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The Interaction of Wood Nanocellulose Dressings and the Wound Pathogen *P. aeruginosa*

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29 **ABSTRACT**

30 Chronic wounds pose an increasingly significant worldwide economic burden (over
31 £1 billion per annum in the UK alone). With the escalation in global obesity and
32 diabetes, chronic wounds will increasingly be a significant cause of morbidity and
33 mortality. Cellulose nanofibrils (CNF) are highly versatile and can be tailored with
34 specific physical properties to produce an assortment of three-dimensional structures
35 (hydrogels, aerogels or films), for subsequent utilization as wound dressing materials.
36 Growth curves using CNF (diameter <20 nm) in suspension demonstrated an
37 interesting dose-dependent inhibition of bacterial growth. In addition, analysis of
38 biofilm formation (*Pseudomonas aeruginosa* PAO1) on nanocellulose aerogels (20
39 g/m²) revealed significantly less biofilm biomass with decreasing aerogel porosity and
40 surface roughness. Importantly, virulence factor production by *P. aeruginosa* in the
41 presence of nanocellulose materials, quantified for the first time, was unaffected
42 (p>0.05) over 24 h. These data demonstrate the potential of nanocellulose materials
43 in the development of novel dressings that may afford significant clinical potential.

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47 **1. Introduction**

48 Normal wound healing follows a defined process involving; coagulation,
49 inflammation, cell proliferation, matrix repair, epithelialization and remodeling
50 (Bjarnsholt et al., 2008). In contrast, chronic wounds are typically characterized by
51 elevated inflammatory responses and tissue breakdown, and are slow or fail to heal,
52 causing a considerable reduction in patient 'quality-of-life'. With a globally ageing
53 population, the biomedical and socioeconomic burdens of chronic wounds are
54 worsening, with annual costs in the US estimated at > \$50 billion per annum and
55 affecting 6.5 million people (2% of the population) (Jung et al., 2016). Chronic
56 wounds are always colonized with bacteria, and the pathogen *Pseudomonas*
57 *aeruginosa* is implicated in non-healing wounds (Davies et al., 2004). The ability of
58 these opportunistic pathogens to produce a thick, mucoid wound biofilm is thought to
59 prevent an effective response by host immune defenses, thereby impairing wound
60 healing (Percival et al., 2012).

61 The most abundant naturally-occurring polymer is cellulose, which can be
62 obtained from a variety of sources including: wood, non-woody plants, agricultural
63 residues, algae and bacteria. In addition to bacterial cellulose (Czaja, Krystynowicz,
64 Bielecki & Brown, 2006; Petersen & Gatenholm, 2011; Portal, Clark & Levinson,
65 2009), nanocellulose from wood pulp has been proposed as an appropriate material
66 for wound dressing applications (Chinga-Carrasco & Syverud, 2014; Powell et al.,
67 2016; Rees et al., 2015). Nanocellulose can be produced in large quantities using
68 effective chemical pre-treatments, which facilitate deconstruction of the wood fiber
69 wall into cellulose nanofibrils (CNF) (Chinga-Carrasco & Syverud, 2014; Saito,
70 Nishiyama, Putaux, Vignon & Isogai, 2006; Wågberg et al., 2008). TEMPO-
71 mediated oxidation is one of the most applied procedures for CNF production, leading
72 to the introduction of carboxyl groups in the C6 position and small numbers of
73 aldehyde groups (Saito & Isogai, 2004; Saito et al., 2006). TEMPO-mediated
74 oxidation produces morphologically homogeneous CNF, having diameters less than
75 20 nm and lengths in the micrometer scale (Chinga-Carrasco, Yu & Diserud, 2011;
76 Saito & Isogai, 2004). Furthermore, this material can be used to produce an
77 assortment of three-dimensional structures e.g. strong, dense and smooth films
78 (Fukuzumi, Saito, Iwata, Kumamoto & Isogai, 2009) and structured hydrogels and
79 aerogels with high porosity, with capacity to absorb large quantities of moisture
80 (Chinga-Carrasco & Syverud, 2014; Syverud, Kirsebom, Hajizadeh & Chinga-

81 Carrasco, 2011). We recently developed a method to produce CNF from *P. radiata*
82 fibers, with low endotoxin levels (<50 endotoxin units/g cellulose) (Nordli, Chinga-
83 Carrasco, Rokstad & Pukstad, 2016) and demonstrated that TEMPO CNF exhibits no
84 toxicity towards 3T3 cells (Alexandrescu, Syverud, Gatti & Chinga-Carrasco, 2013)
85 and primary human skin cells i.e. fibroblasts or keratinocytes (Nordli et al., 2016;
86 Tehrani, Nordli, Pukstad, Gethin & Chinga-Carrasco, 2016). These studies suggested
87 the potential of these materials for wound dressing applications (Nordli et al., 2016).

88 With the increasing economic burden chronic wounds are placing on
89 worldwide healthcare systems, the use of non-toxic, biodegradable biopolymers from
90 an abundant, sustainable source, such as wood CNF, for wound healing applications
91 would be a distinct advantage in our current ‘throw-away’ culture. Many
92 commercially available wound dressings possess a high capacity to absorb moisture
93 due to their porous, three-dimensional structure. However, little attention has been
94 paid of the effect of CNF materials (also with high moisture absorption capabilities)
95 on biofilm formation of typical wound pathogens.

96 This study assessed (i) the effect of CNF hydrogels, at varying concentrations
97 of CNF dispersion, on *Pseudomonas aeruginosa* (PAO1) growth in suspension, (ii)
98 the effect of surface and bulk structure of CNF dressings on biofilm formation of
99 PAO1 and, (iii) for the first time, the effects of these porous structures on
100 pseudomonal virulence factor production.

