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► **To cite this version:**

Muhammad Haseeb Iqbal, Fabienne Quilès, Emeline Pradel, Sarah Benmalek-Kehili, Christine Lancelon-Pin, et al.. Bactericidal and bioinspired chitin-based anisotropic layer-by-layer brushed-nanocoating. *Applied Materials Today*, 2023, 32, pp.101816. 10.1016/j.apmt.2023.101816 . hal-04066255

HAL Id: hal-04066255

<https://hal.science/hal-04066255v1>

Submitted on 12 Apr 2023

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1 Bactericidal and Bioinspired Chitin-Based Anisotropic 2 Layer-by-Layer Brushed-Nanocoating

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4 Muhammad Haseeb Iqbal ^a, Fabienne Quilès^b, Emeline Pradel ^a, Sarah Benmalek-Kehili ^a, Christine
5 Lancelon-Pin ^c, Laurent Heux^c, Florent Meyer^{d,e}, Lydie Ploux^{d,e}, Grégory Francius^b, and Fouzia
6 Boulmedais* ^a

7

8 a. Université de Strasbourg, CNRS, Institut Charles Sadron, UPR 22, Strasbourg, 67034, France

9 b. Université de Lorraine, CNRS, LCPME, F-54000 Nancy, France

10 c. Université Grenoble Alpes, CNRS, CERMAV, Grenoble, 38041, France

11 d. Centre de Recherche en Biomédecine de Strasbourg, Institut National de la Santé et de la

12 Recherche Médicale, UMR 1121, Biomatériaux et Bioingénierie, Strasbourg Cedex, 67085, France

13 e. Université de Strasbourg, Faculté de Chirurgie Dentaire, Strasbourg, 67000, France

14

15 ***Corresponding authors.** E-mail: fouzia.boulmedais@ics-cnrs.unistra.fr

16 Tel: +33 03 88 41 41 60

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19

20 **Abstract**

21 Top-down biomimetic surfaces with micro/nanotopographic features have emerged as a strategy to
22 prevent bacterial attachment and biofilm formation. These nanostructured surfaces mimic the nano-
23 micro topographical features found naturally on cicada wings. However, their development requires
24 expensive equipment and complex processes. Here, inspired by butterfly wings and crustacean
25 exoskeletons, we present the simple design of fully bio-based, anisotropic, and antibacterial layer-by-
26 layer (LbL) coatings based on chitin nanocrystals (ChNCs). Composed exclusively of US Food and
27 Drug Administration-approved materials, the LbL nanocoatings were obtained by manual brushing
28 of ChNC and Tannic Acid (TA), a natural polyphenol, coordinated by iron III (Fe^{3+}). In contrast to
29 those obtained by the dipping method, the brushed $\text{TA}/\text{Fe}^{3+}/\text{ChNC}$ coatings exhibit highly oriented
30 ChNC nanostructures that enable contact-killing antibacterial properties against *Staphylococcus*
31 *aureus* (a Gram-positive bacterium) and *Escherichia coli* (a Gram-negative bacterium). This
32 antibacterial property is due to the orientation of the ChNC into spike-like nanotopographies with a
33 high density of positive charge on the surface. Similar to cicada and dragonfly wings, the positively
34 charged spikes of the oriented ChNC films are likely to damage the bacterial cell wall by (i)
35 puncturing it upon contact with the spikes and/or (ii) stretching and tearing it when a shear force
36 detaches the cell from the highly positive surface. The fully bio-based nature of ChNC-based
37 nanocoatings and their brush-based fabrication method make them a suitable and affordable candidate
38 as a sustainable antibacterial film for (bio)materials in biomedical and environmental applications.

39

40 **Keywords:** Orientation, multilayers, bio-sourced, nanotopography, nanostructured surfaces,
41 antibacterial

42

43

44 **Introduction**

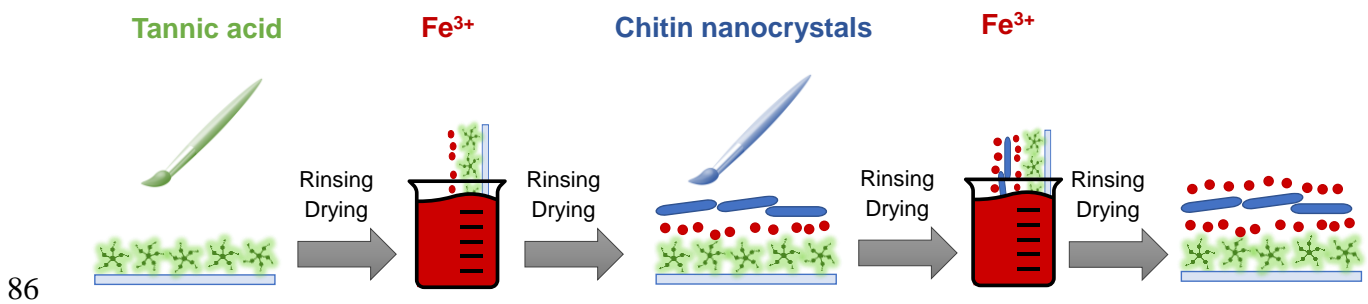
45 Nanostructured surface coatings have attracted great attention in the last decade to develop advanced
46 next-generation materials. Antibacterial surface modification is of paramount importance to address
47 bacterial infections, a major threat to human health worldwide. Antibacterial coatings are developed
48 either by top-down *i.e.*, physicomachanical structuring of surfaces,[1] or bottom-up approaches that
49 typically involve chemical modification of surfaces.[2] Top-down biomimetic surfaces with
50 micro/nano-topographical features have emerged as a strategy to prevent bacterial attachment and
51 biofilm formation.[3] These nanostructured surfaces are inspired by the nano-micro-topographic
52 features found naturally on cicada wings,[3] lotus leaves, gecko feet, and shark skin.[4] However,
53 their development requires expensive equipment and complex processes such as laser ablation,[1]
54 electropolishing, and photolithographic techniques.[4, 5]

55 The chemical modification includes surface functionalization with antimicrobial peptides,[6, 7] silver
56 nanoparticles, antibiotics, and synthetic or bio-based polymers.[8] Among the numerous bottom-up
57 surface functionalization strategies, the layer-by-layer (LbL) method is one of the simplest and most
58 versatile techniques, based on the alternate deposition of positively and negatively charged
59 polyelectrolytes on a substrate via dipping,[9] spin coating,[10] or spray assembly.[11] Because of
60 its ease of application to almost any substrate shape and size, and its mild preparation conditions
61 (aqueous solutions, room temperature), it has been widely used to develop anti-adhesive or
62 bacteriostatic, contact-killing, and release-killing antimicrobial surfaces.[12] Recently, a brush-based
63 LbL method has been introduced using paint brushes, synthetic polyelectrolytes, and soluble
64 polysaccharides.[13] The shear effect of the brushing technique has been reported to align the
65 monolayer of clay nanotubes,[14] hydroxyapatite micro- to nano-fibers,[15] and collagen to develop
66 human muscle fibers *in-vitro*.[16]

67 Extracted by acid hydrolysis from crustacean exoskeletons,[17] highly anisotropic crystalline chitin
68 nanocrystals and nanofibers are commonly used for their excellent mechanical properties to design
69 biodegradable three-dimensional (3D) materials[18] or films.[19] Using expensive types of

70 equipment, chitin-based oriented nanostructures have been designed using electric[20] and magnetic
71 fields,[21] shear,[22] and wet stretching[23] to develop optically anisotropic or mechanically robust
72 films. However, despite the well-known antibacterial properties of chitosan,[24] a deacetylated
73 compound of chitin, few studies have reported chitin-based membranes or 3D materials with
74 antifungal [25] or antibacterial properties.[26, 27] Fungal and bacterial inhibitions have rarely been
75 achieved with pristine chitin,[25] but mostly with chemically modified chitin,[27] or by incorporating
76 chitosan[26] or other antipathogenic agents such as copper.[28] Furthermore, none of the few studies
77 on chitin-based LbL obtained by the dipping method,[29-32] reported antibacterial properties of the
78 bio-sourced film.

