

Non-covalently crosslinked chitosan nanofibrous mats prepared by electrospinning as substrates for soft tissue regeneration

Original

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24 **ABSTRACT**

25 Chitosan (CS) membranes obtained by electrospinning are potentially ideal substrates for soft
26 tissue engineering as they combine the excellent biological properties of CS with the extracellular
27 matrix (ECM)-like structure of nanofibrous mats. However, the high amount of acid solvents
28 required to spun CS solutions interferes with the biocompatibility of CS fibres. To overcome this
29 limitation, a novel CS based solutions were investigated in this work. Low amount of acidic acid
30 (0.5 M) was used and dibasic sodium phosphate (DSP) was introduced as ionic crosslinker to
31 improve nanofibres water stability and to neutralize the acidic pH of electrospun membranes after
32 fibres soaking in biological fluids. Randomly oriented and aligned nanofibres (118 ± 16 nm size)
33 were obtained through electrospinning process (voltage of 30 kV, 30 μ L/min flow rate and
34 temperature of 39 °C) showing mechanical properties similar to those of soft tissues (Young
35 Modulus lower than 40 MPa in dry condition) and water stability until 7 days. C2C12 myoblast
36 cell line was cultured on CS fibres showing that the aligned architecture of substrate induces cell
37 orientation that can enhance skeletal muscle regeneration.

38

39 *Keywords: chitosan, ionic crosslinking, electrospinning, skeletal muscle regeneration*

40

41 **List of abbreviations**

42

43 **CS:** chitosan

44 **DAPI:** 4',6-diamidino-2-phenylindole

45 **DMSO:** dimethyl sulfoxide

46 **DSP:** dibasic sodium phosphate

47 **ECM:** extracellular matrix

48 **EDS:** energy dispersive spectrometer

49 **glass-CTRL:** glass coverslip

50 **GP:** glycerol phosphate

51 **FFT:** Fast Fourier Transform

52 **FTIR-ATR :** attenuated total reflection Fourier transform infrared

53 **P:** phosphorus

54 **PBS:** phosphate buffered saline

55 **PCL:** poly(caprolactone)

56 **PEO:** poly(ethyleneoxide)

57 **PMS:** phenazine methosulphate

58 **PVA:** poly(vinyl alcohol)

59 **SEM:** scanning electron microscopy

60 **TRITC:** tetramethylrhodamine

61 **E:** Young's modulus

62 **UTS:** ultimate tensile strength

63 **$\epsilon_{failure}$:** strain at failure

64

65 **1. Introduction**

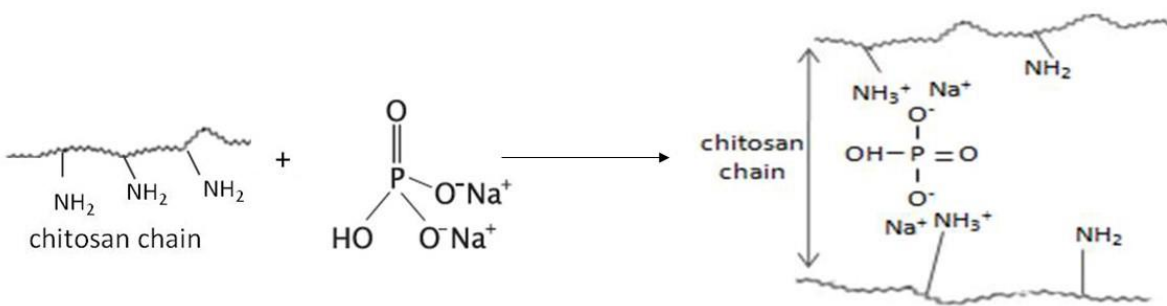
66 Chitosan (CS) is a basic natural polysaccharide obtained by alkaline deacetylation from chitin
67 (Muzzarelli, 2009) having excellent biocompatibility features and antimicrobial activity which
68 foreseen its potential in many medical applications such as drug delivery systems (Bhattarai,
69 Gunn, & Zhang, 2010; J. H. Park, Saravanakumar, Kim, & Kwon, 2010), wound-healing agents
70 (Howling et al., 2001; Murakami et al., 2010) and peripheral nerve repair (Amado et al., 2008; Li
71 et al., 2014). The possibility to process CS into nanofibres has been largely investigated to produce
72 nanofibrous substrate able to mimic the extracellular matrix (ECM) structure (N. Bhattarai, D.
73 Edmondson, O. Veiseh, F. A. Matsen, & M. Zhang, 2005a; Z. G. Chen, Wang, Wei, Mo, & Cui,
74 2010). A number of fabrication techniques have been explored to prepare micro/nanoscale fibrous
75 scaffolds, among which, electrospinning method has been widely accepted as the simplest and
76 least expensive one to fabricate fibrous matrices through the extrusion of the solution from a
77 needle by an high voltage electric field (Agarwal, Wendorff, & Greiner, 2008; Koh, Yong, Chan,
78 & Ramakrishna, 2008; Tonda-Turo et al., 2013b). Random or aligned fibres can be obtained
79 mimicking the ECM architecture of different tissues (e.g. nerves and tendons have an aligned
80 structure while skin and cartilage have a random structure) as many studies have shown that the
81 fibre orientation influences cell adhesion, growth and modulates elongated cellular patterns that
82 are typical of morphology found in native tissue (Choi, Lee, Christ, Atala, & Yoo, 2008; Corey
83 et al., 2007; Gnavi et al., 2015; Gupta et al., 2009; Neal et al., 2012; Qu et al., 2012; Yang,
84 Murugan, Wang, & Ramakrishna, 2005).

85 The electrospinnability of CS is limited mainly by its polycationic nature in solution, rigid
86 chemical structure and specific inter and intra-molecular interactions which makes CS solutions
87 highly viscous at low acid pH and room temperature (Homayoni, Ravandi, & Valizadeh, 2009).

88 However, processing conditions have to be carefully selected as the use of high temperatures and
89 organic solvents may cause CS denaturation and could interfere with CS biocompatibility
90 (Ghasemi-Mobarakeh, Prabhakaran, Morshed, Nasr-Esfahani, & Ramakrishna, 2008). In order to
91 overcome these drawbacks, CS nanofibre fabrication has been attempted using blends with easy
92 spinnable polymers, such as poly(ethyleneoxide) (PEO) (Bhattarai, et al., 2005a; Sarkar, Farrugia,
93 Dargaville, & Dhara, 2013), poly(vinyl alcohol) (PVA) (Charernsriwilaiwat, Opanasopit,
94 Rojanarata, Ngawhirunpat, & Supaphol, 2010; Duan et al., 2006) and poly(caprolactone) (PCL)
95 (Cooper, Bhattarai, & Zhang, 2011), silk fibroin (Z. X. Cai et al., 2010; W. H. Park, Jeong, Yoo,
96 & Hudson, 2004) and collagen (L. Chen et al., 2011; Z. G. Chen, Mo, & Qing, 2007). Although
97 several studies have been reported, the use of CS electrospun nanofibres remains largely
98 unexplored and further experiments are necessary to define process parameter for successful CS
99 nanofibres fabrication. In this work, a novel procedure to electrospun CS nanofibres was
100 developed using low amounts of acetic acid (0.5 M) for CS solubilization in order to reduce the
101 risk of cytotoxic residues and polymer degradation.

