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1 **Silylation of bacterial cellulose to design membranes with intrinsic**
2 **anti-bacterial properties**

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16

17 **Abstract**

18 In this work, we report a convenient method of grafting non-leachable bioactive amine
19 functions onto the surface of bacterial cellulose (BC) nanofibrils, via a simple silylation
20 treatment in water. Two different silylation protocols, involving different solvents and post-
21 treatments were envisaged and compared, using 3-aminopropyl-trimethoxysilane (APS) and
22 (2-aminoethyl)-3-aminopropyl-trimethoxysilane (AEAPS) as silylating agents. In aqueous

23 and controlled conditions, water-leaching resistant amino functions could be successfully
24 introduced into BC, via a simple freeze-drying process. The silylated material remained
25 highly porous, hygroscopic and displayed sufficient thermal stability to support the
26 sterilization treatments generally required in medical applications. The impact of the silylation
27 treatment on the intrinsic anti-bacterial properties of BC was investigated against the growth
28 of *Escherichia coli* and *Staphylococcus aureus*. The results obtained after the *in vitro* studies
29 revealed a significant growth reduction of *S. aureus* within the material.

30 **Keywords:** bacterial cellulose, nanocellulose, aminosilane, anti-bacterial, silylation

31

32 **1. Introduction**

33 In recent years, the field of biomedical materials has witnessed rapid progress,
34 increasingly calling upon the use of efficient sustainable based materials, as cellulose based
35 materials, in applications such as wound healing, drug delivery systems, vascular grafts or
36 scaffolds for *in vivo* tissue engineering (Agarwal, McAnulty, Schurr, Murphy, & Abbott,
37 2011; Jorfi & Foster, 2015). This biopolymer is particularly interesting because of its
38 renewability, biodegradability, and biocompatibility; however, it is not endowed with
39 antibacterial activity, one of the most desired properties in the medical field (O'Neill, 2014).
40 To address this issue, anti-bacterial systems based on cellulose mixed with bioactive
41 compounds have been proposed, such as those with silver nanoparticles (Berndt, Wesarg,
42 Wiegand, Kralisch, & Müller, 2013) or anti-bacterial peptides (Nguyen, Gidley, & Dykes,
43 2008). However, numerous problems may result from the uncontrolled release of bioactive
44 agents, such as the development of multi-resistant micro-organisms (Lewis, 2001; O'Neill,
45 2014). As a consequence, antibiotic-resistant bacterial strains such as *Staphylococcus aureus*
46 (Boswihi & Udo, 2018) or *Escherichia coli* (Milović, Wang, Lewis, & Klibanov, 2005) are
47 now spreading in the environment and becoming one of the greatest health threats of this

48 century (O'Neill, 2014). To circumvent this problem, the immobilization of bioactive agents
49 by chemical bonding offers the opportunity to produce materials, which release no deleterious
50 chemicals and are able to promote bioactivity by simple contact. Until now, essentially
51 materials grafted with active ammonium moieties have been proposed, such as glass grafted
52 with quaternized polyethylenimine (Milović et al., 2005), microcrystalline cellulose grafted
53 with comb-like N,N-dimethyldodecylammonium groups (Bieser, Thomann, & Tiller, 2011) or
54 chitosan grafted with nisin in acidic conditions (X. Zhu et al., 2015). The mechanism of action
55 of these systems is still unclear, but is thought to be related to the positive charge of the
56 ammonium functions, which supposedly interacts with the negative charges at the microbial
57 cell surface (phospholipids), leading to a modification of the membrane permeability and
58 leakage of intracellular components (Bieser & Tiller, 2011; Fernandes et al., 2014; Helander,
59 Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001).

60 Among the cellulose materials with high potential in the biomedical field, bacterial
61 cellulose (BC) is particularly attractive as it naturally displays various properties required for
62 biomedical applications, namely: high water-holding capacity, good mechanical properties,
63 biocompatibility and non-toxicity (Agarwal et al., 2011; Czaja, Young, Kawecki, & Brown,
64 2007). BC is an extracellular polysaccharide produced by the bacteria of the genus
65 *Gluconacetobacter*, *Rhizonium*, *Sarcina*, *Agrobacterium* or *Alcaligenes* (Berlioz, 2007;
66 Chawla, Bajaj, Survase, & Singhal, 2009; Esa, Tasirin, & Rahman, 2014), which has already
67 proved its efficiency as a wound healing membrane (Fontana et al., n.d.; US10425978, 2003),
68 arterial substitute (Wippermann et al., 2009), or matrix for transdermal drug delivery systems
69 (Silva, Rodrigues, et al., 2014; Trovatti et al., 2011). To prepare BC with anti-bacterial
70 activity, the grafting of ammonium moieties by reaction with (3-aminopropyl)-
71 trimethoxysilane (APS) has been proposed as a method to impart anti-bacterial properties to
72 the material (Fernandes et al., 2013; Saini, Belgacem, Salon, & Bras, 2016; Shao et al., 2017;

73 Taokaew, Phisalaphong, & Newby, 2015). Interesting results have been obtained, but it is not
74 clear at this stage if the anti-bacterial activity observed in these pioneering works resulted
75 from a contact mechanism or from the release of the amino moieties after hydrolysis of the
76 grafted silane. It has indeed been reported that such grafting is generally unstable in the
77 presence of water, as the pendant amino group can catalyze the hydrolysis of the chemical
78 bonds formed between APS and the hydroxylated substrate (Etienne & Walcarius, 2003;
79 Smith & Chen, 2008; M. Zhu, Lerum, & Chen, 2012).

