and 22.3 (3.8) MPa respectively for the PCL cage, PCL cage + 8% silk fibroin, and PCL cage + 16% silk fibroin scaffolds. The scaffolds were statistically significantly different from each other in compression modulus in wet state $F_{W(2, 7.12)} = 46.8$, $p < 0.0005$ ($\eta^2 = 0.654$) with the differences of (i) 39.2 MPa (95% CI, 27.0 - 51.5), $p < 0.0005$ between the PCL cage and the PCL cage + 16% silk fibroin scaffolds, and (ii) 27.2 MPa (95% CI, 0.1 - 54.2), $p = 0.05$ between PCL cage + 8% silk fibroin and PCL cage + 16% silk fibroin scaffolds. The water uptake kinetics is shown in Fig. 3 C. Two significant differences were found between the scaffolds: (i) at 12 h, $F(2, 6) = 7.4.0$, $p = 0.024$ ($\eta^2 = 0.712$) with a difference of 17.2% (95% CI, 2.7 - 31.7), $p = 0.025$ between the PCL cage + 8% silk fibroin scaffolds and the PCL cage, and (ii) at 24 h, $F_{W(2, 3.1)} = 45.1$, $p = 0.005$ ($\eta^2 = 0.458$) with a difference of 14.8% (95% CI, 8.5 - 21.1), $p = 0.006$ between the PCL cage + 8% silk fibroin and

the PCL cage + 16% silk fibroin scaffolds. At 72 h and 168 h there were no statistically significant differences between the scaffolds.

Having an anatomically correct shape and size is of critical importance for the orthotopic performance of meniscal scaffolds [2]. In our previous studies, we manufactured the first patient-specific 3D-printed meniscal scaffolds from PCL using the patients' volumetric knee magnetic resonance imaging datasets [28] and quantified the 3D cellular density of human menisci in a segmental and regional manner for first time in the literature [35]. Accordingly, the strategy that was developed and evaluated in the current study, has a high capacity for advancement towards tissue engineering constructs that emulate the native human meniscus cellularity, size and shape which would be needed also for large animal models such as sheep or goat with clinically relevant meniscal defect models [2]

3.2. *In vitro* cytocompatibility assessment

In the present study, two cell types were used, human meniscocytes and Hoffa's fat pad-derived hASCs. *In vitro* evaluation of viability and proliferation of hASCs and human meniscocytes seeded onto the scaffolds and cultured for d 1 and d 21 is depicted in Figs. 4 F-G. For the viability (Fig. 4 F), at both time points and cell types there were statistically significant differences between the scaffolds: (i) at d 1 with hASCs ($p < 0.0005$, $\eta^2 = 0.851$), (ii) at d 1 with human meniscocytes ($p < 0.0005$, $\eta^2 = 0.469$), (iii) at d 21 with hASCs ($p < 0.0005$, $\eta^2 = 0.767$), and (iv) at d 21 with human meniscocytes ($p < 0.0005$, $\eta^2 = 0.516$). For the proliferation (Fig. 4 G), the differences were found only at d 1 for both hASCs and human meniscocytes with respectively $p < 0.0005$, $\eta^2 = 0.513$, and $p < 0.0005$, $\eta^2 = 0.502$. The cell adhesion was observed on the SEM images of the scaffolds at d 21 (Figs. 4-6). The live/dead staining images (Fig. 7)

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confirmed the presence of live cells at d 1 and d 21, and the confocal microscopy images after the immunofluorescence staining for filamentous actin for samples at d 1 and d 21 are presented in Figs. 8-10. The expressed F-actin indicates cellular cytoskeleton development and good cell adhesion while the round or elongated morphologies of the cell nuclei (blue) resemble cells of the native human meniscus, *i.e.*, cells with fibrochondrocyte morphology or fibroblast-like cell morphology [35]. The results showed that the EiC scaffolds had enhanced the cell adhesion, migration, viability, and proliferation as compared to the PCL cage scaffolds. It should be noted that for the use of EiC scaffolds for other tissues, other cell types can be employed. Hoffa's fat pad [52] is an adipose body in the knee, anatomically close to the meniscus, and an intracapsular extrasynovial source for ASCs for meniscus tissue engineering. In the case of a meniscus lesion that could be treated by applying tissue engineering strategies, having a healthy and sufficient number of primary meniscocytes can be a challenge, and this challenge could be overcome by stem cells [1, 2, 12]. Moreover, through their paracrine activity, stem cells that can provide regenerative micro-environments [53, 54]. Future studies should assess the differentiation potential of Hoffa's fat pad-derived hASCs into meniscocytes and compare the obtained neotissue with the one obtained with primary meniscocytes and the native meniscus. The *in vitro* assays showed that the inclusion of silk fibroin enhanced the cell adhesion as shown in SEM images (Figs. 4-6), metabolic activity and proliferation assessed by luminescent cell viability and DNA quantification (Figs. 3 D-E). Along with the autofluorescence from the biomaterials, in Fig. 7 the live cells appear green since Calcein-AM fluoresces green in the case of live cells, and the nucleus of dead cells appear red since PI fluorescence red upon entering through the cytoplasmic membrane and binding to DNA. The cell shape and organization are shown in Figs. 8-10 with the F-actin cytoskeleton appear red while the cell nuclei appear blue.

