

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

*Original*

Non-covalently crosslinked chitosan nanofibrous mats prepared by electrospinning as substrates for soft tissue regeneration / TONDA TURO, C., Ruini, F., Ramella, M., Boccafoschi, F., Gentile, P., Gioffredi, E., Falvo D'Urso Labate, G., Ciardelli, G.. - In: CARBOHYDRATE POLYMERS. - ISSN 0144-8617. - 162:(2017), pp. 82-92.  
[10.1016/j.carbpol.2017.01.050]

*Availability:*

This version is available at: 11583/2665384 since: 2021-04-01T13:42:01Z

*Publisher:*

Elsevier Ltd

*Published*

DOI:10.1016/j.carbpol.2017.01.050

*Terms of use:*

This article is made available under terms and conditions as specified in the corresponding bibliographic description in the repository

*Publisher copyright*

(Article begins on next page)

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

3  
4 Chiara Tonda-Turo<sup>1</sup>, Francesca Ruini<sup>1</sup>, Martina Ramella<sup>2</sup>, Francesca Boccafoschi<sup>2</sup>, Piergiorgio  
5 Gentile<sup>3</sup>, Emilia Gioffredi<sup>1</sup>, Giuseppe Falvo D'Urso Labate<sup>4</sup>, Gianluca Ciardelli<sup>1</sup>

6  
7 <sup>1</sup>Department of Mechanical and Aerospace Engineering, Politecnico di Torino, Corso Duca  
8 degli Abruzzi 24, 10129 Torino, Italy

9 [francesca.ruini@polito.it](mailto:francesca.ruini@polito.it), [emilia.gioffredi@polito.it](mailto:emilia.gioffredi@polito.it), [gianluca.ciardelli@polito.it](mailto:gianluca.ciardelli@polito.it)

10 <sup>2</sup> Department of Health Sciences, University of Piemonte Orientale Novara, Via Solaroli 17,  
11 28100 Novara, Italy

12 [martina.ramella@med.uniupo.it](mailto:martina.ramella@med.uniupo.it), [francesca.boccafoschi@med.uniupo.it](mailto:francesca.boccafoschi@med.uniupo.it)

13 <sup>3</sup> School of Mechanical and Systems Engineering, Newcastle University, Claremont Road,  
14 Newcastle upon Tyne, NE1 7RU, United Kingdom [piergiorgio.gentile@newcastle.ac.uk](mailto:piergiorgio.gentile@newcastle.ac.uk)

15 <sup>4</sup> Biomedical Components s.r.l., Via Calopinace Arg. Dx, 6, 89128 Reggio Calabria , Italy

16 [giuseppe.falvodursolabate@enginlife.com](mailto:giuseppe.falvodursolabate@enginlife.com)

17 [\*] Dr. Chiara Tonda-Turo

18 Department of Mechanical and Aerospace Engineering

19 Politecnico di Torino

20 Corso Duca degli Abruzzi 24, Torino, Italy

21 telephone number: 0039 0903395

22 fax number: 0039 0906999

23 e-mail address: [chiara.tondaturo@polito.it](mailto:chiara.tondaturo@polito.it)

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 ( $118 \pm 16$  nm size)  
33 were obtained through electrospinning process (voltage of 30 kV, 30  $\mu$ 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; Gnani et al., 2015; Gupta et al., 2009; Neal et al., 2012; Qu et al., 2012; Yang,  
84 Murugan, Wang, & Ramakrishna, 2005).

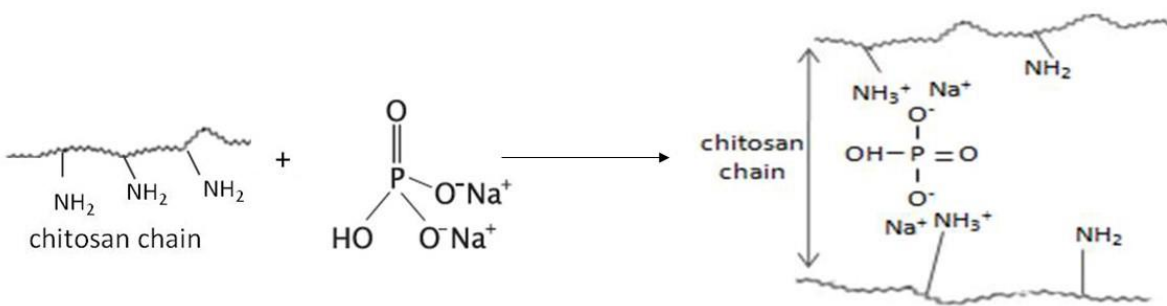
85 The electrospinnability of CS is limited mainly by its polycationic nature in solution, rigid  
86 chemical structure and specific inter and intra-molecular interactions which makes CS solutions  
87 highly viscous at low acid pH and room temperature (Homayoni, Ravandi, & Valizadeh, 2009).

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  $\geq 92.6$  %) 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<sup>-1</sup>).

### 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<sup>-1</sup> to 50 µl min<sup>-1</sup>, (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<sup>-1</sup> with a  
206 resolution of 4 cm<sup>-1</sup> 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).  $2 \times 10^4$  cells/cm<sup>2</sup> 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**