80 In this context, we report here a thorough investigation of the functionalization of BC
81 with two different aminosilanes, APS and (2-aminoethyl)-3-aminopropyl-trimethoxysilane
82 (AEAPS), with the objective of producing a water-stable material with intrinsic anti-bacterial
83 activity. Two different silylation protocols, involving different solvents (acetone or water) and
84 post-treatments (with or without heat curing), were envisaged and compared. The stability of
85 the silane grafting after prolonged soaking in water was investigated in detail. The thermal
86 properties and ultrastructure of the materials with the highest level of non-leachable silane
87 were subsequently characterized by TGA, SEM and porosimetry. Finally, the impact of the
88 silylation treatment on the anti-bacterial activity by contact against the growth of two target
89 bacteria, *E. coli* (Gram-negative) and *S. aureus* (Gram-positive), was investigated and
90 discussed.

91

92 **2. Experimental**

93 *2.1. Materials*

94 (3-Aminopropyl)-trimethoxysilane (APS) was purchased from TCI and (2-aminoethyl)-
95 3-aminopropyl-trimethoxysilane (AEAPS) from Gelest Inc. Tryptose broth was bought from
96 Difco Laboratories and bacteriological agar A1010HA was provided by Biokar Diagnostics.
97 Sodium chloride was obtained from Sigma Aldrich and hydrochloric acid 37% from Fisher

98 Scientific. All chemicals were used without purification. Distilled water Milli-Q Direct 8
99 (Millipore) was used in all experiments. All other reagents used were of analytical grade.

100 2.2. *Preparation of BC*

101 Bacterial cellulose membranes were produced using *Gluconacetobacter sacchari* strain
102 (Silva, Drumond, et al., 2014) in conventional culture media conditions (Hestrin & Schramm,
103 1954). Briefly, a subculture was incubated at 30°C for 48h, in static conditions. The flask was
104 then vigorously agitated and 5 mL of the culture media added to 45 mL HS medium in 250
105 mL Erlenmeyer flasks. The production of BC was carried out under sterile conditions. The
106 flasks were incubated at 30°C, in a static incubator, for 96h. BC membranes were then treated
107 three times with 0.5 M NaOH solution at 90°C for 30 min in order to eliminate attached cells.
108 Next, the membranes were washed with water to remove components of the culture media and
109 other residues. This step was repeated until pH reached neutral value. Wet BC membranes
110 were stored in water at 4°C until use.

111 2.3. *Preparation of silylated BC*

112 2.3.1. Silylation in acetone (Protocol 1)

113 This protocol was inspired by the work of Fernandes et al., 2013 (Fernandes et al., 2013). 5 g
114 of wet BC membranes (about 30-40 mg of dry content) were subjected to solvent exchange in
115 acetone for 1h under stirring (repeated 6 times). Membranes were then placed between two
116 paper sheets and pressed at 39 kPa for 2 min, in order to remove part of the absorbed acetone
117 (between 50 and 70 wt% of acetone removal). 1 ml of a 340 mmol/L APS or AEAPS solution
118 (in acetone) was then deposited on top of the BC membranes, to reach an
119 anhydroglucose/silane molar ratio of 1:1. The material was left for 2h at room temperature,
120 until complete absorption of the aminosilane solution by the substrate. Samples were
121 subsequently dried at room temperature and pressed overnight at 39 kPa (between two paper

122 sheets). Finally, the obtained transparent films were cured at 120°C for 2h. Samples were
123 stored in a desiccator containing phosphorous pentoxide at least 24h before characterization.
124 Samples were named according to the type of silane and concentration used in the process
125 (BC-APS₃₄₀ or BC-AEAPS₃₄₀).

126 2.3.2. Silylation in water (Protocol 2)

127 This protocol was inspired by the work of Zhang *et al.*, 2014 (Zhang, Sèbe, Rentsch,
128 Zimmermann, & Tingaut, 2014). Two types of experiments were performed based on this
129 method, starting from 5g of wet BC membrane (about 30 mg of dry content). In the first set of
130 experiments (Protocol 2a), BC membranes were freeze-dried prior to functionalization, while
131 in the second set (Protocol 2b), the membranes were used in their wet form, after removal of
132 50 to 70% of the water under pressure (same method as in Protocol 1).

133 In both cases, 340 mmol/L (or 280 mmol/L) of an APS or AEAPS water solution
134 (acidified with HCl/ pH = 4.6) was deposited on top of a dry or wet BC membrane, to reach
135 an anhydroglucose/silane molar ratio of 1:1 (or 1:0.75). The material was left for 2h at room
136 temperature until complete absorption of the aminosilane solution by the substrates. Finally,
137 the membranes were soaked in liquid nitrogen and freeze-dried for 24h. Samples were stored
138 in a desiccator with phosphorous pentoxide at least 24h before characterization. Samples were
139 named according to the type of silane and concentration used in the process (BC-APS₃₄₀, BC-
140 AEAPS₃₄₀ or BC-AEAPS₂₈₀).

141 2.4. Leaching experiments

142 Silylated BC samples were soaked in water at room temperature for 20h. After freeze-
143 drying, the samples were stored in a desiccator containing phosphorous pentoxide for at least
144 24h before characterization. The stability of the grafting was assessed by comparing the
145 infrared spectra and nitrogen content in the samples, before and after leaching.

146 2.5. *Characterization of unmodified and silylated BC*

147 2.5.1. Fourier transform infrared spectroscopy (FT-IR)

148 FT-IR spectra were recorded between 400 and 4000 cm^{-1} at a resolution of 4 cm^{-1} (32
149 scans), using a Nicolet FT-IR spectrometer (AVATAR 370) in transmission mode. 2 mg of
150 samples were ground and mixed with 200 mg of KBr to prepare pellets. For comparison
151 purposes, spectra were adjusted with the same baseline correction (polynomial with 1 iteration
152 and 6 points at 3800, 2280, 1900, 1262, 876 and 411 cm^{-1}) and were normalized to the
153 $\delta_{\text{as}}(\text{CH}_2)$ asymmetric bending of cellulose at 1430 cm^{-1} (not affected by the chemical
154 modification).

