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Supported Lysozyme for Improved Antimicrobial Surface Protection

Audrey Beaussart¹, Chloé Retourney², Fabienne Quilès², Raphael Dos Santos Morais³, Claire Gaiani³, Henri-Pierre Fiérobe⁴ and Sofiane El-Kirat-Chatel^{2*}

¹Université de Lorraine, CNRS, LIEC, F-54000 Nancy, France

²Université de Lorraine, CNRS, LCPME, F-54000 Nancy, France

³Université de Lorraine, LIBio, F-54000, Nancy, France

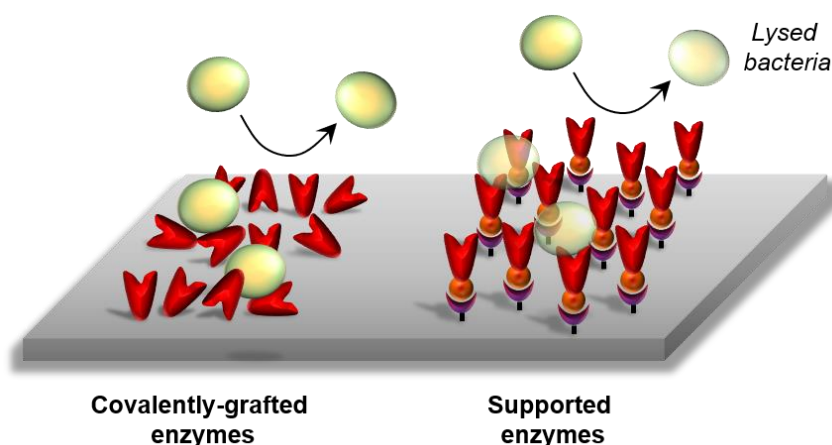
⁴Aix Marseille Univ, CNRS, LCB, F-13420 Marseille, France

*Corresponding author:

Sofiane El-Kirat-Chatel: elkirat1@univ-lorraine.fr

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ToC.



Authors' contributions: AB and SEKC design the research. AB, CR, FQ, RDSM, CG, H-PF and SEKC performed research. AB, CR, FQ, RDSM, CG, H-PF and SEKC analysed the data. AB and SEKC wrote the paper.

1 **Abstract**

2 Surface protection against biofilms is still an open challenge. Current strategies rely on
3 coatings that are meant to guarantee antiadhesive or antimicrobial effects. While it seems
4 difficult to ensure antiadhesion in complex media and against all the adhesive arsenal of
5 microbes, strategies based on antimicrobials lack from sustainable functionalization
6 methodologies to allow the perfect efficiency of the grafted molecules. Here we used the high
7 affinity ligand-receptor interaction between biotin and streptavidin to functionalize surfaces
8 with lysozyme, an enzyme that degrades the bacterial peptidoglycan cell wall. Biotinylated
9 lysozyme was grafted on surfaces coated with streptavidin receptors. Using atomic force
10 microscopy (AFM)-based single molecule force spectroscopy, we showed that grafting
11 through ligand-receptor interaction allows the correct orientation of the enzyme on the
12 substrate for enhanced activity towards the microbial target. The antibacterial efficiency was
13 tested against *Micrococcus luteus* and revealed that surface protection was improved when
14 lysozyme was grafted through the ligand-receptor interaction. These results suggest that bio-
15 molecular interactions are promising for a sustainable grafting of antimicrobial agents on
16 surfaces.

17

18

19 Introduction

20 Biofilm is a cooperative mode of life for microorganisms which can be formed on any
21 type of substratum.[1] It consists of microbial communities adhered on a surface and
22 embedded in a self-produced extracellular polymeric substance (EPS).[2] This three-
23 dimensional organization leads to social and physical cellular interactions that make biofilm a
24 totally different life-style from planktonic cells.[1] Microbes living in such consortium strongly
25 adhere to the substrate and present an increased resistance to external stresses, rendering
26 mature biofilms persistent and hard to eradicate.[3,4] In industry and human health, biofilm
27 contaminations represent a major concern and are associated to important financial burdens
28 and increased mortality.[3-6] Therefore, surface protection against biofilm is a great challenge
29 in many applications and has mobilised important research efforts in the last decades.[7-10]
30 Given the resistance of mature biofilms, their formation has to be tackled at the early stage,
31 *i.e.* during the adhesion of pioneer bacteria. In that sense, different strategies aiming to obtain
32 surfaces with antiadhesive or antimicrobial properties have been developed.[7,8,10,11] The
33 former consists in modifying the physicochemical properties of the substrate to obtain an
34 antifouling effect towards microorganisms. Although encouraging results have been obtained
35 with polymers, *e.g.* polyethylene glycol, the protection efficiency was seen to decrease with
36 time when surfaces were exposed to complex media.[7,10,12] For antimicrobial approaches,
37 the principle is to functionalize surfaces with antimicrobial agents such as enzymes,
38 antibiotics, antimicrobial peptides or disinfectants.[7,8,11] However, most antimicrobials
39 offer a short-term protection and their leaching may be toxic and source of antimicrobial
40 resistances.[13] Antimicrobial enzymes offer promising perspectives for surface protection as
41 they can act continuously as long as their activity is preserved and they are less potent to
42 trigger resistances.[14,15] Although their potentiality for surface protection is gaining more

43 and more interest, the functionalization method used to attach them on substrates is often a
44 limiting step as they usually become less active once anchored to solid substrates due to *e.g.*
45 steric hindrance, masking of their catalytic site, or denaturing interactions with the
46 material.[16,17] Developing new functionalization methods overcoming these limitations is
47 then crucial to exploit the possibility of using enzymes as efficient antimicrobial surface-
48 protective agents. Attempts have been made to coat surfaces with DNase 1 or lysostaphin on
49 polydopamine pre-coating, unfortunately resulting in a non-oriented distribution of the
50 enzymes on the substrate.[18-20] Covalent grafting of lysozyme on stainless steel led also to
51 surfaces with non-oriented enzymes.[21-23] Similarly, pluronic polymers were developed to
52 graft lysozyme on surfaces but this methodology could not ensure the correct orientation of
53 the enzyme on the substrate.[24] Peptidic linkers were used to graft lysostaphin in an oriented
54 manner and to give molecular flexibility to the enzyme interacting with contaminating
55 bacteria.[15] Although the method was original, the grafting strategy was based on weak
56 interactions between the histidine tag and nickel-nitrilotriacetic acid or between silica binding
57 peptide and glass.

