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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-APS<sub>340</sub> or BC-AEAPS<sub>340</sub>).

### 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-APS<sub>340</sub>, BC-  
140 AEAPS<sub>340</sub> or BC-AEAPS<sub>280</sub>).

### 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  $\text{cm}^{-1}$  at a resolution of 4  $\text{cm}^{-1}$  (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  $\text{cm}^{-1}$ ) and were normalized to the  
153  $\delta_{\text{as}}(\text{CH}_2)$  asymmetric bending of cellulose at 1430  $\text{cm}^{-1}$  (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 <sup>1</sup>) 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  $150^{\circ}\text{C}$ . 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  $750^{\circ}\text{C}$  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 =  $130^{\circ}$ ; mercury surface tension =  $485\text{ mN m}^{-1}$ ; 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-APS<sub>340</sub>, BC-AEAPS<sub>340</sub> and BC-AEAPS<sub>280</sub>  
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<sup>-1</sup>  
211 NaCl) to wash the inoculum. This step was repeated 3 times. Dilution was adjusted to place a  
212 9 µL droplet of about 10<sup>2</sup> cells on top of 1 cm<sup>2</sup> 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.

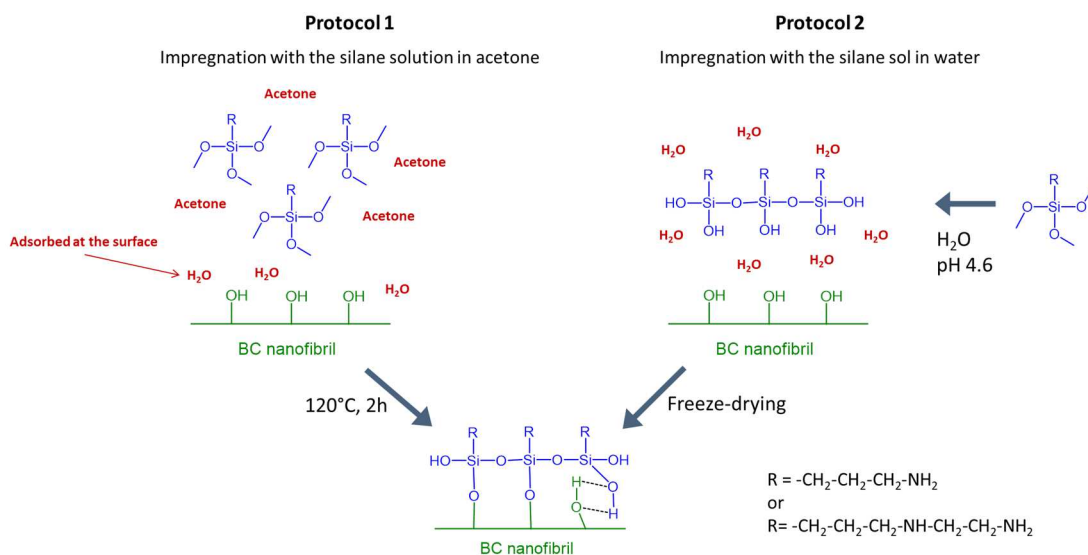
222

### 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  $120^\circ\text{C}$  (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-APS<sub>340</sub>) was confirmed by measuring nitrogen content by elemental analysis  
 249 (Table 1), and observing the  $\delta(\text{NH})$  amine vibration in the 1600-1590  $\text{cm}^{-1}$  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.*