155 2.5.2. Size exclusion chromatography (SEC)

156 SEC analysis was carried out on a Varian apparatus equipped with TosoHaas TSK gel
157 columns and a differential refractometer detector. PBS pH 7.0 served as eluent, at a flow rate
158 of 0.6 $\text{mL}\cdot\text{min}^{-1}$, and calibration was achieved with pullulan/dextran standards.

159 2.5.3. Elemental analysis

160 Elemental analysis data were obtained from SGS Multilab. The atomic weight ratio of
161 the nitrogen present in the silylated samples (N wt%) was measured according to protocol
162 ASTM D5373 (“ASTM D5373-16, Standard Test Methods for Determination of Carbon,
163 Hydrogen and Nitrogen in Analysis Samples of Coal and Carbon in Analysis Samples of Coal
164 and Coke,” 2016). Experiments were conducted in duplicate and results were averaged. The
165 silane content within the material was estimated from N wt%, based on the molecular weight
166 of the monomer unit of the condensed polysiloxane obtained after hydrolysis and
167 condensation of the aminosilane, namely $\text{SiO}_{3/2}\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$ for APS ($M = 109.49 \text{ g}\cdot\text{mol}^{-1}$
168 ¹) and $\text{SiO}_{3/2}\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NH}_2$ for AEAPS ($M = 147.52 \text{ g}\cdot\text{mol}^{-1}$). N% was also
169 used to estimate the amine content, in mmol per gram of silylated BC.

170 2.5.4. Thermogravimetric analyses

171 Thermogravimetric analyses were performed under air, with a TGA-Q500 system from
172 TA instruments operated at a heating rate of $10^{\circ}\text{C}\cdot\text{min}^{-1}$. The residual water content was
173 quantified by measuring the weight loss in the thermo-oxidative curves at 150°C . The 5%
174 weight loss temperature ($T_{5\%}$) was estimated based on the degradation of the dry cellulosic
175 material (i.e. excluding the loss due to water vaporization). The temperature of maximum rate
176 of degradation (T_m) was determined from the maximum of the derivative TG curves. The char
177 yield at 750°C was calculated in terms of weight percentage of dry material.

178 2.5.5. Water absorption assessment

179 The water absorption capacity of the unmodified and silylated BC materials, expressed
180 as g of water/g of dry material, was evaluated by soaking the samples in 50 mL water, and
181 gravimetrically measuring the amount of water absorbed after 72h. The values of four
182 replicates were averaged in each experiment and the standard deviation was calculated.

183 2.5.6. Scanning electron microscopy

184 Scanning electron microscopy (SEM) was performed on a QUANTA 200 microscope
185 from FEI. The voltage was set at 5 kV, in 50 Pa low vacuum atmosphere. Samples for cross-
186 section imaging were prepared by breaking membranes after freezing in liquid nitrogen.
187 Samples were placed on a conducting tape without coating.

188 2.5.7. Porosimetry and density

189 The porosity and density of the BC samples were evaluated by mercury intrusion
190 porosimetry, with a Micromeritics Autopore IV 9500 porosimeter, set with the following
191 parameters: contact angle = 130° ; mercury surface tension = 485 mN m^{-1} ; maximum intrusion

192 pressure = 124 MPa. The Brunauer-Emmett-Teller (BET) specific surface area was measured
193 by nitrogen sorption, with a Micromeritics ASAP 2010.

194 2.5.8. *In vitro* antibacterial properties of silylated-BC

195 To evaluate BC anti-bacterial properties, membranes were specifically autoclaved at
196 120°C for 15 min and the synthesis of silylated BC and the leaching experiment, previously
197 described in sections 2.3 and 2.4, were performed in sterile conditions under a laminar flux
198 hood. To evaluate inherent anti-bacterial activity, and not activity due to the release of
199 aminosilane, BC and silylated BC produced by Protocol 2b were tested after the leaching
200 experiments.

201 In addition, in order to extensively protonate the amine groups of the silylated
202 membranes, chemically-modified BC membranes (10 mg) were soaked for 2h in 5mL of
203 hydrochloric acid solution (pH 2.07). As control materials, unmodified-BC membranes were
204 soaked for 2h in HCl in the same conditions as mentioned before (BC-HCl) or just soaked in 5
205 mL water for 2h (BC), mainly to determine the impact of the acid treatment. Subsequently,
206 membranes were washed three times with water and dried in a vacuum-oven at 60°C.

207 Anti-bacterial activity of BC, BC-HCl, BC-APS₃₄₀, BC-AEAPS₃₄₀ and BC-AEAPS₂₈₀
208 was tested against *E. coli* ATCC 25922 and *S. aureus* ATCC 6538. Overnight cultures in
209 tryptose or nutrient broth for *E. coli* and *S. aureus*, respectively, were centrifuged at 2500 rpm
210 for 8 min and the supernatant was removed and replaced by physiological water (9 g.L⁻¹
211 NaCl) to wash the inoculum. This step was repeated 3 times. Dilution was adjusted to place a
212 9 µL droplet of about 10² cells on top of 1 cm² material samples previously deposited on
213 tryptose or nutrient agar, depending on the target strain. Petri dishes were then stored at 4°C
214 for 4h to allow penetration of the inoculum through the sample in order to reach the agar
215 medium. After 14h of incubation at 30°C, the materials and the agar below were recovered,

216 immersed in 9 mL physiological water and vortexed for 30 s. Successive 10-fold dilutions in
217 physiological water were made up to 10^4 . For each dilution, 0.1 mL aliquots were removed
218 and plated a tryptose or nutrient agar medium for *E. coli* and *S. aureus* numeration,
219 respectively. The number of viable bacterial cells on each material was determined and the
220 results were expressed in $\log_{10}(\text{CFU}/\text{cm}^2)$. The experiments were conducted in triplicate and
221 results averaged.

