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**Polyurethane scaffold with *in situ* swelling capacity for nucleus  
pulposus replacement**

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Running title:

Polyurethane scaffold with *in situ* swelling capacity for nucleus pulposus replacement

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27 **Abstract**

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3 28 Nucleus pulposus (NP) replacement offers a minimally invasive alternative to spinal  
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5 29 fusion or total disc replacement for the treatment of intervertebral disc (IVD)  
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7 30 degeneration. This study aimed to develop a cytocompatible NP replacement  
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10 31 material, which is feasible for non-invasive delivery and tunable design, and allows  
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12 32 immediate mechanical restoration of the IVD. A bi-phasic polyurethane scaffold was  
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15 33 fabricated consisting of a core material with rapid swelling property and a flexible  
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17 34 electrospun envelope. The scaffold was assessed in a bovine whole IVD organ  
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20 35 culture model under dynamic load for 14 days. Nucleotomy was achieved by incision  
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22 36 through the endplate without damaging the annulus fibrosus. After implantation of the  
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25 37 scaffold and *in situ* swelling, the dynamic compressive stiffness and disc height were  
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27 38 restored immediately. The scaffold also showed favorable cytocompatibility for native  
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30 39 disc cells. Implantation of the scaffold in a partially nucleotomized IVD down-  
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32 40 regulated catabolic gene expression, increased proteoglycan and type II collagen  
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34 41 intensity and decreased type I collagen intensity in remaining NP tissue, indicating  
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37 42 potential to retard degeneration and preserve the IVD cell phenotype. The scaffold  
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40 43 can be delivered in a minimally invasive manner, and the geometry of the scaffold  
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42 44 post-hydration is tunable by adjusting the core material, which allows individualized  
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44 45 design.

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50 47 **Keywords**

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53 48 Biphasic polyurethane scaffold; *in situ* swelling; nucleus pulposus replacement; organ  
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55 49 culture; nucleotomy; intervertebral disc degeneration  
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## 52 Introduction

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3 53 The intervertebral disc (IVD) is a complex tissue structure with important mechanical  
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5 54 functions. It consists of a central part, the nucleus pulposus (NP), the surrounding  
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7 55 annulus fibrosus (AF), and the cartilaginous endplates which provide the permeable  
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10 56 connections to the bony vertebrae. The main function of the NP is to absorb and  
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13 57 distribute mechanical load exerted on the spinal column. To fulfil this function, the NP  
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15 58 contains specific extracellular matrix components rich in proteoglycans that allow the  
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17 59 maintenance of a highly hydrated state and high swelling pressure [1].  
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20 60 IVD degeneration is associated with changes in extracellular matrix composition that  
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23 61 often have biomechanical consequences and can lead to painful and debilitating  
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25 62 conditions. The resulting neck and low back pain are major determinants of  
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28 63 discomfort and disability and are the origin of enormous socio-economic health care  
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30 64 problems worldwide [2-4]. Treatment methods range from physical therapies and  
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33 65 pain relieving medication to highly invasive surgical procedures. Discectomy is often  
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35 66 performed in cases of disc bulging or herniation to relieve the painful pressure on  
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38 67 neural elements. Spinal fusion is another standard treatment intended to relieve pain  
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40 68 by reducing motion across the joint. However, removal of disc tissue and fusion alter  
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43 69 the biomechanical function and can accelerate IVD degeneration at adjacent levels  
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45 70 [5]. Due to the limited healing potential and harsh nutritional conditions of adult IVDs,  
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48 71 loss or failure of the extracellular matrix is virtually irreversible and requires  
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50 72 restoration. Implants for total IVD replacement have been developed in order to  
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52 73 preserve the motion at the operated level. However, the procedure is highly invasive  
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55 74 and may not be appropriate at early stages of degeneration; in addition the long term  
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57 75 effects are still uncertain [6].  
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76 Functionally, during IVD degeneration the NP becomes dehydrated, is replaced by  
77 fibrous tissue, and loses its high osmotic pressure [2, 7, 8]. The severe loss of water  
78 content in the NP also causes a reduction in disc height. NP replacement therapy has  
79 therefore been proposed as a less invasive alternative method to spinal fusion or  
80 total IVD replacement [9]. An ideal NP replacement material would allow immediate  
81 redistribution of loads and restoration of disc height, while maintaining the flexibility of  
82 the spine. Various synthetic polymeric materials have been described as potential NP  
83 substitutes that may be delivered as injectable hydrogel or pre-formed material [10-  
84 13]. However, several concerns still exist in their application. While conventional  
85 injectable hydrogels can be delivered through the AF in a minimally-invasive manner,  
86 their generally weak mechanical properties do not allow direct restoration of the disc  
87 height and mechanical function; in addition, their three-dimensional structure is  
88 difficult to control and there is a substantial risk of material leakage with adverse  
89 effects. Pre-formed polymers and scaffolds have the advantage of improved  
90 structural control and faster response to external mechanical forces. Nevertheless,  
91 their application requires a more invasive procedure, and unwanted outcomes such  
92 as material displacement may occur [14-16].

93 The polyurethane (PU) family of biomaterials has been introduced for medical use  
94 because of their elastomeric properties. By varying the composition of the monomer  
95 units and the size of the blocks of the dissimilar monomers within the polymer chain,  
96 these properties can be tailored. As such, PU has been used in applications in which  
97 an elastomeric material is likely to enhance the success or longevity of the implant.  
98 Specifically, PU has been used in cardiovascular applications, where material  
99 flexibility is important, such as for catheters, insulations, vascular prostheses, heart  
100 valves or assist devices [17]. Furthermore, a range of tissue replacement or  
101 augmentation materials are based on PU, especially as wound dressing for skin

102 regeneration [18]. Among the PU family, polycarbonate urethanes have  
103 demonstrated improved biostability and are therefore preferred for many applications  
104 [19, 20]. The function of the intervertebral disc relies on the elastomeric nature of the  
105 matrix components and fluid of which they are constituted; hence, PU is an attractive  
106 material for IVD restoration. In fact PU based materials have been used in spinal  
107 surgery and the overall mechanical and biological properties indicate no long-term  
108 problems to date; though information on their application as pure NP replacement is  
109 scarce [21].

110 Taking into account the requirements for an NP substitute, we have developed a new  
111 bi-phasic polyurethane (PU) scaffold consisting of a highly hydrophilic core material  
112 with significant instant swelling capabilities and an electrospun envelope. The  
113 functions of the envelope are to assure the structural control of the implant after  
114 swelling *in situ* and to facilitate cell attachment and tissue integration to keep the  
115 implant stable also under mechanical load. Moreover, the unique flat geometry and  
116 flexible shape of the bi-phasic scaffold system allow minimally invasive administration  
117 into the NP of the IVD.

118 The aim of this work was to assess the mechanical, swelling properties and the  
119 cytocompatibility of the scaffold materials. A whole IVD organ culture model was then  
120 used to test the hypothesis that the implanted scaffold could restore the disc height  
121 and mechanical stiffness in a nucleotomized IVD. Histological and gene expression  
122 analyses were performed to evaluate the biological response of the disc cells and  
123 tissues to the implanted scaffold.

124

## 125 **Materials and Methods**

### 126 1. Fabrication and characterization of PU scaffolds

#### 127 1.1. Fabrication of PU scaffolds

128 PU scaffolds with swelling capability were manufactured to obtain implants with a flat  
129 discoloid structure. The scaffolds are composed of a core film which swells to a  
130 hydrogel following contact with an aqueous medium, and an envelope composed of  
131 electrospun polymer mixture with a fibrous mat structure (Fig. 1 A). The core solution  
132 was prepared with the ether-based hydrophilic urethane HydroMed™ (HM,  
133 AdvanSource Biomaterials), which was dissolved at a concentration of 20 % (w/w) in  
134 95% ethanol (Bio-Lab) by stirring overnight at room temperature. A predetermined  
135 solution volume was casted on a flat glass plate, dried for three days to ensure  
136 solvent residues evaporation and then cut into discs at a diameter of 3, 5 and 8 mm  
137 for manufacturing scaffold at external diameter of 6, 8 or 9, and 14 mm, respectively.

138 For production of the envelope a mixture was prepared by combining the  
139 polycarbonate urethanes Chronoflex™ (CF, AdvanSource Biomaterials) and HM. In  
140 order to select the optimal ratio of CF to HM in the envelope formulation, several  
141 ratios were tested. Wetting time was visually inspected until color change observed  
142 and mechanical property was evaluated by measurement of the tensile strength. As  
143 shown in Table 1, addition of HM to CF resulted in decrease in the mechanical  
144 strength and reduced the wetting time. Optimal balance between wetting, mechanical  
145 strength and manufacturing considerations by electrospinning resulted in the  
146 selection of CF:HM w/w ratio at 10:1. The polyurethane mixture was then dissolved at  
147 a concentration of 13% (w/w) in a solvent mixture of 1:2 w/w N-N-dimethylformamide  
148 (BioLab) and dioxane (BioLab). Electrospun envelope structures at a thickness of  
149 200 µm were fabricated according to specifications for strong fibers at a range of 0.5-

150 2  $\mu\text{m}$  and porosity of  $\sim 70\%$ . The following electrospinning parameters were applied at  
1 an environment of 40% relative humidity and 28  $^{\circ}\text{C}$ . A collector at diameter of 50 mm  
2 151 an environment of 40% relative humidity and 28  $^{\circ}\text{C}$ . A collector at diameter of 50 mm  
3  
4 152 and length of 150 mm with rotating speed ( $\omega$ ) of 120 rpm was used. A five needles  
5  
6  
7 153 spinneret was used (22 G, 25 mm length) at linear speed of 57 mm/sec and traverse  
8  
9  
10 154 shuttle of 170 mm. The spinneret was placed 260 mm from the collector. A potential  
11  
12 155 of 60 kV, solution flow rate of 14 mL/h and consumption of 10 mL were applied.

156 Following electrospinning the envelope sheet was dried overnight at room  
16  
17 157 temperature and then at  $50\pm 5^{\circ}\text{C}$  for 48 hours. The dried envelope sheets were cut  
18  
19  
20 158 into discs of 20 mm diameter before scaffold assembly.

21  
22  
23 159 Core discs at predetermined content (see 1.3) and size were wrapped by two  
24  
25 160 envelope discs that were heat sealed using custom made tools (Fig. 1 B). The  
26  
27 161 sealing conditions were 4 seconds at pressure of 3 bar and temperature of 118  $^{\circ}\text{C}$ .  
28  
29  
30 162 The PU scaffold was then cut into its final size, at a diameter of 6, 8, 9 or 14 mm. The  
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32 163 scaffolds were sterilized in a cold-cycle (37  $^{\circ}\text{C}$ ) ethylene oxide process and  
33  
34  
35 164 subsequently evacuated at room temperature and 150 mbar for 7 days.

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37  
38 165 A delivery system for non-invasive injection of the PU scaffolds was developed by  
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40 166 Melab Medizintechnik und Labor GmbH (Germany). The PU scaffolds at diameters of  
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43 167 6-14 mm can be rolled into a tube of 2.8-5 mm diameter and delivered through a  
44  
45 168 needle with insertion guide. A demonstration of the delivery process is shown in  
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48 169 Figure 1 C.

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## 52 53 171 1.2. Structure characterization of the PU scaffold envelope

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56 172 The electrospun envelope sheet material was examined using scanning electron  
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58  
59 173 microscopy (SEM). Electron micrographs were recorded for both sides and cross  
60  
61 174 sectional areas of the electrospun envelope. Samples were snap-frozen in liquid



175 nitrogen and fractured into 2 or more pieces to obtain a cross-sectional edge.  
176 Samples were then mounted onto metallic stubs and coated with gold (~1 nm;  
177 Q150T-ES sputter coater, Quorum, UK) and their morphology observed on a Nova  
178 Nano SEM (FEI, Netherlands; Everhart-Thornley Detector in field-free lens mode) at  
179 2,000x and 10,000x magnifications. Fiber dimensions were measured in Q-capture  
180 pro (QImaging, Canada) using the scale bar of the SEM micrograph as a reference  
181 for each image. Measurements were taken from three samples. Approximately 50  
182 fibers were randomly selected for each micrograph.