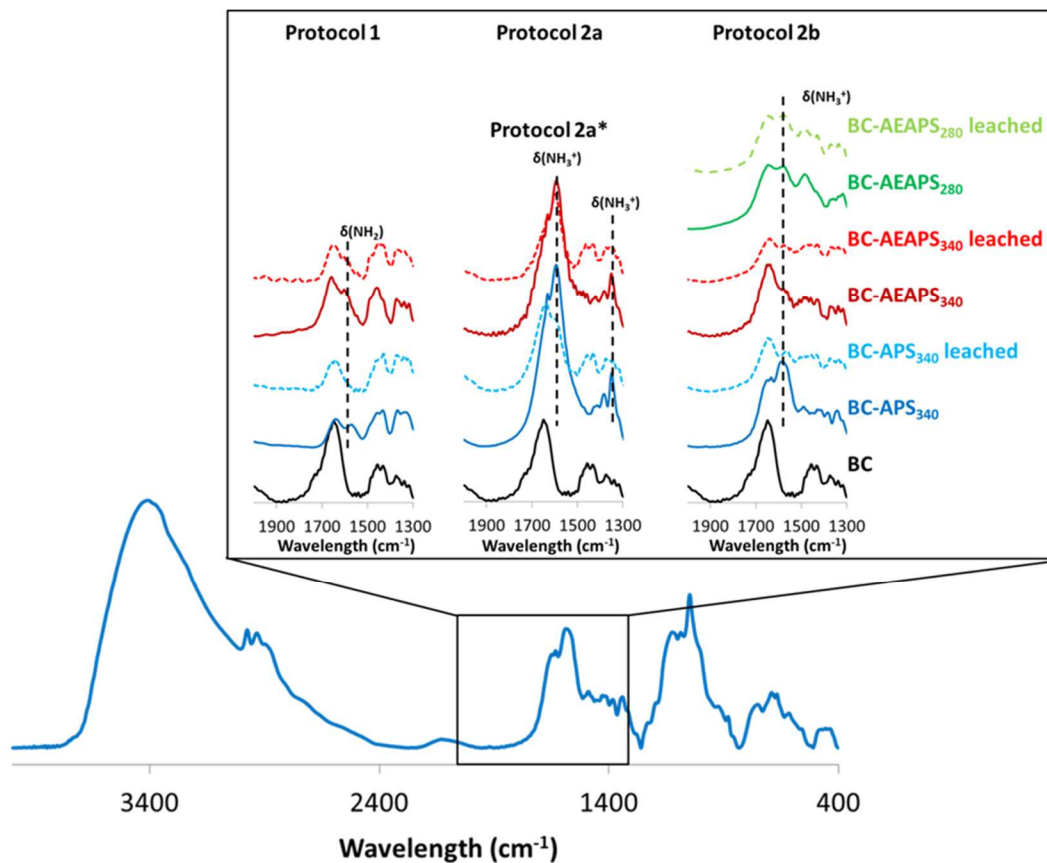
258 (2012) suggested increasing the chain length separating the silicon atom from the primary  
 259 amine moiety, using AEAPS as a silylating agent. This structure prevents the formation of  
 260 five-membered cyclic intermediates, leading to significant improvement in the stability of the  
 261 grafted 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>	<b>Protocol 1</b>		<b>Protocol 2a</b>		<b>Protocol 2b</b>		
	<i>Before</i>	<i>After</i>	<i>Before</i>	<i>After</i>	<i>Before</i>	<i>After</i>	
<b>BC-APS<sub>340</sub></b>	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
<b>BC-AEAPS<sub>340</sub></b>	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
<b>BC-AEAPS<sub>280</sub></b>	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<sup>-1</sup> region of unmodified BC, BC-APS<sub>340</sub> before and after  
 279 water leaching, BC-AEAPS<sub>340</sub> before and after water leaching and BC-AEAPS<sub>280</sub> 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<sup>-1</sup>  
294 and 1350 cm<sup>-1</sup> regions compared with protocol 1, which could be related to the presence of  
295 bicarbonate salts, possibly formed after contact between the grafted NH<sub>2</sub> groups and  
296 atmospheric CO<sub>2</sub> (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  $\delta(\text{NH})$  vibrations in

316 the FT-IR spectra was lower, presumably because fewer bicarbonate salts were formed by  
317 contact with CO<sub>2</sub>. 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-AEAPS<sub>280</sub> 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 ( $\text{SiO}_2$ ). 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**