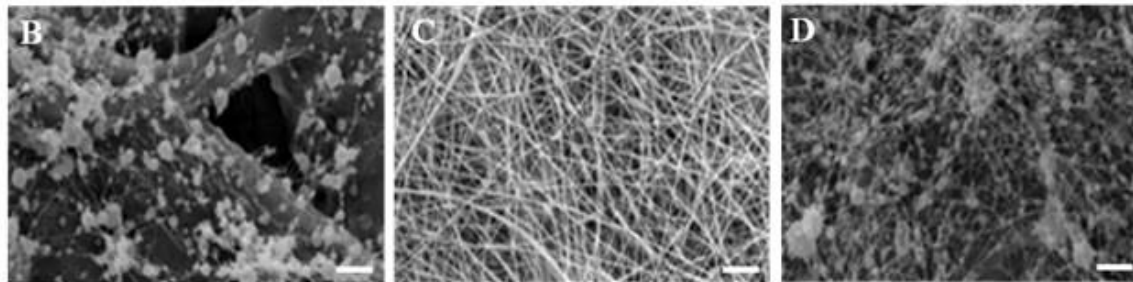
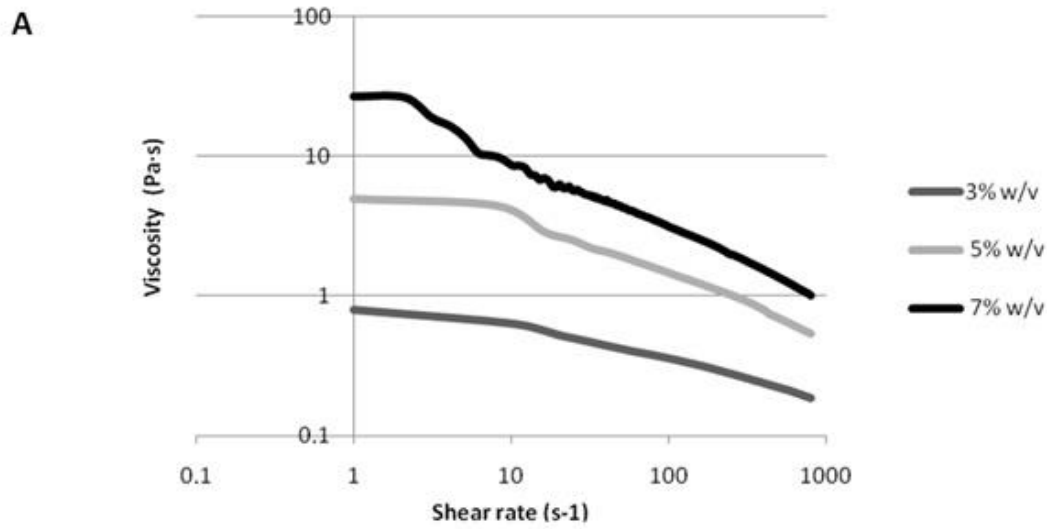
268 **3.1 Optimization of the electrospinning parameters**

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  $\mu$ L/min). Scale bar:

282 2  $\mu$ m.

283

### 284 **3.1.2 Process parameters**

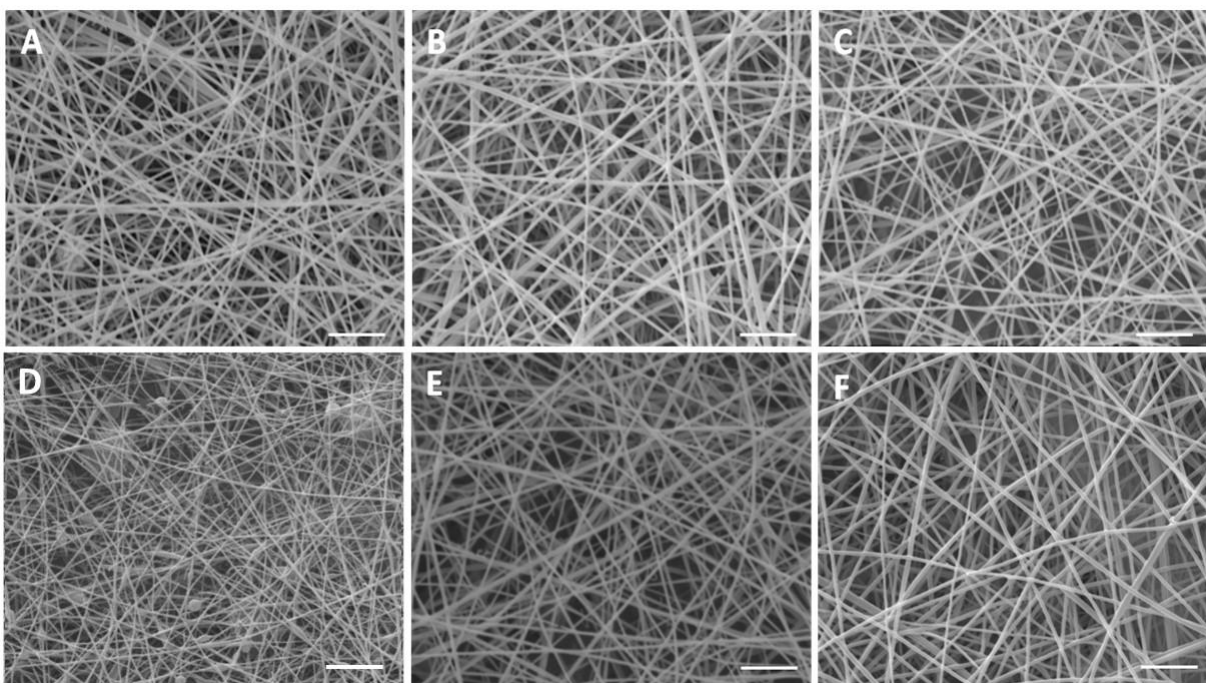
285 The optimization of the process parameters required to vary them in a wide range of sets. For the

286 fabrication of randomly oriented nanofibers, 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