3.3. *In vivo* biocompatibility assessment

The *in vivo* evaluation of the scaffold was performed by subcutaneous implantation in nude mice. The implantation of biomaterials *in vivo* can lead to an inflammatory reaction [55, 56], and macrophages try to digest the foreign substance, *i.e.*, scaffolds. As it is well characterized, athymic nude rodents [57, 58] have intact innate immune system, normal number and activity of macrophages, natural killer cells, B cells, and antigen presenting cells, while they lack a normal thymus and largely deficient in functional mature T-cells. Therefore, it is possible to implant scaffolds that were seeded with xenogenic cells, in this study with human cells. All animals were alive without signs of discomfort up until their sacrifice, and there were no adverse incidents. Figs. 11-13 show the gross appearance and the micro-CT images of the explant. The scaffolds were infiltrated with newly formed tissue after 4 weeks of implantation, and their appearance is homogenous throughout the explants with distinct visibility of the PCL structures (Figs. 11 C3-E3, and Figs. 12-13 A3-C3). The H&E and Masson's trichrome stained images (Figs. 14-16) showed excellent tissue infiltration, collagenous neomatrix synthesis, and formation of new blood vessels. The scaffolds were assessed regarding inflammation, tissue infiltration, and formation of blood vessels. Semi-quantitative analysis revealed that PCL cage scaffolds have a score of 2 (tissue infiltration without scaffold fragmentation) while scaffolds with silk fibroin scored between 2 and 3 indicating partial fragmentation of the silk fibroin. The areas with inflammation were less than the 10% of the entire areas in all scaffolds, and neovascularization was observed in all samples as depicted in Figs. 14-16.

Biological properties and the microstructural features of the scaffolds influence the *in vivo* performance. The cell infiltration into the scaffolds, *i.e.*, neotissue formation, and

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3 1 neovascularization are favorable for tissue remodeling and regeneration. The H&E stained
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5 2 histological images (Figs. 14-16) indicate that inflammatory cells were mildly invaded into the
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7 3 scaffolds. Typically, a scattering of inflammatory cells is commonly found at the silk fibroin
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9 4 areas while denser along the border of the PCL fibers. PCL fibers remained intact, while silk
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11 5 fibroin structures were partially fragmented by the macrophage activities. Since the explants that
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13 6 were acellular or seeded with cells and cultured prior to the implantation were found to have
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15 7 similar outcomes including tissue infiltration and blood vessel formation, the benefit of cell
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17 8 seeding onto scaffolds before implantation is evident. Seeding higher number cells and/or longer
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19 9 culture time may lead to a difference *in vivo*. Also, biologics [59] can be incorporated into *in*
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21 10 *vitro* culture of cell-scaffold constructs, or injected into target tissue environment for the
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23 11 modulation the cell function and the tissue healing environment. The inclusion of silk fibroin
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25 12 into the large pores of PCL fibers, filled the space and contributed formation of collagenous
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27 13 extracellular-matrix (Figs. 15-16 A2-C2). The presence of macrophages also contributes to
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29 14 neovascularization [60]. Therefore, with the semi-quantitative evaluations, tissue infiltration
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31 15 without scaffold fragmentation was observed in the PCL cage, and tissue infiltration with partial
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33 16 fragmentation was observed in the scaffolds with silk fibroin. In the Masson's trichrome stained
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35 17 images (Figs. 14-16) the formation of new blood vessels, and collagenous neomatrix (blue) into
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37 18 the scaffolds can be seen. It should be noted that the tissue environment in the knee joint is
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39 19 different from subcutaneous space. However, subcutaneous implantation is beneficial for
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41 20 biocompatibility assessment, future *in vivo* studies employing an orthotopic meniscus defect
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43 21 model are needed for a better evaluation of the scaffolds. The results of the current study are
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45 22 encouraging to perform an *in vivo* study using a larger model, using ideally animal-specific
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47 23 scaffolds seeded with autologous ASCs.
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5. Conclusion