102 A particular focus of the study was the use of dibasic sodium phosphate (DSP) in novel one-step
103 crosslinking of the CS. DSP is negatively charged in aqueous solution enabling it to bind
104 preferentially with dissolved acidic chitosan quaternary ammonium cation providing ionic
105 crosslinking of the CS (Fig. 1). The one-step crosslinking method offer many advantages
106 compared to the two-step method in terms of repeatability and fine tuning of fibres morphology.
107 DSP was selected as CS non-covalent crosslinker for its ability to increase the CS solution pH
108 without causing CS precipitation and/or increased in solution viscosity. Tailoring the amount of
109 DSP, an increase of CS solution pH from 4 to 5.8-5.9 was achieved. This increase in the solution
110 pH was sufficient to maintain physiological pH (around 7) in the CS membrane surrounding

111 environment after immersion in biological fluids (Ruini, Tonda-Turo, Chiono, & Ciardelli, 2015)
 112 without affecting the viscosity of the CS solution and/or interfering with the crosslinking process
 113 (Ruini, et al., 2015). Ionic crosslinkers, such as DSP and glycerol phosphate (GP), have been
 114 already applied for CS based porous scaffolds and hydrogels (Kim et al., 2010; Ruini, 2015) while,
 115 to the best of our knowledge, the ionic crosslinking combined with low amounts of acid solution
 116 for nanofibres fabrication was applied in this study for the first time. Kielchel et al. reported the
 117 fabrication of GP crosslinked CS nanofibres using a one-step method, however an electrospinnable
 118 solution was obtained with a high amount of trifluoroacetic acid (99%) and fabricated fibres show
 119 a ribbon-like morphology with many defects (Kiechel & Schauer, 2013).
 120 In this work, CS crosslinked nanofibres were produced as randomly oriented or as aligned fibres
 121 (using high speed drum electrode collection) and characterized by scanning electron microscopy
 122 (SEM), infrared spectroscopy (FTIR-ATR), uniaxial tensile mechanical testing, and dissolution
 123 studies. An *in vitro* cell assay was included in the study as a preliminary investigation of the
 124 applicability of the novel CS membrane as a cellular scaffold in tissue engineering applications.
 125 A C2C12 myoblast cell line was used to examine cellular adherence and proliferation on the novel
 126 membranes.



127
 128 **Fig. 1.** Scheme of CS crosslinked DSP (non-covalent crosslinker), reproduced with permission
 129 from (Ruini, et al., 2015).

130 **2. Materials and methods**

131 **2.1 Materials**

132 Medical grade CS (molecular weight 200 – 400 kDa, deacetylation degree ≥ 92.6 %) was
133 purchased from Kraeber GmbH & Co. PEO (M_w 900.000 Da), DSP, dimethyl sulfoxide (DMSO)
134 and solvents were supplied from Sigma Aldrich. All solvents were of analytical grade and were
135 used without further purification.

136

137 **2.2 Electrospun membrane preparation**

138 **2.2.1 Preparation of solutions for electrospinning**

139 Different CS (3, 5 or 7 % (w/v)) and 3% (w/v) PEO solutions were prepared separately by
140 dissolving CS or PEO in 0.5 M acetic acid solution at room temperature by continuous stirring.
141 After complete solubilisation of each components, a 50/50 (v/v) CS/PEO solution was prepared
142 by mixing equal volumes of CS and PEO solutions to obtain the mixtures with weight ratios of
143 CS to PEO of 50/50, 62/38 and 70/30; the resultant mixtures were kept under stirring for about 2
144 hours. A 5% (v/v) of dimethyl sulfoxide (DMSO) was added to the CS/PEO solution as a co-
145 solvent to relax CS chain entanglements and increase the fibre yields and consequently improving
146 the spinnability of the CS-based solution (N. Bhattarai, D. Edmondson, O. Veiseh, F. A. Matsen,
147 & M. Q. Zhang, 2005b). The pH of this solution was around 4. Finally, ionically crosslinked
148 samples (CS/PEO_DSP) were prepared by adding 1M DSP (one drop per second) to the CS/PEO
149 solution with a concentration of 7.5 % v/v with respect to the natural polymer solution volume.
150 One molar DSP solution was used as it represents its maximum solubility in aqueous solution.
151 The amount of DSP solution added to CS solution was selected to avoid CS precipitation (final
152 clear solution) and to reach a final CS solution pH around 5.8-5.9 which guarantees to maintain

153 physiological pH of CS-based scaffold after immersion in physiological solution, as previously
154 described by Ruini et al. (Ruini, 2015; Ruini, et al., 2015). Uncrosslinked solutions were prepared
155 as control samples.

156

157 **2.2.2 Electrospinning of CS nanofibres**

158 The electrospinning system used for fibre preparation was previously described (Tonda-Turo et
159 al., 2013a). Briefly, the electrospinning system was kindly supplied by Biomedical Components
160 s.r.l and it consists of a high voltage generator (PS/EL30R01.5-22 Glassman High Voltage),
161 providing a voltage from 0 to 30 kV; a volumetric pump (KDS210 of KD Scientific); a mobile
162 syringe support and a collector. In this study, two different collectors were used: a 1.5 mm-thick
163 flat aluminium collector for random fibres preparation and a cylindrical rotating drum having a
164 80 mm diameter and a controllable rotating speed from 0 to 2400 rpm. Before characterization,
165 all CS nanofibres were peeled off from the collector.

166

167 **2.3 Membrane preparation and optimization of solution and process parameters**

168 **2.3.1 Solution parameters and viscosity tests**

169 Preliminary tests were performed to optimize the amount of CS in the CS/PEO_DSP solution.
170 Three CS/PEO_DSP solutions were tested having different CS/PEO ratio: 50/50 (coded as 3%
171 CS), 62/38 (coded as 5% CS) and 70/30 (coded as 7% CS). A stress-controlled rheometer
172 (MCR302, Anton Paar GmbH), equipped with 50 mm parallel plates geometry was used. For
173 temperature control a Peltier system was employed. Samples were put on the lower plate at 40
174 °C, maintained in quiescent conditions for 15 minutes to reach the thermal stability and finally

175 isothermally tested (40 °C). The viscosity was checked at constant temperature by means of flow
176 curves with shear rate control (shear rate from 1 to 100 s⁻¹).

177 **2.3.2 Process parameters**

178 Continuous nanofibres were obtained only for CS solution concentration of 5 % (62/38 w/w
179 CS/PEO mixture). Process parameters were varied to reduce fibre defects and maximize the
180 amount of collected material. The parameter values allowing spinnability were: (i) temperature
181 from 25 °C to 39 °C, (ii) flow rate of 25 µl min⁻¹ to 50 µl min⁻¹, (iii) nozzle-collector distance of
182 12 cm, and (vi) voltage of 30 kV. The effect of temperature and flow rate was evaluated to
183 optimize the process and the fibre morphology.

184

185 **2.3.3 Fibres morphology and element distribution**

186 The surface morphology of uncrosslinked and crosslinked CS based nanofibrous membranes was
187 observed by scanning electron microscopy (SEM LEO – 1430, Zeiss) using an accelerating
188 voltage of 15 kV, a working distance of 10 mm and a Tungsten filament. Qualitative
189 compositional analysis and punctual elemental composition of materials were performed using an
190 energy dispersive spectrometer (EDS) on a 40µm x 40 µm area. Samples were sputter coated with
191 gold in an under-vacuum chamber prior to SEM-EDS examination. In EDS analysis the gold peak
192 was omitted using the INCA software prior to elemental mapping.