183

### 1.3. Swelling capacity and kinetics of PU scaffolds

185 PU scaffolds with a diameter of 14 mm containing various dry weights of core (14 to  
186 97 mg, n=18) were incubated in aqueous medium at room temperature for 24 hours.  
187 The swelling capacity was calculated by measurement of the scaffold dry weight  
188 before swelling and the scaffold wet weight after incubation. Results were expressed  
189 as % swelling of core and/or % weight gain of PU scaffold. Swelling capacities of PU  
190 scaffolds with diameter of 6, 8, 9, and 14 mm were also measured at fixed core  
191 content (3.4 mg and 4.5 mg for 6 mm PU scaffold, 12 mg for 8 mm and 9 mm PU  
192 scaffold, 40 mg for 14 mm PU scaffold). Swelling kinetics measurements were  
193 carried out with scaffolds with diameter of 6 and 14 mm using various core contents  
194 (3.4 mg, 4.5 mg and 6.3 mg for 6 mm PU scaffold, 30 mg, 40 mg and 60 mg for 14  
195 mm PU scaffolds) in order to determine the time to reach significant swelling.

196

### 1.4. Cytocompatibility of PU scaffolds

197 To assess the potential release of cytotoxic components that might have remained or  
198 developed from the scaffold production process or the sterilization method, core and

200 envelope discs were incubated in Dulbecco's Modified Eagle Medium (DMEM, with  
201 4.5 g/L glucose, Gibco, Paisley, UK) containing 100 U/mL penicillin and 100 µg/mL  
202 streptomycin (1% Pen/Strep) (all products from Gibco) at 37°C under normoxic  
203 condition. The conditioned medium was collected after 3, 5 and 7 days. Nucleus  
204 pulposus cells (NPCs) were isolated from 4-10 months old bovine caudal IVD and  
205 cultured with DMEM, 1% Pen/Strep, 10% fetal calf serum (FCS, Gibco) at 37°C  
206 under normoxic condition [22]. Human bone marrow aspirates were obtained with  
207 ethical approval from the Swiss cantonal authorities (KEK 188/10) and informed  
208 consent. Bone marrow derived mesenchymal stem cells (MSCs) were isolated by  
209 Ficoll cushion and expanded with alpha modification of Eagle's medium (aMEM,  
210 Gibco), 1% Pen/Strep, 10% FCS, 5 ng/mL recombinant human basic fibroblast  
211 growth factor (Fitzgerald, Acton, USA) at 37°C under normoxic condition [23].  
212 Passage 1 NPCs and passage 2 MSCs were seeded into 96-well plates and cultured  
213 in DMEM with 1% Pen/Strep, 2.5% FCS and 50% conditioned medium of PU core  
214 and envelope for 24 hours or 72 hours at 37°C under normoxic condition. Cell  
215 viability was determined with WST-1 cell proliferation reagent (Roche, Mannheim,  
216 Germany) according to an established protocol [24]. Cell viability of the samples was  
217 normalized to the positive control cells cultured in DMEM with 1% Pen/Strep and 2.5%  
218 FCS.

219 A cell attachment study was performed to examine the capacity of the envelope to  
220 support cell attachment and growth. Passage 1 bovine NPCs were seeded on the top  
221 side of the envelope at a cell density of 100,000 cells per envelope (diameter 14 mm).  
222 Cell-envelope constructs were cultured in DMEM with 1% Pen/Strep and 10% FCS  
223 for 7 days at 37°C under normoxic condition. After 1 and 7 days of culture, samples  
224 were digested with 0.5 mg/mL proteinase-K (2.5 U/mg, Roche, Mannheim, Germany)  
225 at 56°C overnight. DNA content was measured spectrofluorometrically using the

226 Picogreen assay (Invitrogen, Carlsbad, USA) to evaluate the cell proliferation.  
227 Samples were also prepared for SEM imaging of the top surfaces and cross-sections  
228 of the envelopes to evaluate the cell morphology and attachment on the material  
229 surface. Cell-seeded envelopes were fixed in 2.5% glutaraldehyde in 0.1 M PIPES  
230 buffer, and dehydrated in a series of graded concentrations of ethanol before critical  
231 point drying (Quorum Technologies Ltd, East Sussex, UK). Thereafter, samples were  
232 mounted on metallic stubs with silver paint, sputter coated with gold/palladium (10 nm)  
233 and imaged by SEM (Hitachi S-4700, Tokyo, Japan).

234  
235 2. IVD organ culture – IVD dissection, nucleotomy, PU scaffold implantation, and  
236 dynamic loading

237 For assessment of the PU scaffold in organ culture which supplies a  
238 microenvironment close to the *in vivo* situation, bovine caudal spines (4-10 months)  
239 were obtained from local abattoirs and harvested aseptically within three hours after  
240 death. After removal of the soft tissue, IVDs were dissected by means of a band saw  
241 (model 30/833, Exakt Apparatebau, Norderstedt, Germany) proximal and distal to the  
242 cartilaginous endplates, and the endplates of each IVD were cleaned with a Pulsavac  
243 jet-lavage system (Zimmer, IN, USA) [22]. Initial disc height and diameter were  
244 recorded before IVDs were washed with PBS (pH 7.4) containing 10% Pen/Strep for  
245 15 min. Then IVDs were transferred to a six-well plate containing IVD culture medium  
246 and incubated overnight at 37°C, 85% humidity and 5% CO<sub>2</sub>. The IVD culture  
247 medium was composed of DMEM with 4.5 g/L glucose supplied with 2% FCS, 1%  
248 Pen/Strep, 1% ITS+ Premix (Discovery Labware, Inc., Bedford, USA), 50 µg/mL  
249 ascorbate-2-phosphate (Sigma-Aldrich, St. Louis, USA) and 0.1% Primocin. After  
250 dissection, the disc height of the bovine IVDs used for the current study ranged from

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251 7 to 14 mm and the disc diameter ranged from 13 to 20 mm. IVDs from each tail were  
252 randomly assigned among experimental groups to obtain similar average disc size  
253 and distribution of disc levels for each group.

254 A partial nucleotomy model [22] was used to mimic the clinical case of a large NP  
255 defect with relatively healthy non-degenerated surrounding disc tissue. To reach a  
256 nucleotomy fraction of approximately 50% of the total NP volume for different sizes of  
257 IVDs, a NP cavity with diameter of 4 mm was created in small IVDs with diameter of  
258 13-16.5 mm; in IVDs with diameter of 16.5-20 mm, a NP cavity with diameter of 6 mm  
259 was created. IVDs were nucleotomized through the endplate by central incisions with  
260 a biopsy punch of 4 or 6 mm diameter and a number 11 blade. After removing an  
261 endplate core, the NP tissue below the endplate core reaching until the distal  
262 endplate was excised. IVDs and endplate stoppers were preserved in gauze  
263 moistened with IVD culture media. Pilot experiments had revealed that dry PU  
264 scaffolds with 6 mm or 9 mm diameter obtained a diameter of 4 mm or 6 mm after  
265 hydration. Therefore, dry PU scaffolds with 6 mm or 9 mm diameter were implanted  
266 into the NP defects with 4 mm or 6 mm diameter, respectively. The PU scaffolds with  
267 a flat discoid structure were delivered into the NP cavity with forceps, and positioned  
268 parallel to the endplate of the IVD. After implantation of the dry PU scaffold, the  
269 removed endplate stopper was immediately re-inserted, and the crack between the  
270 stopper and the remaining endplate was sealed by polymethyl methacrylate (PMMA,  
Vertecem Mixing Kit, Synthes, Teknimed S.A., Bigorre, France) according to  
manufacturer's instruction. For each IVD, approximately 100–150 mg of PMMA was  
applied.

274 A bioreactor system was used to mimic physiologically relevant loading conditions of  
275 cultured IVDs [25]. IVDs were placed in custom-made chambers filled with 2 mL IVD

276 culture medium, and loaded dynamically for 3 hours/day at 0-0.1 MPa, 0.1 Hz. After  
277 dynamic loading IVDs were transferred to 6-well plates with 5 mL IVD culture medium  
278 for free swelling recovery overnight. It has been reported that the diurnal disc height  
279 loss of human lower lumbar discs was 11.1% [26]. Therefore, a 10% diurnal disc  
280 height loss was considered as physiological for organ cultured discs. In our previous  
281 study, this loading protocol applied on bovine caudal IVDs of similar age has shown  
282 to be physiologically relevant, as indicated by disc height loss and recovery at  
283 physiological level [27].

284

285 3. IVD organ culture - mechanical effect of PU scaffold implantation after 1 day of  
286 loading

287 3.1. Experimental groups

288 To investigate the mechanical repair effect of the PU scaffold, dynamic load was  
289 performed on partial nucleotomized IVDs implanted with PU scaffold (Scaffold group)  
290 for 3 hours. Empty IVDs served as negative controls (Empty group). The experiments  
291 were performed using IVDs from 6 bovine tails, with 2 IVDs from each tail randomly  
292 assigned to one of the two experimental groups.

293

294 3.2. Dynamic compressive stiffness modulus

295 Dynamic compressive stiffness modulus was measured for each IVD at the following  
296 time points: after dissection and overnight free swelling culture (intact), after partial  
297 nucleotomy (defect), after refilling with the PU scaffold and free swelling culture  
298 overnight (refilled), and after dynamic load and free swelling recovery. Measurement  
299 was performed using a custom-made chamber and a Bose 3220 instrument (Bose,  
300 MN, USA) as described previously [22]. IVDs were pre-loaded with 10% strain (based

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301 on initial disc height after dissection) for 3 min, and then loaded with 10 cycles of  
302 sinusoidal compression at 5-15% of strain (based on initial disc height after  
303 dissection) at a frequency of 0.1 Hz for 100 seconds. Dynamic compressive stiffness  
304 modulus was calculated for each loading cycle as the stress segment divided by the  
305 strain segment (10%). Average value of 10 cycles was taken for each IVD at each  
306 time point. The stiffness modulus was then normalized to the value of respective  
307 intact IVD.

### 309 3.3. Disc height change

310 Disc height was measured with a caliper at the following time points: after dissection,  
311 after dissection and free swelling culture overnight (intact), after partial nucleotomy  
312 (defect), after refilling with biomaterial and free swelling culture overnight (refilled),  
313 after dynamic load, and after free swelling recovery. Each IVD was measured at four  
314 positions and the average value was used to calculate the percentage of disc height  
315 change. Disc height change was normalized to the initial dimension after dissection.

## 317 4. IVD organ culture – mechanical and biological effects of PU scaffold 318 implantation after 14 days of loading

### 319 4.1. Experimental Groups

320 The biological repair effect of the PU scaffold was investigated after 14 days of  
321 dynamic load. Empty IVDs served as negative controls. The experiments were  
322 performed using bovine IVDs from 12 tails, with 2 IVDs from each tail randomly  
323 assigned to one of the two experiment groups.

325 4.2. Disc height change

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3 326 The disc height was measured with a caliper at the following time points: day 0 after  
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5 327 dissection, day 1 after 1st load, day 2 after 1st recovery, day 7 after 7th load, day 8  
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7 328 after 7th recovery, day 14 after 14th load and day 15 after 14th recovery. Each IVD  
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9  
10 329 was measured at four positions and the average value was used to calculate the  
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12 330 percentage of disc height change. Disc height change was normalized to the initial  
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15 331 dimension after dissection.

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21 333 4.3. Biochemical and gene expression analysis

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24 334 After free swelling recovery on day 15 cartilaginous endplates of each IVD were  
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26 335 removed and remaining NP and AF tissues were harvested by using a biopsy punch  
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28 336 and a scalpel blade. Approximately 100 mg of each AF and NP tissue was used for  
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31 337 RNA extraction; 50 mg of each AF and NP tissue was digested within 0.5 mg/mL  
32  
33 338 proteinase K at 56°C overnight for glycosaminoglycan (GAG), collagen and DNA  
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36 339 content measurement; 30 mg of NP tissue was used for [<sup>35</sup>S] sulfate incorporation  
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38 340 assay to assess proteoglycan synthesis rate.

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41 341 For RNA extraction, tissue samples were flash frozen and pulverized in liquid  
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43 342 nitrogen and homogenized using a TissueLyser (Qiagen, Venlo, Netherlands). Total  
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46 343 RNA was extracted with TRI Reagent (Molecular Research Center) and reverse  
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48 344 transcription was performed with SuperScript VILO cDNA Synthesis Kit (Life  
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51 345 Technologies, Carlsbad, CA). Quantitative real-time PCR was performed using the  
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53 346 Step-One-Plus instrument (Life Technologies). The sequences of custom designed  
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56 347 bovine primers and TaqMan™ probes are shown in Table 2. For amplification of 18S  
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58 348 ribosomal RNA (18S, 4310893E), Versican (Bt03217632\_m1), and Biglycan (BGN,  
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61 349 Bt03244532\_m1), gene expression assays from Applied Biosystems (Life

350 Technologies) were used. Comparative Ct method was performed for relative  
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2 351 quantification of target mRNA with 18S ribosomal RNA as endogenous control.  
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5 352 The GAG content in the NP and AF tissues was determined by using the 1,9-  
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7 353 dimethylmethylene blue dye (DMMB) method [28]. Quantification of the total amount  
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10 354 of collagen in NP and AF samples was performed using the hydroxyproline assay as  
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12 355 described earlier [29]. DNA content was measured spectrofluorometrically using  
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15 356 Hoechst (33258) dye.