In this work, we successfully produced EiC scaffolds of 3D-printed PCL cage and porous silk fibroin for meniscus tissue engineering applications. The EiC scaffolds were seeded with meniscocytes or Hoffa's fat pad-origin ASCs isolated from human tissues, and characterized *in vitro* and *in vivo*. The results suggest the confirmation of the hypotheses that inclusion of silk fibroin into PCL cage improved the performance of the scaffolds *in vitro* and *in vivo*, and, neotissue formation and neovascularization have been achieved for the scaffolds either seeded with human meniscocytes or hASCs. Moreover, the design of the EiC scaffolds is readily adaptable for the production of scaffolds in anatomically fitting shape and size for future *in vivo* studies using a lagomorph model or a large animal model which are required in order to assess the orthotopic performance of the tissue engineering constructs in a clinically relevant meniscus lesion scenario.

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Disclosures

The authors declare that there is no conflict of interest in relation to this work.

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Tables

Table 1. Evaluation of the tissue infiltration and scaffold intactness using a semi-quantitative scoring

Tissue infiltration and scaffold fragmentation	Score
No tissue infiltration, no fragmentation	1
Tissue infiltration, no fragmentation	2
Small fragments/debris	3
Complete degradation	4

Table 2. Micro-structural and mechanical characteristics of the scaffolds.

	Mean porosity	Mean pore size	Mean wall	Interconnectivity	Compression modulus	
	[%] M (SE)	[µm] M (SE)	thickness	[%] M (SE)	[MPa] M (SE)	
			[µm] M (SE)		Dry	Wet
PCL cage	61.1 (1.7)	679.1 (25.3)	390.2 (4.6)	99.8 (0.0)	53.0 (6.6)	61.6 (0.9)
PCL cage + 8% Silk fibroin	54.6 (1.0)	278.7 (15.5)	387.5 (21.8)	99.0 (0.1)	57.6 (2.0)	49.5 (8.4)
PCL cage + 16% Silk fibroin	50.0 (2.2)	287.2 (25.4)	427.0 (38.4)	98.6 (0.4)	57.8 (6.3)	22.3 (3.8)

Figure legends

Fig. 1. Design and architecture of the scaffolds. (A-C) Schematic illustration of the scaffold design. Fibers indicate the PCL, and the yellow structures indicate the entrapped silk fibroin, and (D-F) 2D cross-sectional micro-CT images. Black color indicates the empty space, gray colors indicate the biomaterials. Scale bars indicate 1 mm. (G-I) 3D images of the scaffolds. The distance between two adjacent white points is 0.5 mm. PCL cage (A, D, and G), PCL cage + 8% silk fibroin (B, E, and H), Silk PCL cage + 16% silk fibroin (C, F, and I).

Fig. 2. SEM images of the scaffolds. PCL cage (A-D), PCL cage + 8% silk fibroin (E-H), and silk PCL cage + 16% silk fibroin (I-L) showing both surface and cross-section of the scaffold at two different magnification. White and yellow scale bars indicate 500 μm and 100 μm , respectively. PCL cage (A-D), PCL cage + 8% silk fibroin (E-H), and silk PCL cage + 16% silk fibroin (I-L).

Fig. 3. Characterizations of the scaffolds. (A) Pore size and (B) wall thickness distributions for each scaffold type. (C) Water uptake behavior of the scaffolds up to 168 h.

Fig. 4. SEM images of the PCL cage scaffold seeded with hASCs (A and C) or with human meniscocytes (B and D) showing adhesion of cells at d 21 on the surface and cross-section, *i.e.*, inside the scaffolds. White, yellow, and red scale bars indicate 500 μm , 100 μm , and 50 μm , respectively. (F) Cell viability assessment through a bioluminescence assay. (G) Cell Proliferation assessed by DNA quantification. Single asterisk and double asterisks in D and E indicate the detected statistically significant and insignificant differences, respectively.

Fig. 5. SEM images of the PCL cage + 8% silk fibroin scaffolds seeded with hASCs (A and C) or with human meniscocytes (B and D) showing adhesion of cells at d 21 on the surface and cross-section, *i.e.*, inside the scaffolds. White, yellow, and red scale bars indicate 500 μm , 100 μm , and 50 μm , respectively.

Fig. 6. SEM images of the PCL cage + 16% silk fibroin scaffolds seeded with hASCs (A and C) or with human meniscocytes (B and D) showing adhesion of cells at d 21 on the surface and cross-section, *i.e.*, inside the scaffolds. White, yellow, and red scale bars indicate 500 μm , 100 μm , and 50 μm , respectively.