101

102 **2. Materials and Methods**

103 *2.1. CNF production*

104 *Pinus radiata* pulp fibers were used as the raw material for CNF production.
105 The carbohydrate composition of the pulp fibers has previously been reported
106 (Chinga-Carrasco et al., 2012). TEMPO (2,2,6,6-tetramethylpiperidiny1-1-oxyl)
107 mediated oxidation was applied as a pretreatment, using 3.8 mM NaClO/g cellulose,
108 pH 10.5 (Saito et al., 2006). Oxidized fibers (1% w/v) were fibrillated following
109 three passes through a Rannie 15 type 12.56X homogenizer. The degree of
110 polymerization (DP 709), aldehyde groups (71 μ M/g) and carboxyl groups (855
111 μ M/g) have previously been quantified (Rees et al., 2015).

112

113

114 *2.2. Sample Preparation*

115 CNF samples were air- or freeze-dried to produce tight (films) or porous
116 structures (aerogels), respectively. Freeze-dried samples were prepared at 0.2, 0.4 or
117 0.6% (w/v) CNF dispersions, frozen in petri dishes at -20°C and freeze-dried for 48 h.
118 Although differing CNF concentrations were used in the liquid dispersions, the actual
119 grammage for all the resulting dried films and aerogels was 20 g/m². All the samples
120 were cut into 1 or 2 cm² sections and sterilized by γ -irradiation (15 kGy).
121 Commercially-available wound dressings AquaCel[®] and AquaCel Ag[®] (ConvaTec
122 Ltd, Deeside) were used as controls.

123

124 *2.3. Absorption Measurements*

125 Fluid absorption of the test and control materials was assessed as previously
126 described (Fulton et al., 2012). Dressing materials were cut into 1 cm² squares and
127 weighed to the nearest 0.001 g. Samples were submerged in phosphate buffer saline,
128 pH 7.4 (PBS) for 24 h, covered to prevent evaporation. Samples were then removed
129 from the PBS and allowed to drip for 30 s and a wet weight obtained. Fluid
130 absorption (g/g) = weight of the fluid absorbed/dressing dry weight.

131

132 *2.4. Taxonomy of the samples*

133 The varying percentages of CNF (wet weight) accounted for varying degrees of
134 porosity in the dried materials. Once dried, there was no difference in the
135 concentration of CNF incorporated in each of the prototype samples (20 g/m²; Table
136 1).

137

138 **Table 1**

139 Characteristics of the nanocellulose (CNF) materials used in this study.

Sample name	Concentration of CNF dispersion (w/v)	Sample preparation (resulting material) ^a
A0.2	0.2%	Freeze-dried (aerogel)
A0.4	0.4%	Freeze-dried (aerogel)
A0.6	0.6%	Freeze-dried (aerogel)
Film	0.2%	Air-dried (film)

140 ^aGrammage of all final dried materials = 20 g/m²

141

142 2.5. Screening for the ability of CNF to support bacterial growth.

143 Bacterial growth and carbon utilization studies were undertaken using CNF
144 dispersions. CNF dispersions were sterilized by γ -irradiation, and then diluted in
145 deionized sterilized water to a final CNF concentration of 0.2, 0.4, 0.6 and 0.8%. The
146 ability of CNF to inhibit or promote growth of *P. aeruginosa* PAO1 in planktonic
147 culture was examined. Overnight cultures of PAO1 were diluted to OD₆₀₀ 0.08
148 (colony forming units = 1×10^8) in either MH broth, PBS or deionized water, and
149 mixed 1:2 (v/v) with CNF dispersion or water in a 24 well plate. Plates were
150 incubated at 37°C aerobically for 24 h measuring OD₆₀₀ every hour at 600 nm (OD₆₀₀)
151 in a FLUOstar Optima plate reader (BMG LABTECH).

152

153 2.6. Log₁₀ reduction assay

154 Time kill assays adapted from Ong, Wu, Moochhala, Tan, & Lu (2008) were
155 used to evaluate the antimicrobial efficacy of aerogel and film materials in
156 comparison to a commercially available wound dressing (AquaCel[®]) control. CNF
157 samples or AquaCel (Ag)[®] were added to a 6-well plate; 2×2 cm² per well in total.
158 MH-broth (6 ml) and 60 μ l of PAO1 overnight culture adjusted to OD₆₀₀ 1.0 were
159 then added to each well. Plates were incubated at 37°C, 24 h (aerobically), with
160 shaking. To enumerate bacterial growth, 10 μ l drops (3 x 10 μ l per dilution) of
161 bacterial suspension was removed from each dilution and dropped onto the surface of
162 an MH-agar plate. The drops were air-dried before being incubated overnight at 37°C
163 to enumerate colony forming units (CFU/ml). Log reduction was calculated as log₁₀
164 CFU/ml (initial bacteria upon challenge) minus log₁₀ CFU/ml (surviving bacteria at
165 time point after challenge). Bactericidal activity was defined as a ≥ 3 log₁₀ CFU/ml
166 (equivalent to $\geq 99.9\%$) reduction in bacterial numbers.

167

168 2.7. Assessment of virulence factors

169 Overnight cultures of *P. aeruginosa* PAO1 were adjusted to OD₆₀₀ 1.0, and 60 μ l
170 were added to MH-broth (6 ml) in a 6-well plate and grown for 24 h \pm CNF samples
171 or AquaCel (Ag)[®] (2×2 cm²). Bacterial cultures were then centrifuged (10000 g) for
172 10 min to produce a cell-free culture supernatant, used for extraction of virulence
173 factors.