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18 357 To determine the proteoglycan synthesis rate NP tissue was incubated for 16-20  
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20 358 hours in IVD culture media supplemented with 2.5  $\mu\text{Ci}$   $^{35}\text{S}$ -sulfate /mL ( $\text{Na}_2$   $^{35}\text{SO}_4$ ;  
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22 359 PerkinElmer, Schwerzenbach, Switzerland). Thereafter NP tissue was digested with  
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25 360 0.5 mg/mL proteinase K at 56°C overnight. Conditioned medium and proteinase K  
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27 361 digested tissue samples were processed with PD-10 desalting columns (GE  
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30 362 Healthcare, Glattbrugg, Switzerland) and radioactive counts were measured by  
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32 363 Wallac 1414 Liquid Scintillation Counter (PerkinElmer, Schwerzenbach, Switzerland).  
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35 364 The counts per minute (cpm) were normalized to the DNA content of the respective  
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37 365 samples.  
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#### 43 367 4.4. Safranin O/Fast Green staining

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46 368 After free swelling recovery on day 15, whole IVDs with implanted PU scaffolds were  
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49 369 fixed in 70% methanol and transferred into PBS with 5% sucrose at 4°C overnight  
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51 370 before cryosectioning. Transverse sections of IVDs were cut at a thickness of 12  $\mu\text{m}$ .  
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53 371 Sections were stained with 0.1% Safranin-O and 0.02% Fast Green to reveal  
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56 372 proteoglycan and collagen deposition respectively, and counterstained with Weigert's  
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58 373 Haematoxylin to reveal cell distribution.  
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#### 4.5. Fourier transform infrared (FTIR) spectroscopic imaging

After free swelling recovery on day 15, a set of IVDs (n=1 per group) were decalcified and subsequently embedded in paraffin. Four micrometre sagittal IVD sections were cut and mounted on custom made reflective 316 stainless steel slides for infrared analysis. FTIR microscopic images were collected using an Agilent 680-IR FTIR spectrometer (ISys50®, Malvern Instruments Limited, Worcestershire, UK) coupled to a FTIR imaging microscope fitted with a liquid nitrogen cooled 64 x 64 mercury-cadmium-telluride focal plane array detector (FPA) and an automated sampling stage. For each sample, an area of 71 x 192 pixels (approximately 6.3 x 16.8 mm) was mapped in transfectance mode. Spectra were attained with a spectral resolution of 4 cm<sup>-1</sup> over a wavenumber range of 950-1900 cm<sup>-1</sup>. Data was pre-processed by performing a second derivative (Savitsky-Golay ISys50 software, Filter order 3, filter length 15) on the spectra to reduce baseline effects and assist the resolution of weaker absorption peaks [30]. Pixels within the tissue section where spectra indicated only the presence of paraffin or the substrate were identified and masked using spectral statistics (ISys50, histogram). Regions of interest (ROI; 10 x 192 pixels) across the middle of each sagittal section were cut and compiled to one data matrix. The data set was analyzed using a multivariate curve resolution-alternating least squares algorithm (MCR-ALS) described by Andrew and Hancewicz [31] and Wang et.al. [32]. MCR-ALS was carried out using the MCR-ALS v1.6 software (MCRv1.6 Copyright © 2003-2004 Unilever) on two wavenumber regions of 950-1600 cm<sup>-1</sup> and 950-1300 cm<sup>-1</sup> with the following settings; initial estimates: 4 -10F, NIPALS, ALS; ALS constraints: MALS-2D, none, 1e-005, 500. General tissue, collagen type I, collagen type II and proteoglycan distributions were estimated according to previous studies that have shown good agreement between (immuno-) histological staining and the integrated peak area of the 2nd derivative of the amide III spectral region

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401 (1186-1297  $\text{cm}^{-1}$ ) as well as MCR-ALS score maps [33]. Additionally, average matrix  
402 component per tissue distribution across ROIs were calculated and are represented  
403 as estimated component per tissue distribution line profiles across the normalized  
404 width (0-100%) of each IVD.

## 405 406 5. Statistical analysis

407 SPSS 21.0 statistical software was used for statistical analysis. One sample  
408 Kolmogorov–Smirnov test was used to define whether the data were normally  
409 distributed (normal distribution at  $p > 0.1$ ). For data that were normally distributed,  
410 unpaired T test was used to determine differences between Empty and Scaffold  
411 groups. For data that were not normally distributed, Mann-Whitney U was used to  
412 determine differences between Empty and Scaffold groups. A p-value  $<0.05$  was  
413 considered statistically significant.

## 414 415 **Results**

### 416 1. Characterization of PU scaffold

#### 417 1.1. Structure characterization of PU scaffold envelope

418 Randomly arranged, homogenous fibers could be observed in the SEM micrographs  
419 of electrospun PU scaffold envelopes, indicating a successful mixing and  
420 electrospinning of the Chronoflex and Hydromed mixture (Fig. 2). The generated  
421 fibers had an average thickness of  $0.84 \pm 0.52 \mu\text{m}$ .

#### 422 423 1.2. Swelling capacity and kinetics of PU scaffolds

424 Swelling capacity of the scaffold (14 mm diameter) at various core contents is shown  
425 in Fig. 3. At the core content range tested, the swelling ratio of the scaffold increased

426 with the increase in core content. Swelling capacity of the different scaffold sizes and  
427 core amounts is summarized in Table 3. At the range tested, the core material  
428 reached similar swelling percentage (~800%) regardless of scaffold size. The  
429 scaffold weight gain increased with the increase in size.

430 Swelling kinetics is shown in Fig. 4. The swelling rate was relatively fast after  
431 immersion in aqueous medium. In the case of the 14 mm scaffold, the scaffold  
432 swelling was about 500% of initial scaffold weight (~70-90% of maximum swelling)  
433 within one hour (Fig. 4 A). In the case of the 6 mm scaffold, the scaffold swelling, at  
434 all core contents tested, reached about 400% of initial scaffold weight (~90% of  
435 maximum swelling) already within one hour (Fig. 4 B). In all scaffolds tested, swelling  
436 in free environment reached more than 90% of maximum capacity within 8 hours.

437

### 438 1.3. Cytocompatibility of PU scaffold component material

439 Viable cell number after culture in the conditioned medium from both core and  
440 envelope material was >85% of control culture medium after up to 72 hours of culture  
441 for NPCs and MSCs (Fig. 5 A and B). Conditioned medium from core material  
442 supported MSC proliferation (viable cell number was above 160-180% of positive  
443 control after 72 hours culture), but did not affect NPC proliferation (viable cell number  
444 was 85-90% of positive control after 72 hours culture).

445 DNA content of cell-envelope constructs after 1 day of culture showed that 88% of  
446 the seeded cells were attached to the envelope 24 hours after seeding, suggesting  
447 high cell attachment ratio. Compared with day 1, the DNA content of samples  
448 increased 4-fold after 7 days of culture, indicating active NPC proliferation on the  
449 envelope (data not shown). Representative SEM images also showed that cells

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2 450 seeded on the top side of the envelope had proliferated and produced extracellular  
3 451 matrix after 7 days of culture (Fig. 5 C).  
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8 453 2. IVD organ culture - mechanical effect of PU scaffold in nucleotomized IVD  
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10 454 after 1 day of dynamic load  
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13 455 The implanted PU scaffold was able to entrap water and swell *in situ* (Fig. 6 A), as  
14 456 indicated by weight increase of  $301 \pm 33\%$  and thickness increase of  $103 \pm 15\%$  already  
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16 457 after 24 h culture in the IVD.  
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21 458 After nucleotomy, the dynamic compressive stiffness modulus dropped to 30%  
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23 459 compared with intact IVDs. After refilling the NP cavity with PU scaffold and free  
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25 460 swelling culture overnight, the disc stiffness modulus was restored to  $73 \pm 21\%$   
26  
27 461 ( $p=0.073$  vs. empty control). After dynamic load and free swelling recovery the  
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29 462 stiffness modulus of PU scaffold refilled IVDs further increased to  $82 \pm 14\%$  ( $p<0.05$  vs.  
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31 463 empty control) (Fig. 6 B). Representative stress/strain curves of the empty control  
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33 464 and PU scaffold refilled IVDs are shown in Figure 6 D and E respectively.  
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39 465 Nucleotomy caused 3% decrease in disc height compared to intact IVDs. After  
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41 466 dynamic load, IVDs implanted with PU scaffold maintained their disc height, while the  
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43 467 disc height of empty controls further dropped by 7% ( $p<0.001$  vs. implant group).  
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45 468 After free swelling recovery overnight, the disc height of the implant group was  
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47 469 restored to the level of the intact IVDs ( $p<0.001$  vs. empty control) (Fig. 6 C).  
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54 471 3. IVD organ culture – mechanical and biological effect of PU scaffold in  
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56 472 nucleotomized IVD after 14 days of dynamic load  
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59 473 3.1. Disc height change  
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474 After the 1<sup>st</sup> cycle of dynamic load, a disc height loss of  $-9.8 \pm 2.3$  % was noticed in  
475 partially nucleotomized IVDs compared with initial dimension of respective intact  
476 IVDs after dissection (Fig. 7 A; Empty Day 1). Implantation of PU scaffold restored  
477 disc height to  $-2.1 \pm 0.5$  % (Fig. 7 A; Scaffold Day 1;  $p < 0.05$  vs. Empty). After free  
478 swelling recovery, all the IVDs swelled and recovered to the height before load. The  
479 differences between partially nucleotomized IVDs and PU scaffold implanted IVDs  
480 were maintained. This diurnal disc height change pattern was observed during the  
481 entire period of 14 days repetitive dynamic load (Fig. 7 A).

482

### 483 3.2. Gene expression

484 After 14 days of culture with dynamic load, gene expression levels of NP and AF  
485 tissues were compared to the expression levels of disc tissues from respective  
486 bovine tails before starting organ culture on day 0 (Fig. 7 B and C). Compared with  
487 day 0, gene expression of IVD extracellular matrix components ACAN, COL2A1,  
488 COL1A2, BGN and Versican in partially nucleotomized IVDs (Empty) did not change  
489 significantly. Implantation of PU scaffold into the partially nucleotomized IVDs  
490 (Scaffold) also did not affect the expression level of these genes. Compared with  
491 healthy tissue of intact IVDs on day 0, gene expression of MMP13 in NP and AF  
492 tissues of partially nucleotomized IVDs increased 24-fold and 134-fold respectively  
493 after 14 days of culture with dynamic load. Implantation of PU scaffold into partially  
494 nucleotomized IVDs down-regulated the MMP13 gene expression in NP and AF by 8-  
495 fold and 7-fold, respectively compared with Empty group.

496

### 497 3.3. Biochemical analysis of disc tissue

498 GAG content, collagen content and proteoglycan synthesis rate per cell of native NP  
499 and AF tissues after 14 days of culture are shown in Table 4. In the remaining NP  
500 tissue after partial nucleotomy, GAG, collagen content and proteoglycan synthesis  
501 rate were not affected by implantation of the PU scaffold. In the AF tissue, neither  
502 GAG content nor collagen content per cell was affected by PU scaffold implantation.

503

### 3.4. Histological analysis of whole IVDs

505 Images of Safranin O/Fast Green stained sections of IVDs cultured for 14 days are  
506 shown in Fig. 8. The overview images (Fig. 8 A, C) clearly show that a partial  
507 nucleotomy was performed in the organ culture model. The swollen PU scaffold  
508 completely filled the nucleotomized region (Fig. 8 C). The envelope of the PU scaffold  
509 was observed as a ring in direct contact with the remaining disc NP tissue (Fig. 8 D).  
510 The remaining native disc NP tissue is still intensely stained with Safranin O after 14  
511 days of dynamic load.