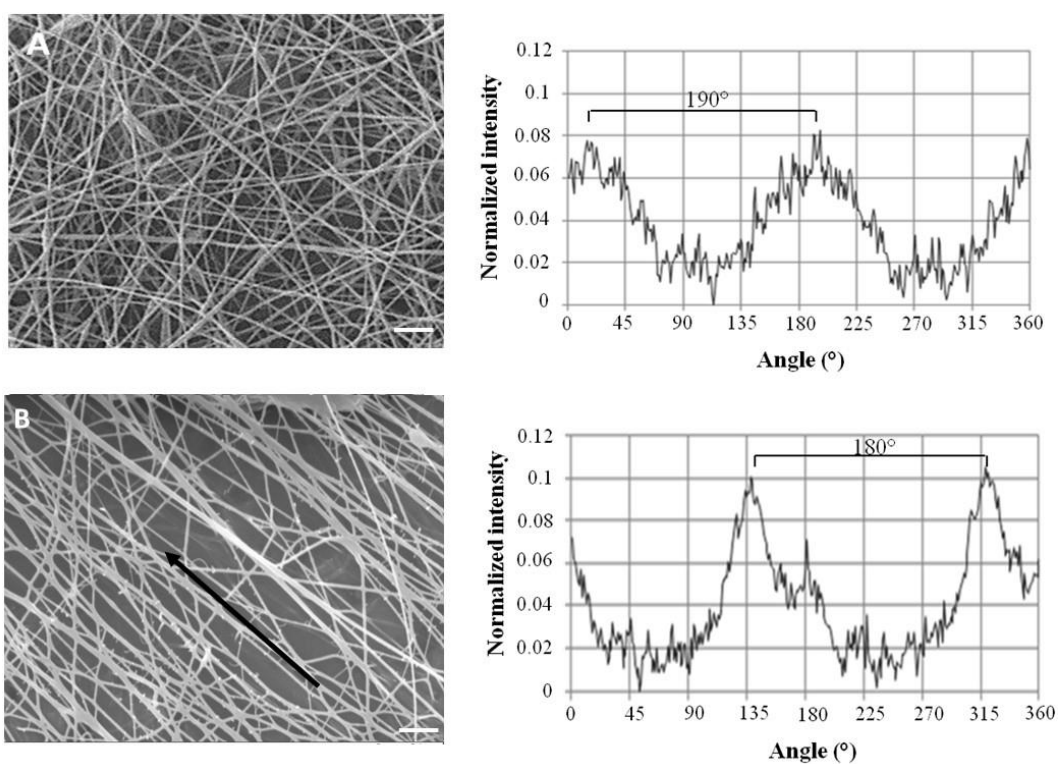
289 the range of 25 to 50  $\mu\text{L}/\text{min}$  and highly homogenous fibres with a diameters of  $118 \pm 16$  nm were  
290 obtained for flow rate of 30  $\mu\text{L}/\text{min}$  (Fig. 3). Concerning temperature, an increase in temperature  
291 allowed to reduce the number of defects on the 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  $\mu\text{m}$ .

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.



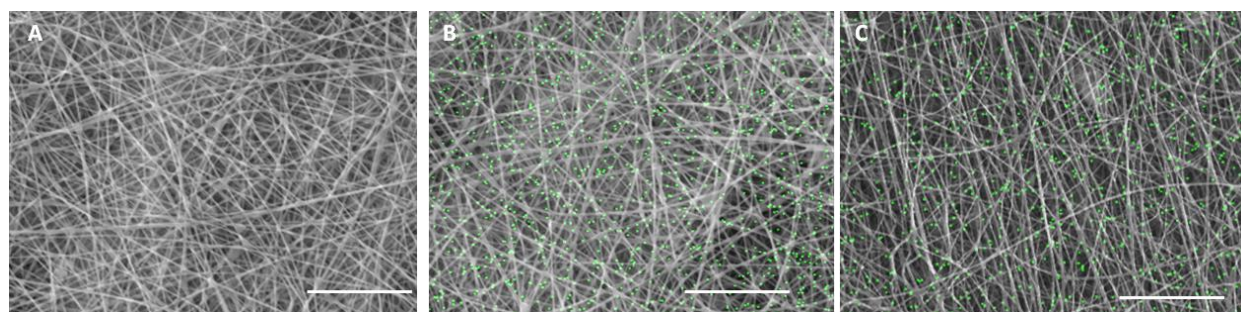
309  
310 **Fig. 4.** SEM micrographs and FTT analysis of nanofibres collected using a rotating mandrel rate  
311 of 300 rpm (A) and 2400 rpm (B). Arrow indicate the fibre alignment direction. Bars 5  $\mu$ m.

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  $\mu$ 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  $128 \pm 19$  nm  
329 and  $140 \pm 41$  nm, respectively, showing comparable values to uncrosslinked CS nanofibrous  
330 samples ( $109 \pm 17$  nm). The pore size was in the range of 1-3  $\mu$ m for both random, aligned,  
331 crosslinked and uncrosslinked membranes. Membrane microporosity guarantees a high number  
332 of site for cell adhesion.

