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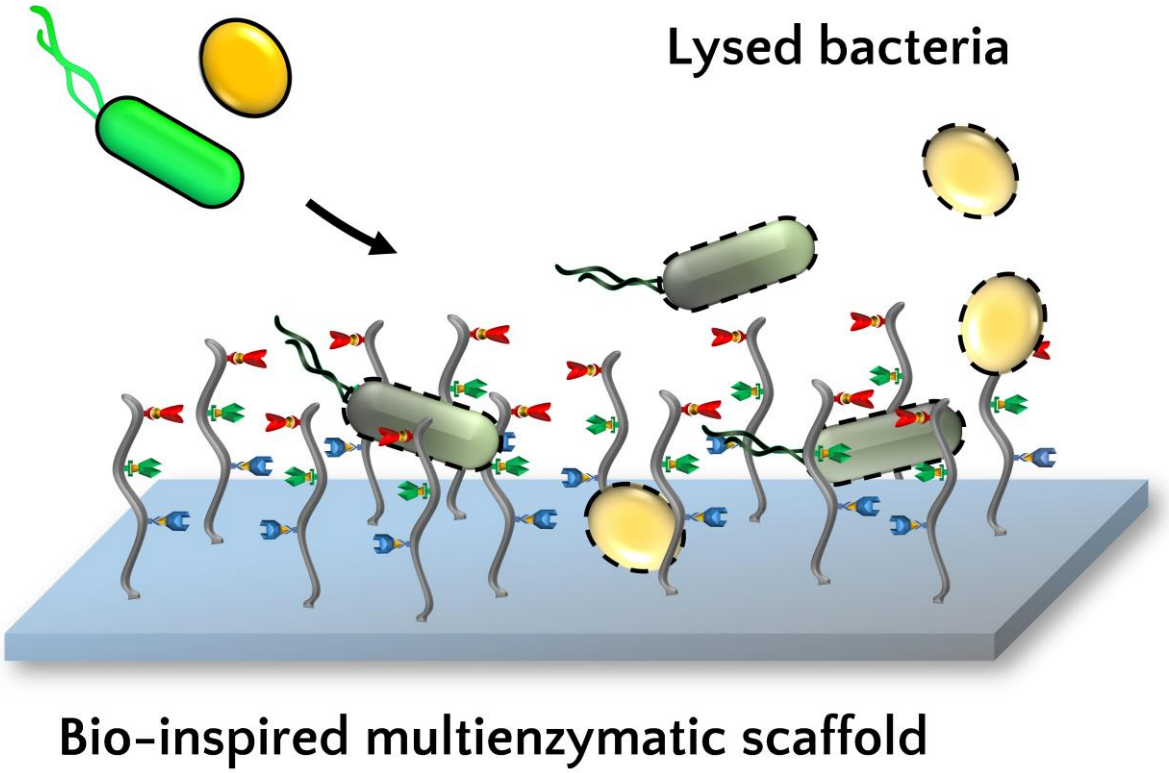
2 GRAPHICAL ABSTRACT



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Simultaneous enzymes grafting on bio-inspired scaffolds for antibacterial protection

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ABSTRACT

Surface bacterial contamination represents a crucial health and industrial concern which requires new strategies to be continuously developed. Successful antibacterial surfaces are characterized by a combination of durable and broad-spectrum antimicrobial actions. Herein, we present a bio-inspired strategy mimicking natural cellulosome to simultaneously immobilize multiple enzymes with antibacterial activity onto surfaces. The grafting strategy leverages the strong biomolecular interaction between receptors on a scaffold protein anchored on the substrate and ligands added to the enzymes. As a proof of concept, lysozyme and lysostaphin were chosen to target the bacterial cell wall, and DNase I to degrade DNA released during cell lysis, known to promote bacterial adhesion which can later lead to biofilm formation. The specificity of the ligand/receptor interaction was confirmed by biochemical and AFM-based single-molecule force spectroscopy assays, thus demonstrating successful co-immobilization of the three enzymes on the protein scaffold. Then, the antibacterial protection was evaluated against *Staphylococcus aureus*, *Escherichia coli* and *Micrococcus luteus* by viability tests which revealed long-term antimicrobial protection of the multi-enzymatic scaffold on both Gram-positive and Gram-negative bacteria. After 24 hours of contact, the system induced lysis of 71 to 85% of bacteria, and its antimicrobial properties remained effective after 5 days even with several cumulative waves of bacterial contaminations. This work demonstrates the relevance of bio-inspired multi-enzymatic scaffolds for antibacterial protection, providing long-term and broad-spectrum action.