174 Pyocyanin pigment was extracted from the cell-free supernatant using chloroform
175 (3:2; v/v). Pyocyanin (in the chloroform-phase) was re-extracted with 0.2 M HCl
176 (2:1; v/v) and the absorbance read at 540 nm (Sarabhai, Sharma & Capalash, 2013).

177 Rhamnolipids were extracted from the cell-free supernatant using ethyl acetate
178 (1:1, v/v), vortexed for 15 sec and centrifuged (10000 g, 4°C, 5 min). The top layer
179 was removed and the extraction repeated (x2) before allowing the ethyl acetate to
180 evaporate overnight. Deionized water was used to dissolve the precipitate and orcinol
181 reagent (0.19% orcinol in 53% H₂SO₄; 1:9, v/v) added. The sample was incubated at
182 80°C for 30 min before reading the absorbance at 421 nm (Smyth, Perfumo, McClean,
183 Marchant & Banat, 2010).

184 Protease activity was determined using 2% azocasein solution in 50 mM PBS, pH
185 7. The azocasein solution was incubated with the cell-free supernatant (1:1; v/v) in a
186 total reaction volume of 400 µl, for 1 h at 37 °C. The reaction was stopped by the
187 addition of 500 µl 10% trichloroacetic acid, and residual azocasein removed by
188 centrifugation (8000 g, 5 min). Protease absorbance was measured at 400 nm
189 (Adonizio, Kong & Mathee, 2008).

190 For elastase determination, cell-free supernatant was mixed (3:1; v/v) with
191 elastin-congo-red solution (5 mg/ml in 0.1 M Tris-HCl pH 8; 1 mM CaCl₂). Samples
192 were incubated at 37 °C, for up to 16 h, at 200 rpm, centrifuged at 3000 g (10 min)
193 before absorbance was read at 490 nm (Sarabhai et al., 2013).

194

195 2.8. *Microscopy characterization of CNF structures*

196 Copper grids were immersed in a 0.01% suspension of the CNF sample and
197 stained with uranyl acetate. Scanning Transmission Electron Microscopy (STEM)
198 using a Hitachi S-5500 electron microscope was used to acquire images in bright field
199 mode (x150000 magnification), using an acceleration voltage of 30 kV.

200 AFM imaging (tapping mode) of the film sample was performed with a
201 Multimode AFM with Nanoscope V controller, (Digital Instruments) and images were
202 acquired in ScanAsyst mode at room temperature. The AFM tips of spring constant
203 value ~0.4 N/m were purchased from Bruker AFM probes. The image size was 2 µm
204 x 2 µm, with a resolution of 1.95 nm/pixel.

205 The aerogels and films were freeze-dried for SEM analysis. Cross-sections of
206 the films and aerogels were prepared by ion milling (using an IM4000 system), where
207 the milling time was 5 hours at 2.5 kV. In addition, samples were cut from the

208 aerogels and films using an 8 mm punch biopsy for surface analysis. All samples
209 were sputter-coated with gold and images acquired using a Hitachi scanning electron
210 microscope (SEM, SU3500), in secondary electron imaging mode.

211

212 2.9. Confocal laser scanning microscopy (CLSM) and scanning electron microscopy 213 (SEM) of biofilm growth on CNF materials

214 CNF samples (1 cm²) were added to a 12-well plate to which contained MH broth
215 (3 ml) and 60 µl of PAO1 overnight culture adjusted to OD₆₀₀ 0.4. Plates were
216 incubated at 37°C in a static aerobic environment for 24 or 48 h before removing the
217 supernatant and then washing the biofilms once with deionised water/PBS. Biofilms
218 were stained with LIVE/DEAD[®] BacLight™ bacterial viability kit (Invitrogen,
219 Paisley, UK) containing SYTO 9 dye (staining LIVE cells, green) and propidium
220 iodide (staining DEAD cells, red) and incubated in the dark (10 min) before mounting
221 on microscope slides with spacers, being set in Vectorshield (Vector Laboratories,
222 UK) and having a coverslip on top, sealed with nail varnish. Biofilms were imaged
223 with a Leica TCS SP2 confocal system (x63). Bacterial growth was quantified using
224 COMSTAT image-analysis software (Heydorn et al., 2000).

225 Biofilm samples were prepared for SEM analyses by immersion in
226 glutaraldehyde (2.5%) for 24 h, before being washed with deionised water (x4) and
227 immersed in fresh deionised water, and then frozen (24 h) and freeze-dried for 24 h.
228 The biofilms were then imaged on the Tescan Vega SEM at 5 kV. Additionally, the
229 A0.6 aerogel with PAO1 biofilm growth was also ion-milled (as described above) and
230 SEM images acquired in secondary electron imaging mode.

231

232 2.10. Statistical analysis

233 GraphPad Prism 3 was used to perform statistical analysis (GraphPad software Inc, La
234 Jolla, USA) including one-way ANOVA using Tukey-Kramer post-test (growth curve
235 data and COMSTAT data) and Dunnet's multiple comparison tests (log₁₀ reduction
236 and virulence data). P<0.05 was considered significant.