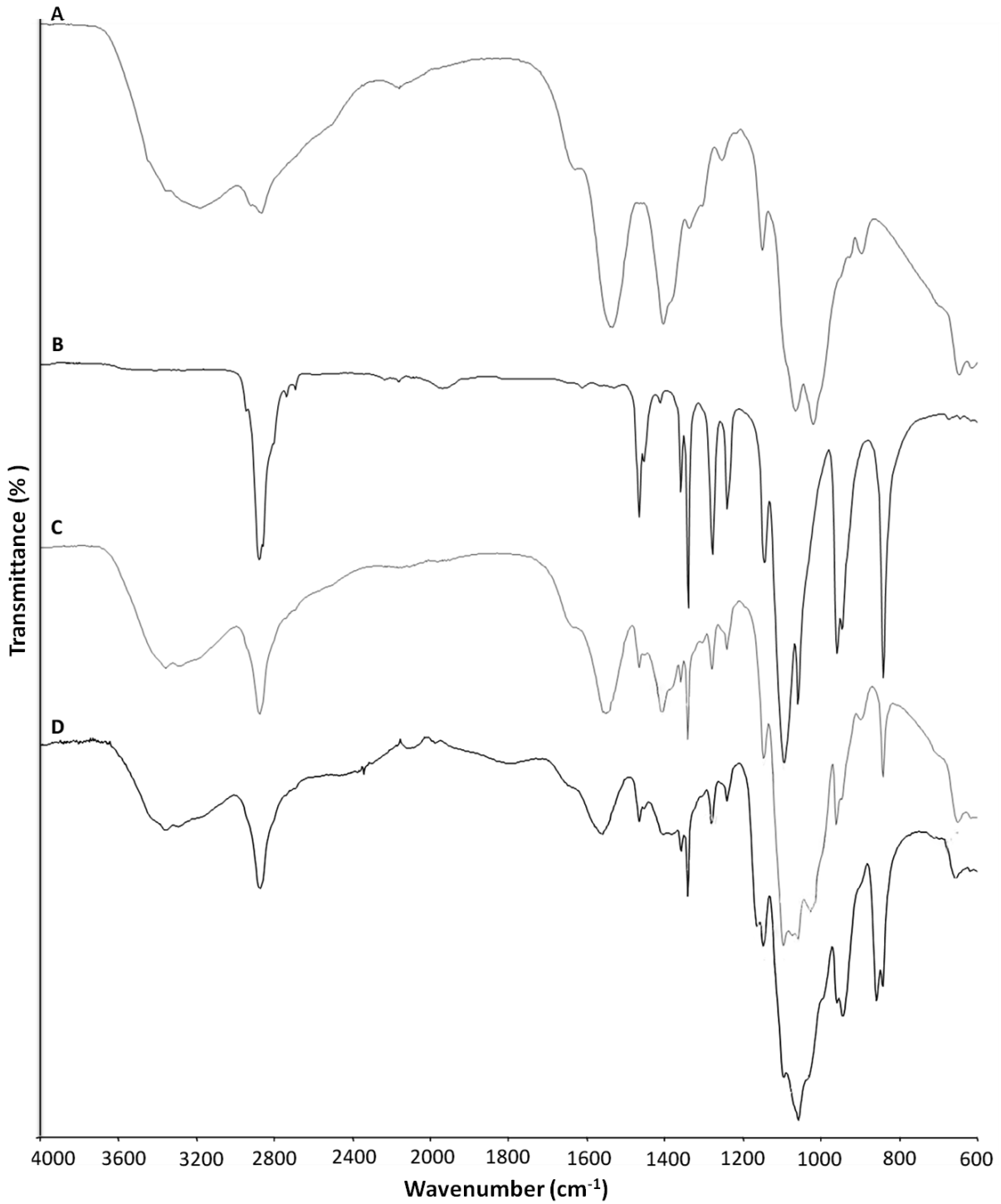


334 **Fig. 5.** EDS elemental mapping and SEM images of uncrosslinked (A) and crosslinked randomly  
335 oriented (B) and aligned (C) CS-based nanofibres. Green spots correspond to phosphorus (P)  
336 elements. Bars 10  $\mu\text{m}$ .

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  $1074\text{ cm}^{-1}$  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  $1059\text{ cm}^{-1}$ ,  $944\text{ cm}^{-1}$  and  $858\text{ cm}^{-1}$   
346 related to  $\text{PO}_3$  stretching ( $\nu_{\text{PO}_3}$ ), 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.

<b>Bond vibration</b>	<b>Wavenumber (cm<sup>-1</sup>)</b>	<b>Material</b>	<b>ref</b>
<b>vO-H</b>	3222	CS	(Rubilar et al., 2013)
<b>vN-H</b>			
<b>vC-H</b>	2883	CS and PEO	(Duan, Dong, Yuan, & Yao, 2004; Ojha et al., 2008)
<b>vC=O</b>	1634	CS	(Duan, et al., 2004; Kjm, Son, Kim, Weller, & Hanna, 2006; Rubilar, et al., 2013)
<b>δN-H</b>	1547	CS	(Duan, et al., 2004; Kjm, et al., 2006; Leceta, Guerrero, & de la Caba, 2013; Rubilar, et al., 2013)
<b>δCH<sub>2</sub></b>	1466	PEO	(Dey, Das, Karan, & De, 2011)
<b>vC-N</b>	1410	CS	(Leceta, et al., 2013)
<b>ωCH<sub>2</sub></b>	1360	PEO	(Dey, et al., 2011)
<b>τCH<sub>2</sub></b>	1280; 1241	PEO	(Dey, et al., 2011)
<b>vC-O-C</b>	114; 1095; 1060	CS and PEO	(Caykara, Demirci, Eroglu, & Guven, 2005; Duan, et al., 2004)
<b>ρCH<sub>2</sub></b>	959; 947	PEO	(Dey, et al., 2011)
<b>δC-O-C</b>	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).

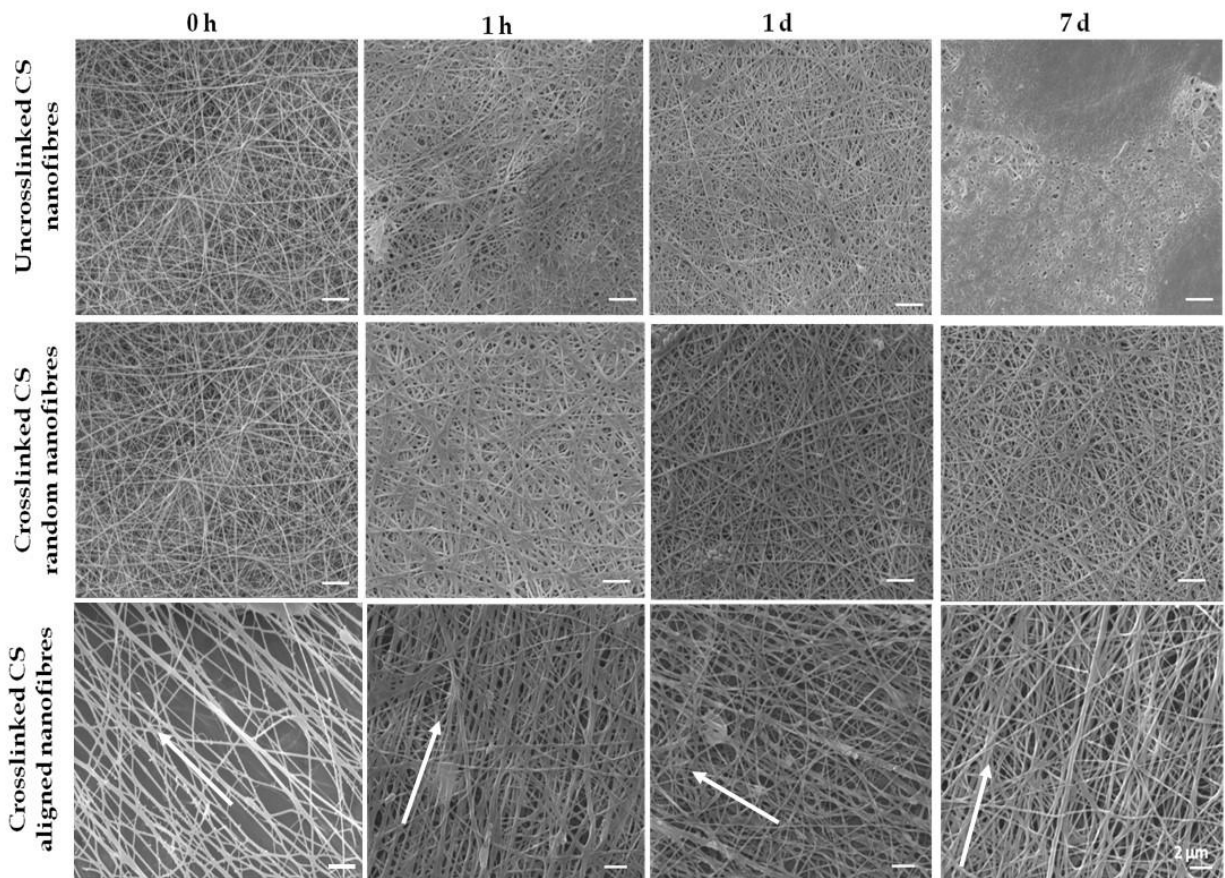
373 Concerning tissue engineering, biomaterial constructs should ideally resemble the *in vivo*  
374 mechanical and structural properties of the tissues that they are intended to replace (Fung 1993  
375 Biomechanics: mechanical properties of living tissues. 2nd ed. New York: Springer).