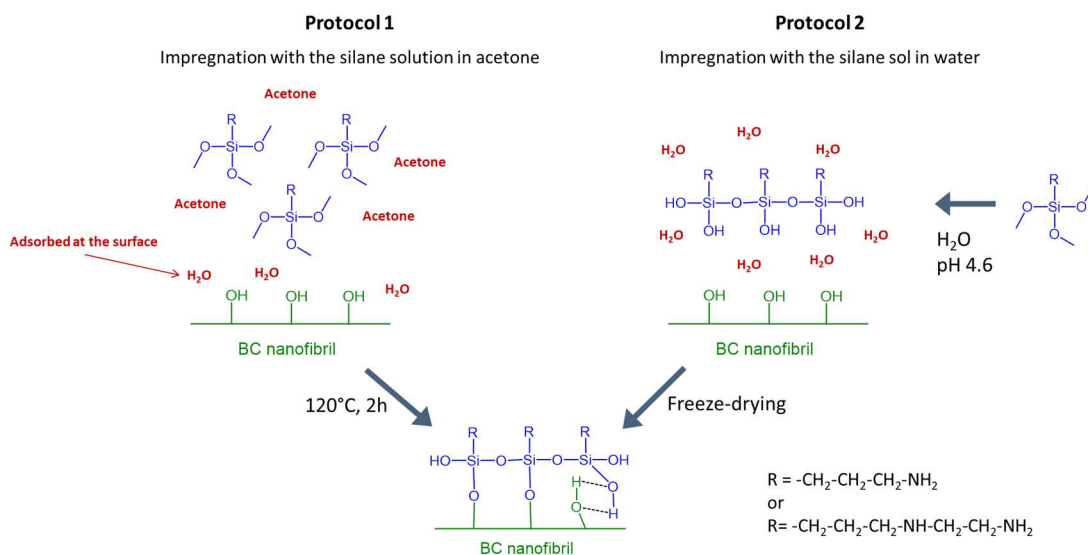
222

223 3. Results and discussion

224 3.1. Silylation of BC with APS and AEAPS

225 In this study, anti-bacterial amino moieties were introduced into BC membranes using
226 two different aminosilanes bearing one or two amine groups, *i.e.* (3-aminopropyl)-
227 trimethoxysilane (APS) and (2-aminoethyl)-3-aminopropyl-trimethoxysilane (AEAPS),
228 respectively. Silylation was performed by reacting BC with the trimethoxysilane functions
229 according to two different protocols inspired by the literature (Protocols 1 & 2) and illustrated
230 in Fig. 1. Irrespective of the protocol, the grafting involves three steps: i) hydrolysis of the
231 alkoxy silanes into silanols, ii) homocondensation of the silanols into oligomers, and iii)
232 condensation of the oligomers with the BC substrate (Fernandes et al., 2013; Zhang et al.,
233 2014; Zhang, Tingaut, Rentsch, Zimmermann, & Sèbe, 2015). The main differences between
234 the two protocols lie in the type of solvent used (acetone or water) and the procedure
235 employed to condense the silanes (with or without heat curing). The water required for the
236 hydrolysis of the aminosilanes in Protocol 1 is provided by a layer of adsorbed water at the
237 surface of the BC nanofibrils, while the condensation with BC is initiated by a heating step at
238 120°C (Fernandes et al., 2013). With Protocol 2, the silanes are readily hydrolyzed and
239 homocondensed in the form of oligomers (silane sol) before contact with BC. The subsequent
240 condensation with BC takes place at a low temperature, when the water solvent is

241 progressively removed from the medium by freeze-drying (Zhang et al., 2014, 2015).
 242 Whatever the protocol used, the stability of the grafting was probed by performing a leaching
 243 test in water (the silylated samples were soaked in water for 20 h).



244
 245 **Fig. 1.** Reaction schemes of the two protocols used for the silylation of BC with APS and AEAPS.

246 A first set of experiments was performed following the procedure of Fernandes *et al.* in
 247 acetone (Protocol 1) (Fernandes et al., 2013). The presence of silane moieties in the modified
 248 samples (BC-APS₃₄₀) was confirmed by measuring nitrogen content by elemental analysis
 249 (Table 1), and observing the $\delta(\text{NH})$ amine vibration in the 1600-1590 cm^{-1} region of the FT-
 250 IR spectrum (Fig. 2, Protocol 1). The silane content was estimated from the nitrogen content,
 251 as described in the experimental section.

252 The $\delta(\text{NH})$ band disappeared after the leaching test in water (Fig. 2, Protocol 1), with
 253 about 80 wt% of the silane being removed (Table 1), indicating that the grafting was not
 254 stable in this aqueous environment. This instability could result from the hydrolysis of the Si-
 255 O-C bonds catalyzed by the amine groups, through the formation of five-membered cyclic
 256 intermediates, as was reported for silica particles grafted with APS (Etienne & Walcarius,
 257 2003; Smith & Chen, 2008; M. Zhu et al., 2012). To circumvent this problem, Zhu *et al.*