193 SEM micrographs were then analysed through Image1.44g software. Fibre diameters and pores
194 were measured on three different SEM micrographs (30 measures were taken for each image) and
195 reported as average value ± standard deviation.

196 Crosslinked nanofibre orientation at different process conditions was examined through 2D Fast
197 Fourier Transform (FFT) ImageJ processing tool. The applied processing tool shows graphical

198 peaks indicating predominant fibre orientation angles. 2D FFT plots having two sharp peaks at a
199 distance of 180° are typical of oriented structures (Jha et al., 2011; Wu, Fan, Chu, & Wu, 2010).

200 **2.4 Electrospun membranes characterization**

201 **2.4.1 Fourier transform infrared-attenuated total reflectance spectroscopy (FTIR- 202 ATR)**

203 Chemical characteristics of the uncrosslinked and crosslinked CS nanofibrous scaffolds were
204 evaluated by an attenuated total reflection Fourier transform infrared (ATR-FTIR)
205 spectrophotometer (Perkin-Elmer). Spectra were obtained in the range of 2000-600 cm⁻¹ with a
206 resolution of 4 cm⁻¹ and 16 scans. A diamond crystal and an angle of incidence of the contact
207 beam of 45° were used. The spectra are reported after blank subtraction (spectrum without
208 sample).

210 **2.4.2 Mechanical properties**

211 The tensile mechanical properties were evaluated on uncrosslinked and crosslinked nanofibrous
212 membranes in dry condition using a MTS QTest/10 device equipped with load cells of 10 N.
213 Rectangular specimens of 30 mm x 5 mm size were cut from each membranes and their thickness
214 were measured using a digital calibrator. Samples were then strained at a constant crosshead speed
215 of 1 mm/min until breaking; for oriented nanofibres the stress direction was parallel to the fibre
216 alignment. Break stress and strain were determined using the associated software Test Works 4
217 while the elastic moduli (E) were calculated from the slope of the linear portion of the stress–
218 strain curve of each sample. Five specimens for each kind of material were tested. The results
219 were expressed as average value ± standard deviation.

220

221 **2.4.3 Fibres dissolution**

222 The dissolution behavior of the uncrosslinked and crosslinked CS samples (randomly oriented
223 and aligned) was evaluated by immersing the samples in phosphate buffered saline (PBS, pH 7.4)
224 at 37°C. After 1, 3, 5 and 7 days immersion, qualitative test was performed analyzing the
225 nanofibres morphology by SEM. Prior to morphological analysis, samples were removed from
226 PBS at each time step and freeze-dried for 24 hours. The solution pH was measured at the same
227 time intervals and three measurements were performed at each time points using a pH meter (XS
228 Instruments).

229

230 **2.5 *In vitro* characterization using C2C12 myoblast cell line**

231 C2C12 myoblast cell line (ATCC CRL1772), isolated from mouse muscle was used. Cells were
232 cultured in DMEM enriched with 10% fetal bovine serum, glutamine (2mM), penicillin (100
233 U/ml), and streptomycin (100mg/ml) (Euroclone, Italy). 2×10^4 cells/cm² cells were cultured on
234 randomly oriented and aligned crosslinked CS fibres for 3 and 6 days. Tests were performed in
235 triplicate. Cells cultured on glass coverslips (glass-CTRL) were used as control.

236 Cell viability has been measured using a colorimetric method (CellTiter 96® Aqueous Non-
237 Radioactive Cell Proliferation Assay — Promega, Italy). The CellTiter 96® AQueous Assay is
238 composed of solutions of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-
239 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron
240 coupling reagent phenazine methosulphate (PMS). MTS is bioreduced by cells into a formazan
241 product that is soluble in culture medium. The absorbance of the formazan product at 490 nm can
242 be measured directly in 96-well assay plates. The conversion of MTS into the aqueous soluble
243 formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells.

244 The quantity of formazan product as measured by the amount of 490 nm absorbance is directly
245 proportional to the number of living cells.

246 Briefly, at each time point cell culture medium was removed and MTS solution was added into
247 each assay-plate; after 4 h incubation of cells with MTS solution, the UV-vis absorbance of the
248 solution at 490 nm was measured.

249 Cell morphology on different surfaces was observed through fluorescent microscopy (Leica
250 Microsystems DM2500) at 20X and 40X magnifications. At 3 and 6 days, cells were fixed in
251 formaldehyde 4% for 60 min at room temperature. After rinsing, phalloidin -
252 tetramethylrhodamine (TRITC) conjugated (Sigma, Italy) was incubated for 45 min at 37°C in
253 the dark. For nuclear staining and 4',6-diamidino-2-phenylindole (DAPI) was used.

254 The stability of the nanofibrous mats after cell culture was confirmed using a scanning electron
255 microscope (SEM, LEO – 1430, Zeiss). To perform SEM analysis, the medium was removed and
256 samples were washed twice in 0.15M cacodylate buffer and fixed for 30 minutes at 4°C with
257 Karnovsky solution (2% paraformaldehyde and 2,5% gluteraldehyde in 0.15M cacodylate buffer,
258 pH 7.2-7.4). Following fixation, samples were treated for 30 minutes with 1% osmium tetroxide
259 in 0.15M cacodylate buffer solution. Samples were then dehydrated with graded ethanol (25%,
260 50%, 75%, 90% and 100%), dried and sputter-coated with gold-palladium prior to SEM analysis.

261

262 **2.6 Statistical Analysis**

263 Statistical analysis was performed applying t-Student for two group comparisons and one-way
264 ANOVA for multiple analysis using GraphPad Prism 6.0 software. Data were considered
265 statistically different for p value < 0.05.

266

267 **3. Results and discussion**

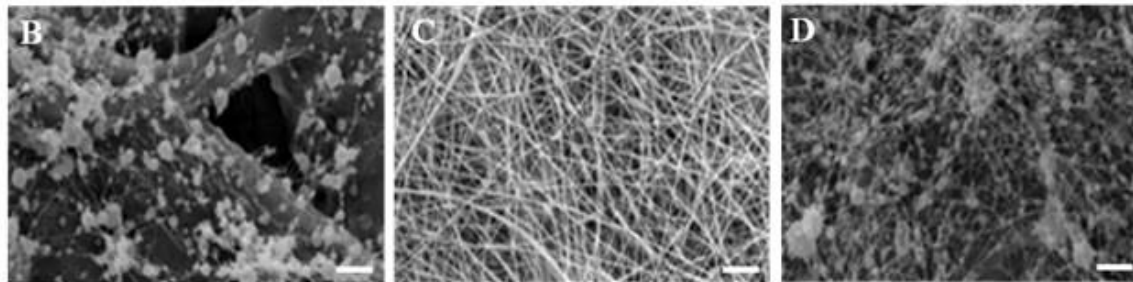
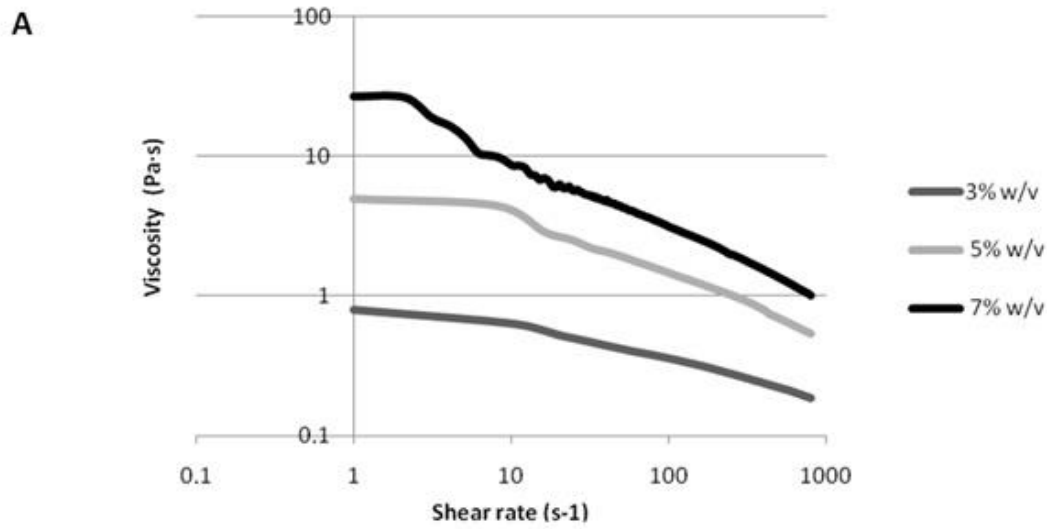
268 **3.1 Optimization of the electrospinning parameters**