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  $7.2 \pm 0.2$  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

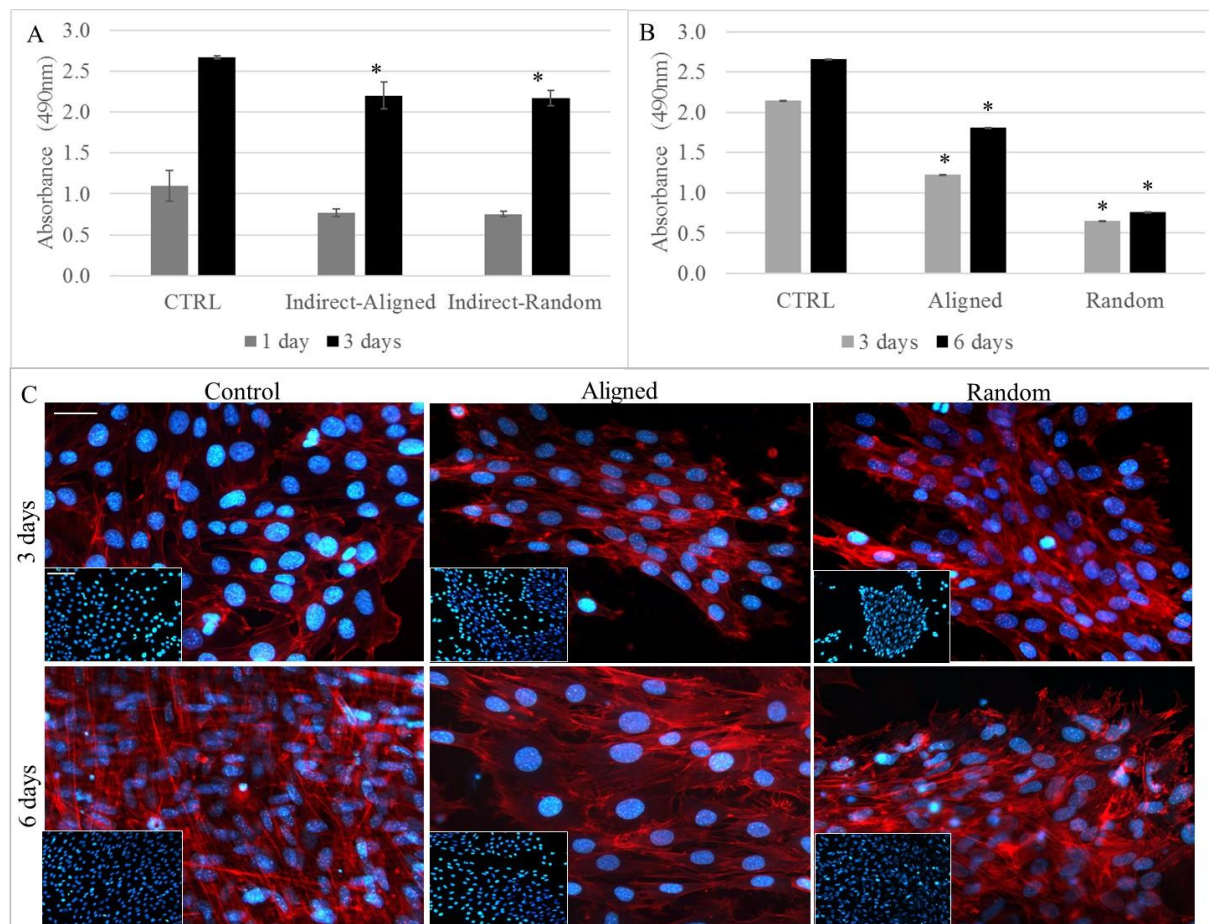
### 397 **3.3 *In vitro* characterization using C2C12 myoblast cells: cell viability and morphology**

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  $\mu\text{m}$  and 20  $\mu\text{m}$  (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  $\mu\text{m}$ ) 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

#### 474 **Acknowledgements**

475 Dr P. Gentile, is member of the UK EPSRC Centre for Innovative Manufacturing of Medical  
476 Devices. Susanna Sartori is acknowledged for SEM analysis.