58 The biotin-streptavidin interaction is one of the strongest noncovalent biomolecular
59 bond known in nature.[25-28] Its high affinity (K_d of $\sim 10^{-13}$ M) and stability make it of high
60 interest for controlled and oriented molecular grafting.[25,29] Here, we took advantage of
61 this high affinity ligand-receptor interaction to functionalize surfaces with lysozyme, an
62 antibacterial enzyme that hydrolyses β -(1,4) linkages between N-acetylmuramic acid and N-
63 acetyl-D-glucosamine residues of the peptidoglycan present in bacterial cell walls (Figure
64 1a).[30] We first demonstrate that enzymes are grafted in an oriented manner by probing the
65 interaction between streptavidin surfaces and biotinylated lysozyme decorating atomic force
66 microscopy (AFM) tips (Figure 1b). Then, we showed that lysozyme supported by the biotin-

67 streptavidin complex achieves greater antibacterial activity against the opportunistic
68 pathogen *Micrococcus luteus*[31] than surfaces functionalized with covalently-bond lysozyme.
69 This work demonstrates that grafting through bio-molecular interaction is a good strategy to
70 prospect when developing coatings that bear oriented and preserved antimicrobials.

71 **Results and discussion**

72 **Biotinylated lysozyme binds to streptavidin surfaces.**

73 To preserve the antibacterial activity of the enzymes upon surface functionalization,
74 biotinylated-lysozyme have been grafted through the ligand-receptor interaction between
75 biotin and streptavidin (Figure 1a). For that, streptavidin was first covalently attached to gold
76 surfaces. AFM topography images of the surface revealed that streptavidin coating was
77 homogeneous and stable upon repeated scanning, indicating strong attachment of the
78 receptor on the substrate (Figure 2). Scanning a small area at large forces removed the
79 streptavidin layer and the scratched square was visible by imaging larger area at small forces.
80 This allowed to determine that the streptavidin layer thickness was ~ 2 nm which correlates
81 with previous thickness reported for streptavidin coated surfaces.[32] It also demonstrated
82 that the first functionalization step resulted in a dense streptavidin layer which can be further
83 used to graft biotinylated enzymes.

84 The second step of the grafting strategy consists in depositing biotinylated-lysozyme onto the
85 streptavidin surface. We have assessed the amount of lysozyme attached to the surface in this
86 way, and compared it to the amount of lysozyme when the enzyme is directly and covalently
87 attached to the metal support. To do so, the concentration of absorbed proteins has been
88 estimated *via* ultraviolet (UV)-visible spectroscopic quantification, revealing that lysozyme
89 density was more than 4 times higher when directly grafted covalently on the surface (~ 59
90 $\text{ng}\cdot\text{mm}^{-2}$ for covalently grafted lysozyme vs ~ 13 $\text{ng}\cdot\text{mm}^{-2}$ for streptavidin-supported lysozyme).

91 The density of grafted streptavidin was $\sim 27 \text{ ng.mm}^{-2}$. Regarding the molecular weights of
92 biotinylated lysozyme and streptavidin (14300 and 52800 g.mol^{-1} , respectively), we obtain a
93 respective molar density grafted on surfaces of $\sim 9 \times 10^{-13} \text{ mol.mm}^{-2}$ and $\sim 5 \times 10^{-13} \text{ mol.mm}^{-2}$,
94 corresponding to a molecular ratio of ~ 1.8 , which is in good agreement with a stoichiometry
95 of 2 expected for grafted streptavidin with biotinylated molecules (compared to the
96 tetravalence of streptavidin in solution).[35]

97 Our hypothesis is that attaching lysozyme *via* receptor-ligand interaction allows to control the
98 orientation of the enzyme on the surface. To verify this assumption, the specific binding of
99 biotinylated-lysozyme to the streptavidin surface was assessed by single-molecule force
100 spectroscopy (SMFS) measurements. For that, the adhesion frequency of the biotinylated-
101 lysozyme-decorated tips toward streptavidin surface was quantified, and compared to the
102 adhesion of other biotinylated or non-biotinylated molecules to the same support (Figure 1b).
103 In details, approaching a functionalized tip from the streptavidin-coated surface results in a
104 force-distance curves. On such curve, either the force remains close to zero independently of
105 the distance, and the event is considered as non-adhesive, or the two proteins interact, which
106 results in an increasing force until the link between the coated-tip and the streptavidin
107 ruptures. The distance at which this link breaks informs on the extension/flexibility of the
108 proteins. Figure 3 shows adhesion force and rupture length histograms, together with
109 representative force curves, obtained between streptavidin surfaces and AFM tips
110 functionalized with biotinylated lysozyme, non-biotinylated lysozyme and biotinylated
111 albumin. The first bin corresponds to the frequency of non-adhesive events. The colours
112 correspond to 3 independent measurements obtained with different coated-tips. For
113 biotinylated lysozyme, 21 to 32 % of force curves presented simple peak adhesive events with
114 unbinding forces ranging from 50 to 350 pN (mean value of $230 \pm 124 \text{ pN}$) and rupture lengths

115 from 5 to 100 nm (Figure 3a,b). Similar interactions were recorded for biotinylated albumin
116 (Figure 3c,d). These values are in good accordance with previous SMFS studies on biotin-
117 streptavidin interaction.[27,28,32-34] By contrast, most force curves featured no adhesive
118 events when surfaces were probed with non-biotinylated lysozyme (adhesion frequency <7%,
119 Figure 3e,f). This SMFS analysis demonstrates that, as expected, adhesion to the streptavidin
120 pre-coated surface is modulated by the presence of a biotin group on the proteins (lysozyme
121 and albumin). It confirms that biotin-streptavidin interaction can be used to graft lysozyme on
122 surfaces in an oriented and specific manner.

123 To reinforce the idea that supported-lysozyme grafting preserves the conformation of the
124 enzyme, we also performed infrared reflection absorption spectroscopy (IRRAS) on the
125 different functionalized surfaces. This infrared-based technique allows chemical
126 characterization of thin monolayers deposited on a metal surface. Figure 4 depicts the 1720-
127 1600 cm^{-1} spectra, corresponding to the amide band I region and particularly sensitive to
128 proteins secondary structures. The spectrum of streptavidin-coated surface (Fig. 4, black line)
129 shows the occurrence of a band at 1637 cm^{-1} and a shoulder at 1686 cm^{-1} assigned to
130 streptavidin β -sheet.[36] For the covalently-grafted lysozyme (Fig. 4, blue line), the amide I
131 band attributed to lysozyme was more intense as compared to the two other surfaces, with
132 an integrated intensity 1.5 time higher than the other spectra. This result correlates with the
133 higher protein amount on covalently-grafted lysozyme surfaces detected by (UV)-visible
134 spectroscopy, although care should be taken in quantitative comparison as changes of
135 proteins orientation may also influence the IRRAS spectra. The secondary structure of native
136 lysozyme consists mainly in α -helices.[37] The amide I band of covalently grafted lysozyme
137 showed a band near 1670 cm^{-1} (Fig. 4, blue line) instead of $\sim 1650 \text{ cm}^{-1}$ expected for the α -
138 helix conformation. This shift may be attributed to a denaturation and/or aggregation of

139 lysozyme, as already described for heated lysozyme in solution,[38] and could result here from
140 the covalent-grafting of lysozyme on the surface. Such alteration was not observed for the
141 supported-lysozyme surface (Fig. 4, red line), for which the amide I band region showed two
142 main bands at 1655 and 1638 cm^{-1} assigned to lysozyme α -helices and streptavidin β -sheets,
143 respectively, consistent with the native conformations of both proteins. It confirms that
144 supported-lysozyme grafting strategy favours a proper orientation of the lysozyme towards
145 its target.