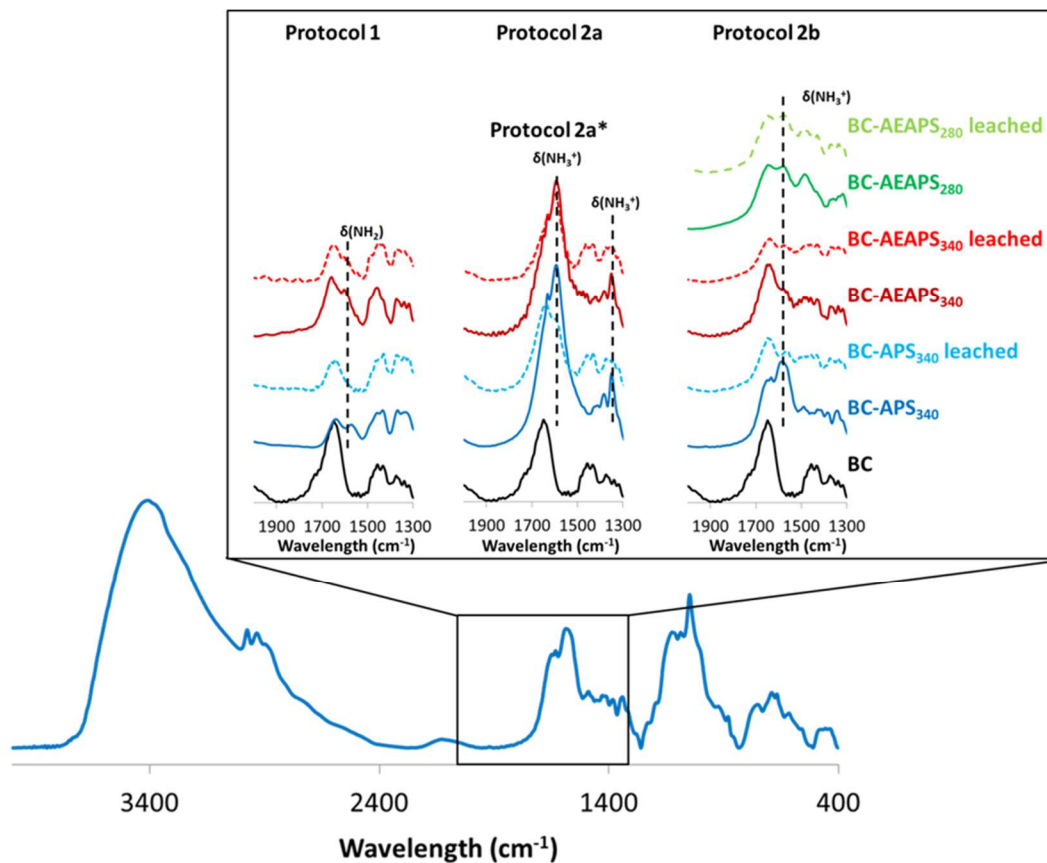
258 (2012) suggested increasing the chain length separating the silicon atom from the primary
 259 amine moiety, using AEAPS as a silylating agent. This structure prevents the formation of
 260 five-membered cyclic intermediates, leading to significant improvement in the stability of the
 261 grafted silane in their case. We therefore envisaged silylating our BC membrane with AEAPS,
 262 using the same molar concentration as with APS (Table 1 and Fig. 2). Using AEAPS instead
 263 of APS led to a significant improvement in silane grafting, probably because the high
 264 molecular weight AEAPS was vaporized less during the heating step at 120°C. The $\delta(\text{NH})$
 265 vibrations of the primary and secondary amine functions of AEAPS were identified in the
 266 same FT-IR region as for APS (Fig. 2), however, contrary to expectations, the silylated
 267 material still lost about 80 wt% of the silane after the leaching test in water. In view of these
 268 results, we inferred that the AEAPS molecules may have been poorly condensed with the
 269 cellulose substrate in our experimental conditions. Nevertheless, a significantly higher amount
 270 of non-leachable amine groups was grafted within the BC material when AEAPS was used
 271 (about seven times more than with APS), both because more silane was introduced initially,
 272 and because each monomer contained two amine functions.

273 **Table 1**

274 Amount of nitrogen (wt%), silane (wt%) and amine functions (mmol/g) within the silylated BC
 275 materials before and after water leaching (determined by elemental analysis).

<i>Leaching experiment</i>	Protocol 1		Protocol 2a		Protocol 2b		
	<i>Before</i>	<i>After</i>	<i>Before</i>	<i>After</i>	<i>Before</i>	<i>After</i>	
BC-APS₃₄₀	N(wt%)	1.3	0.3	4.0	0.5	4.3	0.7
	Silane (wt%)	9.9	2.2	31.4	3.6	33.6	5.9
	Amine (mmol/g)	0.9	0.2	2.9	0.3	3.1	0.5
BC-AEAPS₃₄₀	N(wt%)	5.8	1.0	5.0	1.0	5.9	2.63
	Silane (wt%)	30.6	5.0	26.4	5.2	31.0	13.8
	Amine (mmol/g)	8.3	1.4	7.2	1.4	8.4	3.8
BC-AEAPS₂₈₀	N(wt%)					4.6	3.4
	Silane (wt%)					24.3	18.2
	Amine (mmol/g)					6.6	4.9

276



277

278 **Fig. 2.** FT-IR spectra in the 2000-1300 cm⁻¹ region of unmodified BC, BC-APS₃₄₀ before and after
 279 water leaching, BC-AEAPS₃₄₀ before and after water leaching and BC-AEAPS₂₈₀ before and after
 280 water leaching.

281 In an alternative approach, we envisaged silylating the BC membranes (with both APS
 282 and AEAPS) following the procedure of Zhang *et al.* (Protocol 2), who reported a simple
 283 method to graft alkoxy silane molecules onto the surface of nanofibrillated cellulose in water
 284 medium (Fig. 1) (Zhang et al., 2015). A first set of experiments was performed starting from
 285 freeze-dried BC (Protocol 2a), in order to control the silane/BC and silane/water ratios during
 286 the treatment. Before being introduced into the BC network, the aminosilanes were
 287 hydrolyzed at pH 4.6, leading to a silane sol composed of polysiloxane oligomers, which were
 288 analyzed by SEC in the case of APS (Fig. S1 of supporting information). Results indicated
 289 that the APS sol was composed mostly of a mixture of two oligomers, with average molecular
 290 weights of 1391 g/mol (10-15 condensed units) and 5108 g/mol (35-60 condensed units).