477

478

479

480

481

482 **References**

- 483 Agarwal, S., Wendorff, J. H., & Greiner, A. (2008). Use of electrospinning technique for  
484 biomedical applications. *Polymer*, 49(26), 5603-5621. doi: DOI  
485 10.1016/j.polymer.2008.09.014
- 486 Amado, S., Simoes, M. J., da Silva, P. A. S. A., Luis, A. L., Shirosaki, Y., Lopes, M. A., . . .  
487 Geuna, S. (2008). Use of hybrid chitosan membranes and N1E-115 cells for promoting  
488 nerve regeneration in an axonotmesis rat model. *Biomaterials*, 29(33), 4409-4419. doi:  
489 DOI 10.1016/j.biomaterials.2008.07.043
- 490 Bhattarai, N., Edmondson, D., Veiseh, O., Matsen, F. A., & Zhang, M. (2005a). Electrospun  
491 chitosan-based nanofibers and their cellular compatibility. *Biomaterials*, 26(31), 6176-  
492 6184. doi: S0142-9612(05)00262-0 [pii]  
493 10.1016/j.biomaterials.2005.03.027
- 494 Bhattarai, N., Edmondson, D., Veiseh, O., Matsen, F. A., & Zhang, M. Q. (2005b). Electrospun  
495 chitosan-based nanofibers and their cellular compatibility. *Biomaterials*, 26(31), 6176-  
496 6184. doi: 10.1016/j.biomaterials.2005.03.027
- 497 Bhattarai, N., Gunn, J., & Zhang, M. Q. (2010). Chitosan-based hydrogels for controlled,  
498 localized drug delivery. *Advanced Drug Delivery Reviews*, 62(1), 83-99. doi: DOI  
499 10.1016/j.addr.2009.07.019
- 500 Cai, Y. Z., Zhang, G. R., Wang, L. L., Jiang, Y. Z., Ouyang, H. W., & Zou, X. H. (2012). Novel  
501 biodegradable three-dimensional macroporous scaffold using aligned electrospun  
502 nanofibrous yarns for bone tissue engineering. *Journal of Biomedical Materials Research*  
503 *Part A*, 100A(5), 1187-1194. doi: 10.1002/jbm.a.34063
- 504 Cai, Z. X., Mo, X. M., Zhang, K. H., Fan, L. P., Yin, A. L., He, C. L., & Wang, H. S. (2010).  
505 Fabrication of Chitosan/Silk Fibroin Composite Nanofibers for Wound-dressing  
506 Applications. *International Journal of Molecular Sciences*, 11(9), 3529-3539. doi: Doi  
507 10.3390/Ijms11093529
- 508 Caykara, T., Demirci, S., Eroglu, M. S., & Guven, O. (2005). Poly(ethylene oxide) and its blends  
509 with sodium alginate. *Polymer*, 46(24), 10750-10757. doi: DOI  
510 10.1016/j.polymer.2005.09.041
- 511 Charernsriwilaiwat, N., Opanasopit, P., Rojanarata, T., Ngawhirunpat, T., & Supaphol, P. (2010).  
512 Preparation and characterization of chitosan-hydroxybenzotriazole/polyvinyl alcohol  
513 blend nanofibers by the electrospinning technique. *Carbohydrate Polymers*, 81(3), 675-  
514 680. doi: DOI 10.1016/j.carbpol.2010.03.031
- 515 Chen, L., Zhu, C., Fan, D., Liu, B., Ma, X., Duan, Z., & Zhou, Y. (2011). A human-like  
516 collagen/chitosan electrospun nanofibrous scaffold from aqueous solution: electrospun  
517 mechanism and biocompatibility. [Research Support, Non-U.S. Gov't]. *Journal of*  
518 *Biomedical Materials Research Part A*, 99(3), 395-409. doi: 10.1002/jbm.a.33202
- 519 Chen, Z. G., Mo, X. M., & Qing, F. L. (2007). Electrospinning of collagen-chitosan complex.  
520 *Materials Letters*, 61(16), 3490-3494. doi: DOI 10.1016/j.matlet.2006.11.104
- 521 Chen, Z. G., Wang, P. W., Wei, B., Mo, X. M., & Cui, F. Z. (2010). Electrospun collagen-chitosan  
522 nanofiber: a biomimetic extracellular matrix for endothelial cell and smooth muscle cell.  
523 [Research Support, Non-U.S. Gov't]. *Acta Biomaterialia*, 6(2), 372-382. doi:  
524 10.1016/j.actbio.2009.07.024
- 525 Choi, J. S., Lee, S. J., Christ, G. J., Atala, A., & Yoo, J. J. (2008). The influence of electrospun  
526 aligned poly(epsilon-caprolactone)/collagen nanofiber meshes on the formation of self-

527 aligned skeletal muscle myotubes. *Biomaterials*, 29(19), 2899-2906. doi: DOI  
528 10.1016/j.biomaterials.2008.03.031

529 Christopherson, G. T., Song, H., & Mao, H. Q. (2009). The influence of fiber diameter of  
530 electrospun substrates on neural stem cell differentiation and proliferation. [Research  
531 Support, U.S. Gov't, Non-P.H.S.]. *Biomaterials*, 30(4), 556-564. doi:  
532 10.1016/j.biomaterials.2008.10.004

533 Cooper, A., Bhattarai, N., & Zhang, M. (2011). Fabrication and cellular compatibility of aligned  
534 chitosan–PCL fibers for nerve tissue regeneration. *Carbohydrate Polymers*, 85(1), 149–  
535 156.

536 Corey, J. M., Lin, D. Y., Mycek, K. B., Chen, Q., Samuel, S., Feldman, E. L., & Martin, D. C.  
537 (2007). Aligned electrospun nanofibers specify the direction of dorsal root ganglia neurite  
538 growth. *Journal of Biomedical Materials Research Part A*, 83A(3), 636-645. doi: Doi  
539 10.1002/Jbm.A.31285

540 Dey, A., Das, K., Karan, S., & De, S. K. (2011). Vibrational spectroscopy and ionic conductivity  
541 of polyethylene oxide-NaClO<sub>4</sub>-CuO nanocomposite. *Spectrochimica Acta Part a-  
542 Molecular and Biomolecular Spectroscopy*, 83(1), 384-391. doi: DOI  
543 10.1016/j.saa.2011.08.050

544 Duan, B., Dong, C. H., Yuan, X. Y., & Yao, K. D. (2004). Electrospinning of chitosan solutions  
545 in acetic acid with poly(ethylene oxide). *Journal of Biomaterials Science-Polymer  
546 Edition*, 15(6), 797-811. doi: Doi 10.1163/156856204774196171

547 Duan, B., Yuan, X. Y., Zhu, Y., Zhang, Y. Y., Li, X. L., Zhang, Y., & Yao, K. D. (2006). A  
548 nanofibrous composite membrane of PLGA-chitosan/PVA prepared by electrospinning.  
549 *European Polymer Journal*, 42(9), 2013-2022. doi: DOI  
550 10.1016/j.eurpolymj.2006.04.021

551 Fregnan, F., Ciglieri, E., Tos, P., Crosio, A., Ciardelli, G., Ruini, F., . . . Raimondo, S. (2016).  
552 Chitosan crosslinked flat scaffolds for peripheral nerve regeneration. *Biomedical  
553 Materials*, 11(4). doi: Artn 045010  
554 10.1088/1748-6041/11/4/045010