146 Next, we wanted to determine the capability of the different surfaces functionalized with
147 enzymes to degrade bacterial cells.

148 **Grafting lysozyme through biotin-streptavidin interaction preserves antibacterial activity.**

149 One advantage of using enzymes as antimicrobial agents is that they can degrade bacteria
150 continuously as long as their activity is preserved. Therefore, enzyme stability is a prerequisite
151 for long-term antibiofilm surface protection. An ideal antibacterial activity would eliminate
152 contaminating bacteria and would be reusable, *i.e.* able to face repetitive contaminations. To
153 test whether grafting through biotin-streptavidin improves the capability of lysozyme to face
154 surface contamination, we have determined the antibacterial effect of different surfaces
155 toward *M. luteus* by standard plating assays. Bacteria were exposed to surfaces and viability
156 was determined over time and consecutive challenges (*i.e.* contamination with fresh bacteria).
157 For consecutive challenges, $\sim 5 \times 10^7$ bacteria were added on surfaces and viability was
158 determined after 0h, 2h, 4h, 6h and 24h. After 24h, surfaces were rinsed and fresh bacteria
159 were reloaded for the next challenge. Figure 5a shows the evolution of bacterial viability over
160 3 consecutive challenges on surfaces functionalized with streptavidin-supported biotinylated
161 lysozyme, non-supported lysozyme and streptavidin alone. For control surfaces with
162 streptavidin only, bacterial viability was $\sim 100\%$ after 24h for all challenges. The slight decrease

163 observed upon time (<10 %) during the two first challenges could be attributed to bacteria
164 adhering to the plastic well plates in which the surfaces are immersed to expose them to the
165 cells. Surfaces functionalized with biotin-streptavidin-supported lysozyme killed 70-75 % of
166 bacteria within 24h of all challenges. By contrast, the killing efficiency of surfaces with non-
167 supported lysozyme decreased from 65 % to 50 % and 35 % during challenge 1, 2 and 3,
168 respectively. During the 24h of challenge 1, bacterial concentration decreased constantly for
169 both surfaces coated with lysozyme, yet with less efficiency for surfaces with non-supported
170 lysozyme. While the killing efficiency remained constant over the 3 challenges for surfaces
171 with biotin-streptavidin-supported lysozyme, it started to decrease during the challenge 2 and
172 was almost abolished after the first 2h of challenge 3 for surfaces with non-supported
173 lysozyme. This improved killing activity for lysozyme grafted by biotin-streptavidin interaction
174 was confirmed up to 5 challenges (Fig. 5b). For clarity, only the bacterial survival values at 24h
175 are indicated on Fig. 5b. The killing capacity of the supported-lysozyme surface after 5
176 challenges of 24h was ~55% (Fig. 5b, red dots) *versus* only ~20 % for surfaces with non-
177 supported lysozyme (Fig. 5b, blue dots).

178 It could be argued that the decrease in antimicrobial performance is due to lysozyme removal
179 during the rinsing steps. To exclude this hypothesis, we have analysed the topography of
180 surfaces before and after the rinsing steps (Fig. S1). No significant difference in the height of
181 the coating layer was observed after surface rinsing, suggesting that molecules grafted
182 covalently or through the biotin-streptavidin interaction were not removed between the
183 contamination challenges.

184 The bacterial killing capability of surfaces may decrease with time if enzyme activity decreases
185 or if surfaces are fouled with bacterial debris. To determine the kinetics of bacterial killing by
186 the different surfaces, the viability of $\sim 1 \times 10^8$ bacteria was assessed over 7 days (Figure 6). On

187 surfaces bearing only streptavidin, bacterial viability remained constant at ~100 % over the
188 total test duration. Surfaces functionalized with biotin-streptavidin-supported lysozyme killed
189 ~75 % of bacteria after 24 h and 90 % after 3 days. By contrast, surfaces with non-supported
190 lysozyme killed 60 % of bacteria after 24 h and a survival plateau was reached after 2 days
191 with a maximum of 80 % of bacteria killed, suggesting that lysozyme was inactivated and/or
192 not able to degrade bacteria after this time point. This improved antibacterial effect of
193 surfaces with supported-lysozyme suggests that enzyme conformation and activity are
194 preserved and that surface fouling by bacterial debris is less potent to inhibit enzymes grafted
195 through biotin-streptavidin interaction.

196 To further demonstrate that ligand-receptor grafting improves the activity of grafted enzymes,
197 we analysed the morphology of cells exposed to the different functionalized surfaces (Figure
198 7). As lysozyme degrades bacterial cell wall, we postulated that bacteria exposed to surfaces
199 with preserved enzymatic activity would present more damages than bacteria exposed to
200 surfaces with non-oriented enzymes or with streptavidin alone. Cells from the 5th challenge
201 (see above) were collected after 24 h of exposure to the different surfaces and deposited on
202 gold substrates for AFM topographic imaging. Figure 7 shows low- and high-resolution images
203 of *M. luteus* cells exposed to surfaces with streptavidin alone (Figure 7a), non-supported
204 lysozyme (Figure 7b) and biotin-streptavidin supported lysozyme (Figure 7c). Large scale
205 images revealed numerous aggregated cells for streptavidin surfaces whereas aggregates
206 were smaller for the two surfaces functionalized with lysozyme, further confirming that the
207 enzyme kills bacteria and decreases the cell density. At high resolution, all cells exposed to
208 streptavidin surfaces presented smooth and regular cell walls (Figure 7a). By contrast, all cells
209 exposed to surfaces with supported lysozyme presented substantial alterations of their
210 structure and appeared bumped and rough (Figure 7c). Finally, two populations were

211 observed for cells exposed to surfaces with non-supported lysozyme: rough cells and smooth
212 cells, documenting that lysozyme failed to degrade the cell wall of bacteria in this case. The
213 potency of surfaces with supported lysozyme to degrade *M. luteus* cell wall as compared to
214 surface with non-supported lysozyme was further confirmed through roughness calculation.
215 Cells exposed to streptavidin, non-supported lysozyme and supported lysozyme presented
216 roughness of 1.28 ± 0.43 nm, 3.06 ± 1.24 nm and 6.05 ± 3.05 nm, respectively (calculated on
217 12 cells from 3 independent experiments). This increased roughness directly reflects the
218 activity of the enzyme on the bacterial cell wall and demonstrates that grafting through biotin-
219 streptavidin interaction preserves lysozyme activity.