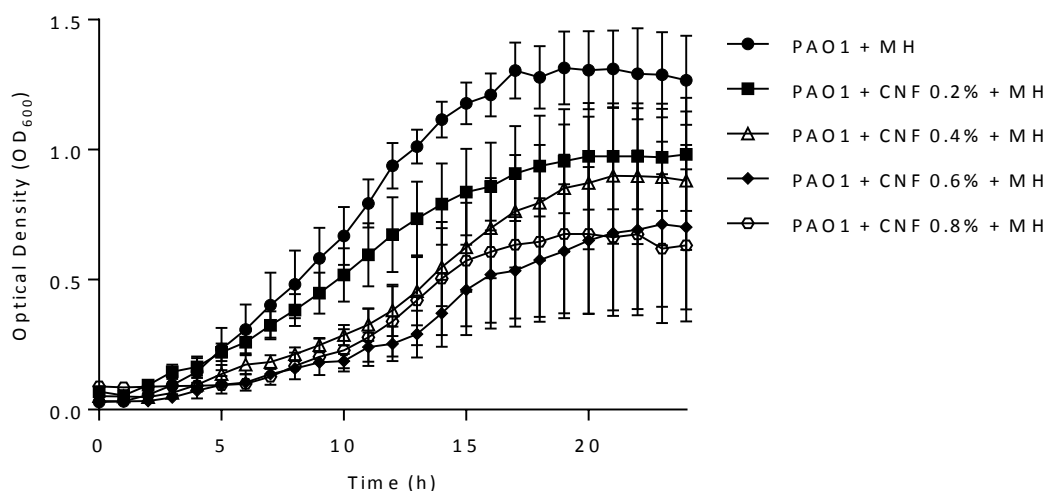
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238 3. Results

239 3.1. Growth of *P. aeruginosa* PAO1 in CNF hydrogel suspensions

240 Irradiated CNF samples at ≥0.4% concentrations indicated dose-dependent
241 inhibition of *P. aeruginosa* PAO1 (Fig. 1), possibly reflecting increased viscosity of

242 these dispersions or direct inhibition by CNF. No change in optical density was
243 observed for CNF in either PBS or water after 24 h, indicating *P. aeruginosa* did not
244 grow in, nor could it utilize CNF as a carbon source; growth only being observed in
245 the presence of MH broth (Fig. S1).



246
247 **Fig. 1.** Growth characteristics for *P. aeruginosa* PAO1 in CNF. *P. aeruginosa* PAO1
248 grown in either MH-broth/dH₂O or MH-broth/ γ -irradiated CNF (adjusted to give final
249 concentrations of CNF at 0.2, 0.4, 0.6 or 0.8%). (n=3).

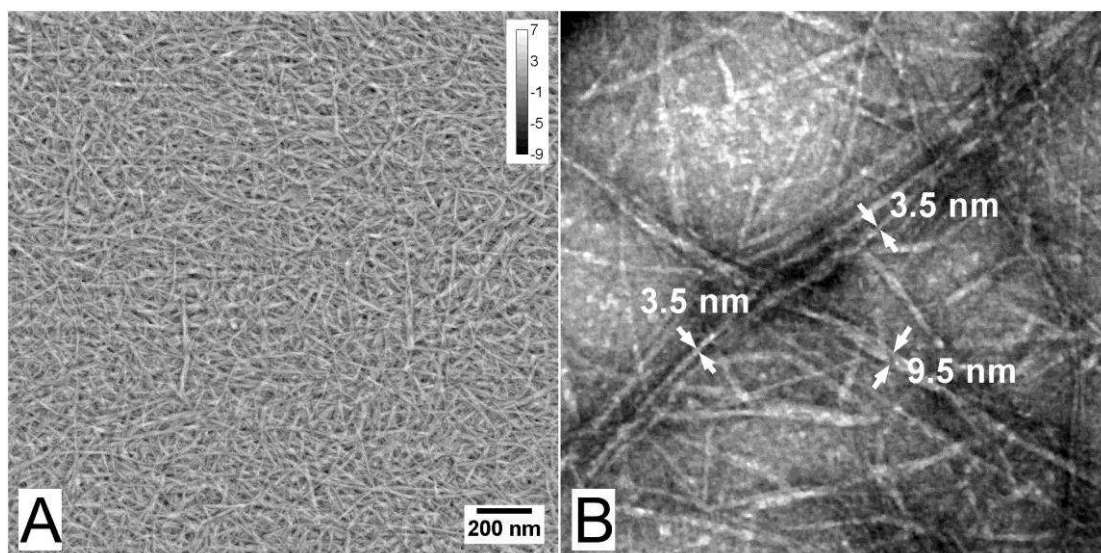
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251 3.2. Characterization of the Cellulose Materials

252 The CNF material was highly fibrillated and structurally homogenous (Rees et
253 al., 2015), containing CNF with diameters in the nanoscale (<20 nm) and with a high
254 aspect ratio, as exemplified in this study (Fig. 2).

255 The aerogel, A0.2 (manufactured from a 0.2% CNF dispersion) possessed the
256 largest thickness, porosity and surface roughness, as demonstrated by SEM and fluid
257 absorption (Fig. 3A; Table S1). The porosity and surface roughness of the other
258 aerogels decreased with the increasing CNF concentration; A0.6 (manufactured from
259 a 0.6% CNF dispersion) showing the lowest (Figs 3A-C). In contrast, the film was far
260 more dense and extremely thin, with no apparent porosity (Fig. 3D). The commercial
261 dressings AquaCel[®] and AquaCel Ag[®] displayed rough surfaces, which during the
262 course of the experiment altered, following fluid absorption. The more porous
263 (thicker) CNF aerogels absorbed more moisture when compared to the film;
264 resembling absorbancy of AquaCel[®] and AquaCel Ag[®].