512

### 3.5. FTIR imaging analysis of discs tissue

514 An example of an image reflecting the tissue distribution (sagittal direction) of a  
515 partially nucleotomized IVD cultured for 14 days under dynamic load without PU  
516 scaffold implantation is shown in Fig. 9 A. Tissue distribution maps of ROI of partially  
517 nucleotomized IVDs without (top) and with (bottom) PU scaffold implantation cultured  
518 for 14 days under dynamic load are shown in Fig. 9 B. The ROI tissue image of the  
519 empty IVD shows fissures caused by the nucleotomy and native tissue across the  
520 whole width of the IVD. The ROI tissue image of the PU implanted IVD shows a  
521 cavity in the center of the IVD where the implant was situated, surrounded by native  
522 disc tissue. Estimated component / tissue distribution line profiles of proteoglycan,

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523 collagen type II and collagen type I across the IVD sections show distinctive trends,  
524 with proteoglycan and collagen type II profiles showing an overall increase and  
525 collagen type I profiles showing a decrease towards the NP region (Fig. 9 C). A  
526 comparison of the line profiles between the empty and PU scaffold implanted IVD  
527 shows very similar overall trends for proteoglycan, collagen type II and collagen type  
528 I. However, both proteoglycan and collagen type II show localized higher component  
529 to tissue ratios (sharp positive features) at around 25 %, 30 % and 67 % IVD width in  
530 the PU implanted IVDs. Collagen type I line profiles show localized lower component  
531 to tissue ratios (sharp negative features) at around the same areas (25 %, 30 % and  
532 67 % IVD width) in the PU implanted IVDs.

## 533 534 **Discussion**

535 The aim of this study was to develop a nucleus pulposus replacement material, which  
536 is feasible for non-invasive delivery and custom-made design, restores the  
537 mechanical property of nucleotomized IVDs and sustains cytocompatibility at the  
538 same time. The bi-phasic PU scaffold developed in this work has a flat discoid shape  
539 after manufacturing (Fig. 1 A front) and will swell after insertion into the IVD. Swelling  
540 rate is important for scaffold development and implementation, and enables the  
541 selection of the optimal core content. The PU scaffolds showed favorable *in vitro*  
542 swelling capability (Fig. 3 and Table 2) with a fast swelling rate (Fig. 4) post-hydration.  
543 Based on these results the extent of swelling could be tailored to the desired external  
544 scaffold dimensions. Hence a custom-made design is feasible depending on the  
545 individual geometry of NP material to be replaced. Implantation of the PU scaffolds  
546 into nucleotomized IVDs further confirmed the swelling capability of the PU scaffolds  
547 *in situ*. The implanted PU scaffolds expanded anisotropically to fill the nucleotomy

1 548 defect (Fig. 6 A and Fig. 8 C). In addition, stress relaxation tests revealed higher toe  
2 549 and linear modulus for these PU scaffolds than for human NP and no significant  
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4 550 difference in percentage relaxation between the PU scaffold and human NP, further  
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7 551 indicating the suitability of the material for NP replacement (unpublished results from  
8  
9 552 consortium partner).

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12 553 The cytocompatibility of the PU scaffold was assessed with NP cells and bone  
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14 554 marrow derived MSCs. Viable cell numbers after culturing in the conditioned medium  
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17 555 from the core or the envelope material were higher than 85% of cell numbers after  
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20 556 culture in control medium (Fig. 5 A and B). In addition, NP cells were seeded on top  
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22 557 of the outer envelope material, and presented effective adhesion after 1 day and  
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25 558 active cell proliferation and extracellular matrix synthesis after 7 days (Fig. 5 C).  
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27 559 These results indicate that the cytocompatibility of the PU scaffolds is advantageous  
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30 560 with respect to both NP cells and MSCs. Another interesting finding is that the  
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32 561 conditioned medium of the core material stimulated MSC growth compared to the  
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35 562 control medium. The underlying reason of this observation should be further  
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37 563 examined in future studies.

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40 564 The main challenge in developing an NP replacement material is to immediately  
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42 565 restore the mechanical property in a nucleotomized IVD. Emerging numbers of NP  
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45 566 replacement devices/biomaterials have been tested for their mechanical restoration  
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47 567 effect in nucleotomized IVDs [9, 34-37]. However, all the materials were tested in *ex*  
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50 568 *vivo* or *in vivo* nucleotomy models with an incision through the AF. Reitmaier *et al.*  
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52 569 compared two annulus closure methods after NP replacement with the native NP  
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55 570 tissue removed during nucleotomy. They found that the disc height loss and disc  
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57 571 stiffness differ between the two conditions. This demonstrates that the preservation of  
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59 572 the disc's mechanical function following NP replacement is partially dependent on the  
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573 quality of the closure of the annulus defect [38]. This may also explain some failures  
574 of NP replacement materials in the case of dislocation or protrusion through the AF  
575 incision. To assess the mechanical repair effect of the NP replacement material  
576 solely, an alternative nucleotomy approach in organ culture was used in this study,  
577 whereby an incision was made through the endplate while preserving an intact  
578 annulus (Fig. 6 A right) [22]. This model is highly useful for evaluation and screening  
579 NP replacement materials in whole organ culture under load before pre-clinical *in*  
580 *vivo* testing.

581 In the current study, partial nucleotomy through the endplate in the organ cultured  
582 IVD substantially altered the mechanical property of the IVD, as indicated by a 70%  
583 reduction in the axial stiffness (Fig. 6 B). Implantation of the PU scaffold significantly  
584 restored the axial stiffness and disc height of the nucleotomized IVD after cyclic  
585 dynamic load and recovery culture (Fig. 6 B and C). The restoration in axial stiffness  
586 and disc height occurred relatively fast, already after the first cycle of 3 hours load,  
587 which indicates an immediate repair effect. Furthermore, the disc height restoration  
588 was maintained through 14 days of repetitive dynamic load and recovery culture (Fig.  
589 7 A), which indicates that the PU scaffolds implanted *in situ* are able to restore the  
590 mechanical property of a nucleotomized IVD in organ culture. In our previous study  
591 [22], a fibrin-hyaluronan hydrogel was delivered into nucleotomized IVD and the  
592 mechanical repair function was assessed with the same method. It was found that  
593 the fibrin-hyaluronan hydrogel restored the disc height after dynamic loading, but not  
594 the dynamic compressive stiffness of the nucleotomized IVD. These results  
595 demonstrate that the PU scaffolds developed in this study are superiorly able to  
596 restore the mechanical function of the nucleotomized IVD compared to a biomimetic  
597 hydrogel. In the current study, the mechanical characterization was focused on the  
598 compressive modulus and the disc height change. The assessment of the

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5 601 viscoelastic parameters and the neutral zone of the IVD before and after scaffold  
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8 600 implantation will also be of interest for us in future studies to explore the effects of the  
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13 601 scaffolds in more detail.

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602 Another advantage of our organ culture model is that the IVDs can be maintained  
603 viable under dynamic load in a bioreactor for several weeks [25], after which the  
604 biological property of the nucleotomized IVDs with/without NP replacement can be  
605 assessed. In this study, after 14 days culture with dynamic load, we investigated the  
606 phenotype of the disc cells, the extracellular matrix components, and the structure of  
607 the disc tissue in nucleotomized IVDs with/without implanted PU scaffold. In  
608 nucleotomized IVDs without NP replacement, the MMP13 gene expression level of  
609 the cells in the AF tissue and remaining NP tissue increased dramatically after 14  
610 days of culture (Fig. 7 B and C), which demonstrates an increase in catabolic signals  
611 in the disc tissue. MMP13 is known to be overexpressed in cartilage from patients  
612 with osteoarthritis compared with healthy cartilage [39]. The expression and activity  
613 of MMP13 are also increased during disc degeneration [40]. This indicates that a IVD  
614 after partial nucleotomy is prone to a degenerative pathology in the remaining native  
615 disc tissue. Implantation of the PU scaffold into the nucleotomized IVD markedly  
616 down-regulated the MMP13 expression in the NP and AF tissues (Fig. 7 B and C).  
617 This may slow down further degeneration of the native disc tissue compared with the  
618 unrepaired IVD.

619 The content and distribution of proteoglycan and collagen in the disc tissue were  
620 analyzed by biochemical measurement, Safranin O/Fast Green staining, and FTIR on  
621 disc sections. The disc matrix composition was not changed significantly in the NP  
622 and AF regions of the IVDs in the presence of the PU scaffold. However, in the native  
623 NP tissue surrounding the implanted PU scaffold, proteoglycan and type II collagen

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624 showed localized higher expression, while type I collagen showed localized lower  
625 expression compared with the nucleotomized IVD without repair (Fig. 9 C). This  
626 indicates that the implanted PU scaffold is beneficial in maintaining the NP cell  
627 phenotype in the surrounded disc tissue.

628 The underlying reason for the above mentioned biological changes is likely to result  
629 from the restoration in the geometry and load pressure on the remaining native disc  
630 tissue. In the nucleotomized IVD without repair, the remaining disc tissue swells into  
631 the nucleotomized region. Therefore, the tissue is less compact and the pressure on  
632 the tissue is disturbed. The implantation of the PU scaffold decreased the native  
633 tissue volume in the disc space by occupying the NP cavity, which is likely to lead to  
634 a higher local intensity of proteoglycan and type II collagen. Furthermore, the  
635 implanted PU scaffold restored the pressure in the nucleotomized region and the  
636 stiffness of the IVD, therefore the remaining disc tissue experienced higher load  
637 pressure compared with the tissue in the non-repaired IVD. Our previous study has  
638 shown that dynamic load down-regulated the MMP13 gene expression level in AF  
639 tissue and COL1 gene expression level in NP tissue from organ cultured IVD  
640 compared with free swelling condition [27]. Other *ex vivo* studies also revealed that  
641 dynamic load preserves disc cell phenotype compared to unloaded or static load  
642 condition [41, 42]. Taken together, these data corroborate that mechanical pressure  
643 is essential to maintain the function and homeostasis of the disc tissue. The PU  
644 scaffold developed in this study, as an NP replacement material, can rebalance the  
645 pressure in the nucleotomized IVD and has the potential to prevent further  
646 degeneration of the remaining disc tissue.

647 The dry PU scaffolds can be rolled into a tube and delivered through a needle with  
648 insertion guide (Fig. 1 C). Such injectable design allows less invasive delivery

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649 compared to the mechanical NP replacement devices, including NUBAC™ [43],  
650 REGAIN™ [34], and BUCK™ [9]. Furthermore, a transpedicular approach has  
651 recently been developed to deliver therapeutic factors to the IVD without further  
652 damage to the AF [44, 45]. With this approach, our PU scaffolds could be delivered  
653 through the pedicle and the endplate to the NP while preserving the mechanical  
654 function of AF in cases of early disc degeneration with an intact annulus. In case of  
655 patients with AF defects, proper AF closure approach is needed to prevent herniation  
656 of the implanted NP replacement material. Suture [46] and glue [47, 48] may be  
657 sufficient to close small annular defects. For large annular failure, tissue engineered  
658 material mimicking native AF tissue may be needed to fully restore the mechanical  
659 property of the AF [27]. Regardless of mechanical properties, the nucleotomy  
660 approach through the endplate might also induce a biological degenerative cascade  
661 according to literature data. Damage of the EP by drilling has been shown to  
662 decrease the water content in the outer AF and proteoglycan content in the NP [49]  
663 and may trigger Schmorl's nodes [45]. Further investigation to optimize the  
664 transpedicular approach is essential to fully reach the requirements of a safe, non-  
665 invasive, and functional NP replacement therapy.

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666 Limitations of the current study remain in the following aspects: 1) the PU scaffolds  
667 were assessed in nucleotomized IVD under healthy conditions; hence exposure to  
668 the pathological environment occurring in a degenerative IVD is lacking; 2) the *ex*  
669 *vivo* organ culture model differs from *in vivo* conditions where additional systemic  
670 responses may arise. More research work will therefore be necessary to assess the  
671 effects of the material before clinical translation can be considered. Whole organ  
672 culture models mimicking IVD degeneration through enzymatic treatment of the NP  
673 [50, 51], high frequency mechanical loading [52], or addition of pro-inflammatory  
674 cytokines [53] may be used to evaluate the material under pathological conditions.

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2 675 Ultimately, *in vivo* large animal models would provide further insight into the delivery,  
3 676 stability and effect of the PU scaffolds for NP replacement at longer term after  
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5 677 implantation.  
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11 679 **Conclusions**  
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13 680 In this study, an injectable and cytocompatible bi-phasic PU scaffold was developed  
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15 681 for NP replacement. The implant was assessed in whole organ culture under  
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18 682 dynamic load for its mechanical and biological repair effect in nucleotomized IVD.  
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21 683 The nucleotomy model through the endplate enabled pure assessment of the PU  
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23 684 scaffold, independent of the closure method for an annulus incision. The designed  
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25 685 PU scaffold with swelling capacity *in situ* is able to restore the mechanical property of  
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28 686 nucleotomized IVDs, and shows potential to retard further degeneration of native disc  
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31 687 tissue. This work provides a new approach to facilitate future progress in the  
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33 688 development of an NP replacement material, and the screening of materials using  
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35 689 whole organ culture. The bi-phasic PU scaffold developed in the current study  
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38 690 warrants further pre-clinical investigation to evaluate its potential for functional, non-  
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40 691 invasive and custom designed NP replacement.  
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46 693 **Acknowledgement**  
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**Table 1.** The effect of Chronoflex™ (CF) : Hydromed™ (HM) w/w ratio on the function of the envelope material.