220 Altogether, these assays demonstrate an enhanced antimicrobial efficiency for the supported-
221 lysozyme grafting strategy. As i) the supported-lysozyme surfaces performance cannot be
222 attributed to a higher amount of enzymes, ii) the chemistry of the covalent grafting used for
223 non-supported-lysozyme results in a random orientation of the protein on the surface with
224 conformational changes and iii) the supported-lysozyme binds to the streptavidin-coated
225 support *via* its biotin-group, the supported-lysozyme strategy most likely improves
226 antibacterial surface protection by ensuring the correct orientation of enzymes on the surface,
227 preserving enzyme conformation and limiting denaturing interactions with the substrate.

228

229 **Conclusion**

230 Surface protection against microbial biofilms remains an open challenge for which new
231 strategies are needed.[4,6-10] In this work, we have demonstrated that the enzyme lysozyme
232 could be grafted on surfaces in a manner that offers antimicrobial protection over consecutive
233 contaminations. Our strategy is based on the strong-affinity bio-molecular ligand-receptor
234 interaction -namely biotin-streptavidin- used here to support grafted enzymes and to improve

235 their activity against the bacteria *Micrococcus luteus*. Compared to previously reported
236 approaches,[21-24] the supported-lysozyme surfaces showed improved antimicrobial
237 protection over several days of continuous bacterial challenge. The efficiency of the bacterial
238 killing effect as compared to surfaces coated with covalently-grafted lysozyme most likely
239 arises from an improve orientation and conformation of the enzyme catalytic site towards its
240 target, as demonstrated by AFM-based single molecule and IRRAS experiments.
241 Further developments of grafting strategies are under way to attach on the same surface
242 various enzymes that would degrade diverse cell-wall components in order to reach a broad-
243 spectrum activity. These results are encouraging for the future development of long-term
244 surface protection by antimicrobial enzymes.

245

246 **Materials and methods**

247 **Surface functionalization.** Proteins were attached covalently on self-assembled monolayers
248 (SAM) of carboxyl-terminated alkanethiols. Round glass coverslips (\varnothing 12 mm) coated by
249 plasma sputtering with a thin layer of chromium (\sim 10 nm) and a thin layer of gold (\sim 30 nm)
250 were cleaned by UV-ozone treatment for 15 min, rinsed with ethanol and dried under N_2 flow.
251 SAM were formed on clean surfaces by immersion overnight in a 1 mM solution containing
252 10% of 16-mercaptohexadecanoic acid (>90%, Sigma) and 90% of 11-mercapto-1-undecanol
253 (>97%, Sigma) and then rinsed with ethanol and dried under N_2 flow. Carboxylic groups were
254 activated by immersion for 30 min in a solution of 20 mg mL⁻¹ N-hydroxysuccinimide (NHS,
255 >97%, Sigma) and 50 mg mL⁻¹ 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, >99%,
256 Sigma). Activated surfaces were rinsed in water and immersed for 2h with 0.2 mg mL⁻¹ of
257 streptavidin (>13 units/mg, Sigma) (for control surfaces functionalized with only streptavidin
258 and for surfaces functionalized with supported lysozyme) or with 0.2 mg mL⁻¹ of lysozyme

259 from chicken egg white (>90%, >40 units/mg, Sigma) (for surfaces functionalized with non-
260 supported lysozyme) and then rinsed with phosphate-buffered saline (PBS, Sigma). For
261 supported-lysozyme surfaces, streptavidin functionalized surfaces were further immersed for
262 30 min in 0.2 mg mL⁻¹ of biotinylated lysozyme (>20 units/mg, Genetex) and rinsed again with
263 PBS. All surfaces were prepared and used within the same day. For protein quantification,
264 ultraviolet (UV)-visible measurements were performed in solution before and after deposition
265 on the surfaces with a NanoDrop 2000c spectrophotometer (Thermo Fischer Scientific) and
266 using molar extinction coefficients at 280 nm of 38940 and 41326 M⁻¹.cm⁻¹ and MWs of 14300
267 and 52800 g.mol⁻¹ for lysozyme and streptavidin, respectively.

268 **AFM imaging and force spectroscopy.** Unless stated otherwise, all AFM experiments were
269 achieved in PBS at room temperature using a Bioscope Resolve AFM (Bruker corporation,
270 Santa Barbara, CA). Before force measurements, surfaces functionalized with proteins were
271 imaged with bare AFM tips (MSCT, Bruker) under minimal applied force. To measure
272 streptavidin layer thickness, surfaces were first scratched with the AFM tip by applying large
273 forces on an area of 1 x 1 μm² and then a larger area of 5 x 5 μm² was imaged under small
274 forces. For force spectroscopy, gold coated AFM tips (NPG, Bruker) were functionalized with
275 biotinylated lysozyme, biotinylated albumin (>80%, Sigma) or lysozyme following the same
276 protocol as described for surfaces. Without dewetting, surfaces were immobilized in a glass-
277 bottomed Petri dish with double side tape and covered with PBS. Force measurements were
278 performed by recording 32 x 32 force-distance curves on areas of 1 x 1 μm² with a maximum
279 applied force 250 pN, 200 ms contact time and a constant approach and retraction velocity of
280 1.5 μm s⁻¹. The spring constants of the cantilevers were measured by the thermal noise
281 method. Adhesion force and rupture length histograms were obtained by calculating for each
282 force curve the last adhesion peak and rupture distance. For cell imaging, 200 μl of cell

283 suspension from the challenge 5 were collected at 24h and deposited on gold substrates. After
284 2h, surfaces were gently rinsed by immersion in ultrapure water and dried overnight at 30°C
285 before imaging in peak-force tapping mode using a gold-coated AFM tips (NPG, nominal spring
286 constant 0.24 N/m, Bruker corporation) on a Fastscan Dimension-Icon AFM (Bruker
287 corporation, Santa Barbara, CA). Rms (root mean square) roughness values correspond to the
288 Rq values given by the Nanoscopy analysis software on 400 nm x 400 nm square at the surface
289 of the bacteria after 2nd-order-flattening of the height image. Given values have been
290 averaged over at least 12 bacteria per conditions.