79 Here, inspired by chitin-based butterfly wings[33] and iron-rich crustacean exoskeletons,[34] we
80 report the simple design of fully bio-sourced, anisotropic, and antibacterial LbL coatings based on
81 chitin nanocrystals (ChNCs) using the brushing method (Figure 1 and Supporting Information Figure
82 S1). Composed exclusively of FDA-approved materials (FDA: United States Food and Drug
83 Administration),[35, 36] the LbL nanocoatings were obtained by manual brushing of ChNC and
84 tannic acid (TA), an abundant natural polyphenol with intermediate dipping in an iron III (Fe^{3+})
85 solution to ensure coordination bonding with both TA and ChNC.[37]



87 **Figure 1: The LbL brushing method.** Schematic representation of anisotropic ChNC film's buildup
88 by the brushing LbL method based on the cycling deposition of (TA/ Fe^{3+} /ChNC/ Fe^{3+}). After the
89 deposition of TA or ChNC by the paintbrush, an adlayer of Fe^{3+} is deposited by dipping alternated by
90 the rinsing step with ultrapure water and the drying step by compressed air along the brushing
91 direction. Paintbrush image from stockio.com for commercial use.

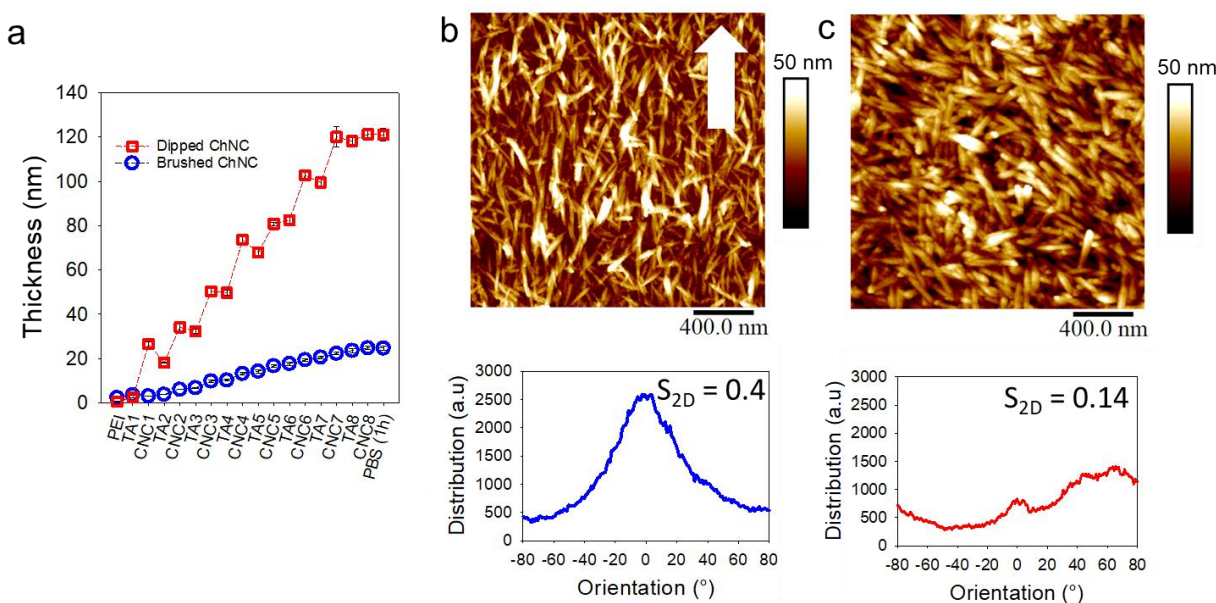
92 In contrast to those obtained by the dipping method, the brushed TA/Fe³⁺/ChNC coatings exhibit
93 highly oriented ChNC nanostructures, which enable contact-killing antibacterial properties against
94 *Staphylococcus aureus* (*S. aureus*, a Gram-positive bacterium) and *Escherichia coli* (*E. coli*, a Gram-
95 negative bacterium). The main focus of this study is on a comprehensive evaluation of the
96 physicochemical and antibacterial properties of brushed and dipped TA/Fe³⁺/ChNC/Fe³⁺ coatings to
97 decipher their mechanism of action.

98 **1. Results and discussion**

99 **1.1. The brushing method aligns chitin nanocrystals in LbL films**

100 Anisotropic elongated nanostructured ChNCs suspension was obtained from chitin powder using a
101 previously described protocol.[17] The ChNC nanostructure size was measured at 116 nm by
102 dynamic light scattering measurements and confirmed by atomic force microscopy (AFM) with 116
103 ± 8 nm in length and 10 ± 2 nm in width (Supporting Information Figure S2). They showed strong
104 cationic character with a zeta potential of +48.3 mV due to the presence of protonation of some amino
105 groups that have been formed by deacetylation of surface N-acetyl amido groups.[18] Since the ChNC
106 suspension was at pH 3 to ensure its long-term stability during storage, all the LbL buildups were
107 performed with all solutions prepared at pH ≤ 3. Based on chitosan/TA[38] and chitosan/Fe³⁺[39]
108 interactions, ChNC/Fe³⁺ and ChNC/TA LbL films were built up on a poly(ethylene imine) (PEI)
109 precursor layer by manual brushing, which showed limited thickness growth (Supporting Information
110 Figure S3-S4). Thus, the deposition of (TA/Fe³⁺/ChNC/Fe³⁺) quadrilayers was enabled by the
111 coordination bonds known to allow TA to interact with Fe³⁺,[37] resulting in the so-called brushed
112 ChNC films. The dipping method was used for a control surface, which was named dipped ChNC
113 films. The thickness of the brushed ChNC film increases linearly as a function of the last deposition
114 step reaching up to 24.8 ± 0.8 nm after 8 quadrilayers, *i.e.* PEI-(TA/Fe³⁺/ChNC/Fe³⁺)₈. In contrast,
115 irregular growth of the dipped films was obtained reaching a plateau of 121.3 ± 2.8 nm after 7
116 quadrilayers (Figure 2a). The main thickness increase comes from ChNC (about 15-20 nm
117 corresponding to 1-2 ChNC thick). The reduced amount of suspension and the high shear effect of

118 the brushing method lead to a smaller thickness increment of ChNC compared to the dipping method
 119 as reported for other systems.[13] The small increment may also result from the different refractive
 120 index values in a direction parallel or perpendicular to their long axis[40] which can explain the small
 121 thickness increment of ChNC. The dipped and brushed ChNC/TA films were stable in PBS with no
 122 significant loss of dry thickness (Figure 2a).



123
 124 **Figure 2. Thickness and surface characterization of ChNC LbL films.** (a) Evolution of the
 125 thickness as a function of the last deposited layer followed by contact with PBS. For sake of clarity,
 126 the incremental thickness (< 1 nm) of Fe^{3+} is not reported. The standard deviations are not visible due
 127 to the scale of the y-axis. Typical atomic force microscopy (AFM) topography images and their
 128 orientation distribution graphs, with the order parameter S_{2D} of (b) the brushed and (c) the dipped
 129 ChNC films, *i.e.* $\text{PEI}-(\text{TA}/\text{Fe}^{3+}/\text{ChNC}/\text{Fe}^{3+})_7\text{-TA}/\text{Fe}^{3+}/\text{ChNC}$. The brushing direction, visible on the
 130 video camera, is indicated by the white arrow.