291 After the treatment, the amount of nitrogen in BC was again evaluated by elemental analysis
292 (Table 1, Protocol 2a) and the presence of silane was confirmed by FT-IR spectroscopy (Fig.
293 2, Protocol 2a). The FT-IR spectra displayed stronger absorption bands in the 1600-1500 cm⁻¹
294 and 1350 cm⁻¹ regions compared with protocol 1, which could be related to the presence of
295 bicarbonate salts, possibly formed after contact between the grafted NH₂ groups and
296 atmospheric CO₂ (Culler, Naviroj, Ishida, & Koenig, 1983). The infrared vibrations of these
297 salts are indeed expected in the same region. Compared with the treatment in acetone, the
298 Protocol 2a in water allows a greater amount of APS to be grafted (about three times more in
299 Table 1), probably because the vaporization of APS was prevented in that case, as the sample
300 was not heated during the process. In addition, no significant difference in grafting level was
301 noted between the two protocols when the higher molecular weight AEAPS was used. Here
302 again, a significant amount of silane was leached out after prolonged contact with water (~ 90
303 wt% with APS and ~ 80 wt% with AEAPS), suggesting once again that the silane oligomers
304 were poorly condensed with the cellulose substrate, and/or were partly hydrolyzed in the
305 presence of water. We reasoned at this stage that the use of dry BC might have reduced the
306 efficiency of the treatment when the silane sol was deposited at the surface of this highly
307 hygroscopic substrate. Indeed, the water surrounding the silane oligomers is expected to be
308 absorbed within seconds after contact with the dry cellulosic substrate. As a result, the
309 homocondensation of the oligomers at the surface of the film should be highly favored,
310 thereby preventing their in-depth penetration into the BC network, and limiting the possibility
311 for condensation with the bulk cellulose fibrils. To address this problem, the previous
312 experiments were reproduced, but this time starting from never-dried BC (Protocol 2b). The
313 elemental analysis and FT-IR data obtained with Protocol 2b are reported in Table 1 and Fig.
314 2, respectively. The silylation levels obtained with wet BC were not significantly different
315 from those obtained with Protocol 2a (Table 1), yet the intensity of the δ(NH) vibrations in

316 the FT-IR spectra was lower, presumably because fewer bicarbonate salts were formed by
317 contact with CO₂. Compared with Protocol 2a, the resistance to water leaching was
318 significantly improved when AEAPS was used. About 55 wt% of the silane was lost after
319 prolonged contact with water in that case, instead of the 80 wt% loss previously noted with
320 dry BC. The BC grafted with APS was also more resistant to water leaching, but to a much
321 lower extent (loss of ~ 80 wt% silane instead of ~ 90 wt%). The better stability obtained with
322 AEAPS was attributed to the structure of AEAPS, which prevents the amine-catalyzed
323 hydrolysis generally noted with APS (Zhu *et al.* 2012). In an attempt to further limit the
324 homocondensation of the hydrolyzed silane, i.e. further promote its condensation with the
325 cellulosic scaffold, an additional sample was silylated with a lower concentration of AEAPS
326 (BC-AEAPS₂₈₀ in Table 1). As expected, the resistance to water leaching was further
327 increased using this strategy, only 25% wt% of the silane introduced initially being now
328 leached out after 20h in water.

329 In view of the encouraging results obtained with Protocol 2b, only the silylated
330 samples obtained in these conditions were selected for further investigations. In addition, only
331 the water-leached samples, i.e. those containing only non-leachable silanes, were thoroughly
332 characterized.

333 3.2. *Thermal properties of silylated BC*

334 The TGA thermograms under air flow of the unmodified and water-leached silylated
335 samples (Protocol 2b) are presented in Fig. S2 of supporting information, and the degradation
336 data are summarized in Table 2. The amount of residual water adsorbed by the dry material
337 was evaluated from the weight loss at 150°C, and is also reported in Table 2. The thermogram
338 of unmodified BC is consistent with the thermal behavior generally observed for bacterial
339 cellulose (Cheng, Catchmark, & Demirci, 2009; Fernandes *et al.*, 2013). The introduction of
340 amine moieties into BC led to an increase in the hygroscopic character of the material, the

341 water content increasing concomitantly with the nitrogen content. No significant modification
 342 of the thermal stability of the material was noted after the APS treatment, but samples treated
 343 with AEAPS displayed a 5 % lower weight loss temperature ($T_{5\%}$), while the temperature of
 344 maximum rate of degradation (T_m) was only slightly modified. The reduced $T_{5\%}$ noted with
 345 AEAPS could result from the release of volatile compounds, by cleavage of the AEAPS
 346 moieties between 200 and 240°C (through nucleophile reactions initiated by the terminal
 347 amine group for instance). The solid residue at 750°C obtained after degradation of the
 348 silylated samples (Table 2), was assigned to silicon inorganic species formed at high
 349 temperature (SiO_2). In any case, the silylated materials displayed sufficient thermal stability to
 350 support the sterilization treatments generally required in the medical applications targeted.