291 **Bacterial growth and viability tests.** *M. luteus* ATCC4698 cells were grown routinely on Luria-
292 Bertani (LB, Sigma) plates at 30°C. For viability experiments, a single colony of *M. luteus* was
293 grown in 5 mL of LB at 30°C with shaking at 200 rpm. Overnight cultures were harvested by
294 centrifugation at 5000 *g* for 5 min, washed twice in PBS. For consecutive challenges, 1.2 mL of
295 bacterial suspension containing $\sim 5 \times 10^7$ bacteria were deposited on functionalized surfaces
296 in 12-well plates and gently agitated at 30°C. At defined time points (0h, 2h, 4h, 6h and 24h),
297 100 μ l were pipetted, serially diluted and plated on LB before cultivation at 30°C to determine
298 bacterial viability. Before reloading with 1.2 mL of fresh bacteria for the next challenge,
299 surfaces were gently rinsed in 3 baths of PBS. For continuous experiments without bacterial
300 reload, 2 mL of bacterial suspension containing $\sim 1 \times 10^8$ bacteria were deposited on surfaces
301 and 50 μ L were pipetted at different time points (from 0 to 7 days) for viability analysis. Results
302 presented for viability tests correspond to three independent experiments.

303 **Infrared reflection absorption spectroscopy (IRRAS).** Infrared spectra were recorded in the
304 mid-infrared range on a Bruker Vertex 70v spectrometer equipped with a KBr beamsplitter
305 and a MCT detector. An advanced grazing angle specular reflectance accessory (Pike
306 technologies Inc.) with a fixed 80° angle of incidence was used for acquiring spectra of the

307 functionalized gold-coated surfaces. The spectra resolution was 4 cm^{-1} and the accumulation
308 time was ~ 2.5 min (256 scans). Compartments containing the detector and the specular
309 reflectance accessory were under vacuum (< 1 hPa). IR spectra are displayed in the scale
310 corresponding to $\log(1/R)$, where R is the collected reflectance.

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318

319 **Conflict of interest**

320 The authors declare no conflict of interest.

321

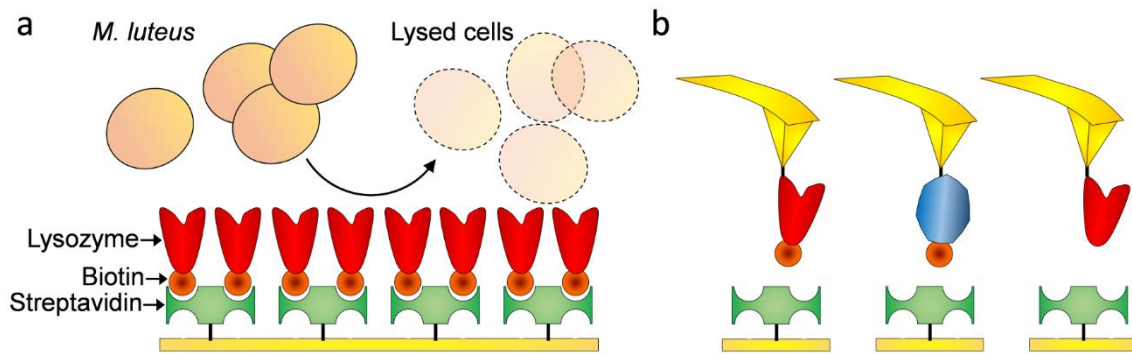
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452 **Figures**

453

454 **Figure 1. Principle of surface protection with biotin-streptavidin grafted enzymes and of**455 **single-molecule force spectroscopy.** (a) Lysozyme was grafted on surface through biotin-

456 streptavidin interaction to obtain antimicrobial surfaces. Streptavidin was covalently attached

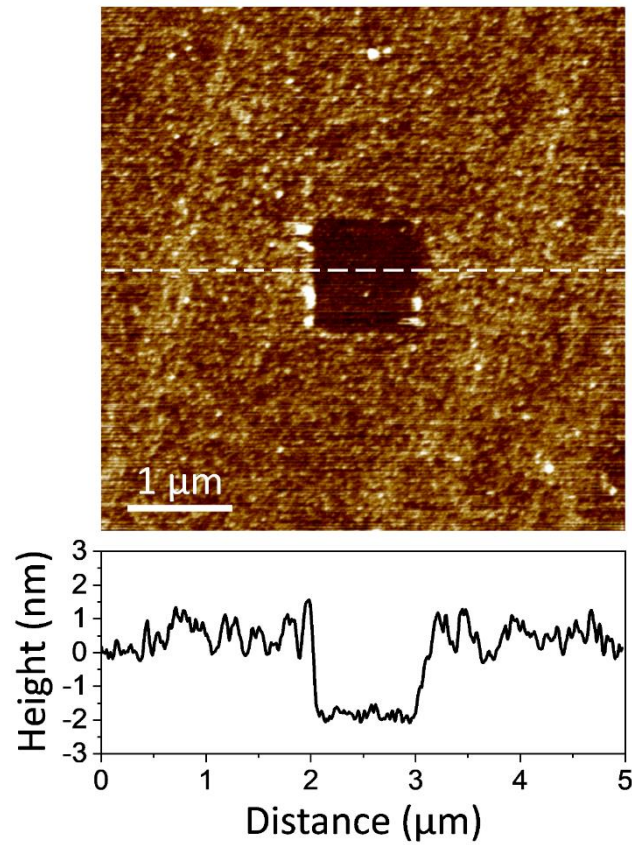
457 to carboxyl-terminated surfaces *via* NHS/EDC chemistry and biotinylated lysozyme was added.458 When bacterial cells, here *M. luteus*, approach the surface, the supported lysozyme degrades

459 their cell wall, leading to bacterial death. (b) To demonstrate the specific interaction between

460 biotin and streptavidin, AFM tips were functionalized with biotinylated lysozyme (left),

461 biotinylated albumin (center) and non-tagged lysozyme (right) and probed towards

462 streptavidin surfaces.