269 **3.1.1 Solution viscosity and its effect on electrospun nanofibres**

270 The effect of CS solution concentration on viscosity and, consequently, spinnability was
271 evaluated. The three solutions analysed showed a non-newtonian behavior and an increase in
272 viscosity for more concentrate CS solutions (Fig. 2A). Homogenous nanofibres were obtained
273 only for 5% solution (Fig. 2C), while less viscous solution caused the formation on beads instead
274 of fibres (Fig. 2B) and highly concentrated solution impeded the flow of the solution from the
275 needle (Fig. 2D).

276

277



278

279 **Fig. 2.** Viscosity versus shear rate for three different concentration of the CS-based solutions (A).

280 SEM micrographs of electrospun CS mats obtained with a 3% (B), 5% (C) and 7% (D) CS

281 solutions (Parameters: 30kV, temperature 39°C, distance 12 cm, flow rate 30 μ L/min). Scale bar:

282 2 μ m.

283

284 3.1.2 Process parameters

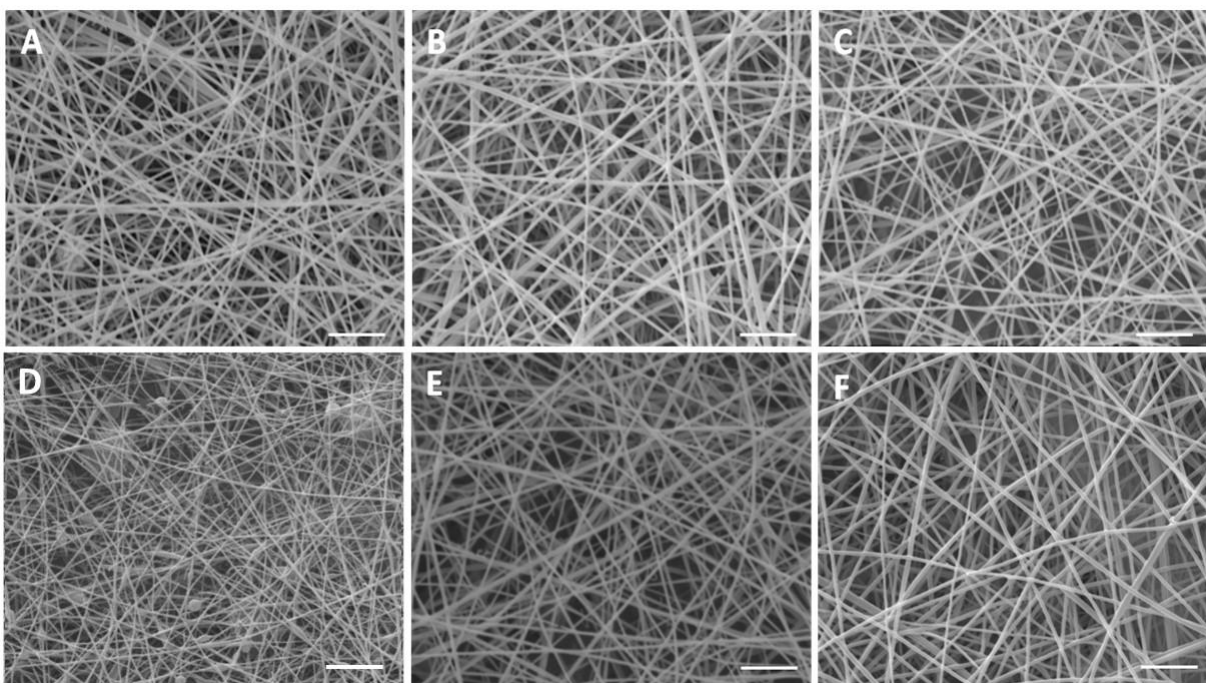
285 The optimization of the process parameters required to vary them in a wild range of sets. For the

286 fabrication on randomly oriented nanofibres, the voltage applied was fixed at 30 kV and the

287 distance between needle and collector was 12 cm. The influence of temperature and flow rate was

288 analyzed to maximize the formation of homogeneous fibres. The 5% solution was spinnable in

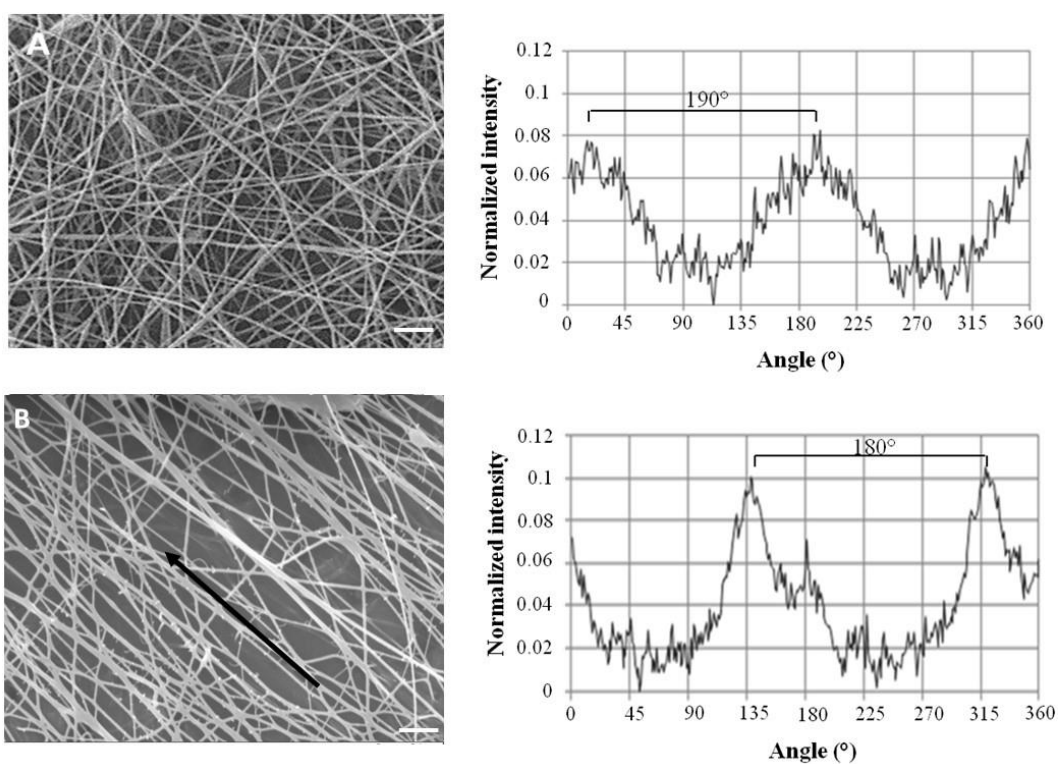
289 the range of 25 to 50 $\mu\text{L}/\text{min}$ and highly homogenous fibres with a diameters of 118 ± 16 nm were
290 obtained for flow rate of 30 $\mu\text{L}/\text{min}$ (Fig. 3). Concerning temperature, an increase in temperature
291 allowed to reduce the number of defects on the fibres as reported in Figure 3. The electrospinning
292 process was set at 39°C.