131 The low magnification light microscopy and scanning electron microscopy (SEM) images of the
 132 brushed ChNC films show the presence of parallel patterns in the brushing direction (Supporting
 133 Information, Figure S5). In contrast, the dipped films appeared homogeneous with a dense coverage
 134 of the surface by ChNCs. The ChNC films were then imaged by atomic force microscopy (AFM) in
 135 the dry state to characterize their topography (Figure 2b-c). In contrast to the dipped films, the ChNCs

136 of the brushed ChNC/TA films were preferentially oriented along the brushing direction. To
137 characterize their degree of orientation, the AFM topography images were analyzed using
138 OrientationJ (Supporting Information Figure S6). The orientation distribution curve of the brushed
139 ChNC/TA films shows a maximum at 0° (*i.e.* along the brushing direction) and nanocrystals oriented
140 between -40° and $+40^\circ$ (Figure 2b). In contrast, the dipped films result in randomly distributed
141 nanocrystals with an almost flat distribution curve (Figure 2c). The shearing effect of the brushing
142 allowed an efficient orientation of the ChNC with the order of orientation “ S_{2D} ” (values ranging from
143 0 to 1, random orientation when $S_{2D} = 0$, perfect alignment when $S_{2D} = 1$) at 0.40 and 0.14 for the
144 brushed and dipped films, respectively.[14] The cross-section of the AFM topography images shows
145 peaks of 20-30 and 15-25 nm in height for the brushed and dipped films, respectively (Figure 2d).
146 The thickness of the brushed ChNC films was verified by AFM imaging of the cross-section of a
147 scratched film. The thickness of the 8 brushed quadrilayers was 23.1 ± 2.7 nm (Supporting
148 Information Figure S7) in agreement with the value obtained by ellipsometry. TA, TA/Fe³⁺, and
149 ChNC/Fe³⁺-terminated brushed films showed similar topography features with S_{2D} values of 0.57,
150 0.34, and 0.44, respectively (Supporting Information Figure S8). In the following, the brushed and
151 the dipped ChNC films of the eight ChNC terminated quadrilayers, *i.e.* PEI-(TA/Fe³⁺/ChNC/Fe³⁺)₇-
152 TA/Fe³⁺/ChNC, will be referred to as oriented and nonoriented ChNC films, respectively.

153 **1.2. Antibacterial activity of ChNC LbL films**

154 Bacterial adhesion is the first step before surface colonization leads to biofilm (with a polysaccharide-
155 rich matrix), which protects bacteria from the action of antibiotics. *S. aureus* is known to cause
156 nosocomial and device-associated infections[41] and *E. coli* is the most common cause of catheter-
157 associated infections.[42] Therefore, the antibacterial properties of the oriented and nonoriented
158 ChNC films were evaluated against *S. aureus* and *E. coli* by assessing bacterial adhesion at a short
159 time (1.5 h) and proliferation during 24 h as well as biofilm formation after 48 h of *S. aureus*
160 incubation. A Live/Dead™ assay was performed to observe bacterial growth on the surface of the
161 films, with live bacteria stained green (SYTO®-9 stain) and damaged bacteria stained red (propidium

162 iodide stain, PI). Note that no reduction in bacterial growth of both species was observed in the
163 supernatant of either ChNC film compared to uncoated glass (Supporting Information Figure S9).

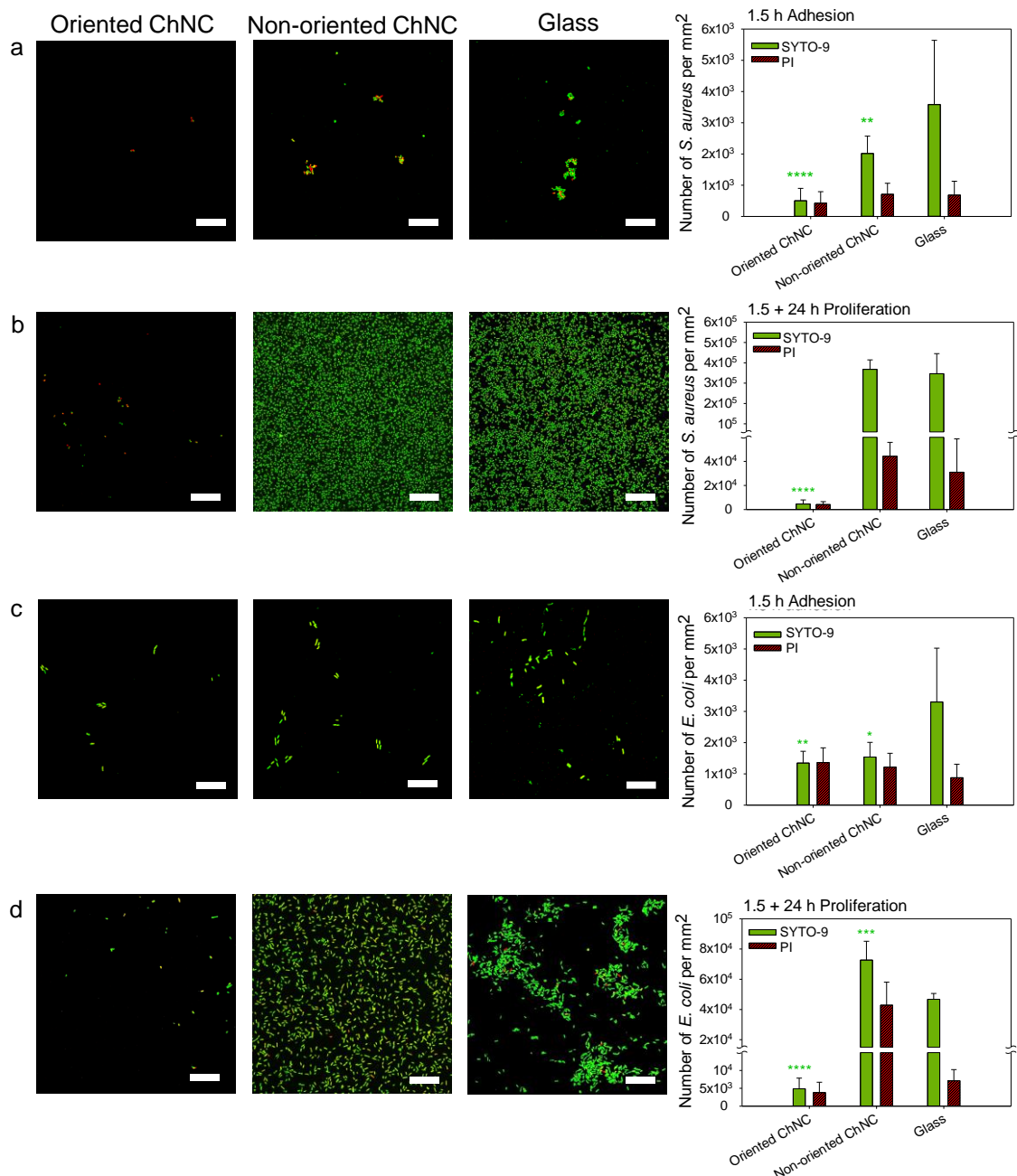
164 *1.2.1. Oriented ChNC film prevents bacterial adhesion*

165 For both bacterial species, the confocal optical microscopy images clearly show fewer bacteria
166 adhering to the oriented ChNC films compared to the nonoriented films and uncoated glass (Figures
167 3a and c). The oriented ChNC films show approximately 86% reduction in the number of live *S.*
168 *aureus* (green staining) compared to uncoated glass (with $p\text{-value} \leq 0.0001$) (Figure 3a). The
169 nonoriented films present only about 43% reduction vs the uncoated glass (with $p\text{-value} \leq 0.01$). The
170 viability of bacteria was evaluated by calculating the ratio of the number of permeabilized and
171 damaged bacteria (red staining) and all bacteria (SYTO®-9 green staining + PI red staining).
172 Approximately 46% of adherent *S. aureus* were damaged on the oriented ChNC films compared to
173 only 26% and 16% on the nonoriented films and uncoated glass, respectively. For *E. coli*, bacterial
174 adhesion on the oriented ChNC films was reduced by 46% compared to the uncoated glass (with $p\text{-}$
175 $value \leq 0.01$), with 50% of the bacteria damaged (vs. 20% for uncoated glass) (Figure 3c). The
176 nonoriented films showed similar results with a 41% reduction in live bacteria and the presence of
177 44% damaged bacteria. The oriented ChNC films show better prevention of bacterial adhesion than
178 the nonoriented films for both bacterial strains. In this effect, contact-killing plays an important role
179 since a significant fraction of bacteria observed on the surface are damaged, while no inhibition of
180 bacterial growth is observed in the supernatant (Supplementary Information Figure S9ac).