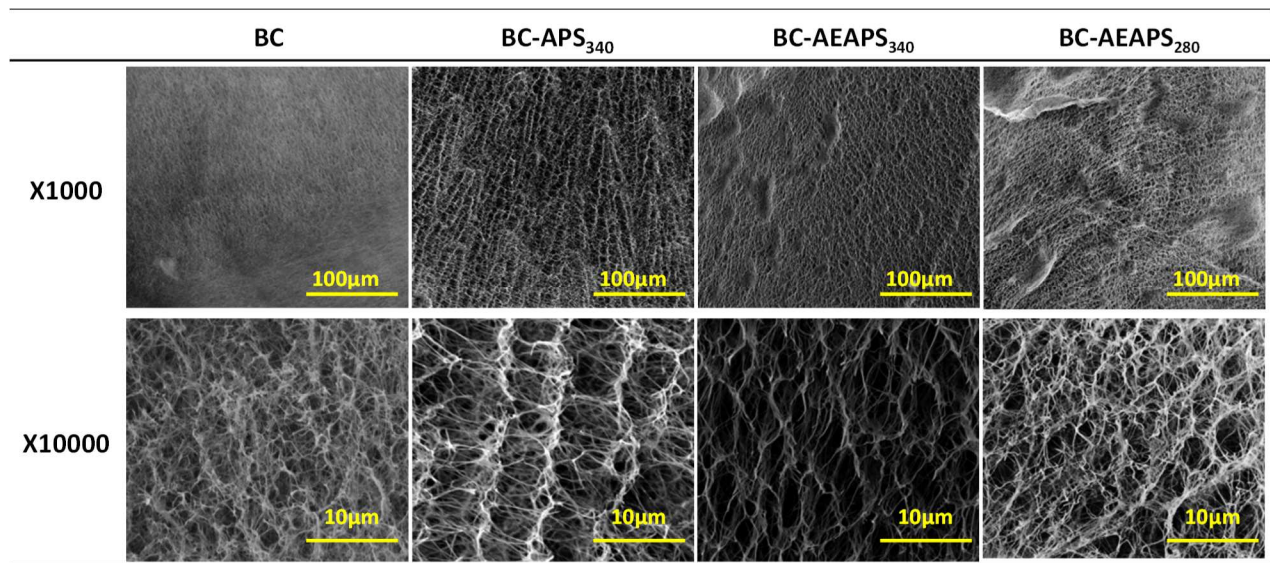
352 TGA data obtained after analysis under air flow of BC, BC-APS<sub>340</sub>, BC-AEAPS<sub>340</sub> and BC-AEAPS<sub>280</sub>,  
 353 produced according to Protocol 2b (water-leached samples). Water and amine content are also  
 354 reported.

	$T_{5\%}$ (°C)	$T_{\text{max}}$ (°C)	Residue at 750 °C (%)	Water content (%)	Amine content (mmol/g)
BC	288	327	1.7	1.5	0
BC-APS <sub>340</sub>	284	329	5.1	3.6	0.5
BC-AEAPS <sub>340</sub>	207	320	17.1	9.9	3.8
BC-AEAPS <sub>280</sub>	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-AEAPS<sub>340</sub> and BC-AEAPS<sub>280</sub>. 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-AEAPS<sub>340</sub> or BC-AEAPS<sub>280</sub> 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-AEAPS<sub>340</sub> 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 <sup>3</sup> )	Porosity (%)	BET surface area (m <sup>2</sup> /g)	Absorbed water (g H <sub>2</sub> O / g dry material)
<b>BC</b>	11	95	87	82
<b>BC-AEAPS<sub>340</sub></b>	28	92	52	37
<b>BC-AEAPS<sub>280</sub></b>	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 log<sub>10</sub>(CFU/cm<sup>2</sup>). 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 log<sub>10</sub>(CFU/cm<sup>2</sup>) for BC-APS<sub>340</sub>, BC-AEAPS<sub>340</sub> and  
 400 BC-AEAPS<sub>280</sub>, 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-APS<sub>340</sub>, BC-AEAPS<sub>340</sub> and BC-AEAPS<sub>280</sub> 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 log<sub>10</sub>(CFU/cm<sup>2</sup>) within 14 days of  
414 incubation. On BC-APS<sub>340</sub>, BC-AEAPS<sub>340</sub> and BC-AEAPS<sub>280</sub> previously treated by HCl, the  
415 viable cell charges after incubation were 9.8, 7.0 and 6.3 log<sub>10</sub>(CFU/cm<sup>2</sup>), 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 log<sub>10</sub>(CFU/cm<sup>2</sup>) 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
<b>BC</b>	8.92 ± 0.08	9.07 ± 0.06	10.34 ± 0.12	10.29 ± 0.10
<b>BC-APS<sub>340</sub></b>	9.59 ± 0.07	8.52 ± 0.01	ND	9.85 ± 0.07
<b>BC-AEAPS<sub>340</sub></b>	9.54 ± 0.05	8.16 ± 0.03	ND	7.02 ± 0.09
<b>BC-AEAPS<sub>280</sub></b>	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 AEAPS<sub>280</sub> 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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