293
294 **Fig. 3.** SEM micrographs of CS randomly oriented nanofibres fabricated at different flow rates
295 (fixed parameters: voltage 30kV, distance nozzle-collector 12 cm, T=39°C): 25 $\mu\text{L}/\text{min}$ (A), 27,5
296 $\mu\text{L}/\text{min}$ (B), 30 $\mu\text{L}/\text{min}$ (C), and at different temperature (fixed parameters: voltage 30kV, distance
297 nozzle-collector 12 cm, flow rate 30 $\mu\text{L}/\text{min}$): 25 °C (D), 32°C (E) and 39 °C (F). Bars: 2 μm .

298
299 The optimized parameters (solution concentration 5%, voltage 30kV, temperature 39°C, distance
300 12 cm, flow rate 30 $\mu\text{L}/\text{min}$) were applied to fabricate aligned CS-based nanofibres. The mandrel
301 rotation was varied from 300 to 2400 rpm to analyze the influence of this parameter on fibre
302 alignment. The FFT analysis of the SEM images was also used to quantitatively analyze the

303 degree of the CS based nanofibre alignment. A graphical plot of the FFT frequency distribution
304 was generated by summing the pixel intensities encountered along the radius of the FFT output
305 image obtained from the original SEM image. Rotating speed around 300 rpm did not allow fibre
306 orientation as confirmed by SEM image and FTT analysis (Fig. 4A). On the other hand, for
307 rotating speed of 2400 rpm (Fig. 4B), two sharp peaks can be observed at a distance around 180°,
308 confirming that a high amount of fibres is aligned along a preferential direction.



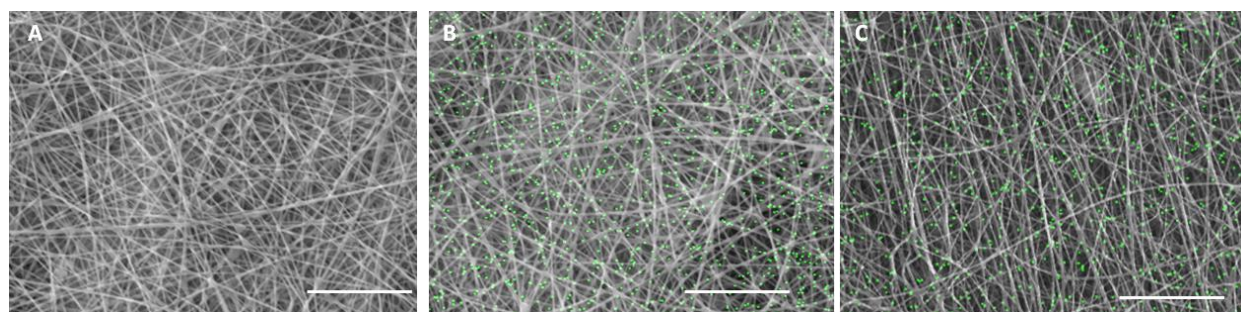
309
310 **Fig. 4.** SEM micrographs and FTT analysis of nanofibres collected using a rotating mandrel rate
311 of 300 rpm (A) and 2400 rpm (B). Arrow indicate the fibre alignment direction. Bars 5 μm .

312
313
314
315

316 **3.2 Characterization of the CS based nanofibres**

317 **3.2.1 Fibres morphology and element distribution**

318 Uncrosslinked and crosslinked fibres obtained using optimized parameters (solution
319 concentration 5%, voltage 30 kV, temperature 39 °C, distance 12 cm, flow rate 30 μ L/min) were
320 visualized through SEM and qualitative analysis of phosphorus (P) element was performed using
321 EDS. EDS analysis confirmed the presence of carbon, oxygen and nitrogen, the main elemental
322 components of CS, in all samples (data not shown). Green spots representing phosphorus were
323 found to be homogeneously distributed within both randomly oriented and aligned crosslinked
324 samples confirming the presence of DSP into nanofibers produced using both plane and rotating
325 collectors (Fig. 5B and C). No green spots were detected on uncrosslinked samples (Fig. 5A). The
326 insets in figure 5 display the corresponding morphologies. Highly uniform and smooth nanofibres
327 were formed without the occurrence of bead defects for all the nanofibrous scaffolds. Randomly
328 oriented and aligned nanofibres size was no significantly different with values of 128 ± 19 nm
329 and 140 ± 41 nm, respectively, showing comparable values to uncrosslinked CS nanofibrous
330 samples (109 ± 17 nm). The pore size was in the range of 1-3 μ m for both random, aligned,
331 crosslinked and uncrosslinked membranes. Membrane microporosity guarantees a high number
332 of site for cell adhesion.