181 *1.2.2. Oriented ChNC film inhibits bacterial proliferation on the film surface*

182 After 1.5 h of initial incubation followed by 24 h of incubation in fresh culture media, confocal
183 microscopy images showed a dramatic effect of the oriented ChNC films with only a few *S. aureus*
184 and *E. coli* bacteria on the surface, in contrast to that observed on the nonoriented films and uncoated
185 glass (Figure 3b, d).

186

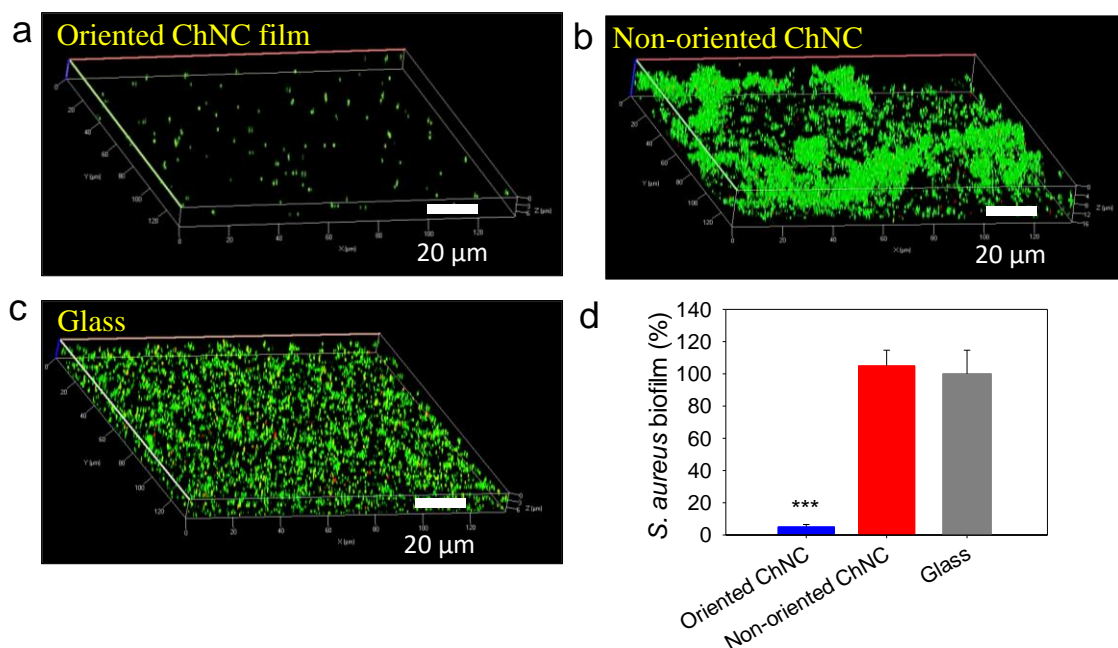


187 **Figure 3. The contact-killing activity of the oriented ChNC films.** Antibacterial properties of
 188 ChNC films determined by the Live/Dead™ assay, which stains live bacteria green (SYTO®-9) and
 189 damaged ones red (PI). Typical confocal microscopy images in combined channels (scale bar: 20 μm)
 190 and the corresponding number of bacteria adhered per mm² (at least 7 samples, 21 images) (a, b) of
 191 *S. aureus* and (c, d) *E. coli* after (a, c) 1.5 h incubation and (b, d) followed by 24 h proliferation at
 192 37°C. The two-tail student t-test was calculated on the number of SYTO-9 labeled bacteria in
 193 comparison to the uncoated glass (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001). As the
 194 total number of adherent bacteria is dramatically different, the statistical test on the number of PI-
 195 labelled bacteria does not seem relevant and was not performed.

196 Compared to the uncoated glass, the oriented ChNC films decreased the number of live *S. aureus*
197 (green staining) by almost 2-log (98.6%, $p\text{-value} \leq 0.0001$) and *E. coli* by 1-log (90%, $p\text{-value} \leq$
198 0.0001), with 48% and 44% damaged adherent bacteria, respectively (Figure 3b-d). The nonoriented
199 films showed unchanged and increased proliferation of *S. aureus* and *E. coli*, respectively. The
200 metabolic activity of *S. aureus* was assessed using the SYTO®-24/CTC Live/Dead™ assay, staining
201 all bacteria green and respiratory active bacteria red (CTC) (Supplementary Information Figure S10)
202 after 24 h of proliferation. As observed previously, few bacteria adhered to the oriented ChNC films
203 with 48% of *S. aureus* having a respiratory activity (90% on the uncoated glass), thus confirming the
204 detrimental effect on live bacteria (Figure 3b). Taken together, these results confirm that the reduction
205 in colonization on the oriented ChNC is due, at least in a large part, to a surface-localized contact-
206 killing effect rather than an antiadhesive effect, and demonstrate that it also affects the bacterial cells
207 resulting from the proliferation of the pioneer sessile bacteria. In addition, the results show that
208 contact-killing not only damages the cell wall but also a viable function such as respiration.

209 210 *1.2.3. Oriented ChNC film prevents biofilm formation*

211 To investigate the long-term surface activity, biofilm assays were performed with 48 h incubation of
212 high *S. aureus* density. The SYTO®-24/CTC staining showed the presence of only a few bacteria on
213 the oriented ChNC films in contrast to the nonoriented films and the uncoated glass (Figure 4a). The
214 biomass (i.e. the number of cells) on the oriented ChNC films was approximately 96% lower than on
215 the uncoated glass (with a $p\text{-value} \leq 0.001$) (Figure 4b).