Experimental Group	CF:HM w/w ratio	Tensile Strength (N/mm <sup>2</sup> )	Wetting time (minutes)
1	1:0	5.32	300
2	15:1	5.31	210
3	12:1	4.80	30
4	10:1	4.71	18
5	9:1	4.65	5
6	8:1	4.28	5
7	6:1	4.1	2
8	4:1	3.7	1

**Table 2.** Oligonucleotide primers and probes (bovine) used for real-time PCR.

Gene	Primer/probe type	Sequence
COL1A2	Primer fw (5'-3')	TGC AGT AAC TTC GTG CCT AGC A
	Primer rev (5'-3')	CGC GTG GTC CTC TAT CTC CA
	Probe (5'FAM/3'TAMRA)	CAT GCC AAT CCT TAC AAG AGG CAA CTG C
COL2A1	Primer fw (5'-3')	AAG AAA CAC ATC TGG TTT GGA GAA A
	Primer rev (5'-3')	TGG GAG CCA GGT TGT CAT C
	Probe (5'FAM/3'TAMRA)	CAA CGG TGG CTT CCA CTT CAG CTA TGG
ACAN	Primer fw (5'-3')	CCA ACG AAA CCT ATG ACG TGT ACT
	Primer rev (5'-3')	GCA CTC GTT GGC TGC CTC
	Probe (5'FAM/3'TAMRA)	ATG TTG CAT AGA AGA CCT CGC CCT CCA T
MMP13	Primer fw (5'-3')	CCA TCT ACA CCT ACA CTG GCA AAA
	Primer rev (5'-3')	GTC TGG CGT TTT GGG ATG TT
	Probe (5'FAM/3'TAMRA)	TCT CTC TAT GGT CCA GGA GAT GAA GAC CCC
ACAN: aggrecan; COL1A2: collagen type I; COL2A1: collagen type II; MMP13: matrix metalloproteinase 13; fw: Forward; rev: Reverse; FAM: Carboxyfluorescein; TAMRA: Tetramethylrhodamine		

**Table 3.** Swelling capacity (at saturation) of PU scaffolds with various sizes in aqueous media, Mean  $\pm$  SD.

Core content (mg)	Scaffold diameter (mm)	Increase in scaffold weight (% of initial)	Increase in core weight (% of initial)
3.4 (n=10)	6	409.96% $\pm$ 21	825.68% $\pm$ 34
4.5 (n=6)		444.49% $\pm$ 25	769.40% $\pm$ 17
12 (n=5)	8	563.07% $\pm$ 26	783.01% $\pm$ 40
12 (n=10)	9	559.22% $\pm$ 18	847.85% $\pm$ 20
40 (n=7)	14	635.40% $\pm$ 13	846.20% $\pm$ 25

**Table 4.** Biochemical analysis of native disc tissue in partially nucleotomized IVDs without/with PU scaffold implantation after 14 days of dynamic load. Total glycosaminoglycan (GAG) content, total collagen content and proteoglycan (PG) synthesis rate were measured and normalized to the DNA content of the respective samples. Empty – partially nucleotomized IVD, Scaffold – partially nucleotomized IVD implanted with PU scaffold. GAG/DNA ratio in  $\mu\text{g} / \mu\text{g}$ ; Collagen/DNA ratio in  $\mu\text{g} / \mu\text{g}$ , PG/DNA in counts per minute (CPM) /  $\mu\text{g}$ ; PG/DNA was performed for NP tissue only. Mean  $\pm$  SD, n=6.

Biochemical analysis of disc tissue		
NP tissue		
Group	Empty	Scaffold
GAG / DNA	1151.6 $\pm$ 224.7	1166.8 $\pm$ 369.1
Collagen / DNA	639.9 $\pm$ 474.3	1017.2 $\pm$ 944.5
PG / DNA	98.4 $\pm$ 45.1	79.1 $\pm$ 50.2
AF tissue		
Group	Empty	Scaffold
GAG / DNA	628.9 $\pm$ 348.1	502.8 $\pm$ 281.2
Collagen / DNA	1070.4 $\pm$ 653.9	1276.8 $\pm$ 640.6



Figure1  
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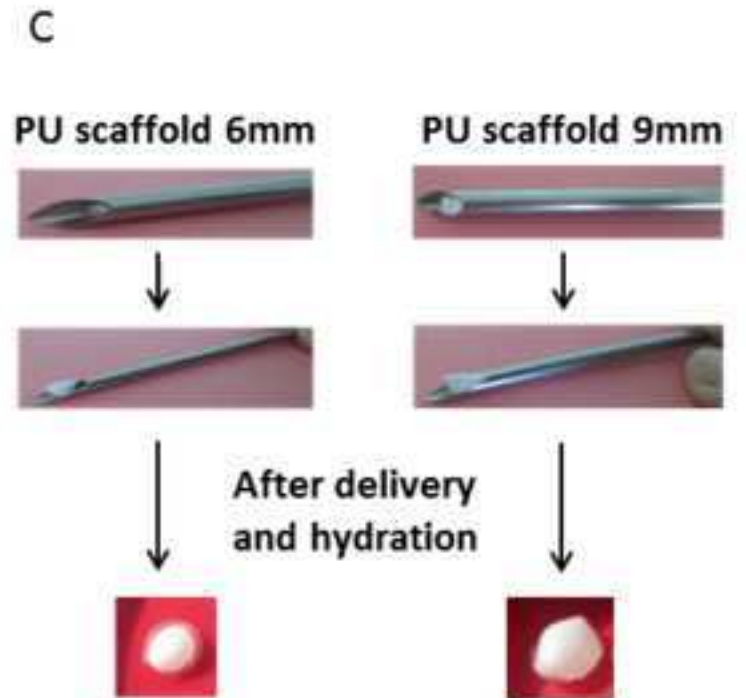
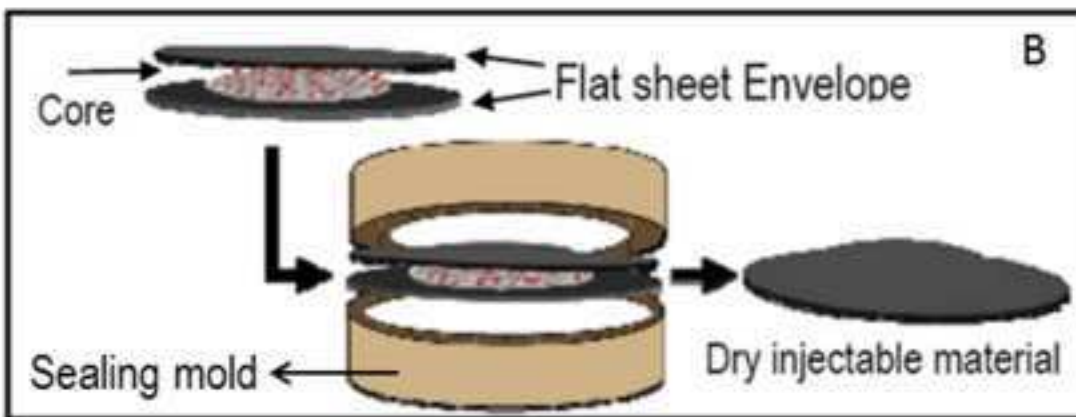
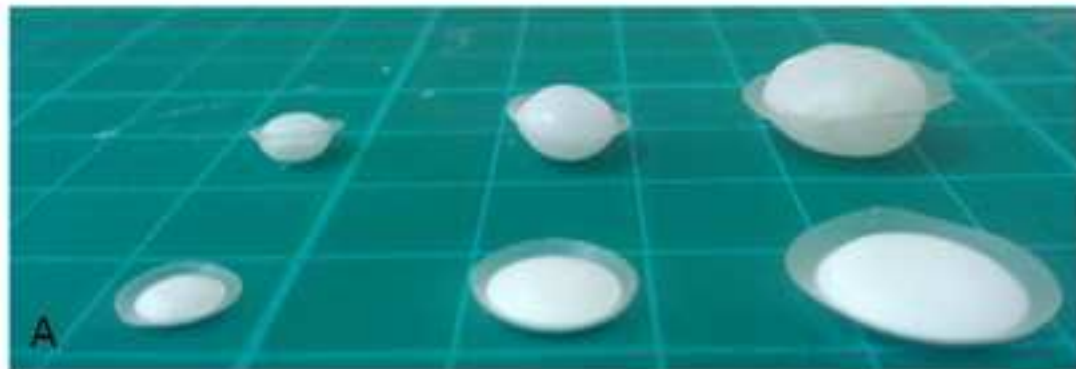


Figure 2  
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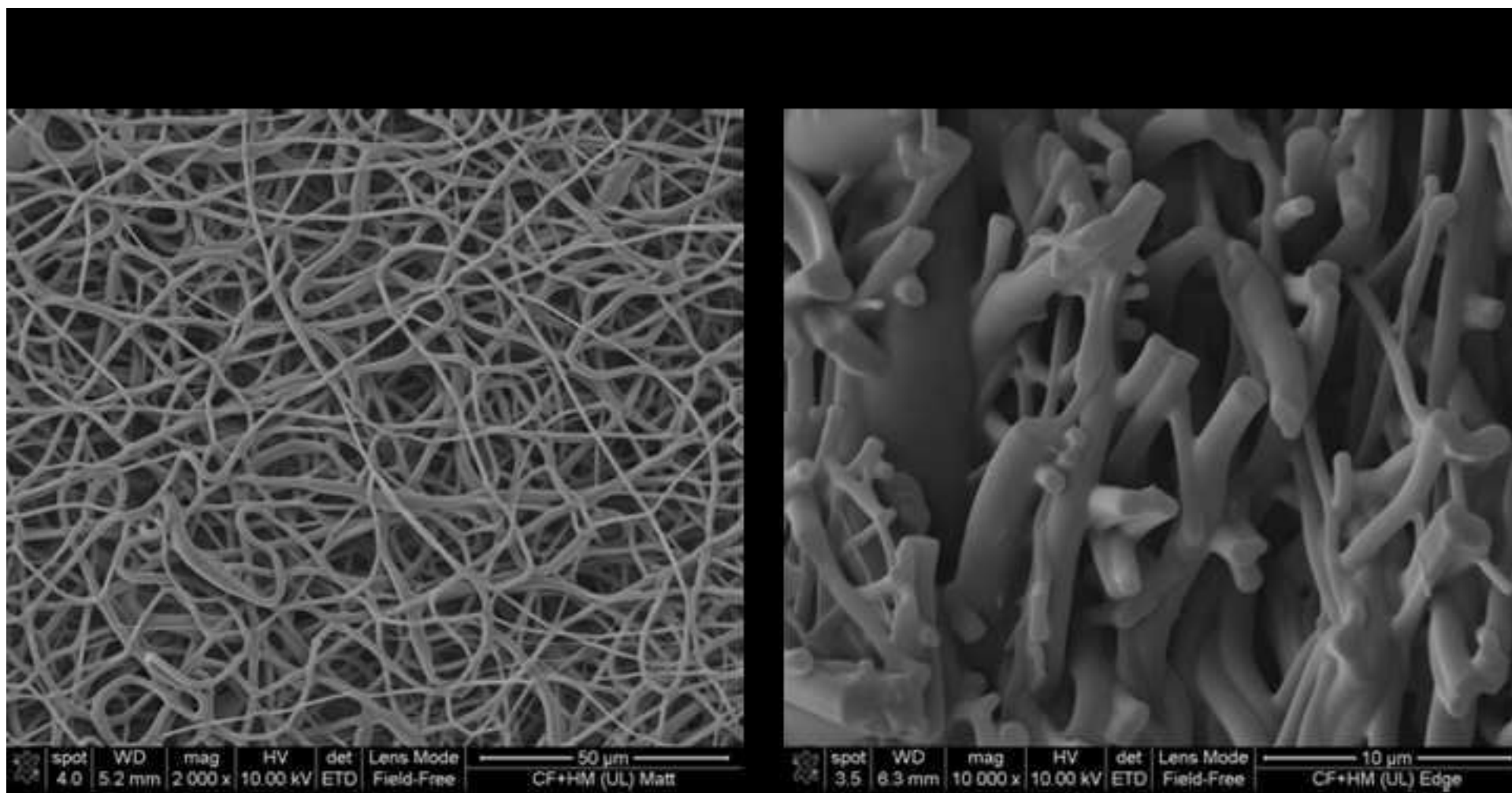