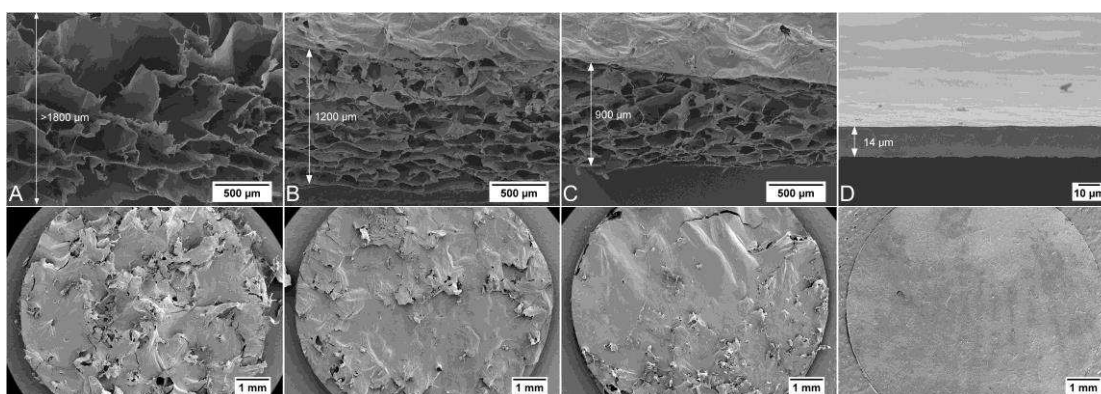
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266

267 **Fig. 2.** Highly fibrillated nanocellulose. (A) Atomic force microscopy imaging
268 (AFM). (B) Scanning Transmission Electron Microscopy imaging (STEM). Arrows
269 indicate individual nanofibrils.

270



271

272 **Fig. 3.** Scanning electron microscopy of aerogels and films (cross-sectional and
273 aerial views) made with CNF. (A) A0.2. (B) A0.4. (C) A0.6. (D) Film. Arrows
274 indicate local thickness of the materials. (N.B. A0.2 was too thick to be visualized in
275 its entirety within the maximum field of view).

276

277 3.3 Log₁₀ reduction assay

278 The ability of CNF films and aerogels (with differing porosities) to inhibit the
279 growth of planktonic *P. aeruginosa* PAO1 showed that none of the CNF materials,
280 (both aerogels, 0.29-0.37±0.33-0.37 and film samples, 0.25±0.21) demonstrated
281 significant change in Log₁₀ CFU, being comparable to the negative controls
282 AquaCel[®] dressing (0.24±0.16) and MH (0.38±0.37). AquaCel Ag[®] (the positive

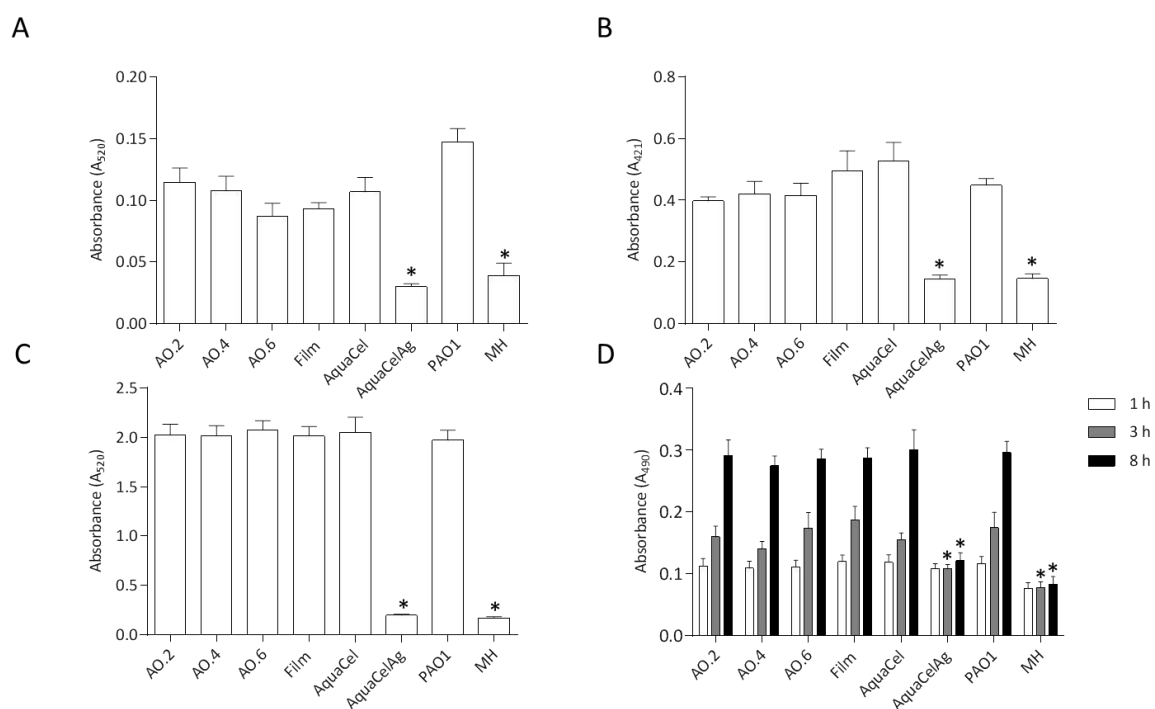
283 control) containing ionic silver however, demonstrated significant bactericidal activity
 284 (≥ 3 log-fold) reduction in Log_{10} CFU compared to the control (6.62 ± 0.94).