STATEMENT OF SIGNIFICANCE

With the raise of resistance among pathogenic bacteria, it became crucial to find alternatives to conventional antibiotics. Antimicrobial enzymes present many advantages as they are “re-usable”, less associated with resistance issues, non-toxic and can withstand a wide range of contaminations. In this article, we focus on grafting antibacterial enzymes on materials surfaces. We used a bio-inspired approach to simultaneously graft different yet complementary enzymes on a protein scaffold and to achieve broad-spectrum antibacterial effect. The resulting multi-enzymatic scaffold enhanced

antibacterial effects compared to a strategy where the enzymes were directly grafted on the material's surface. This was a proof of concept of antibacterial strategies bio-inspired by cellulosomes as our system provided long-lasting protection concurrently against Gram + and Gram – bacteria.

1. INTRODUCTION

Bacterial infections pose a significant threat in both medical and industrial contexts. They represent an ever-growing global scourge which caused the death of 7.7 million patients in 2019, about one eighth of the total number of deaths worldwide [1]. Infections often take the form of surface contaminations forming on medical devices such as wound dressings, urethral and intravascular catheters, prosthetic grafts, prosthetic joints, and shunts [2, 3]. This leads to increased mortality and morbidity which causes an important economic burden on health care systems [2, 4-6].

Surface contamination starts with the initial adhesion of pioneer bacteria to the substrate which then multiply to form microcolonies and later biofilm, the most predominant and successful lifestyle of bacteria and highly resistant to external stress, including antibiotics [7-9]. Antibacterial effect (or contact-killing) is often used on surfaces to inactivate pioneer cells and therefore prevent infections by inhibiting bacterial growth and biofilm formation [10-12]. Initially, antibiotics and synthetic/natural antimicrobial peptides were considered promising candidates but the emergence of multi-resistant strains has rapidly put a curb on this prospect [13, 14]. In this context, antimicrobial enzymes have been considered as an interesting alternative as they are less associated with resistance issues. Their activity is "re-usable" *i.e.* they can act several times provided their catalytic site is active which can ensure long-lasting antimicrobial effect. Moreover, some enzymes can withstand broad-spectrum contaminations and are non-toxic [12, 13, 15-20].

In this work, we present an antimicrobial strategy based on a multi-enzymatic system bio-inspired by natural cellulosomes, which are cell-associated supramolecular structures or "nanomachines" consisting in several cellulolytic enzymes organized on a protein scaffold [21, 22]. Enzymes displayed on cellulosomes have complementary roles that enable efficient degradation and absorption by cellulolytic bacteria of nutrients from cellulose, a highly recalcitrant polysaccharide. Our proposed bio-inspired strategy was to mimic the supramolecular organization of natural cellulosomes by simultaneously assembling three enzymes with complementary antimicrobial roles on a protein

scaffold grafted on material surfaces. Enzymes were chosen to favor broad-spectrum antimicrobial protection while concurrently preventing the accumulation of lysed cell debris on the surface. The first enzyme is lysozyme which cleaves the β-(1,4) bond between the N-acetyl-muramic acid and the N-acetyl-D-glucosamine residue of the peptidoglycan (PG) chains present in bacterial cell walls [23, 24]. This enzyme was also reported to act against Gram-negative bacteria through membrane disruption. For instance, it has the capability to permeabilize both the outer and inner membranes of *E. coli*, achieving this effect with and without the formation of pores, respectively [24-27]. The second enzyme is lysostaphin, an endopeptidase that hydrolyses the pentaglycine chains linking the PG fibrils in the cell wall of staphylococci, a major pathogenic genus [28]. The third enzyme is deoxyribonuclease I (DNase I), which aims to degrade the extracellular DNA, an important polymer released during cell lysis by cell wall degrading enzymes, and known to promote microbial adhesion and bacterial cohesion [29].

Immobilizing antimicrobial enzymes is a crucial and challenging step for antibacterial strategies. Ideally, the grafting technique should preserve the tridimensional conformation of the enzymes to ensure their antimicrobial effects. It should also provide an optimized accessibility of the catalytic site once loaded onto the surface. A wide range of immobilization methods rely on physical adsorption or direct covalent attachment on the substrate which often lead to steric hindrance of randomly orientated enzymes, loss of activity of antimicrobial compounds due to conformational changes and possibly leaching of adsorbed molecules [13, 19, 30]. Previously, we demonstrated that strategies based on strong-affinity biomolecular ligand/receptor interactions present many advantages for antibacterial and antibiofilm properties as they ensure a controlled orientation of the grafted enzymes [30]. This controlled immobilization preserves the orientation and the conformation of the enzymes and thus contributes to optimal and long-lasting antimicrobial properties. Lysozyme, for instance, was successfully grafted on a substrate and showed enhanced antimicrobial effects over several consecutive cycles of contaminations when grafted in an ordered manner, via ligand/receptor interactions, rather than randomly orientated [30]. Yet, this approach cannot be applied to the simultaneous and controlled grafting of several enzymes with complementary activities in order to