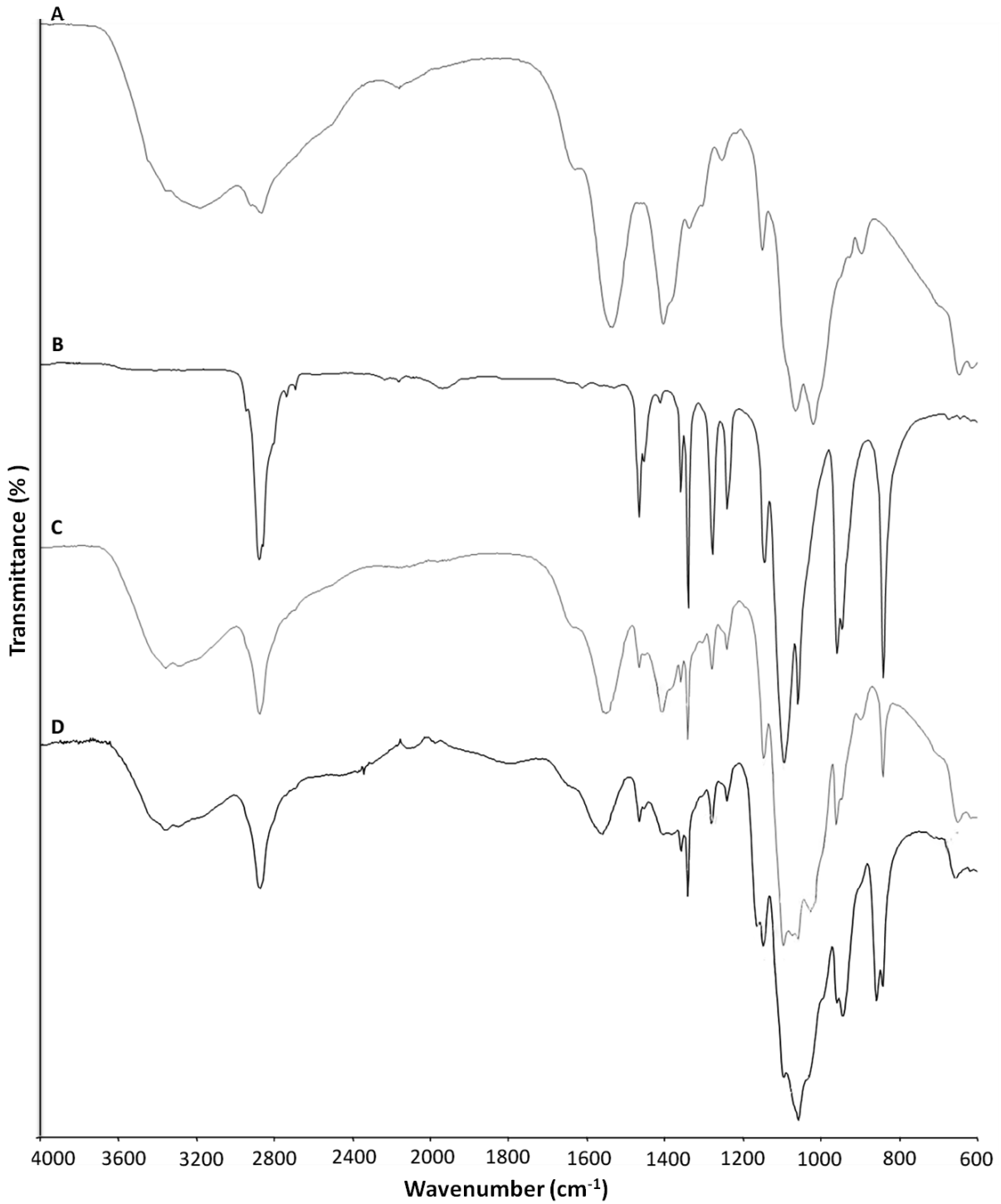


334 **Fig. 5.** EDS elemental mapping and SEM images of uncrosslinked (A) and crosslinked randomly
335 oriented (B) and aligned (C) CS-based nanofibres. Green spots correspond to phosphorus (P)
336 elements. Bars 10 μm .

337

338 **3.2.2 Fourier transform infrared-attenuated total reflectance spectroscopy (FTIR-ATR)**

339 FTIR spectra of uncrosslinked and crosslinked randomly oriented CS-based nanofibres presented
340 the peaks related to CS and PEO, which are present in the CS-based nanofibres (figure 6). Peak
341 wavenumbers and their relative bond vibrations are reported in Table 1. For CS-based nanofibres,
342 the appearance of the peak at 1074 cm^{-1} is related to the stretching of S=O bonds ($\nu_{\text{S=O}}$) due to the
343 presence of DMSO residues in the nanofibres, in accordance to results obtained by Markarian et
344 al. (Markarian, Gabrielyan, & Grigoryan, 2004). Furthermore, in the crosslinked nanofibres the
345 crosslinking was confirmed by the appearance of peaks at 1059 cm^{-1} , 944 cm^{-1} and 858 cm^{-1}
346 related to PO_3 stretching (ν_{PO_3}), O-P-O bending ($\delta_{\text{O-P-O}}$) and P-OH bending ($\nu_{\text{P-OH}}$),
347 respectively (Larkin, 2011). No differences were observed between FTIR-ATR spectra of
348 randomly oriented and aligned nanofibres.



349

350 **Figure 6.** FTIR spectra of CS (A), PEO (B), uncrosslinked CS nanofibers (C), crosslinked CS

351 nanofibers (D).

352

353 **Table 1.** FTIR peaks and their relative bond vibrations.

Bond vibration	Wavenumber (cm⁻¹)	Material	ref
vO-H	3222	CS	(Rubilar et al., 2013)
vN-H			
vC-H	2883	CS and PEO	(Duan, Dong, Yuan, & Yao, 2004; Ojha et al., 2008)
vC=O	1634	CS	(Duan, et al., 2004; Kjm, Son, Kim, Weller, & Hanna, 2006; Rubilar, et al., 2013)
δN-H	1547	CS	(Duan, et al., 2004; Kjm, et al., 2006; Leceta, Guerrero, & de la Caba, 2013; Rubilar, et al., 2013)
δCH₂	1466	PEO	(Dey, Das, Karan, & De, 2011)
vC-N	1410	CS	(Leceta, et al., 2013)
ωCH₂	1360	PEO	(Dey, et al., 2011)
τCH₂	1280; 1241	PEO	(Dey, et al., 2011)
vC-O-C	114; 1095; 1060	CS and PEO	(Caykara, Demirci, Eroglu, & Guven, 2005; Duan, et al., 2004)
ρCH₂	959; 947	PEO	(Dey, et al., 2011)
δC-O-C	842	PEO	(Caykara, et al., 2005)

354

355

356 3.2.3 Mechanical properties

357 The mechanical behaviour of uncrosslinked and crosslinked (randomly oriented and aligned) CS
358 fibrous matrices was determined in dry condition. A stress-strain plot for the CS based nanofibres
359 was obtained and the average Young's modulus (tensile elastic modulus), ultimate tensile strength
360 (UTS) and strain at failure ($\epsilon_{\text{failure}}$) were determined. Young's moduli were calculated from the
361 slope of the linear elastic region of the stress-strain curve (Table 2) while UTS and $\epsilon_{\text{failure}}$ were the
362 values at break.

363 **Table 2.** Young's modulus (E), ultimate tensile strength (UTS) and strain at failure ($\epsilon_{\text{failure}}$) of
364 the electrospun membranes with random and aligned fibres.

Sample	E (MPa)	UTS (MPa)	$\epsilon_{\text{failure}}$ (%)
Uncrosslinked random	35±7	1.8±0.5	3.5±0.6
Crosslinked random	42 ±12	1.1±0.3	3.2±0.5
Crosslinked aligned	14±6	0.9±0.2	8.1±0.9

365
366 Uncrosslinked and crosslinked randomly oriented CS nanofibres showed comparable results to
367 nanofibrous membranes. However, following the alignment of crosslinked nanofibres, the E
368 decreased significantly (*p < 0.05). During the fabrication of the aligned structure, the high speed
369 rotation of the mandrel caused a fibres pre-loading which consequently resulted in a reduction of
370 the measured E. Concerning the $\epsilon_{\text{failure}}$ values, an increased in elongation was obtained for aligned
371 samples as a consequence of the ordered structure parallel to the stress direction (Cooper, et al.,
372 2011).