285

286 3.4. Virulence factor assays

287 The production of virulence factors (pyocyanin, rhamnolipids, total proteases and
 288 elastase) by *P. aeruginosa* PAO1 was not affected by CNF (Fig. 4). However, for
 289 elastase (measured at 1, 3 and 8 h) a significant difference to the control was
 290 detectable at ≥ 3 hours of incubation. A modest decreasing trend in pyocyanin
 291 production was noted based on the porosity of the aerogel materials, with A0.2
 292 (largest pores, Fig. 3A) producing the greatest amount, whereas the A0.6 (smallest
 293 pores, Fig. 3C) and film (no pores at the assessed scale) produced the least, however
 294 this was found not to be significant.

295



296

297 **Fig. 4.** The effect of the different CNF film and aerogels on virulence factor
 298 production by *Pseudomonas aeruginosa* PAO1 (A) pyocyanin. (B) rhamnolipid. (C)
 299 total protease. (D) elastase, when compared to the negative controls MH broth and
 300 AquaCel Ag[®]. (*significantly different as compared to the PAO1 control; n=3;
 301 p<0.05).

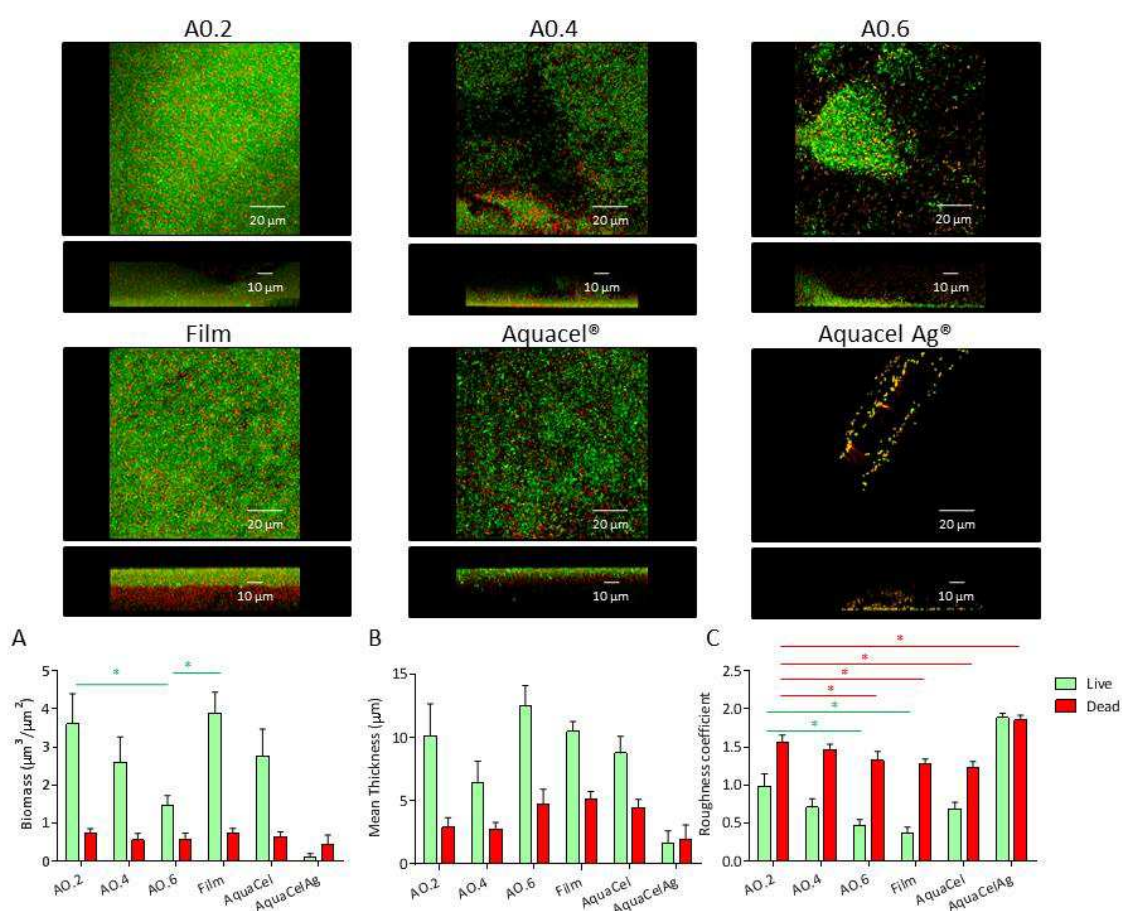
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304 3.5. Confocal Laser Scanning Microscopy (CLSM)

305 *P. aeruginosa* PAO1 was able to form established biofilms on all the CNF and
 306 commercial (AquaCel®-based) dressing materials (Fig. 5, and Fig. S2 in the
 307 supporting information) at 24 and 48 h. LIVE/DEAD®-staining confirmed bacterial
 308 viability in the established biofilms. Little difference in bacterial viability was
 309 evident for all the dressings, (apart from AquaCel Ag® which showed sparse biofilm
 310 growth at 24 h). COMSTAT image analysis was employed to quantify biomass,
 311 surface roughness (using a roughness coefficient) and mean biofilm thickness (Fig. 5
 312 and Fig. S2) in test samples and control AquaCel Ag®.