broaden the antimicrobial protection towards various contaminants. In the strategy proposed herein, chimeric ligand-tagged enzymes were grafted on receptors located on a recombinant protein scaffold, a supramolecular organization similar to those of natural cellulosomes. Cellulosomal enzymes anchored on supramolecular structures found in natural cellulosomes show optimal synergistic activity which is significantly enhanced compared to the corresponding free enzymes [31]. These supramolecular assembly relies on a highly specific and strong ligand/receptor interactions between domains named dockerin and cohesin, respectively [32]. The calcium-dependent ligand/receptor pairs in cellulosomes were found to be one of the strongest non-covalent biomolecular bonds known in nature where a duplicated calcium-binding loop-helix motif of the ligand interacts with the receptor site [32-36].

Here we demonstrated that bio-inspired multi-enzymatic scaffold is an attractive strategy to prevent surfaces from being colonized by biofilm-forming bacteria. Far western blot based on scaffold detection confirmed that all three enzymes displayed functional dockerin modules and were successfully immobilized on the scaffold *via* ligand/receptor (dockerin/cohesin) binding. Atomic force microscopy (AFM)-based single-molecule force spectroscopy (SMFS) revealed the calcium-dependent nature of the ligand/receptor interactions used to dock the enzymes on the protein scaffold, similarly to natural cellulosome. It also demonstrated a strong binding and the specificity of these interactions. Lastly, viability assays on planktonic bacteria indicated the antimicrobial effect of the multi-enzymatic scaffold over five consecutive cycles of contamination by biofilm-forming bacteria, namely *Staphylococcus aureus*, *Escherichia coli* and *Micrococcus luteus*.

2. MATERIALS AND METHODS

2.1. Reagents and proteins

Lysozyme from chicken egg white (>90%, >40 units/mg), recombinant lysostaphin and DNase I (≥2,000 Kunitz units/mg protein) from bovine pancreas were purchased from Sigma and BioVendor (France). Recombinant scaffold protein as well as lysozyme, lysostaphin and DNase I enzymes tagged with ligands were produced and purified by the Protein Production and Purification Plateforme of the

Institut de Biologie Moléculaire et Cellulaire (IBMC)-CNRS of the Université de Strasbourg (details below).

2.2. Scaffold expression and purification

BL21 (DE3) *E. coli* strain transformed with pET9d-Scaf6-6xHis plasmid was grown at 37°C and in 2L lysogeny broth medium supplemented with 1,2% glycerol and Carbenincilin until reaching an optical density of 1.5. The temperature was lowered to 20°C and the expression induced by 0.2 mM IPTG during 20 hours.

Bacteria were collected by centrifugation at 5000 *g*, resuspended in 50 mL of 50 mM $NaPO_4$ (pH = 7) with 20 µg/mL DNase I and protease inhibitors mix (Roche), and lysed with a Microfluidizer LM-20 (Microfluidics).

The lysate was clarified by centrifugation at 18000 *g* for 20 min at 4°C, the supernatant collected and incubated with 15 g of micro-crystalline AvicelPH101 cellulose (SIGMA) for 90 min at 4°C under constant agitation. The cellulose was recovered by filtration on 2 µm glass fiber filter and washed three times with 50 mL of 50 mM $NaPO_4$ (pH = 7) and twice with 50 mL of 12.5 mM $NaPO_4$ (pH = 7).

Protein was sequentially eluted by three 50 mL volumes of cold water, followed by two sequential 50 mL volumes of 1 % triethanolamine, immediately buffered by the addition of 0.1 volume of 500 mM Tris-HCl (pH = 8). Elution fractions were assessed by SDS-PAGE and those containing the protein were pooled and filtered at 0.2 µm. The protein was further purified on an Akta Pure FPLC system with a MonoQ 5/150 GL anion exchange column (Cytiva) equilibrated in 25 mM Tris-HCl (pH = 8), 10 % glycerol and eluted with a gradient 0-1M NaCl, 25mM Tris-HCl (pH = 8) and 10 % glycerol. Fractions containing Scaf6 were pooled and loaded onto a HiLoad 16/60 Superdex S200 size exclusion column (Cytiva) equilibrated in PBS (pH = 7.4). Fraction corresponding to the monomeric protein were pooled and concentrated on a Vivaspin 20 concentrator (MWCO 30kDa; Sartorius) to a final concentration of 3.5 mg/mL, aliquoted, snap frozen in liquid nitrogen and stored at -80°C.