351 **Table 2**

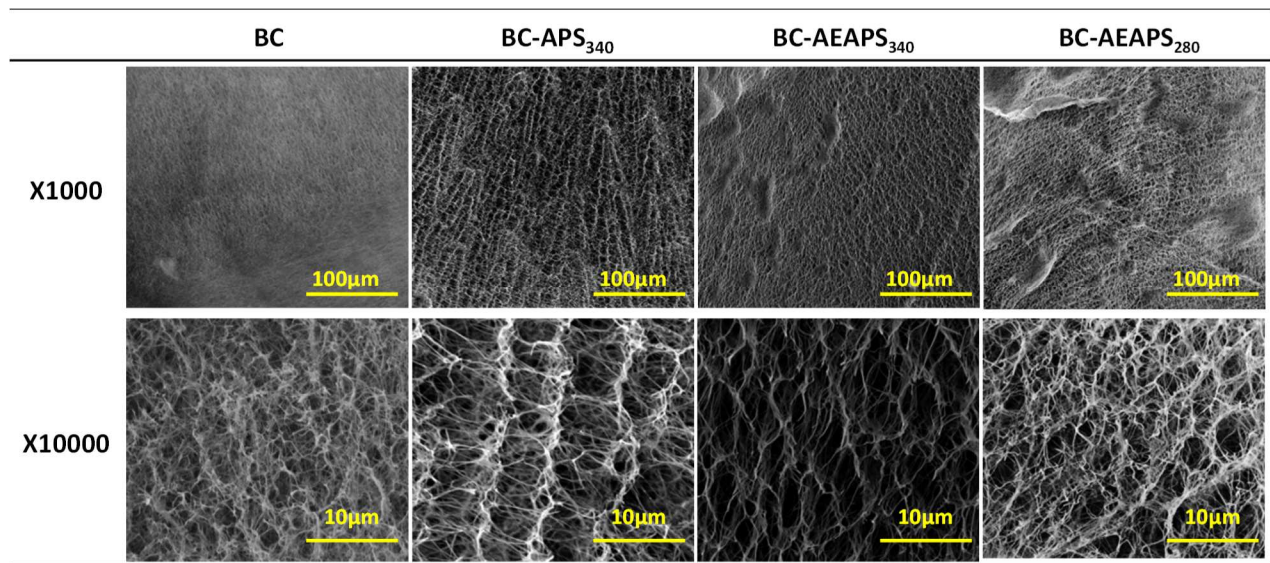
352 TGA data obtained after analysis under air flow of BC, BC-APS₃₄₀, BC-AEAPS₃₄₀ and BC-AEAPS₂₈₀,
 353 produced according to Protocol 2b (water-leached samples). Water and amine content are also
 354 reported.

	$T_{5\%}$ (°C)	T_{max} (°C)	Residue at 750 °C (%)	Water content (%)	Amine content (mmol/g)
BC	288	327	1.7	1.5	0
BC-APS ₃₄₀	284	329	5.1	3.6	0.5
BC-AEAPS ₃₄₀	207	320	17.1	9.9	3.8
BC-AEAPS ₂₈₀	238	333	18.0	7.1	4.9

356 **3.3. Ultrastructure**

357 The cross-sections of the freeze-dried unmodified and silylated samples were
 358 examined by SEM (Fig. 3). Irrespective of the treatment, the fibrillar structure of the BC
 359 material was preserved after silylation. All samples consisted of a 3D network of nanofibrils
 360 of varying diameters ($\varnothing = 10$ to 200 nm) forming a porous structure. The porosity and density
 361 of the freeze-dried samples were evaluated by mercury intrusion porosimetry, while the BET

362 specific surface area was determined by nitrogen sorption (Table 3 and Fig. S3 of supporting
363 information).



364

365 **Fig. 3.** SEM micrographs of the cross-sections of unmodified and water-leached silylated samples.

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Two magnifications are shown with scale bars of 100 and 10 μm.

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The silylation treatment did not significantly modify the porosity of the samples, which remained relatively high for both BC-AEAPS₃₄₀ and BC-AEAPS₂₈₀. Therefore, the presence of silane during the freeze-drying step did not significantly modify the structure of the membrane. However, the BET surface area decreased significantly after silylation, which can be partly attributed to the fact that 1 g of BC-AEAPS₃₄₀ or BC-AEAPS₂₈₀ contained only 0.69 g or 0.74 g of cellulosic material, respectively (Table 1). Hence, the BET surface area per gram of cellulose should be closer to 67 and 65 %, respectively. The difference still noted compared with the unmodified sample can then be reasonably attributed to a thickening of the cellulosic scaffold after condensation of the silane at the surface of the BC nanofibrils (a coarser structure is generally observed with silylated samples in Fig. 3). In all cases, the nitrogen adsorption isotherms of the samples, Fig. S3 of supporting information, displayed a type II profile, characteristic of a macro-porosity (> 50 nm) with no micro-pores (< 2 nm) (Thommes et al., 2015). The increase in density noted with the silylated samples, was

380 attributed to a compression of the specimen during the mercury intrusion experiments (clearly
 381 observed in the case of the BC-AEAPS₃₄₀ sample). Therefore, pore size distribution was not
 382 evaluated with this technique, as it would probably vary during measurements. Although the
 383 BC material was more hygroscopic after silylation (Table 2), its water absorption capacity
 384 was reduced (Table 3), which can again be attributed to the fact than the cellulose content in 1
 385 g of silylated material is less than in 1 g of unmodified BC. The water absorption capacity of
 386 the silylated BC scaffold nevertheless remained high.

387 **Table 3**

388 Density, porosity, specific surface area and water absorption capacity of the unmodified and water-
 389 leached silylated samples.

	Mercury intrusion data		Nitrogen data	Water abs. ass. data
	Density (kg/m ³)	Porosity (%)	BET surface area (m ² /g)	Absorbed water (g H ₂ O / g dry material)
BC	11	95	87	82
BC-AEAPS₃₄₀	28	92	52	37
BC-AEAPS₂₈₀	15	96	46	49

390

391 3.4. Anti-bacterial properties of silylated-BC

392 Antibacterial activities of the silylated-BC were first assessed by the plate-counting
 393 method against *E. coli* and *S. aureus* as the gram-negative and gram-positive model strains,
 394 respectively (Table 4).