313



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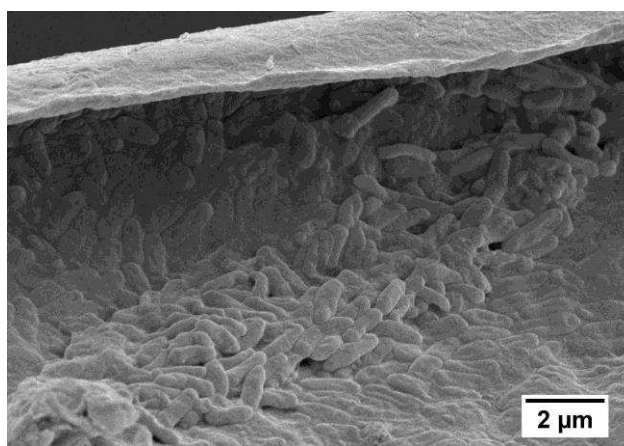
316 **Fig. 5.** CLSM images and COMSTAT analysis of LIVE/DEAD® staining of 24 h
 317 biofilms grown on CNF aerogels A0.2, A0.4 and A0.6 (0.2, 0.4 and 0.6%
 318 respectively), film and commercial dressings (Aquacel and Aquacel Ag) showing
 319 mean: (A) biomass; (B) thickness and (C) roughness coefficient (n=3, *p>0.001).
 320 All materials were significantly different to AquaCel Ag (data not shown on graphs).

321 COMSTAT analysis (n=7 images from n=3 replicates) revealed the viability of
322 the biofilms and that a distinct decrease in viable cell numbers was observed between
323 24 and 48 h, (Fig. 5A and Fig. S2A in the supporting information). In the aerogels,
324 biomass reflected material porosity; the greatest biomass being observed in A0.2 and
325 decreased to A0.6 (the least porous aerogel; $p < 0.05$). AquaCel Ag[®] had very little
326 growth at both 24 and 48 h, reflecting the CLSM.

327 Biofilm thickness varied between samples tested, although at 24 h these
328 differences were less apparent (Fig. 5B). As expected, mean thickness was higher on
329 all the materials compared to AquaCel Ag[®] (Fig. 5B and Fig. S2B in the supporting
330 information), with differences more apparent at 48 h.

331 There were significant differences in roughness coefficient between biofilms
332 grown on the different materials, with biofilms grown on A0.2 being the roughest,
333 while biofilms grown on A0.6 and Film were significantly smoother ($p < 0.05$; 24 h);
334 AquaCel Ag[®] being the roughest of all at both 24 and 48 h (Fig. 5C and Fig. S2C in
335 the supporting information). Generally, at 48 h there were less distinguishable
336 differences between surface roughness, probably reflecting the large proportion of
337 non-viable cells present on all the materials at this time.

338



339

340 **Fig. 6.** SEM image of cross-section following ion milling of a *P. aeruginosa* biofilm
341 growing on the A0.6 aerogel.

342

343 3.6. Scanning electron microscopy (SEM) of biofilm growth on CNF materials.

344 SEM imaging of a cross-section through a CNF aerogel following ion milling
345 confirmed that a large part of the biofilms were formed inside the aerogel structure

346 (Fig. 6), probably due to the relatively large porosity of the samples. SEM imaging of
347 the surface of the materials can be found in Figs. S3-S6 in the supporting information.

348

349 **4. Discussion**

350 It has become increasingly recognized that chronic wounds are able to sustain
351 a substantial bacterial biofilm which (both directly and indirectly) influences clinical
352 outcome (Hill et al., 2010). The CNF materials did not support bacterial growth of
353 *Pseudomonas aeruginosa* PAO1, confirming that the CNF could not be used as a
354 bacterial carbon source by the wound pathogen PAO1 (Powell et al., 2016). Materials
355 that significantly impair microbial growth would be a distinct advantage for a wound
356 dressing and, interestingly, a dose-dependent inhibition of bacterial growth by the
357 CNF hydrogel was evident. This inhibition may, in part, reflect the apparent viscosity
358 of the medium, as motile species, such as *P. aeruginosa*, move through media
359 obtaining available nutrients (Sampedro, Parales, Krell & Hill, 2015). In keeping
360 with this notion, the Log₁₀ reduction assay revealed that CNF aerogels and films
361 failed to inhibit microbial planktonic growth of *P. aeruginosa* PAO1 in these studies
362 (as did the commercial wound dressing AquaCel®). The CNF materials may,
363 therefore, exhibit distinct properties in hydrogel, aerogel or film forms.

364 *P. aeruginosa* PAO1 produces the blue pigment, pyocyanin, which is a
365 secondary metabolite that interrupts cellular function and is also a virulence factor
366 (Lau, Hassett, Ran & Kong, 2004) affecting mammalian cells by inhibiting cell
367 respiration and ciliary function (Ran, Hassett & Lau, 2003; Sorensen & Klinger,
368 1987). Rhamnolipids are also virulence factors produced by *P. aeruginosa* which aid
369 colonisation by acting as a surfactant, reducing surface and interfacial tension
370 (Soberón-Chávez, Lépine & Déziel, 2005). In addition, the production of
371 rhamnolipids in *P. aeruginosa* can enable the utilization of alternative carbon sources
372 (such as alkanes) by pseudosolubilisation of insoluble substrates that would not
373 normally be broken-down (Beal & Betts, 2000). *P. aeruginosa* also secretes proteases
374 and elastase that have been implicated as virulence factors (Caballer et al., 2001),
375 both of which are known to damage dermal matrix proteins and hinder cell migration
376 and wound healing (Oldal & Tranfny, 2005). The lack of effect of CNF wound
377 dressing materials on these virulence factors was characterized for the first time in the
378 present study.