Figure 3  
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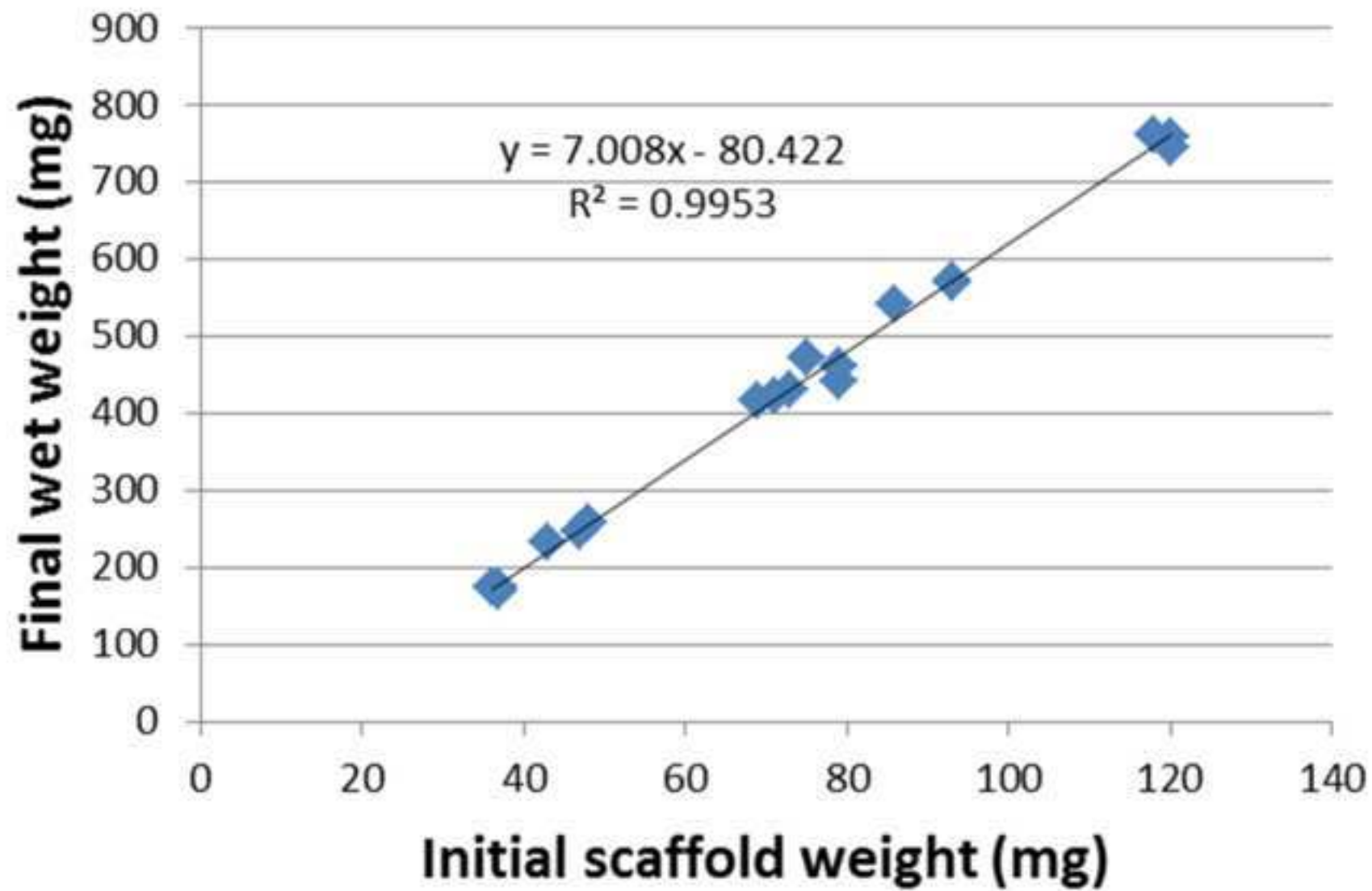


Figure 4

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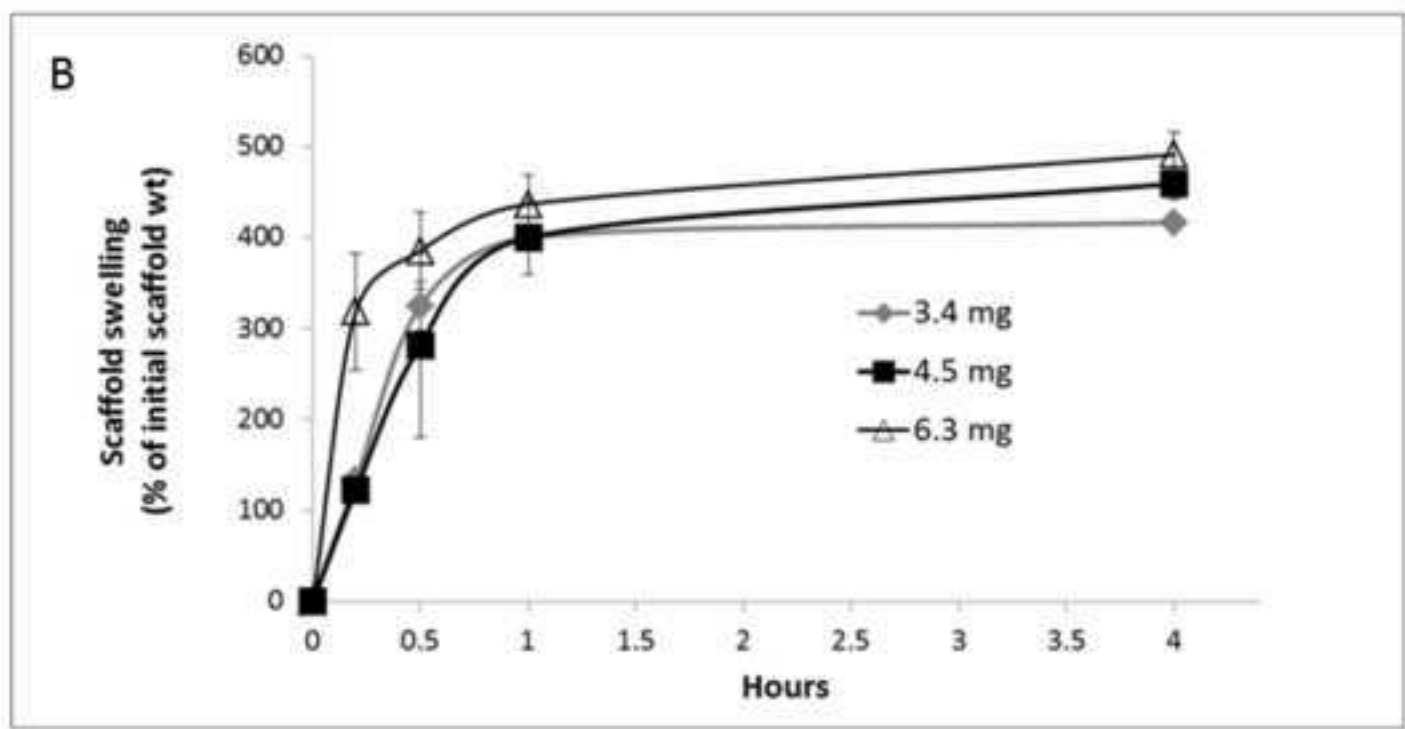
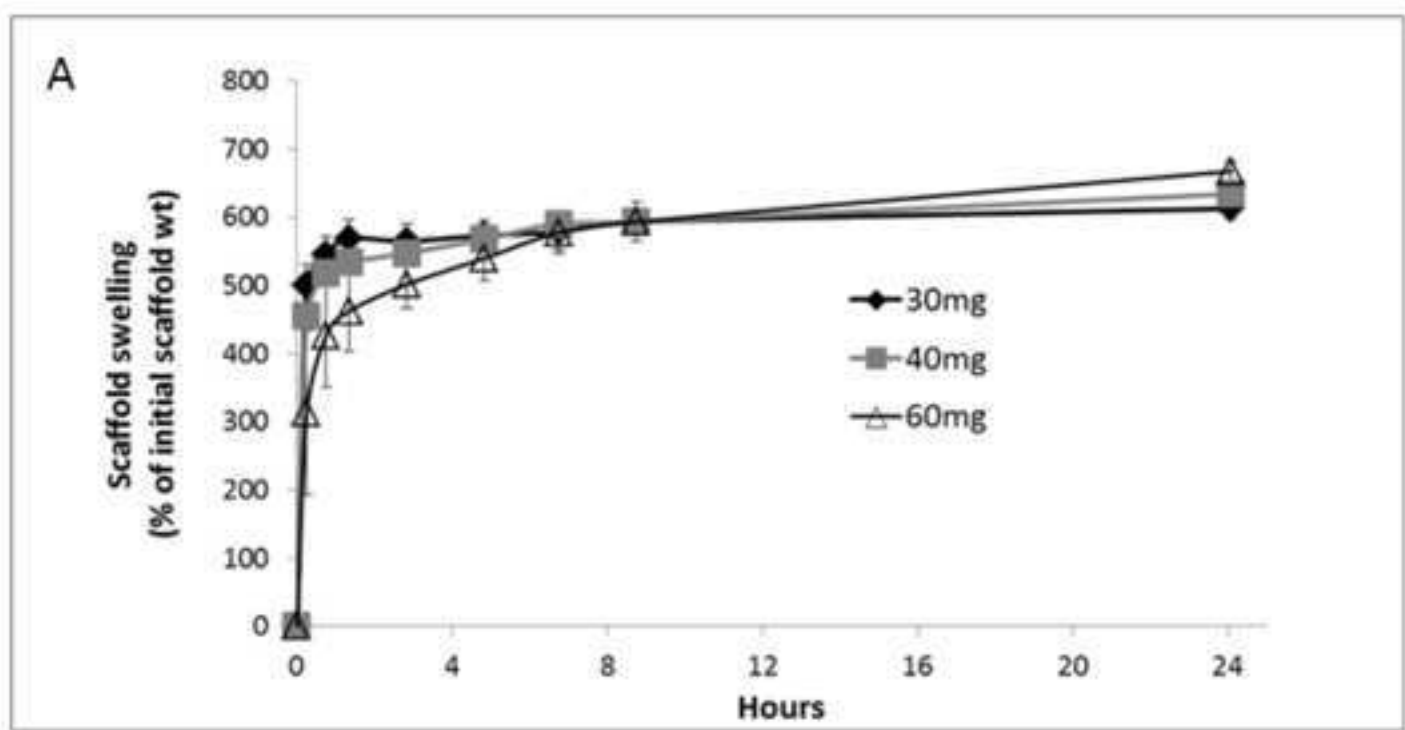
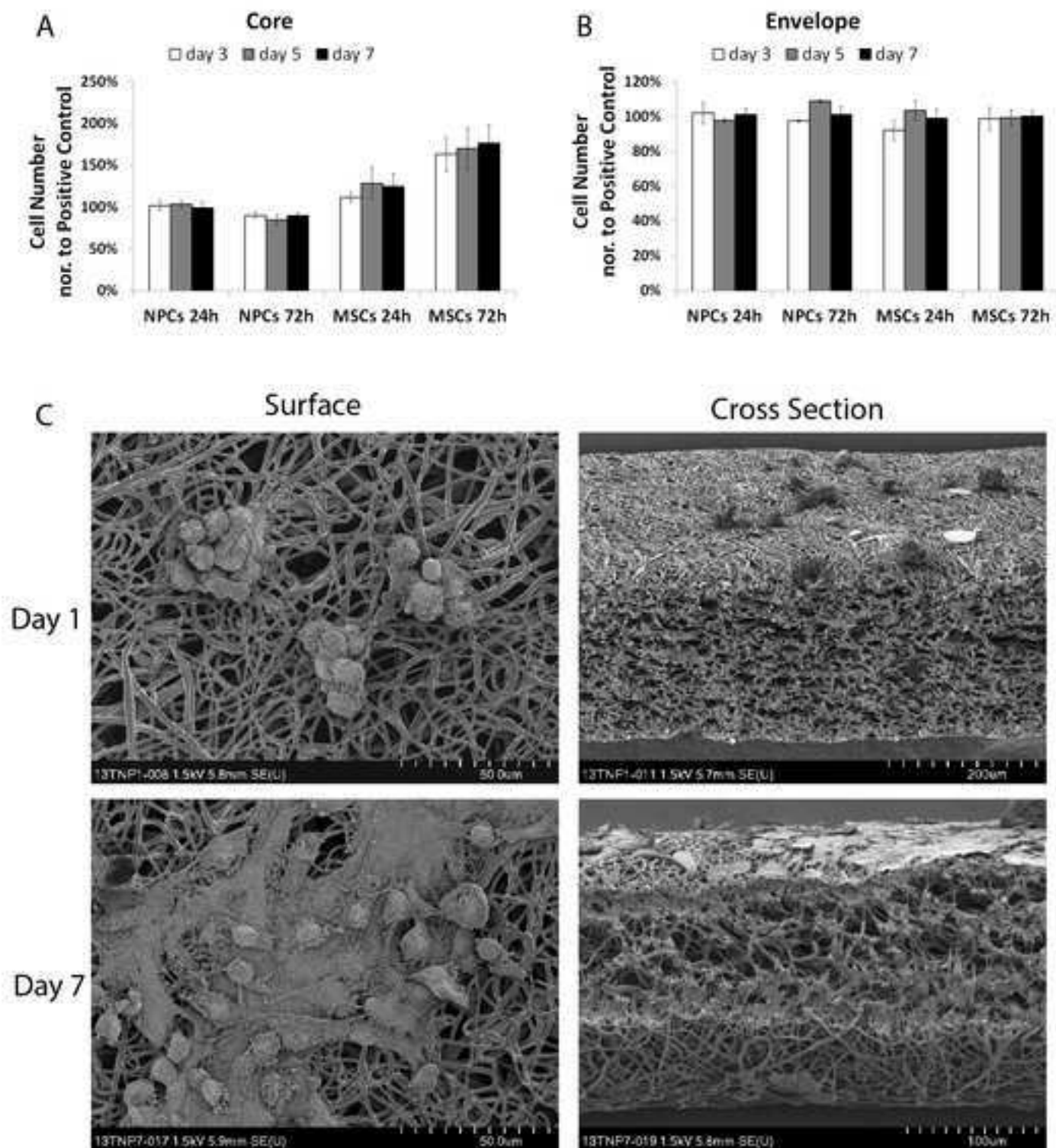