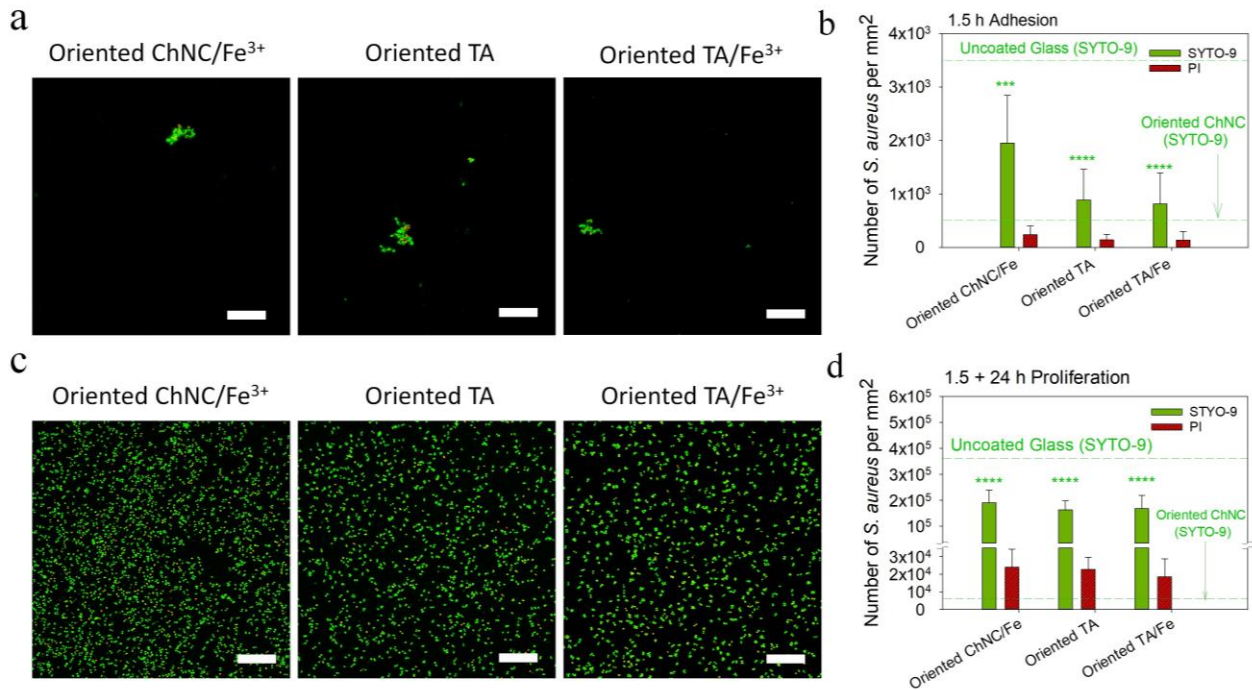
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217 **Figure 4: The antibiofilm property of ChNC LbL films.** *S. aureus* biofilm on ChNC films and
 218 uncoated glass after 48 h of incubation. (a-c) Typical 3D confocal microscopy images after SYTO-
 219 24®/CTC staining, which stains all bacteria green (SYTO-24®) and metabolically active ones red
 220 (CTC). (d) Normalized biofilm quantification obtained by Safranin staining with the significance
 221 level in t-test in comparison to the uncoated glass with *** $p \leq 0.001$.

222 1.2.4. The crucial role of the terminating layer of ChNC-oriented films on the antibacterial activity

223 Knowing that they have a similar nanotopography (Supplementary Information Figure S8), the
 224 antibacterial properties of ChNC-based oriented films with different terminating layers were
 225 evaluated against *S. aureus*. Bacterial adhesion and proliferation were assessed using the SYTO®-
 226 9/PI Live/Dead™ assay on the oriented ChNC/Fe³⁺, TA, and TA/Fe³⁺-terminated films corresponding
 227 to (TA/Fe³⁺/ChNC/Fe³⁺)₈, (TA/Fe³⁺/ChNC/Fe³⁺)₇-TA, and TA/Fe³⁺/ChNC/Fe³⁺)₇-TA/Fe³⁺ LbL films,
 228 respectively. The oriented ChNC/Fe³⁺, TA, and TA/Fe³⁺ films showed higher colonization with more
 229 adherent bacteria compared to the oriented ChNC films terminated by ChNC (Figure 5a-b). The
 230 percentage of damaged bacteria after 1.5 h of culture was between 11-14 % (similar to glass at 16%).
 231 In terms of proliferation, a weaker reduction effect was obtained on the three oriented films compared
 232 to the ChNC terminated films, but with a statistically lower number of bacteria than on the

233 nonoriented ChNC films and uncoated glass (with p -value ≤ 0.0001) and 10% damaged bacteria
 234 (similar to uncoated glass) (Figure 5c-d).



235
 236 **Figure 5. The crucial role of the ending layer on the antibacterial activity of the oriented ChNC**
 237 **films against *S. aureus*.** Typical confocal microscopy images (combined green and red channels,
 238 scale bar: 20 μ m) and the corresponding number of bacteria adhered per mm² (at least 7 samples, 21
 239 images) for the bacterial (a, b) adhesion and (c, d) proliferation after SYTO®-9/PI Live/Dead assay
 240 of live bacteria green (SYTO®-9) and damaged ones red (PI). The values obtained in SYTO®-9
 241 staining for the uncoated glass and the oriented ChNC films are represented by two dashed green
 242 lines. The significance level in the t-test was performed on SYTO®-9 staining in comparison to the
 243 uncoated glass *** $p \leq 0.001$ and **** $p \leq 0.0001$.

244 Thus, the results showed a clear effect of the ChNC terminated layer on the antibacterial properties
 245 of the oriented ChNC films. In the case of Fe³⁺-terminated films, iron could affect the antibacterial
 246 activity as it is an essential nutrient for bacteria. On TA-terminated films, even though TA is known
 247 to be antibacterial in solution,[43] but its immobilized form is usually not.[44]

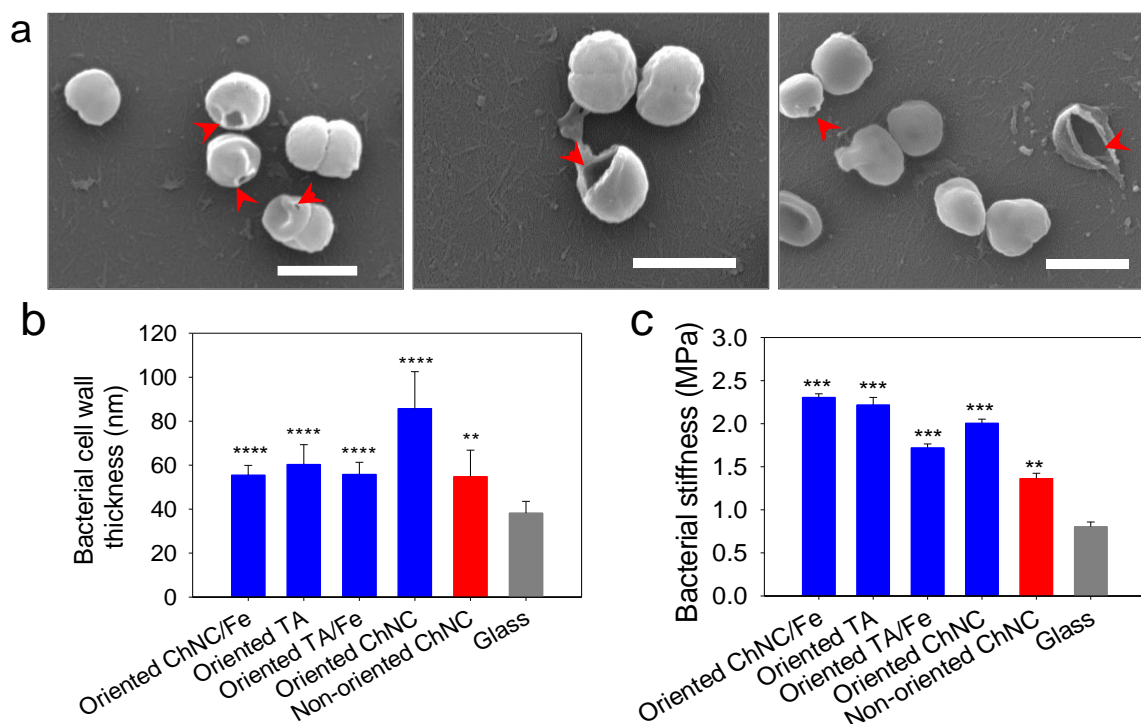
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249 **1.3. Insight into the mode of action of the oriented ChNC films**

250 ChNC-terminated oriented films showed strong contact-killing antibacterial activity with a possible
251 antiadhesive property against *S. aureus* and *E. coli*. Few studies have reported the antibacterial
252 property of chitin-based films or materials. ChNC/starch blend membranes have been reported to be
253 bacteriostatic against *L. monocytogenes* (Gram-positive) but not against *E. coli* (Gram-negative).[45]
254 The antibacterial activity of ChNC is described as being due to its interaction with the negatively
255 charged bacterial cell wall, altering permeability and causing membrane rupture and leakage of
256 intracellular material, followed by cell death.[46] To gain more insight into the mechanism of action,
257 we characterized the properties of adherent *S. aureus* on ChNC films and the surface properties of
258 the films in comparison to the glass substrate.