2.3. Tagged-enzymes expression and purification

BL21 (DE3) *E. coli* strains transformed with pT7 expression vectors bearing 6His-enzymes (lysozymes, lysostaphin, and DNase I) tagged with the ligand motifs were grown at 37°C and in 1L Terrific Broth medium supplemented with kanamycin until reaching an optical density of 2.5. The temperature was lowered to 20°C and the expression induced by 0.5 mM IPTG during 18 hours.

Bacteria were collected by centrifugation at 5000 *g*, resuspended in 100 mL 25mM Tris-HCl (pH = 8), 150 mM NaCl, 0,1% CHAPS, 3mM β-mercapto-ethanol, and lysed with a Microfluidizer LM-20 (Microfluidics).

The lysate was clarified by centrifugation at 18000 *g* for 20 min at 4°C, and the supernatant purified on an Akta Pure FPLC system by binding on a HisTrap HP Ni-NTA affinity column (Cytiva) with 25 mM imidazole. The protein was eluted by a 25 to 500 mM imidazole gradient in 25 mM Tris-HCl (pH = 8), 150mM NaCl, 0,1% CHAPS, 3 mM β-mercapto-ethanol. Fractions enriched in tagged enzymes were further purified on a Hi-Load 16/60 Superdex 75 column (Cytiva) equilibrated in PBS (pH = 7.4). Fractions containing monomeric tagged enzymes were pooled and concentrated on an AMICON Ultra 4 (MWCO 3kDa, Millipore) to a final concentration of 1.5 mg/mL, aliquoted, snap frozen in liquid nitrogen and stored at -80°C.

2.4. Far western blots

Ligand-tagged and non-tagged proteins (20 µL at 0.2 mg/mL) were mixed with 6 µL of denaturing buffer, prior boiling for 5 min. 5-µL of boiled samples were subjected to denaturing polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-rad precast gels (gradient 4-15%) and analyzed by far western blot, after transfer on nitrocellulose (GE healthcare), using biotinylated Scaf6 and streptavidin-POD (Roche) as formerly described in [37].

2.5. Surface and AFM tip functionalization

169 Silicon wafers and round glass coverslips (Ø12 mm) were coated by plasma sputtering with an under
170 layer of chromium (~10 nm) and an upper layer of gold (~30 nm). They were then cleaned by UV-ozone
171 treatment for 15 min, rinsed with ethanol and dried under N_2 flow.
172 Enzymes and scaffolds were covalently attached on self-assembled monolayers (SAMs) of carboxyl-
173 terminated alkanethiols. Briefly, gold-coated substrates and gold-coated NPG-D (Bruker) AFM tips
174 were immersed overnight in a solution of 0.1 mM 16-mercaptododecahexanoic acid and 0.9 mM 11-
175 mercapto-1-undecanol to form SAMs. They were then rinsed with ethanol and dried under N_2 flow.
176 Next, carboxylic groups were activated by immersion 30 min in a solution of N-hydroxysuccinimide
177 (NHS, 10 mg/mL) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, 25 mg/mL). AFM tips and
178 substrates were then rinsed with ultrapure water and immersed in protein solutions (0.2 mg/mL in
179 PBS) for 2 hours. Enzymes-decorated tips and scaffold-coated surfaces were stored in Tris-buffered
180 saline (TBS) supplemented 1 mM $CaCl_2$ and 1 mM $ZnCl_2$ (TBS^+) at 4°C for 4 days maximum before they
181 were used for SMFS. Calcium chloride and zinc chloride were used to ensure calcium-dependent
182 ligand/receptor interactions and zinc-dependent lysostaphin activity.

183 For bacterial viability experiments, substrates coated with scaffolds were immersed in a solution
184 containing 0.2 mg/mL of tagged lysozyme, tagged lysostaphin and tagged DNase I. Control surfaces
185 were prepared by grafting directly non-tagged enzymes on SAMs activated substrates (without
186 scaffold layer) or by grafting only the scaffold (no enzyme addition). All grafting lasted for 2 hours at
187 0.2 mg/mL in PBS. Prepared surfaces were rinsed in TBS^+ and directly used for bacterial viability tests.