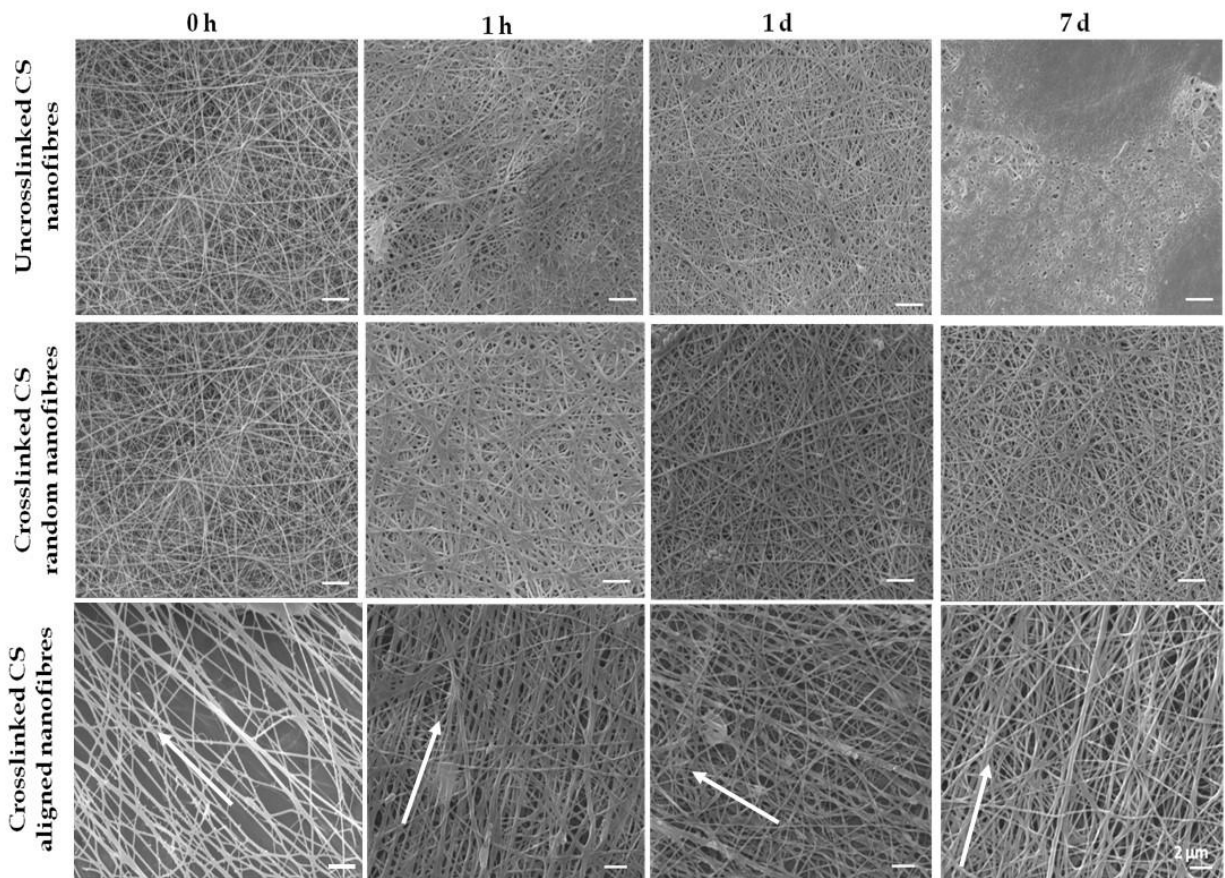
373 Concerning tissue engineering, biomaterial constructs should ideally resemble the *in vivo*
374 mechanical and structural properties of the tissues that they are intended to replace (Fung 1993
375 Biomechanics: mechanical properties of living tissues. 2nd ed. New York: Springer).

376 Based on the mechanical properties of the scaffolds, the electrospun nanofibrous matrices are
377 indicated for soft tissue applications, such as skin, cartilage and nerve (Hung, Chang, Lin, Walter,
378 & Bunegin, 1981; Mow & Guo, 2002).

379

380 **3.2.4 Fibres dissolution**

381 To confirm the effectiveness of the crosslinking process and to evaluate the stability of CS based
382 nanofibres in aqueous environment, a qualitative analysis of the dissolution behaviour of
383 uncrosslinked and crosslinked CS based nanofibres was performed in PBS at 37°C. The soaking
384 solution pH was monitored and values of 7.2 ± 0.2 were detected at each time point. Figure 7
385 illustrates the morphological changes in nanofibres during *in vitro* dissolution. All nanofibrous
386 matrices showed swelling during the first hour after immersion in aqueous solutions, though
387 significant morphological changes were not observed. After 7 days incubation in PBS, partial
388 dissolution of the fibres was observed for uncrosslinked nanofibres. On the other hand, both
389 crosslinked aligned and randomly oriented nanofibres showed a stable morphology at 7 days
390 confirming the effect of the crosslinker on nanofibres water stability. Furthermore, the aligned
391 structure of CS based nanofibres was maintained after one week of immersion in PBS.



392

393 **Fig. 7.** SEM images of uncrosslinked and crosslinked nanofibres before immersion in PBS (0 h)
 394 and after 1 hour (1 h), 1 day (1 d) and 7 days (7 d) dissolution in PBS. Arrows indicate the fibre
 395 alignment direction.

396

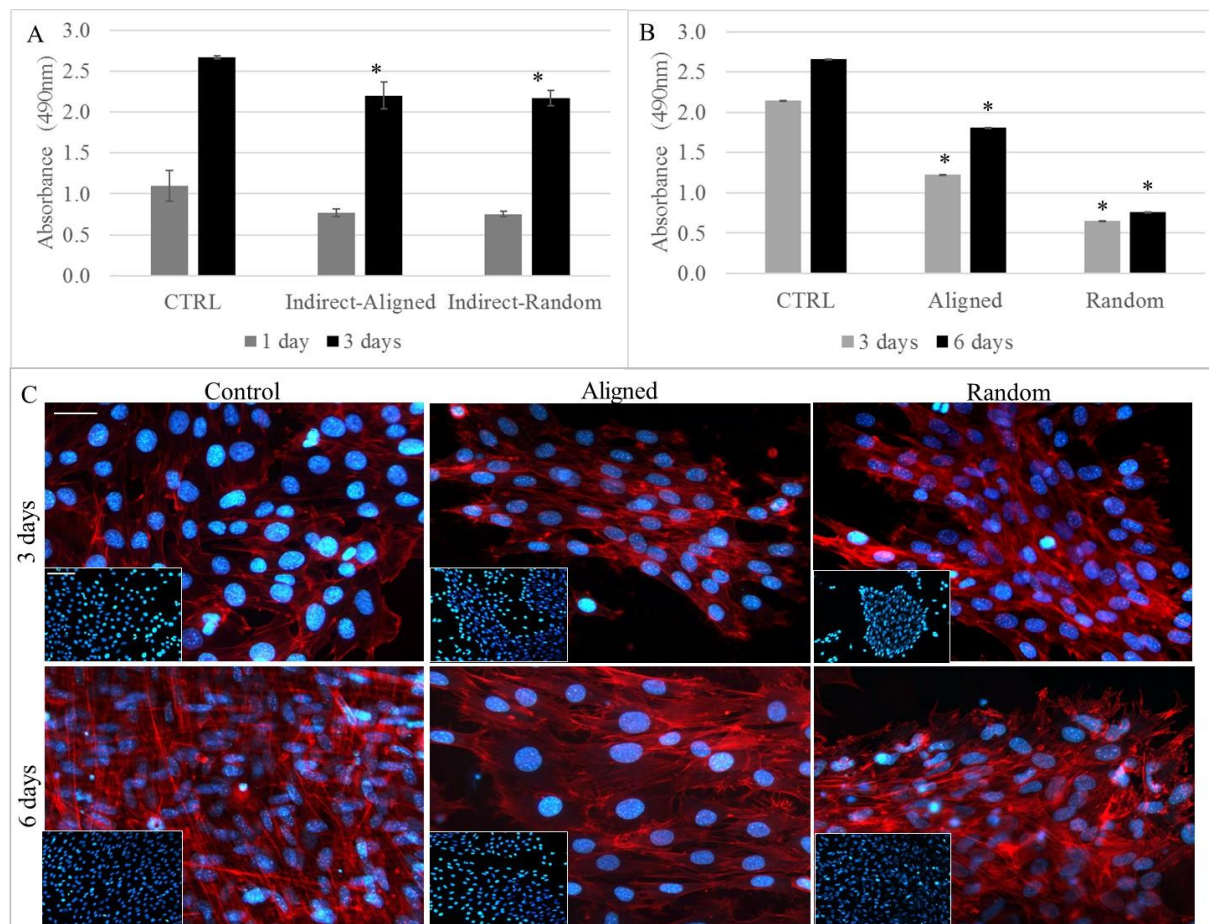
397 **3.3 *In vitro* characterization using C2C12 myoblast cells: cell viability and morphology**