Figure 5  
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**Figure6**  
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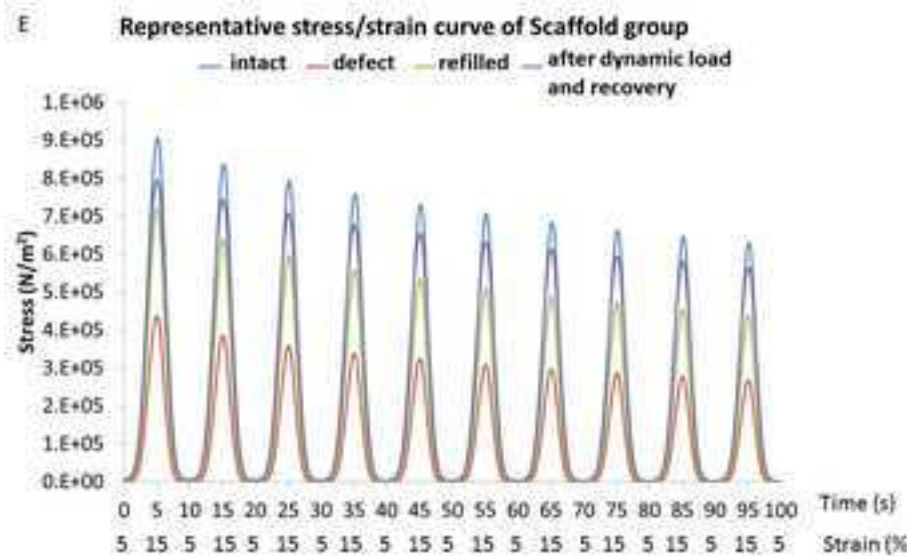
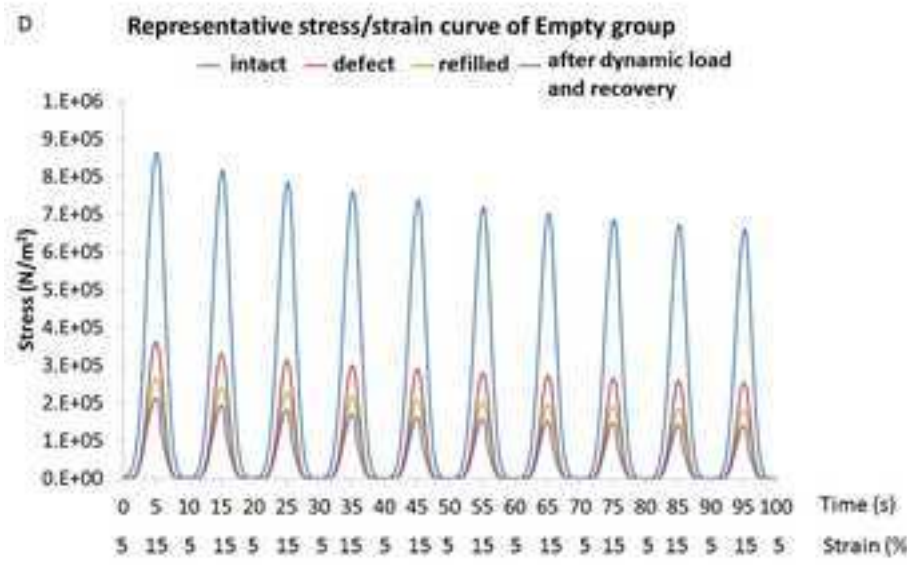
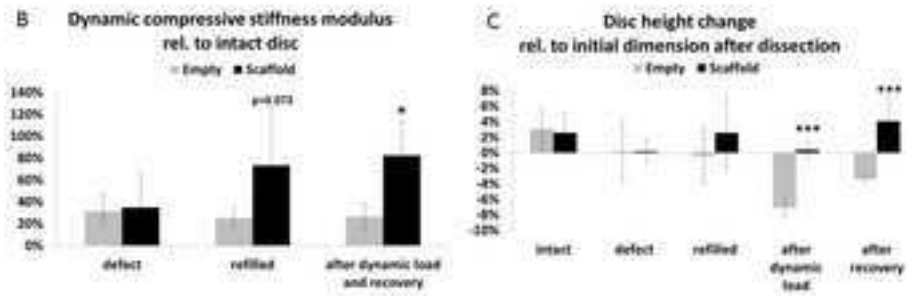


Figure 7

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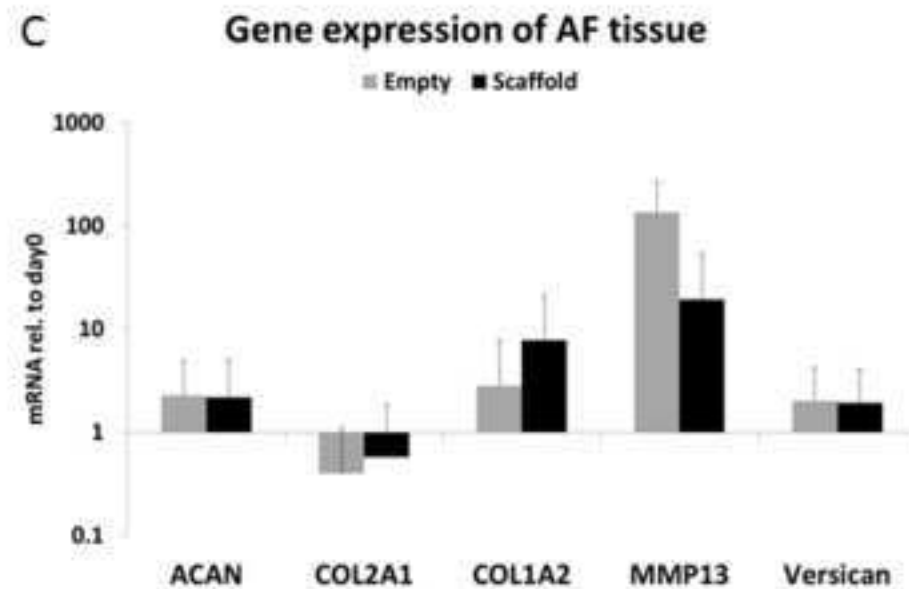
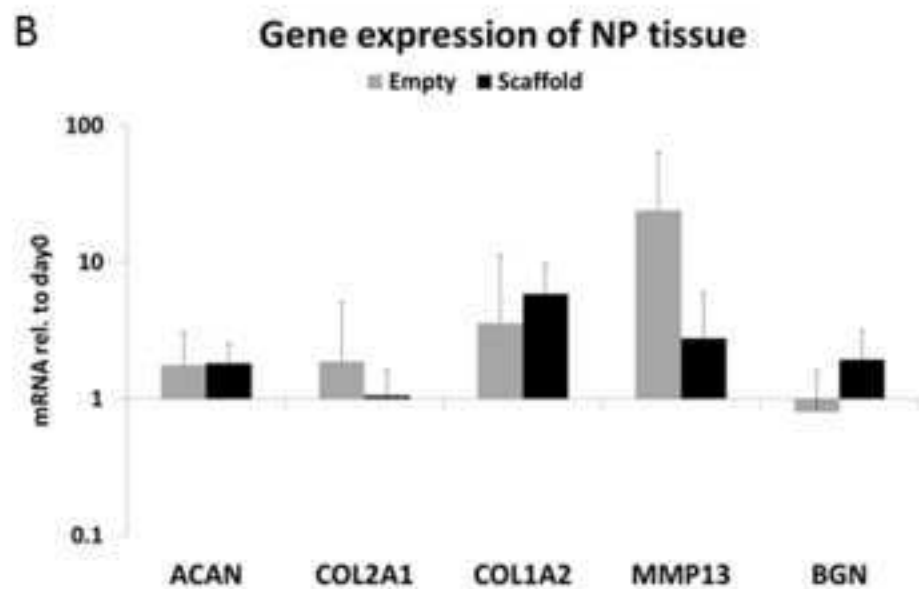
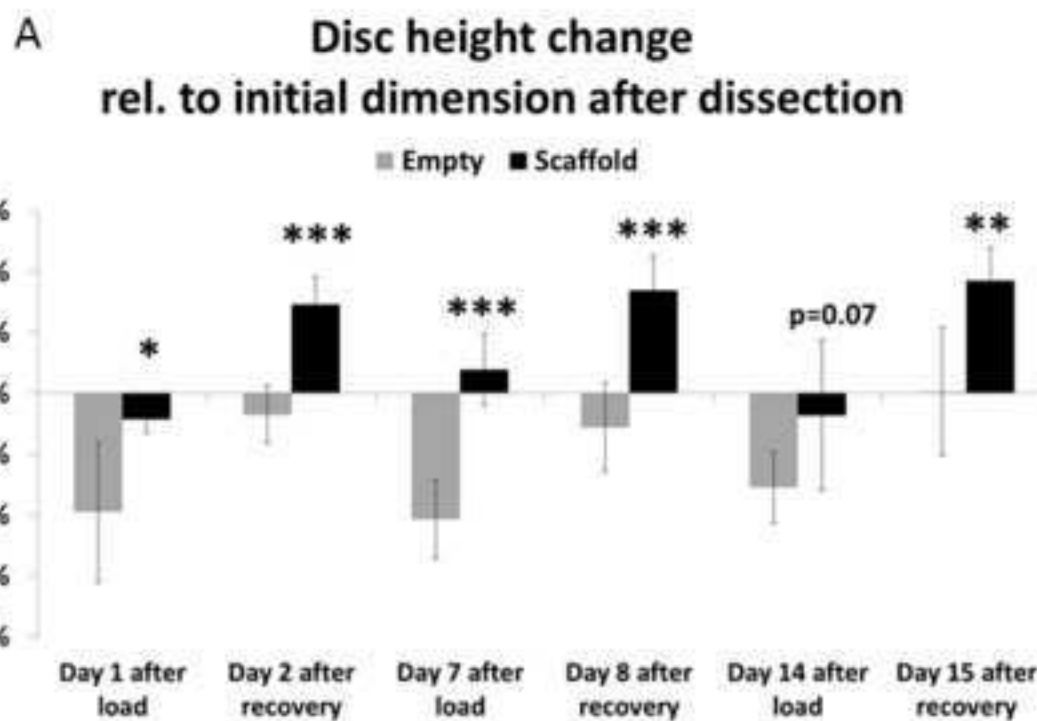