259 *1.3.1. Membrane integrity and mechanical properties of adherent S. aureus*

260 The membrane integrity of adherent *S. aureus* was assessed by SEM after their proliferation on the
261 different ChNC-based films compared to uncoated glass. The few adherent bacteria found on oriented
262 ChNC films had severely damaged cell wall membranes (Figure 6a). More bacteria were present on
263 the nonoriented ChNC films, oriented ChNC/Fe³⁺, TA/Fe³⁺, and TA-terminated films as well as
264 uncoated glass with less damaged bacteria (Supplementary Information Figure S11). Interestingly,
265 the cell-wall thickness of the adherent *S. aureus* was significantly higher on the oriented ChNC films
266 (86 ± 17 nm, p values < 0.0001) than on glass (38 ± 5 nm) and about 60 nm on the nonoriented ChNC
267 films and the other oriented films (Figure 6b). This increase in cell-wall thickness can probably
268 explain the higher stiffness values of bacteria in contact with the oriented ChNC film (2.00 ± 0.05
269 MPa), and the other films tested, compared to those on the uncoated glass (0.80 ± 0.05 MPa) as shown
270 in Figure 6c. Thickening of the *S. aureus* cell wall is commonly associated with antibiotic
271 resistance.[47] This bacterial strain can reversibly switch from the wild-type to a different phenotype
272 (small colony variants) resulting in an almost twofold increase in cell wall thickness in an attempt to
273 survive in harsh environmental conditions, usually after a few days.[48]



274 **Figure 6: Characterization of adherent *S. aureus* on the ChNC-based films vs glass after 24 h**
 275 **proliferation. (a)** Morphology, observed by SEM, on the oriented ChNC films (Scale bar: 1 μm) and
 276 **(b)** Bacterial cell wall thickness, calculated from SEM images, and **(c)** stiffness, determined by AFM
 277 nanoindentation technique, with the significance level in ANOVA test in comparison to the uncoated
 278 glass with * $p \leq 0.05$, ** $p \leq 0.05$, *** $p \leq 0.001$, and **** $p \leq 0.0001$.

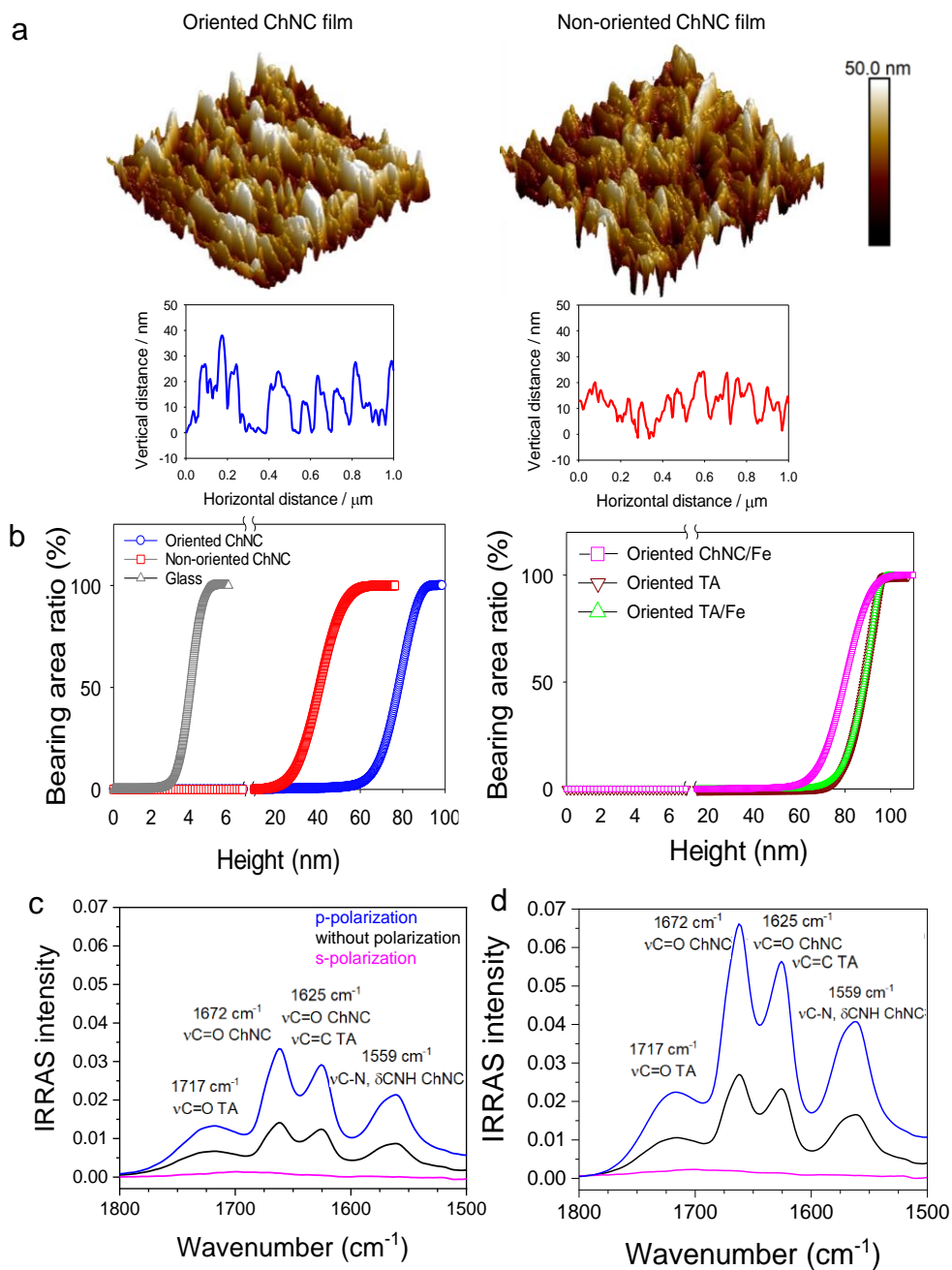
279 Nanopillar-structured surfaces have been reported to cause stiffening of bacterial cell walls due to
 280 induced mechanical stresses superseding the cell wall elasticity, leading to membrane perforation,
 281 loss of cell contents, and ultimately cell death.[51] The increase in cell wall thickness on the oriented
 282 ChNC films may therefore be due to an adaptation and survival strategy of *S. aureus* to the harsh
 283 environment provided by the films. The increase in cell wall stiffness may be the result of this change
 284 in thickness or the direct effect of the mechanical stresses exerted on the cell membrane by the ChNC
 285 on the film surface.

286

287 1.3.2. Nanotopography characterization of ChNC-based films

288 Spike-like topographic features of the oriented ChNC films were observed from the three-
 289 dimensional (3D) AFM images as well as the cross-sectional profiles, where the oriented ChNC films

290 had more and higher peaks (20-30 up to 40 nm in height) than the nonoriented ChNC films (less than
 291 25 nm in height) (Figure 7a and Supplementary Information Figure S8b).



292 **Figure 7: Nanotopography and chemical characterization of the ChNC-based films. (a)** 3D AFM
 293 images ($1 \times 1 \mu\text{m}^2$) of the topography of ChNC films with their respective cross-section profiles. **(b)**
 294 Bearing area ratio, calculated from AFM topography images, as a function of the height of the ChNC-
 295 based films and the uncoated glass. Infrared spectra in reflection absorption mode (IRRAS) of **(c)** the
 296 oriented and **(d)** nonoriented ChNC films.