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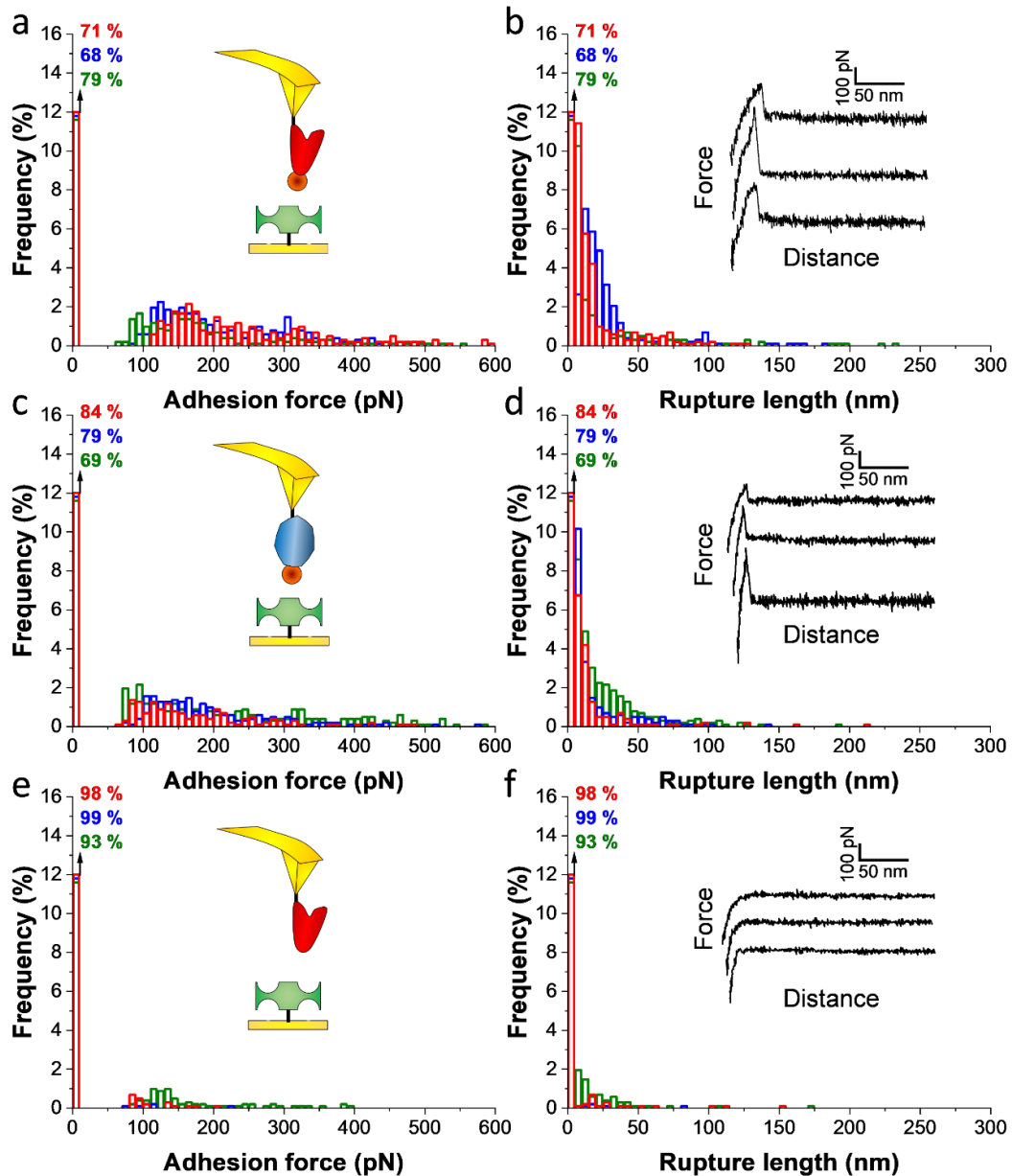
464 **Figure 2. Topography of streptavidin surfaces.** AFM height image ($5 \times 5 \mu\text{m}^2$; z scale = 8 nm)

465 of a surface with covalently attached streptavidin showing a smooth and homogeneous

466 topography. A vertical cross section taken along the dashed line is shown below the image.

467 The streptavidin thickness ($\sim 2 \text{ nm}$) was determined by imaging a square area of $1 \times 1 \mu\text{m}^2$ at468 large forces ($>10 \text{ nN}$) before imaging $5 \times 5 \mu\text{m}^2$ of the same area at small forces.

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471 **Figure 3. Single-molecule force spectroscopy demonstrates specific interaction between**472 **streptavidin surface and biotinylated proteins.** Adhesion force (a, c, e) and rupture length

473 histograms together with representative force curves (b, d, f), obtained by recording force-

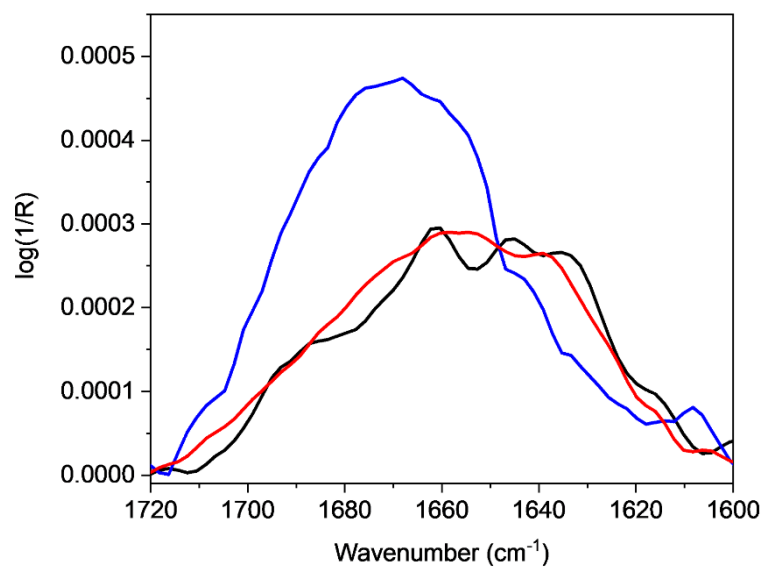
474 volume maps of 32 x 32 pixels on areas of $1 \times 1 \mu\text{m}^2$ between streptavidin surfaces and AFM

475 tips bearing biotinylated lysozyme (a, b), biotinylated albumin (c, d) and non-tagged lysozyme

476 (e, f). Blue, red and green colours correspond to the adhesion frequency (calculated from 1024

477 force-curves each) from 3 independent experiments using different functionalized tips. The

478 numbers on top of the first bin indicate the number of non-adhesive events for each tip.



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480 **Figure 4. IRRAS spectra of functionalized surfaces.** Amide I band region (1720-1600 cm⁻¹) of
481 gold surfaces functionalized with streptavidin (black line), lysozyme by covalent grafting (blue
482 line) and biotin-streptavidin-supported lysozyme (red line).