398 Cell viability in contact with nanofibrous membranes was evaluated with MTS assay, using direct
 399 contact tests (cells cultured on biomaterials) and indirect tests (cells cultured with eluates obtained
 400 from media maintained for 24 hours at 37°C together with tested materials). Indirect tests did not
 401 show any toxic effect due to the present of leached products confirming the biocompatibility of

402 CS nanofibers (Fig. 8A). During direct contact tests, random and aligned biomaterials were less
403 performing as compared to control (Fig. 8B). After 3 days, the number of cells adhered to the CS
404 fibres is significantly lower compared to cells on control surfaces. However, the number of cells
405 increased with increasing incubation time on both CS nanofibres and control. Therefore, the CS
406 nanofibres developed were biocompatible substrates for the attachment and proliferation of
407 C2C12, even though significantly higher adhesion of C2C12 were observed within the first 3 days
408 on glass coverslips compared to the CS nanofibres. Low cells adhesion followed by cell growth
409 in the next days has been reported on for CS based flat films (Fregnan et al., 2016), porous sponges
410 (Seol et al., 2004) and nanofibres (Kang et al., 2010). The ability of natural polymers to adsorb
411 water, and consequently swell after immersion into physiological media, causes a softening of the
412 material (Ruini, et al., 2015) that could be related to the reduction in initial cell adhesion compared
413 to rigid glass or polypropylene cell culture plates.

414 Cells cultured on aligned fibres showed a good viability and significantly increase up to 6 days
415 without showing substantial differences with respect to proliferation trend of control. Random
416 fibres resulted as non-ideal substrates for myoblast proliferation as fluorescence analysis showed
417 well spread cells growing as cellular cluster. Cells seeded on aligned fibres showed an adequate
418 spread morphology as well and cell oriented with fibres. The analysis of cell morphology on the
419 investigated substrates revealed that C2C12 cultured onto nanofibres displayed markedly different
420 cell morphologies, with respect to cells cultured onto aligned fibres as shown by phalloidin staining
421 (Fig. 8C). After 3 and 6 days, cells cultured on nanofibres mainly presented elongated aspect.
422 Furthermore, aligned biomaterials led to cell alignment on a preferential direction reproducing the
423 ordered structure on muscular tissue. The same morphology was maintained up to 6 days and cell
424 proliferation was observed. The effect of aligned structures on cell morphology and, consequently,

425 the possibility to instruct cells *in-vitro* to organize their morphology in a specific structure is a
 426 promising tool to recreate physiological-like biological tissue.



427
 428 **Fig. 8.** MTS assay on C2C12: indirect test (A) and direct test (B). Fluorescent microscopy images
 429 after TRITC - phalloidin (actin filaments) and DAPI (nuclei) staining after 3 and 6 days of culture
 430 (B). Figures are representative of three different experiments. Random and aligned refer to cells
 431 cultured directly on CS based nanofibres. Cover glass is used as control. * indicates statistical
 432 significance with respect to control with $p \leq 0.01$. Bars: 10 μm and 20 μm (insert).

433 Finally, SEM images show a stable nanofibrous structure within cell culture time points. The
 434 presence of non-degraded nanofibres after cell tests confirmed the ability of DSP crosslinking to
 435 enhance CS fibres water stability increasing the fibres dissolution time (Fig.S1).

436 **4. Conclusion**

437 Development of functional tissue engineering products requires an appropriate scaffolding for the
438 treatment of injury and disease to mimic the structure and the biological cues of the native ECM.
439 In this study, electrospun CS based nanofibres were prepared in the form of non-woven and
440 aligned nanofibrous matrices with high specific surface areas and relatively small fibre diameters.
441 Compared to previous work in literature (Ghasemi-Mobarakeh, et al., 2008; Homayoni, et al.,
442 2009) (Kiechel & Schauer, 2013), CS was solubilized in slightly acid solution and without the use
443 of potentially cytotoxic organic solvents. Optimized electrospinning parameters, such as solution
444 concentration, CS/PEO ratio, electric field and temperature allowed to obtained homogenous
445 nanofibres without defects. Finally, an innovative ionic crosslinker (DSP) was used to improve
446 the stability of CS electrospun nanofibres in aqueous environment and to neutralize the residual
447 acid present into the CS nanofibres. A one-step DSP crosslinking method was applied to fabricate
448 crosslinked nanofibrous mats without subsequent post-processing steps (as required in the two-
449 step crosslinking method) that could affect the nanofibrous mats morphology (Kiechel & Schauer,
450 2013). The crosslinked nanofibres were deposited as a nonwoven membrane or as a highly aligned
451 bundle mimicking the morphology of different tissue characterized by non-oriented or oriented
452 ECM fibrils. Both uncrosslinked and crosslinked CS-based nanofibres were produced showing
453 fibre diameters in a hundred nanometre range which has been reported to be advantageous for
454 chondrocyte (Bhattacharai, et al., 2005a; Subramanian, Vu, Larsen, & Lin, 2005), human
455 keratinocyte and fibroblast (Noh et al., 2006) as well as for glial cell adhesion and proliferation
456 (Bhattacharai, et al., 2005b; Christopherson, Song, & Mao, 2009). Furthermore, the developed
457 nanofibres showed mechanical properties similar to those of several biological soft tissues such
458 as skin, nerve, muscle. After 7 days in physiological solution, developed nanofibrous mats still

459 show fibrous morphology. Furthermore, the pH values of soaking solution were maintained into
460 physiological range (7-7.4) thanks to the presence of DSP.

461 The biocompatible composition of the developed nanofibrous membranes and their biomimetic
462 structure and mechanical properties were assessed through *in vitro* tests using C2C12 myoblast
463 cell line. Our results demonstrated that the topographical constraint generated by the aligned fibres
464 induced the alignment and elongation of C2C12 by contact guidance, also enabling an adequate
465 proliferation on the surfaces. This cell behaviour is important for skeletal muscle regeneration as
466 a pre-requisite for myotubes formation. The CS based membranes are promising surface for
467 muscular cells proliferation and organization into physiological-like structure. Electrospun
468 membranes lack high spacial interconnectivity as only small pores (1-3 μm) can be obtained. To
469 fabricate a macroporous nanofibrous-based scaffold further post-spinning process are required as
470 recently proposed by Cai et al. (Y. Z. Cai et al., 2012). Further work will be addressed to the
471 application of these membranes in the fabrication of 3D macroporous structures to produce
472 scaffold for soft tissue regeneration.

473

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