297

298 We evaluated the difference in nanotopography between the ChNC LbL films using the bearing
299 analysis on the AFM topography images. This method allows analyzing the distribution of the heights
300 over a surface by plotting the percentage of the surface (*y*-axis), *i.e.* the bearing area ratio, which is
301 above the selected height (*x*-axis) (Figure 7b). [52] A sharp increase in the bearing area ratio was
302 observed at 60 nm for all the oriented films regardless of the final layer, 20 nm for the nonoriented
303 CNC films, and 2 nm for uncoated glass. The increase in bearing area ratio at higher values of heights
304 is related to a rougher topography. Infrared reflection absorption spectra (IRRAS) were recorded on
305 the oriented and nonoriented ChNC films, deposited on a gold substrate, to have information on the
306 molecular orientation of ChNC (Figure 7c-d and Supplementary Information Figure S12). The bands'
307 wavenumbers and assignments are gathered in Supplementary Information Table S1. The band
308 assignments were found in the literature[53, 54] and the spectra of pure ChNC and TA compounds
309 (Supplementary Information Figure S12). The spectral features show the presence of ChNC and TA
310 in both films with different overall intensities. In the 1800-1480 cm^{-1} region, the spectra are dominated
311 by band absorptions at 1717 cm^{-1} from TA (C=O, esters of gallic acid units), 1662 cm^{-1} from ChNC
312 (C=O, amide I band), 1625 cm^{-1} from ChNC (C=O, acetyl group) and TA (C=C aromatic skeletal
313 rings), and 1559 cm^{-1} from ChNC (amide II band) (Figure 7c-d). The high reflectivity of p-polarized
314 light on metals allows the measurement of only vibrations with a component of the transition dipole
315 moment oriented perpendicular to the surface.[55] Considering the fingerprint region (1900-700 cm^{-1})
316 without polarized or with a p-polarized IR beam, the calculated integrated intensities were about 2
317 times higher for the nonoriented ChNC films compared to the oriented ones. With the film thickness
318 measured to be 121.3 ± 2.8 nm for the nonoriented films and 24.8 ± 0.8 nm for the oriented ones
319 (Figure 1a), the integrated intensity ratio should be about 5 times higher instead of 2. Thus, despite a
320 lower amount of ChNC deposited, the oriented films have more vibrational modes oriented
321 perpendicular to the surface plane than the nonoriented films, showing a higher spatial organization
322 in this direction. This confirms the spike-like topography of the oriented ChNC films.

323

324 1.3.3. Surface charge plays a critical role in bacterial death

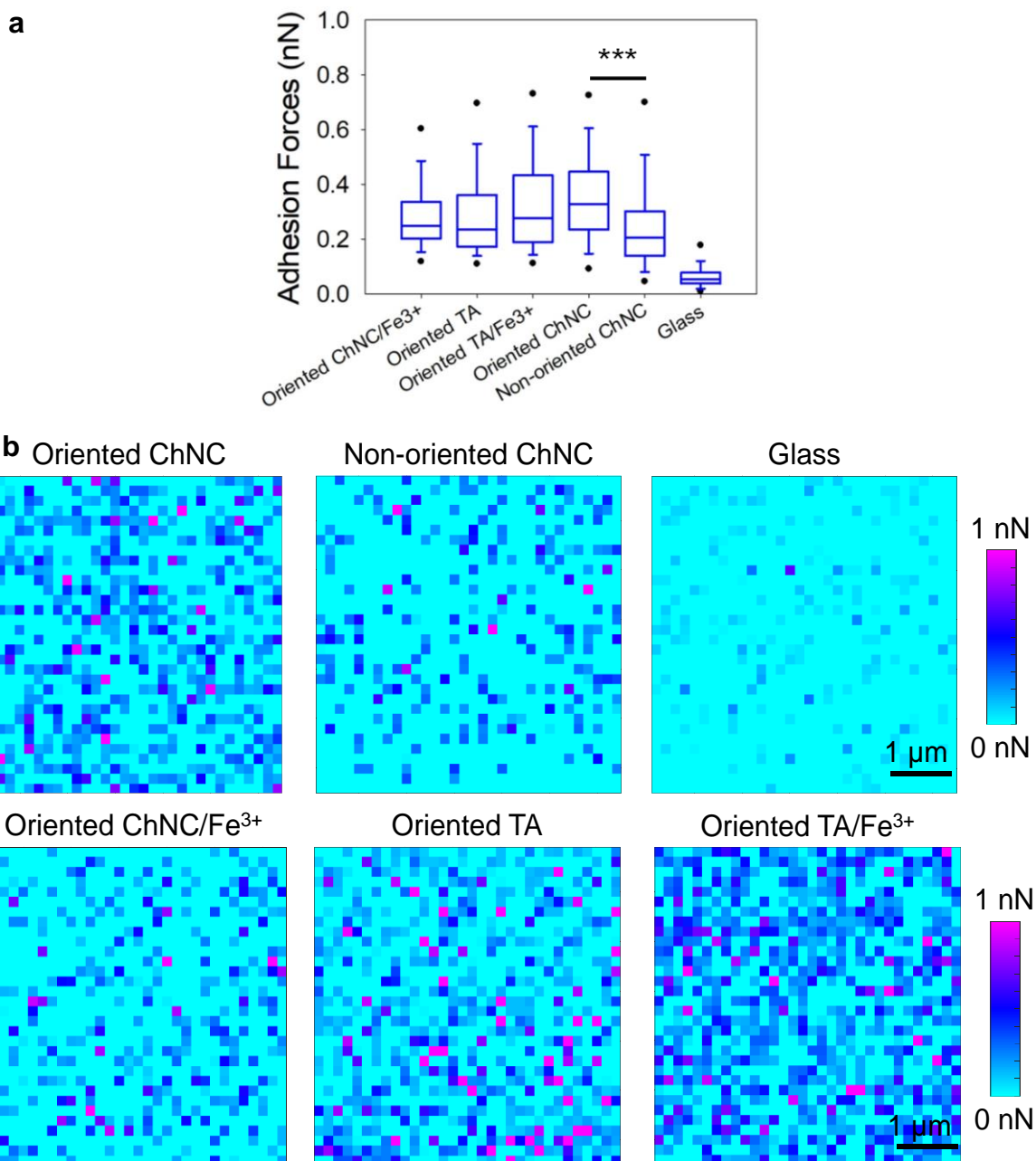
325 The nature of the terminal layer plays a crucial role as the most effective antibacterial activity is
326 obtained by terminating with the ChNC layer. Chemical force microscopy (CFM) was used to
327 characterize the surface charge density of the different studied films.[56] The chemical interaction
328 between the tip and the surface was measured by converting the deflection of the AFM cantilever
329 during the AFM tip withdrawal into adhesion force.[52, 57] The positive surface charge of the films
330 was characterized using a phosphate-functionalized AFM probe, to mimic the bacterial cell
331 membranes, in PBS at pH 7.4. Since the radius of the contact area of *S. aureus* on flat surfaces is in
332 the range of several hundred nanometers,[58] the mapping of the adhesion force was determined on
333 the $5 \times 5 \mu\text{m}^2$ surface area of the samples. A statistically higher adhesion force, *i.e.* interactions
334 between the phosphate tip and the surface, was observed for the non-oriented and oriented ChNC-
335 based films independently of the final layer compared to the uncoated glass (Figure 8a,
336 Supplementary Information Table S2). With a median value of 0.35 nN (p-value < 0.001), a higher
337 density of positive charges and adhesion force were observed on the oriented ChNC films compared
338 to the nonoriented films (Figure 8 and Supplementary Information Table S2). This result is probably
339 related to the rougher topography of the oriented ChNC films, resulting in the availability of a higher
340 amount of surface charges of the deposited ChNC compared to the nonoriented ChNC films. The
341 density of charges on ChNC/Fe³⁺, TA/Fe³⁺, and TA-terminated films was either similar to or higher
342 than the ChNC-terminated film with statistically similar adhesion force (Figure 8b). This could be
343 explained by electrostatic interactions of Fe³⁺ and hydrogen bonding interactions of TA with the
344 phosphate-functionalized tip, respectively.[59]

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350 **Figure 8: Surface charge density of ChNC-based films.** Adhesion forces, measured by chemical
 351 force microscopy (CFM) using a phosphonate-functionalized AFM tip, **(a)** box-chart showing the
 352 median (line), the 5% and 95% quartiles, with the significance level in ANOVA test in comparison
 353 to the uncoated glass with *** $p \leq 0.001$ for all films and **(b)** mapping over a $5 \times 5 \mu\text{m}^2$ area of the
 354 surface of different films where each pixel represents the adhesion force measured by the
 355 functionalized AFM tip as it retracts from the sample surface.