Figure 8  
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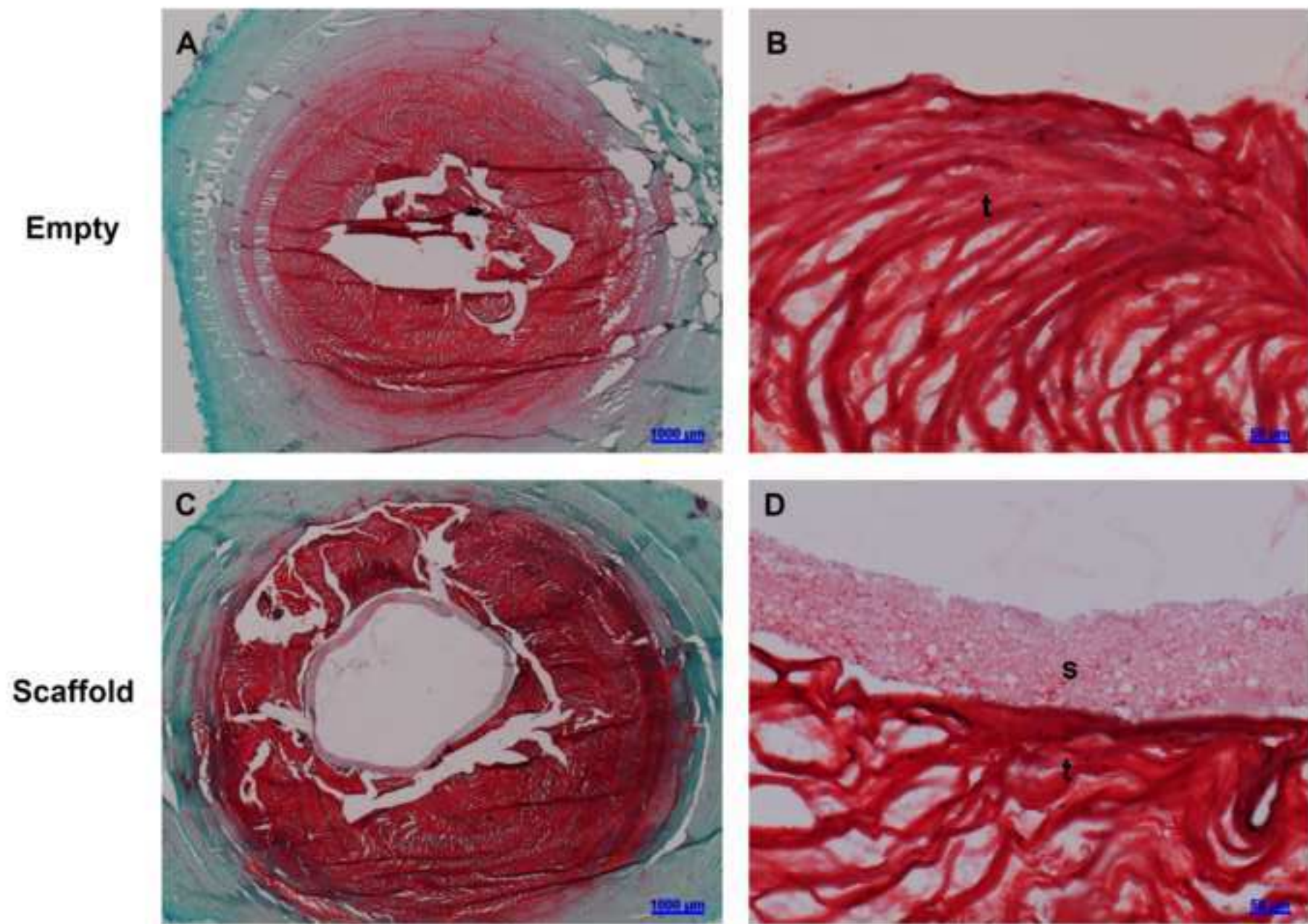
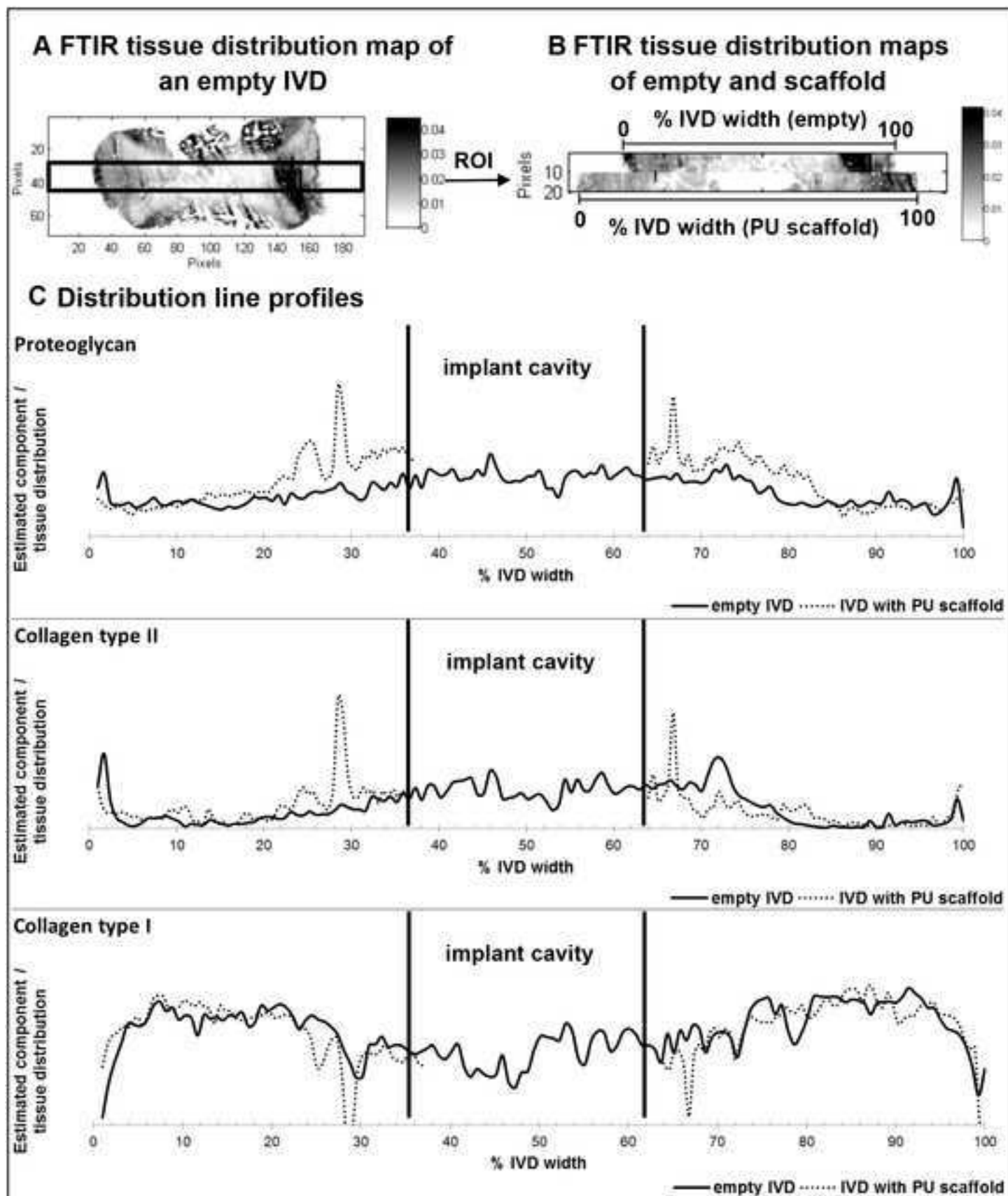




Figure 9

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## Figure Legends

**Figure 1.** (A) Flat discoid shaped PU scaffold before (front) and after (back) swelling at various sizes (from left to right 6, 9 and 14 mm). Each size has defined core content. (B) Scheme of PU scaffold assembly process: core disc is wrapped by two envelope discs, and heat sealed within a custom made sealing mold. (C) Non-invasive delivery system for PU scaffold: a demonstration of the scaffold insertion and swelling function after delivery and hydration.

**Figure 2.** SEM micrographs of electrospun PU scaffold envelope. Magnifications: left 2,000x; right 10,000x. Scale bar: left 50  $\mu\text{m}$ , right 10  $\mu\text{m}$ .

**Figure 3.** Swelling of PU scaffolds after 24 hours incubation in aqueous media tested at different core contents. Scaffold size: 14 mm in diameter.

**Figure 4.** Swelling kinetic of PU scaffolds at a diameter of 14 mm (A) and 6mm (B). For each scaffold diameter different amounts of core material were tested. Mean  $\pm$  SD, n=3.

**Figure 5.** (A-B) Cell number after culture in core and envelope conditioned media measured by WST-1 assay and normalized to positive control (cells cultured with DMEM/2.5% FBS). Mean  $\pm$  SD, n=3. (A) NPCs and MSCs cultured in conditioned medium of core for 24 hours or 72 hours. (B) NPCs and MSCs cultured in conditioned medium of envelope for 24 hours or 72 hours. (C) Representative SEM

images showing morphological structures of top surface and cross section of cell-seeded envelope constructs after 1 or 7 days of culture.

**Figure 6.** (A) Left: Macroscopic view of dry PU scaffold (diameter 6 mm) before implantation into partial nucleotomized IVD. Middle: Macroscopic sagittal view of partial nucleotomized IVD. Right: Macroscopic sagittal view of partial nucleotomized IVD with implanted PU scaffold after 24 hours of culture. PU scaffold swelled *in situ* and completely filled the nucleotomized region. (B) Dynamic compressive stiffness modulus of partial nucleotomized IVD before (defect) and after (refilled) refilling with PU scaffold, and after dynamic load and free swelling recovery (after dynamic load and recovery). Data were normalized to the stiffness modulus of respective intact IVD. (C) Disc height change of IVDs after dissection and free swelling culture overnight (intact), after partial nucleotomy (defect), after refilling with PU scaffold and free swelling culture overnight (refilled), after dynamic load (after dynamic load), and after free swelling recovery (after recovery). Data were normalized to initial disc height after dissection. (D and E) Representative stress/strain curves of IVDs from Empty and Scaffold groups at different time points. Mean  $\pm$  SD, n=6, \* p<0.05, \*\*\* p<0.001 Empty *versus* Scaffold. Empty – partially nucleotomized IVDs, Scaffold – partially nucleotomized IVDs with implanted PU scaffold.

**Figure 7.** (A) Disc height change of partially nucleotomized IVDs without/with PU scaffold implantation under repetitive dynamic load. Mean  $\pm$  SD, n=6, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 Empty *versus* Scaffold. (B-C) Relative mRNA expression of cells from remaining native NP and AF tissue in nucleotomized IVDs without/with PU scaffold implantation after 14 days of dynamic load. Data were normalized to the

gene expression level of disc tissue from respective bovine tail before starting organ culture on day 0. Mean + SD, n=8. Empty – partially nucleotomized IVDs, Scaffold – partially nucleotomized IVDs with implanted PU scaffold.

**Figure 8.** Representative Safranin O/Fast Green stained transverse sections of partially nucleotomized IVDs without/with PU scaffold implantation after 14 days of culture under dynamic load. (A, C) Overview of IVD without/with PU scaffold implantation, scale bar: 1000  $\mu\text{m}$ . (B, D) Interface between remaining NP tissue and nucleotomized region without/with PU scaffold implantation, scale bar: 50  $\mu\text{m}$ . Empty – partially nucleotomized IVDs, Scaffold – partially nucleotomized IVDs implanted with PU scaffold, s – PU scaffold, t – native disc NP tissue.

**Figure 9.** Representative FTIR tissue distribution map of a sagittal section of a partially nucleotomized IVD cultured for 14 days under dynamic load without PU scaffold (empty) (A). Image contrast for tissue distribution map is generated by integration of the 2nd derivative of the Amide III peak ( $1186\text{-}1297\text{ cm}^{-1}$ ). The black square indicates a region of interest (ROI) which was used to investigate the matrix distribution of native disc tissue surrounding the implants cavity. FTIR tissue distribution maps of ROIs of an empty IVD (top) and an IVD implanted with the PU scaffold (bottom) cultured for 14 days under dynamic load (scale bar: white to black indicative of a low to high tissue content) (B). Estimated component per tissue distribution line profiles across the ROI normalised to 100 % IVD width of proteoglycan, collagen type II and collagen type I are shown for an empty IVD and a PU scaffold implanted IVD (C).