555 Ghasemi-Mobarakeh, L., Prabhakaran, M. P., Morshed, M., Nasr-Esfahani, M. H., &  
556 Ramakrishna, S. (2008). Electrospun poly(epsilon-caprolactone)/gelatin nanofibrous  
557 scaffolds for nerve tissue engineering. *Biomaterials*, 29(34), 4532-4539. doi: DOI  
558 10.1016/j.biomaterials.2008.08.007

559 Gnavi, S., Fornasari, B. E., Tonda-Turo, C., Laurano, R., Zanetti, M., Ciardelli, G., & Geuna, S.  
560 (2015). The Effect of Electrospun Gelatin Fibers Alignment on Schwann Cell and Axon  
561 Behavior and Organization in the Perspective of Artificial Nerve Design. *International  
562 Journal of Molecular Sciences*, 16(6), 12925-12942. doi: 10.3390/ijms160612925

563 Gupta, D., Venugopal, J., Prabhakaran, M. P., Dev, V. R. G., Low, S., Choon, A. T., &  
564 Ramakrishna, S. (2009). Aligned and random nanofibrous substrate for the in vitro culture  
565 of Schwann cells for neural tissue engineering. *Acta Biomaterialia*, 5(7), 2560-2569. doi:  
566 DOI 10.1016/j.actbio.2009.01.039

567 Homayoni, H., Ravandi, S. A. H., & Valizadeh, M. (2009). Electrospinning of chitosan  
568 nanofibers: Processing optimization. *Carbohydrate Polymers*, 77(3), 656-661. doi: DOI  
569 10.1016/j.carbpol.2009.02.008

570 Howling, G. I., Dettmar, P. W., Goddard, P. A., Hampson, F. C., Dornish, M., & Wood, E. J.  
571 (2001). The effect of chitin and chitosan on the proliferation of human skin fibroblasts and

572 keratinocytes in vitro. *Biomaterials*, 22(22), 2959-2966. doi: Doi 10.1016/S0142-  
 573 9612(01)00042-4  
 574 Hung, T. K., Chang, G. L., Lin, H. S., Walter, F. R., & Bunegin, L. (1981). Stress-Strain  
 575 Relationship of the Spinal-Cord of Anesthetized Cats. *J Biomech*, 14(4), 269-276. doi: Doi  
 576 10.1016/0021-9290(81)90072-5  
 577 Jha, B. S., Colello, R. J., Bowman, J. R., Sell, S. A., Lee, K. D., Bigbee, J. W., . . . Simpson, D.  
 578 G. (2011). Two pole air gap electrospinning: Fabrication of highly aligned, three-  
 579 dimensional scaffolds for nerve reconstruction. *Acta Biomaterialia*, 7(1), 203-215. doi:  
 580 DOI 10.1016/j.actbio.2010.08.004  
 581 Kang, Y. M., Lee, B. N., Ko, J. H., Kim, G. H., Kang, K. N., Kim, D. Y., . . . Kim, M. S. (2010).  
 582 In Vivo Biocompatibility Study of Electrospun Chitosan Microfiber for Tissue  
 583 Engineering. *International Journal of Molecular Sciences*, 11(10), 4140-4148. doi:  
 584 10.3390/ijms11104140  
 585 Kiechel, M. A., & Schauer, C. L. (2013). Non-covalent crosslinkers for electrospun chitosan  
 586 fibers. *Carbohydrate Polymers*, 95(1), 123-133. doi: 10.1016/j.carbpol.2013.02.034  
 587 Kim, S., Nishimoto, S. K., Bumgardner, J. D., Haggard, W. O., Gaber, M. W., & Yang, Y. Z.  
 588 (2010). A chitosan/beta-glycerophosphate thermo-sensitive gel for the delivery of ellagic  
 589 acid for the treatment of brain cancer. *Biomaterials*, 31(14), 4157-4166. doi:  
 590 10.1016/j.biomaterials.2010.01.139  
 591 Kjm, K. M., Son, J. H., Kim, S. K., Weller, C. L., & Hanna, M. A. (2006). Properties of chitosan  
 592 films as a function of pH and solvent type. *Journal of Food Science*, 71(3), E119-E124.  
 593 Koh, H. S., Yong, T., Chan, C. K., & Ramakrishna, S. (2008). Enhancement of neurite outgrowth  
 594 using nano-structured scaffolds coupled with laminin. *Biomaterials*, 29(26), 3574-3582.  
 595 doi: DOI 10.1016/j.biomaterials.2008.05.014  
 596 Larkin, P. (2011). *Infrared and Raman Spectroscopy; Principles and Spectral Interpretation* (1st  
 597 ed.): Elsevier.  
 598 Leceta, I., Guerrero, P., & de la Caba, K. (2013). Functional properties of chitosan-based films.  
 599 *Carbohydrate Polymers*, 93(1), 339-346. doi: DOI 10.1016/j.carbpol.2012.04.031  
 600 Li, G. C., Zhang, L. Z., Wang, C. P., Zhao, X. Y., Zhu, C. L., Zheng, Y. H., . . . Yang, Y. M.  
 601 (2014). Effect of silanization on chitosan porous scaffolds for peripheral nerve  
 602 regeneration. *Carbohydrate Polymers*, 101, 718-726. doi: DOI  
 603 10.1016/j.carbpol.2013.09.064  
 604 Markarian, S. A., Gabrielyan, L. S., & Grigoryan, K. R. (2004). FT IR ATR study of molecular  
 605 interactions in the urea/dimethyl sulfoxide and urea/diethyl sulfoxide binary systems.  
 606 *Journal of Solution Chemistry*, 33(8), 1005-1015. doi: Doi  
 607 10.1023/B:Josl.0000048050.47474.Fc  
 608 Mow, V., & Guo, X. E. (2002). Mechano-electrochemical properties of articular cartilage: Their  
 609 inhomogeneities and anisotropies. *Annual Review of Biomedical Engineering*, 4, 175-209.  
 610 doi: 10.1146/annurev.bioeng.4.110701.120309  
 611 Murakami, K., Aoki, H., Nakamura, S., Nakamura, S., Takikawa, M., Hanzawa, M., . . . Ishihara,  
 612 M. (2010). Hydrogel blends of chitin/chitosan, fucoidan and alginate as healing-impaired  
 613 wound dressings. *Biomaterials*, 31(1), 83-90. doi: DOI  
 614 10.1016/j.biomaterials.2009.09.031  
 615 Muzzarelli, R. A. A. (2009). Chitins and chitosans for the repair of wounded skin, nerve, cartilage  
 616 and bone. *Carbohydrate Polymers*, 76(2), 167-182. doi: DOI  
 617 10.1016/j.carbpol.2008.11.002