379 Wound dressings represent, not only a physical barrier to infection and
380 trauma, but also incorporate antimicrobials to inhibit bacterial growth and provide an
381 optimal (moist) environment to facilitate wound healing (i.e. cell proliferation,
382 migration and differentiation). In close proximity to damaged skin, for considerable
383 periods of time, they must be biocompatible. A number of natural polymers are
384 already used as dressing materials including polysaccharides e.g. alginates and
385 chitins, and proteins e.g. collagen (Mogoşanu & Grumezescu, 2014). Unlike bacterial
386 nanocellulose, CNF has not traditionally been used for biomedical applications (Lin
387 & Dufresne, 2014). Whilst a number of researchers have investigated CNF
388 interactions in immune/inflammatory responses (Hua et al., 2015; Nordli et al., 2016),
389 as scaffolds for cell culture (Lou et al., 2014; Ninan et al., 2013) and as spray-dried
390 coatings (or stabilisers) for tablet production (Kolakovic et al., 2011), few have
391 studied their interaction with the bacteria and biofilms which characterize human
392 infection. This study clearly advances our previous studies with nanocellulose for
393 wound dressings (Nordli et al., 2016; Powell et al., 2016), and represents novel
394 findings in this area.

395 TEMPO-mediated oxidization of fibers is used to facilitate breakage of native
396 hydrogen bonding of individual cellulose fibrils from wood cellulose fibers without
397 causing extensive damage to the materials which would otherwise occur (Isogai, Saito
398 & Fukuzumi, 2011). This chemical pre-treatment is followed by physical
399 homogenization of the materials; the fibrillation process determining the
400 characteristics of the resulting material, *i.e.* fibril size. This resultant uniform, highly-
401 fibrillated material is suitable for a range of medical applications and may be
402 functionalized/modified in individual biomedical applications (Lin & Dufresne,
403 2014). An important consideration for clinical use is sterility. The lack of structural
404 changes in aerogels and films following γ -irradiation reflects its suitability for large-
405 scale manufacturing/processing. The recent demonstration confirming that ultrapure
406 CNF (lipopolysaccharide content <50 endotoxin units/gram cellulose) meets the
407 endotoxin limits required in medical devices for wound management (Nordli et al.,
408 2016), was also reassuring.

409 Three-dimensional visual assessment of the CNF CLSM images proved
410 difficult and subjective due to the inherently rough surfaces and heterogeneous nature
411 of the biofilms which formed. COMSTAT image analysis was therefore employed to
412 characterize and study the biofilms formed on the materials; a technique that proved

413 useful. However, in terms of biomass and thickness, COMSTAT was only able to
414 quantify changes in bacteria which remained adherent to the material surface and
415 were visualised by CLSM. It was evident that biofilms infiltrate the dressing
416 materials. Ion milling of biofilms growing on the materials showed that this was
417 evident in the aerogels used in this study. Hence, the biomass and thickness data for
418 the aerogels (being extremely porous) will have been substantially underestimated
419 compared to those of the film (possessing no apparent pores for the biofilms to
420 invade).

421 Surface roughness plays a significant role in bacteria adhesion (Hsu, Fang,
422 Borca-Tasciuc, Worobo & Moraru, 2013; Yoda et al., 2014). This was confirmed in
423 our study as the aerogel with the lowest surface roughness and porosity (A0.6)
424 demonstrated the lowest biofilm growth (in terms of both thickness and biofilm
425 height) and this was also markedly less than that observed on the commercial
426 AquaCel[®] dressings. Aerogels are likely to have a clinical advantage over the film for
427 wound applications due to their enhanced absorbency; chronic wounds often
428 producing large amounts of exudate. This study showed that, despite having identical
429 grammage, the aerogels (A0.2, A0.4 and A0.6) possessed distinctly different material
430 properties (e.g. surface roughness, fluid absorption). The commercial dressings
431 AquaCel[®] and AquaCel Ag[®] displayed an inherently rough surface, which
432 progressively altered during the course of the experiment to become smoother with
433 increased absorption of fluids as noted by Walker, Hobot, Newman & Bowler (2003).

434 Our results indicate an ability to fabricate the desired surface/pore structure of
435 the CNF materials and their absorbance, which will be important in distinct clinical
436 applications. For example, wound dressings should have a smooth surface (to reduce
437 bacterial adhesion), with adequate porosity to enhance absorbency (and retain wound
438 fluid), whilst maintaining a moist environment to promote healing. These
439 microbiological studies show the potential use of CNF as a wound dressing material.
440 Not only was it demonstrated that CNF dispersions did not support bacterial growth
441 but, when the CNF dispersions were challenged with *P. aeruginosa* PAO1, bacterial
442 growth was inhibited. Using CNF as a potential wound dressing material represents
443 an important clinical use for this sustainable resource, with properties comparable to
444 those of commercially-available dressings.

445

446 5. Conclusions

447 Our previous studies have importantly shown these materials to be non-toxic to
448 human cells. This study confirms that CNF produced using differing manufacturing
449 processes generates a potential wound dressing material, which allows for absorbency
450 based on porosity to be controlled. Bacterial virulence was unaltered when grown on
451 these materials, showing equivalency with other commercially available wound
452 dressings already on the market. Moreover, these materials are biodegradable and
453 may be renewably sourced; both of which are important for biosustainability. Also as
454 the physical and chemical composition of CNF is highly malleable, the development
455 of nanocellulose dressings offers significant clinical potential.

456

457

458 **ASSOCIATED CONTENT**

459 **Supporting Information**

460 Growth characteristics for *P. aeruginosa* PAO1 in CNF; Absorbancy of the CNF
461 materials used in this study; CLSM images and COMSTAT analysis of
462 LIVE/DEAD[®] staining of 48 h biofilms grown on CNF aerogels; and SEM images of
463 24 and 48 h *P. aeruginosa* PAO1 biofilms grown on the CNF materials.

464

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469 NanoHeal: Bio-compatible cellulose nanostructures for advanced wound healing
470 applications.

471

472

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