395 For *E. coli*, BC and BC-HCl resulted in similar bacterial growth, around 9.0
 396 log₁₀(CFU/cm²). This showed that the possible residual acid after BC HCl-treatment did not
 397 lead to bacterial inhibition. Silylated BC without the protonation step in acidic conditions did
 398 not show any bacterial inhibition compared to the BC control. After HCl-treatment, silylated
 399 BC reduced the *E. coli* to 8.5, 8.1 and 7.9 log₁₀(CFU/cm²) for BC-APS₃₄₀, BC-AEAPS₃₄₀ and
 400 BC-AEAPS₂₈₀, respectively, but unfortunately at a very low level. The commonly accepted

401 mode of action of anti-bacterial activity of amino groups is related to the positive charge of
402 the corresponding ammonium functions (Bieser & Tiller, 2011; Fernandes et al., 2014;
403 Helander et al., 2001). Thanks to elemental analysis, the number of moles of primary amines
404 in BC-APS₃₄₀, BC-AEAPS₃₄₀ and BC-AEAPS₂₈₀ membranes after leaching experiments is
405 known. The APS solution had pH values of 10.16 (in water) and 2.23 (in HCl) and the
406 AEAPS solution had pH values of 10.08 (in water) and 6.80 (in HCl). Knowing the pKa of
407 free APS (9.6) and AEAPS (9.3), without HCl treatment, only 21.6% of amine functions are
408 protonated with APS and 14.2% with AEAPS, while the protonation is higher than 99.6% for
409 both APS and AEAPS treated by the HCl solution. Thus, contrary to what was expected, the
410 selected *E. coli* strain showed low sensitivity to chemically modified-BC, even after the
411 protonation step.

412 Compared to *E. coli*, *S. aureus* demonstrated high sensitivity to silylated-BC. First, BC
413 and BC-HCl showed similar behavior and reached about 10 log₁₀(CFU/cm²) within 14 days of
414 incubation. On BC-APS₃₄₀, BC-AEAPS₃₄₀ and BC-AEAPS₂₈₀ previously treated by HCl, the
415 viable cell charges after incubation were 9.8, 7.0 and 6.3 log₁₀(CFU/cm²), respectively. As has
416 been frequently observed with various active agents or materials, silylated-BC showed much
417 greater efficacy against the gram-positive pathogen than the gram-negative one. This could be
418 due to differences in the outer cell walls and membranes of gram-negative and gram-positive
419 bacteria. Gram-negative bacteria have a hydrophilic outer membrane and degradative and
420 detoxifying enzymes in the periplasmic space (Beveridge, 1999), while Gram-positive
421 bacteria do not have an outer membrane or periplasmic space containing protective enzymes
422 (Beveridge, 1999; Shan, Cai, Brooks, & Corke, 2007).

423 In addition, by contrast with *E. coli*, a strong difference was observed between APS-
424 containing BC (no significant log reduction compared to controls) and AEAPS-containing BC
425 (3 to 4.0 log reduction compared to controls). In addition to the variable aminosilane

426 concentration, this could be due to the longer distance between the cellulosic backbone and
427 the ammonium groups in BC-AEAPS. Indeed, Bieser *et al.* suggested that different
428 mechanisms can allow bioactivity by contact, depending on the side-chain length (Bieser &
429 Tiller, 2011).

430 **Table 4**

431 Number of *E. coli* and *S. aureus* colonies in the BC-materials in log₁₀(CFU/cm²) after 14h incubation.
432 Experiments were conducted in triplicate and results averaged.

	<i>E. coli</i>		<i>S. aureus</i>	
	Untreated	HCl treatment	Untreated	HCl treatment
BC	8.92 ± 0.08	9.07 ± 0.06	10.34 ± 0.12	10.29 ± 0.10
BC-APS₃₄₀	9.59 ± 0.07	8.52 ± 0.01	ND	9.85 ± 0.07
BC-AEAPS₃₄₀	9.54 ± 0.05	8.16 ± 0.03	ND	7.02 ± 0.09
BC-AEAPS₂₈₀	9.77 ± 0.04	7.87 ± 0.04	ND	6.33 ± 0.24

433 ND: Not determined

434 **4. Conclusion**

435 In conclusion, we have developed a convenient method of grafting non-leachable
436 bioactive amine functions onto the surface of bacterial cellulose nanofibrils, *via* a simple
437 silylation treatment in water. Under a controlled set of conditions (never-dried material, water
438 medium, room temperature, freeze-drying), water-leaching resistant (2-aminoethyl)-3-
439 aminopropylsilyl functions were successfully introduced into BC, after reacting with the
440 corresponding trimethoxysilane reagent (AEAPS). The success of the reaction was
441 determined by i) the use of BC in its wet state, and ii) the use of sufficiently low initial
442 concentrations of silane, to limit homocondensation and promote the reaction with
443 hydroxylated substrate. The relatively good stability of the grafting was attributed to the
444 structure of the AEAPS, which prevents the amine-catalyzed hydrolysis generally noted with

445 aminosilanes such as (3-aminopropyl)-trimethoxysilane (APS). The silylated material
446 remained highly porous, hygroscopic and displayed sufficient thermal stability to support the
447 sterilization treatments generally required in medical applications. The nanofibrillar structure
448 and macro-porosity of the cellulose membranes was preserved after the silylation treatment,
449 although a decrease in specific surface area was, attributed to a thickening of the fibrils after
450 the silane condensation. After protonation of the amino sides by an acidic treatment, BC-
451 AEAPS₂₈₀ was found to be an effective inherent antibacterial material against *S. aureus* in *in*
452 *vitro* studies.

453 Further investigations would be interesting in order to understand better the impact of
454 the concentration of aminosilanes in water on their condensation on BC nanofibrils. Further
455 work on the mechanism responsible for the bioactivity by contact of protonated amino
456 functions could help to give a better understanding of the critic parameters for the
457 functionalization of BC with aminosilanes. Further study is also required to test the
458 antibacterial properties of such materials against other microbial strains and in *in vivo*
459 conditions. Finally, the heterogeneous functionalization of BC with accessible amine groups
460 opens up a range of opportunities for the grafting of numerous compounds, such as peptides
461 in order to target anti-bacterial activity against specific stains.

462

463 **Supporting information**

464 Figures supplied in the supporting information include size exclusion chromatographs
465 of APS solution, TGA thermograms and nitrogen adsorption isotherms of unmodified and
466 silylated-BC.

467

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