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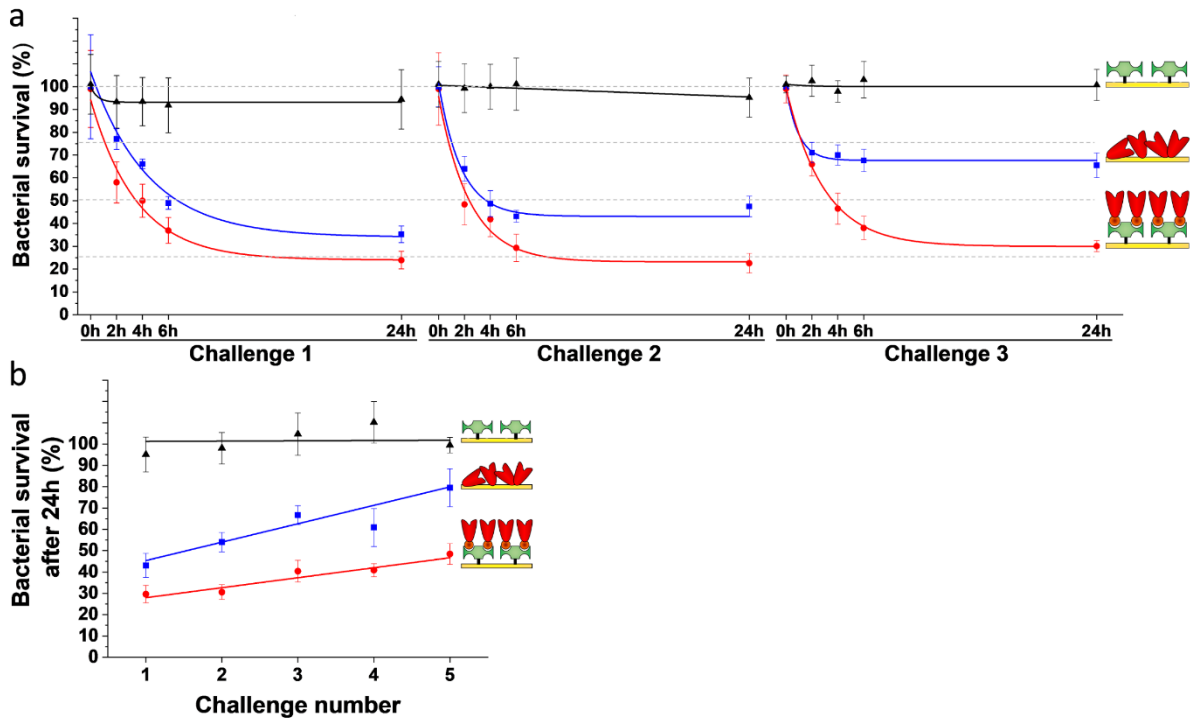
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495 **Figure 5. Antimicrobial activity of surfaces with supported lysozyme is preserved over**496 **consecutive challenges.** (a) Viability of *M. luteus* cells determined by counting the number of

497 colony forming units (CFU) on plates after 3 consecutive challenges of 24 h on surfaces

498 functionalized with streptavidin (black triangles), non-supported lysozyme (blue squares) and

499 streptavidin-supported biotinylated lysozyme (red disks). For each challenge, viability was

500 determined after (a) 0 h, 2 h, 4 h, 6 h and 24 h and (b) 24 h exposure time, then surfaces were

501 rinsed and fresh cells were reloaded. Bacterial survivals decrease exponentially, as revealed

502 by the exponential decay fit (plain lines). (b) Values of bacterial survival after 24h for surfaces

503 exposed to 5 consecutive challenges. Plain lines have not physical meaning, they are depicted

504 for eye guidance, highlighting the clear capacity of supported-lysozyme surface to handle

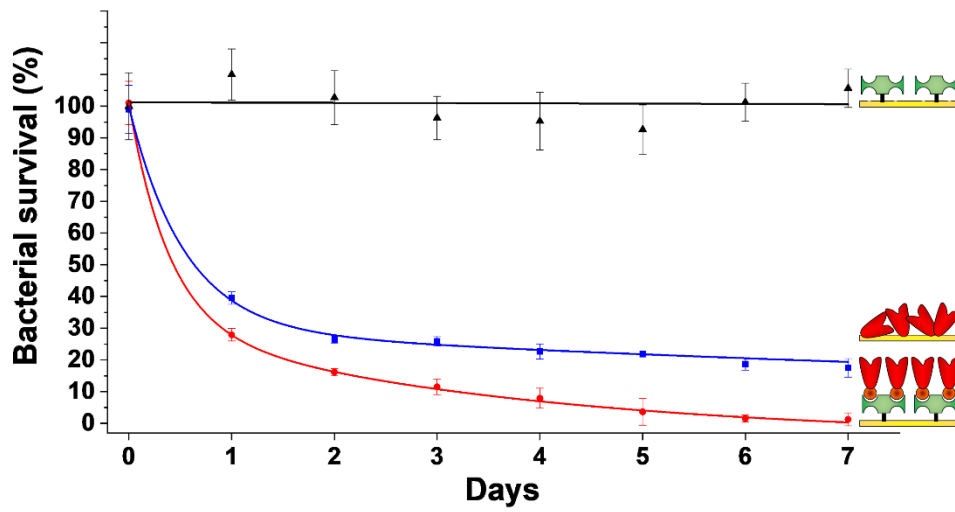
505 multiple contaminations. Experimental values correspond to the average over 3 experiments.