618 Neal, R. A., Tholpady, S. S., Foley, P. L., Swami, N., Ogle, R. C., & Botchwey, E. A. (2012).  
619 Alignment and composition of laminin-polycaprolactone nanofiber blends enhance  
620 peripheral nerve regeneration. *Journal of Biomedical Materials Research Part A*, *100A*(2),  
621 406-423. doi: Doi 10.1002/Jbm.A.33204

622 Noh, H. K., Lee, S. W., Kim, J. M., Oh, J. E., Kim, K. H., Chung, C. P., . . . Min, B. M. (2006).  
623 Electrospinning of chitin nanofibers: Degradation behavior and cellular response to  
624 normal human keratinocytes and fibroblasts. *Biomaterials*, *27*(21), 3934-3944. doi:  
625 10.1016/j.biomaterials.2006.03.016

626 Ojha, S. S., Stevens, D. R., Hoffman, T. J., Stano, K., Klossner, R., Scott, M. C., . . . Gorga, R. E.  
627 (2008). Fabrication and characterization of electrospun chitosan nanofibers formed via  
628 templating with polyethylene oxide. *Biomacromolecules*, *9*(9), 2523-2529. doi: Doi  
629 10.1021/Bm800551q

630 Park, J. H., Saravanakumar, G., Kim, K., & Kwon, I. C. (2010). Targeted delivery of low  
631 molecular drugs using chitosan and its derivatives. *Advanced Drug Delivery Reviews*,  
632 *62*(1), 28-41. doi: DOI 10.1016/j.addr.2009.10.003

633 Park, W. H., Jeong, L., Yoo, D. I., & Hudson, S. (2004). Effect of chitosan on morphology and  
634 conformation of electrospun silk fibroin nanofibers. *Polymer (Guildf)*, *45*(21), 7151-7157.  
635 doi: DOI 10.1016/j.polymer.2004.08.045

636 Qu, J., Zhou, D. D., Xu, X. J., Zhang, F., He, L. H., Ye, R., . . . Zhang, H. X. (2012). Optimization  
637 of electrospun TSF nanofiber alignment and diameter to promote growth and migration of  
638 mesenchymal stem cells. *Applied Surface Science*, *261*, 320-326. doi: DOI  
639 10.1016/j.apsusc.2012.08.008

640 Rubilar, J. F., Cruz, R. M. S., Silva, H. D., Vicente, A. A., Khmelinskii, I., & Vieira, M. C. (2013).  
641 Physico-mechanical properties of chitosan films with carvacrol and grape seed extract.  
642 *Journal of Food Engineering*, *115*(4), 466-474. doi: DOI 10.1016/j.jfoodeng.2012.07.009

643 Ruini, F. (2015). *Chitosan based biomaterials: soft tissue engineering applications*. Doctoral  
644 Thesis, Politecnico di Torino, Turin.

645 Ruini, F., Tonda-Turo, C., Chiono, V., & Ciardelli, G. (2015). Chitosan membranes for tissue  
646 engineering: comparison of different crosslinkers. *Biomedical Materials*, *10*. doi:  
647 10.1088/1748-6041/10/6/065002

648 Sarkar, S. D., Farrugia, B. L., Dargaville, T. R., & Dhara, S. (2013). Physico-chemical/biological  
649 properties of tripolyphosphate cross-linked chitosan based nanofibers. *Materials Science  
650 & Engineering C-Materials for Biological Applications*, *33*(3), 1446-1454. doi: DOI  
651 10.1016/j.msec.2012.12.066

652 Seol, Y. J., Lee, J. Y., Park, Y. J., Lee, Y. M., Young-Ku, Rhyu, I. C., . . . Chung, C. P. (2004).  
653 Chitosan sponges as tissue engineering scaffolds for bone formation. *Biotechnology  
654 Letters*, *26*(13), 1037-1041. doi: Doi 10.1023/B:Bile.0000032962.79531.Fd

655 Subramanian, A., Vu, D., Larsen, G. F., & Lin, H. Y. (2005). Preparation and evaluation of the  
656 electrospun chitosan/PEO fibers for potential applications in cartilage tissue engineering.  
657 *Journal of Biomaterials Science-Polymer Edition*, *16*(7), 861-873. doi: Doi  
658 10.1163/1568562054255682

659 Tonda-Turo, C., Cipriani, E., Gnani, S., Chiono, V., Mattu, C., Gentile, P., . . . Ciardelli, G.  
660 (2013a). Cross linked gelatin nanofibres: Preparation, characterisation and in vitro studies  
661 using glial-like cells. *Materials Science & Engineering C-Materials for Biological  
662 Applications*, *33*(5), 2723-2735. doi: 10.1016/j.msec.2013.02.039

663 Tonda-Turo, C., Cipriani, E., Gnani, S., Chiono, V., Mattu, C., Gentile, P., . . . Ciardelli, G.  
664 (2013b). Crosslinked gelatin nanofibres: preparation, characterisation and in vitro studies  
665 using glial-like cells. [Research Support, Non-U.S. Gov't]. *Mater Sci Eng C Mater Biol*  
666 *Appl*, 33(5), 2723-2735. doi: 10.1016/j.msec.2013.02.039  
667 Wu, H. J., Fan, J. T., Chu, C. C., & Wu, J. (2010). Electrospinning of small diameter 3-D  
668 nanofibrous tubular scaffolds with controllable nanofiber orientations for vascular grafts.  
669 *Journal of Materials Science-Materials in Medicine*, 21(12), 3207-3215. doi: DOI  
670 10.1007/s10856-010-4164-8  
671 Yang, F., Murugan, R., Wang, S., & Ramakrishna, S. (2005). Electrospinning of nano/micro scale  
672 poly(L-lactic acid) aligned fibers and their potential in neural tissue engineering.  
673 *Biomaterials*, 26(15), 2603-2610. doi: DOI 10.1016/j.biomaterials.2004.06.051  
674  
675