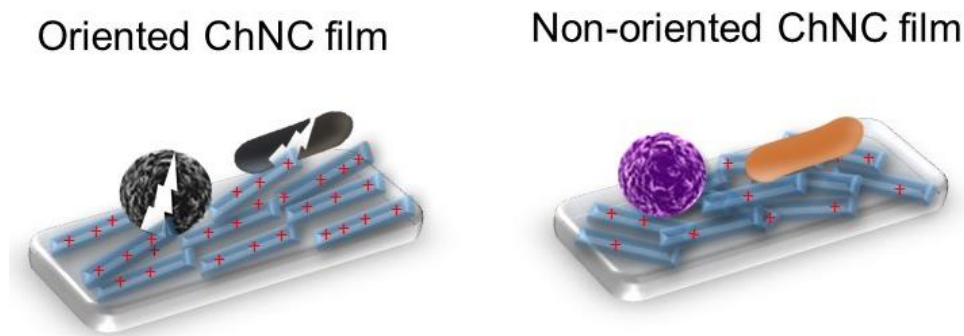
356

357

358 *1.4.4 Mode of action of the oriented ChNC films*

359 The bactericidal effect of nanostructured surfaces, consisting of metals, organic/inorganic materials,
360 or synthetic polymers, is usually associated with the mechanical rupture of cells, independent of the
361 surface chemistry and possible antiadhesive properties resulting from the nanoscale topography. Two
362 mechanisms of bacterial cell rupture on nanostructured surfaces have been described in the literature:
363 (i) compression of the bacteria on the nanopillars under sedimentation forces, leading to puncture or
364 rupture of the bacterial cell wall[3] and (ii) a combination of strong adhesion between the cells and
365 the nanopillars, and a shear force exerted on the adherent bacteria as they move away from their initial
366 adhesion site. The shear force can be due to their motility or to the hydrodynamic or thermodynamic
367 motion of the fluid environment. Under this stress, the cell wall of the highly adherent bacteria is
368 thought to rupture. Recently, Elena P. Ivanova and coworkers demonstrated that the mechanical
369 injury caused is not sufficient to kill the bacteria immediately, due to the survival of the inner plasma
370 membrane, but leads to apoptosis-like death. This work also showed that the mechano-bactericidal
371 actions have sustained physiological effects on the bacterium, due to the self-accumulation of reactive
372 oxygen species.[60] As an example of the first mechanism, it has been reported that cicada wings
373 have bactericidal activity only against Gram-negative bacteria due to a specific nanopillar topography
374 responsible for the stretching and further rupture of the adherent bacterial wall between two adjacent
375 nanopillars.[4, 5] It can be hypothesized that the contact-killing property of the oriented ChNC films
376 is due to similar mechanisms, thanks to the spike-like nanotopography (20-30 up to 40 nm in height).
377 This leads to a thickening of the bacterial cell wall to resist the rupture and also increases its rigidity
378 for all the ChNC-based oriented films that have the same nanotopography features. The second
379 mechanism may also play an important role in the ChNC-terminated oriented films, which has been
380 proposed to occur on strongly adhesive nanostructured,[4, 61] or strongly positive surfaces.[62] The
381 higher charge of the ChNC-oriented films leads to stronger and multiple interactions between the
382 positively charged spikes and the negatively charged bacterial cell wall. Thus, the ChNC-terminated
383 oriented films are likely to cause more stretching and further rupture of the cell wall when bacteria

384 are forced to move away from their attachment location compared to the other films. As a result, they
385 have the strongest contact-killing property (with between 44-50% damaged *S. aureus* and *E. coli*),
386 whereas the other oriented films have only between 11-14% damaged *S. aureus*. Finally, the steeper
387 spike-like character and the higher positive charge of the oriented ChNC film are both likely to cause
388 the antibacterial property of this surface by leading to the cell wall puncture induced either by the
389 compression of bacterial cells on the spikes and/or by a shear force applied to highly electrostatically
390 adherent cells (Figure 9).



391

392 **Figure 9: Contact-killing mechanism of the oriented ChNC film.** A schematic representation of
393 the nanotopographical orientation of ChNC in spike-like features with a high surface density of
394 positive charges of the oriented ChNC films, explaining its antibacterial activity by contact-killing
395 against *S. aureus* and *E. coli* and the nonoriented ChNC films, with a smoother surface and less
396 surface density of positive charge.

397

398 **1.4. Conclusions**

399 Inspired by chitin-based butterfly wings and iron-rich crustacean exoskeletons, we developed the first
400 ChNC-based LbL films with contact-killing properties against both *S. aureus* and *E. coli* as well as
401 antibiofilm properties. The antibacterial property of the films is related to spike-like nanotopographies
402 with a high density of positive charge on the surface, which are obtained by orienting ChNC by the
403 brushing method in combination with TA and Fe^{3+} as partners. The puncture and associated damage
404 of the adherent bacteria on the oriented ChNC films are likely caused by (i) the compression of the
405 bacterial cell wall on the spike-like structures and/or (ii) the stress exerted on the adherent bacteria

406 cell wall by the positively charged spikes under the shear force due to bacterial motility or movement
407 of the liquid environment. This hypothesis is supported by the morphology and mechanical properties
408 of bacterial cell walls. The fully bio-based nature of ChNC-based nanocoatings and their brush-based
409 fabrication method make them a suitable and affordable candidate as a sustainable antibacterial film
410 for (bio)materials.

411 **CRedit authorship contribution statement**

412 **Muhammad Haseeb Iqbal**: Methodology, Investigation, Validation, Formal Analysis, Funding
413 acquisition, Writing original draft. **Fabienne Quilès**: Methodology, Investigation, Formal Analysis,
414 Writing - review & editing. **Emeline Pradel**: Investigation. **Sarah Benmalek-Kehili**: Investigation.
415 **Christine Lancelon-Pin**: Investigation. **Laurent Heux**: Investigation, Resources. **Florent Meyer**:
416 Conceptualization, Resources, Supervision, **Lydie Ploux**: Supervision, Resources, Writing - review
417 & editing, Supervision. **Gregory Francius**: Methodology, Investigation, Resources, Formal
418 Analysis, Writing - review & editing. **Fouzia Boulmedais**: Conceptualization, Supervision,
419 Resources, Funding acquisition, Writing - review & editing, Project administration.

420 **Declaration of Competing Interest**

421 The authors declare that they have no known competing financial interests or personal relationships
422 that could have appeared to influence the work reported in this paper.

423 **Acknowledgments**

424 The authors thank the CarMAc of ICS <https://www.ics-cnrs.unistra.fr/plateforme-carmac.html>, the
425 Spectroscopy and Microscopy Service Facility (SMI) of LCPME
426 www.lcpme.ul.cnrs.fr/equipements/smi/, where the AFM and infrared experiments were performed
427 and the NanoBio- ICMG Platform (UAR 2607, Grenoble) for granting access to the Electron
428 Microscopy facility. M.H.I. thanks the Higher Education Commission (HEC) Pakistan for his Ph.D.
429 scholarship. Fonds Régional de Coopération pour la Recherche of Région Grand Est ERMES project

430 and International Centre for Frontier Research in Chemistry (icFRC) are acknowledged for financial
431 support.

432 **Appendix A. Supplementary material**

433 Supplementary data to this article can be found online at.

434 **Data availability statement.**

435 Data available upon request.

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437

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Graphical abstract

Bio-inspired & bio-sourced antibacterial coating