506 Errors bars indicate the standard deviation between experiments.

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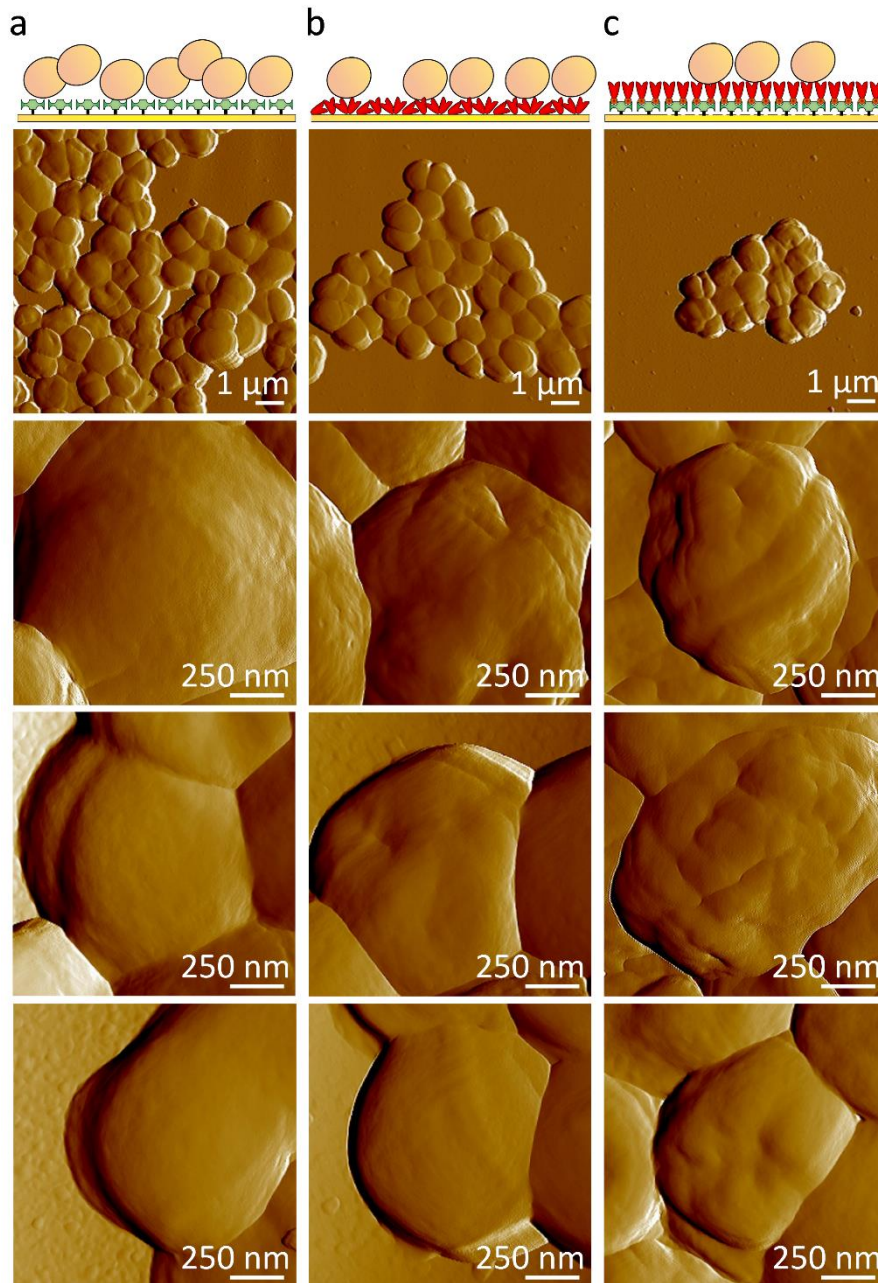
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511 **Figure 6. Antimicrobial activity of surfaces with supported lysozyme is preserved during**
 512 **continuous challenge.** Viability of *M. luteus* cells determined each 24 h over 7 days by
 513 counting the number of colony forming units (CFU) on plates after exposure to surfaces
 514 functionalized with streptavidin (black triangles), non-supported lysozyme (blue squares) and
 515 streptavidin-supported biotinylated lysozyme (red disks). Experimental values correspond to
 516 the average over 3 experiments. Errors bars indicate the standard deviation between
 517 experiments.



518

519 **Figure 7. AFM imaging reveals structural damages on cells exposed to surfaces with**
 520 **supported lysozyme.** Low and high resolution peak force error images of *M. luteus* cells
 521 collected from the 5th challenge after 24 h of exposure. (a) Cells exposed to surfaces with
 522 streptavidin alone, (b) cells exposed to surface with non-supported lysozyme and (c) cells
 523 exposed to surfaces with biotin-streptavidin supported lysozyme. High resolution images
 524 correspond to cells from 3 independent experiments. Similar images have been obtained for
 525 at least 12 bacteria per condition.

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Supplementary Information

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Supported Lysozyme for Improved Antimicrobial Surface Protection

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Audrey Beaussart, Chloé Retourney, Fabienne Quilès, Raphael Dos Santos Morais, Claire

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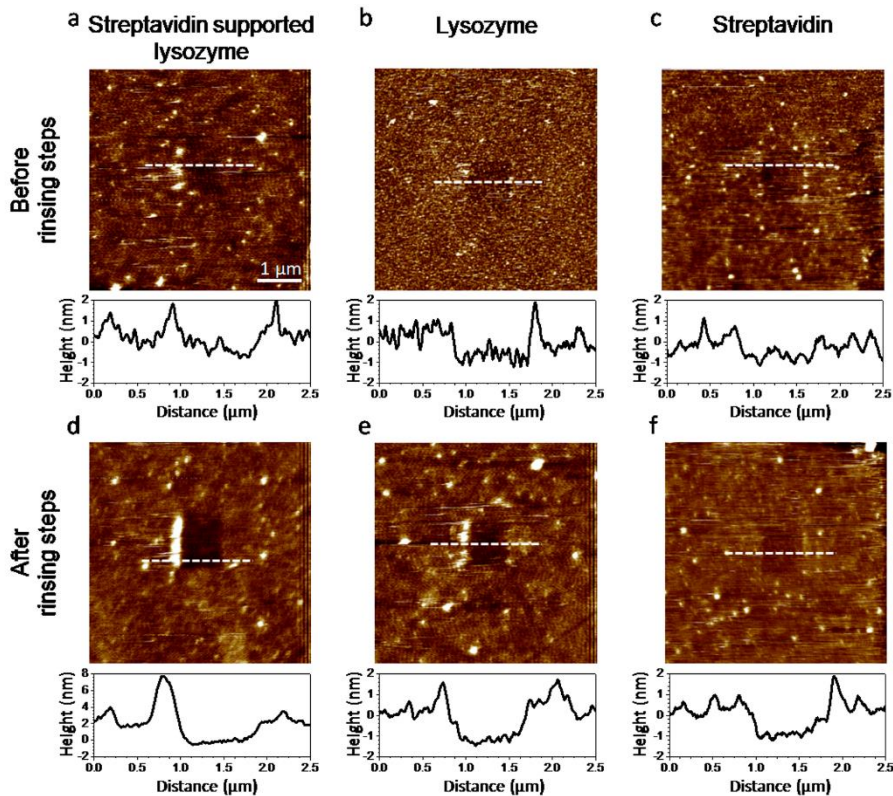
Gaiani, Henri-Pierre Fiérobe and Sofiane El-Kirat-Chatel

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Figure S1. Topography of functionalized surfaces before and after rinsing steps. AFM height images ($5 \times 5 \mu\text{m}^2$; z scale = 5 nm) of surfaces functionalized with streptavidin-supported lysozyme (a, d), with covalently grafted lysozyme (b, e) and with covalently grafted streptavidin (c, f). Surfaces were imaged just after functionalization (a, b and c) or after 5 consecutive rinsing steps to mimic the washing steps applied between bacterial challenges (d, e and f). Vertical cross sections taken along the dashed lines and bridging the scratched squares are shown below the images. No significant differences were observed after rinsing steps indicating that protein layers are not removed over consecutive challenges.