Fig. 7. The live/dead images of the scaffolds seeded with hASCs (A, C, and E) or with human meniscocytes (B, D, and F) at d 1 and d 21 obtained by calcein/propidium iodide staining. The white and yellow scale bars indicate 200 μm , and 50 μm , respectively.

Fig. 8. Confocal microscopy images of the PCL cage scaffolds seeded with hASCs (A and C) or with human meniscocytes (B, and D) at d 1 and d 21 at two different magnifications after immunofluorescence staining for filamentous actin. Along with the autofluorescence of the scaffold, the F-actin cytoskeletons appear red while the cell nuclei appear blue. The white and green scale bars indicate 100 μm , and 50 μm , respectively.

Fig. 9. Confocal microscopy images of the PCL cage + 8% silk fibroin scaffolds seeded with hASCs (A and C) or with human meniscocytes (B, and D) at d 1 and d 21 at two different magnifications after immunofluorescence staining for filamentous actin. Along with the

autofluorescence of the scaffold, the F-actin cytoskeletons appear red while the cell nuclei appear blue. The white and green scale bars indicate 100 μm , and 50 μm , respectively.

Fig. 10. Confocal microscopy images of the PCL cage + 16% silk fibroin scaffolds seeded with hASCs (A and C) or with human meniscocytes (B, and D) at d 1 and d 21 at two different magnifications after immunofluorescence staining for filamentous actin. Along with the autofluorescence of the scaffold, the F-actin cytoskeletons appear red while the cell nuclei appear blue. The white and green scale bars indicate 100 μm , and 50 μm , respectively.

Fig. 11. *In vivo* characterization of the PCL cage scaffolds. (A) A photograph of the scaffold prior to implantation. (B) A photograph of a mouse right after the scaffold implantation. Photographs of explants (C1, D1, and E1), overall 3D micro-CT images (C2, D2 and E2), and cropped 3D micro-CT images showing the inside of the scaffold (C3, D3, and E3). The distance between two adjacent white points is 0.5 mm. Acellular scaffold (C), seeded with hASCs (D), and human meniscocytes (E) prior to implantation.

Fig. 12. *In vivo* characterization of the PCL cage + 8% silk fibroin scaffolds. Photographs of explants (A1, B1, and C1), overall 3D micro-CT images (A2, B2, and C2), and cropped 3D micro-CT images showing the inside of the scaffold (A3, B3, and C3). The distance between two adjacent white points is 0.5 mm. Acellular scaffold (A), seeded with hASCs (B), and human meniscocytes (C) prior to implantation.

Fig. 13. *In vivo* characterization of the PCL cage + 16% silk fibroin scaffolds. Photographs of explants (A1, B1, and C1), overall 3D micro-CT images (A2, B2, and C2), and cropped 3D

1 micro-CT images showing the inside of the scaffold (A3, B3, and C3). The distance between two
2 adjacent white points is 0.5 mm. Acellular scaffold (A), seeded with hASCs (B), and human
3 meniscocytes (C) prior to implantation.

4
5 **Fig. 14.** Histological images of the PCL cage scaffold explants. (A1, B1, and C1) H&E staining.
6 (A2, B2, and C2) Masson's trichrome staining. Acellular scaffold (A), seeded with hASCs (B),
7 and human meniscocytes (C) prior to implantation. Black asterisks indicate PCL structures and
8 yellow arrow indicate tissue infiltration. The black and yellow scale bars indicate 500 μ m, and
9 200 μ m, respectively.

10
11 **Fig. 15.** Histological images of the PCL cage + 8% silk fibroin scaffold explants. (A1, B1, and
12 C1) H&E staining. (A2, B2, and C2) Masson's trichrome staining. Acellular scaffold (A), seeded
13 with hASCs (B), and human meniscocytes (C) prior to implantation. Black asterisks indicate
14 PCL structures, yellow asterisks indicate silk fibroin structures, and yellow arrows indicate tissue
15 infiltration. The black and yellow scale bars indicate 500 μ m, and 200 μ m, respectively.

16
17 **Fig. 16.** Histological imaged of the PCL cage + 16% silk fibroin scaffold explants. (A1, B1, and
18 C1) H&E staining. (A2, B2, and C2) Masson's trichrome staining. Acellular scaffold (A), seeded
19 with hASCs (B), and human meniscocytes (C) prior implantation. Black asterisks indicate PCL
20 structures, yellow asterisks indicate silk fibroin structures, and yellow arrows indicate tissue
21 infiltration. The black and yellow scale bars indicate 500 μ m, and 200 μ m, respectively.

Figures

Fig. 1

Fig. 2

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Fig. 4

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1 **Fig. 14**

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1 **Fig. 16**

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