188 **2.6. AFM imaging and force spectroscopy**

189 All AFM experiments were achieved at 21±1°C in an air-conditioned room, in TBS^+ buffer using a
190 Bioscope Resolve AFM (Bruker corporation, Santa Barbara, CA). Cantilevers spring constants were
191 determined by the thermal noise method [38]. Measurement of scaffold coating thickness was
192 performed by scanning a 1 x 1 μm^2 area at high forces and high scan rate and then imaging a larger
193 area (of 5 x 5 μm^2) under small forces and reduced frequencies. To perform SMFS, adhesion maps of

32 x 32 force-distance (FD) curves on 5 x 5 µm² areas were recorded on the scaffold-coated substrates with the enzyme-decorated AFM tips with an applied force of 500 pN, 1 µm/s constant approach and retraction speeds and a contact time of 500 ms. For control experiments, after acquiring adhesion in presence of calcium (TBS^+), 20 mM of ethylenediaminetetraacetic acid (EDTA, Carlo Erba Reagents) was added in the buffer and SMFS was performed again to acquire the adhesion in the presence of the chelating agent. The media was then replaced by fresh TBS^+ and SMFS was performed one last time. Positive control was achieved by performing the AFM measurements with the cellulase Cel9T from *R. cellulolyticum* tagged with its native dockerin.

The adhesion force and rupture length of the last adhesive peaks were extracted from each force curve with Nanoscope Analysis 1.8 (Bruker). Adhesion forces and rupture lengths of the last adhesive peaks for each curve were ordered in bins to form the frequencies histograms displaying adhesion frequencies and rupture frequencies. These histograms were processed with MATLAB R2016b (MathWorks) and designed in figures with Origin 2023 (OriginLab). Results presented for SMFS correspond to the average over three independent experiments of 1024 force-distance curves each.

2.7. Bacterial growth and viability tests

All bacterial cells were grown routinely at 37°C. *Staphylococcus aureus* strain 187 (HER 1239) were grown on tryptic soy broth (TSB, Sigma) plates and *Micrococcus luteus* (ATCC4698) and *Escherichia coli* (ATCC25922), on lysogeny broth Lennox (LB, Sigma) plates. For viability experiments, a single colony of *S. aureus*, *M. luteus* and *E. coli* was grown in 5 mL of respective broth media under gentle agitation (150 rpm on an agitation plate). Overnight cultures were washed twice in TBS^+ by centrifugation at 5000 g for 5 min before being retrieved. For the consecutive challenges, 1.2 mL of bacterial suspension containing ~5 x 10^7 bacteria were deposited on functionalized surfaces in 12-well plates and gently agitated at 37°C. Multiple waves of contaminations (challenges) were performed by replacing the bacterial suspension in the plates with fresh suspension containing ~5 x 10^7 bacteria every 24h for a total of 5 challenges. Samples were pipetted (100 µL) at 0h, 2h, 4h, 6h and 24h of every 24h-challenge

and serially diluted then plated on agar plates before culture at 37°C to determine bacterial viability by colony forming units counting. Results presented for viability tests correspond to the average on four serial dilutions for each time and over four independent experiments. Results are presented with exponential decay fittings on Origin 2023 (OriginLab).

2.8. Statistical analysis

All statistical analysis was performed using GraphPad Prism 7.0. Standard deviations of each data group of the adhesion frequency for each enzyme (n=3) were not found significantly different with a Brown-Forsythe test. A one-way ANOVA was therefore used followed by a Tukey's multicomparison test for pair-wise comparison between conditions.

3. RESULTS

3.1. Ligand-tagged enzymes bound to the receptors located on the scaffolds

To concurrently graft three different enzymes on surfaces, we decided to construct and purify after heterologous expression an interspecific hybrid scaffold made of receptors (cohesin modules) from three different species, namely *Acetivibrio thermocellu*s (formerly known as *Clostridium thermocellum*), *Ruminiclostridium cellulolyticum* and *Ruminococcus flavefaciens*, and separated by spacers of 30 amino acids. Enzymes of interests were tagged with the ligands corresponding to their scaffold receptors, *i.e* lysostaphin, lysozyme and DNase I were tagged with ligands (dockerin modules) from *A. thermocellu*s, *R. cellulolyticum* and *R. flavefaciens*, respectively. More precisely, Cel48S dockerin from *A. thermocellu*s was anchored on the C-terminus of lysostaphin to form the tagged lysostaphin; lysozyme tagged enzymes carried Man5K dockerin from *R. cellulolyticum* on the N-terminus; and tagged DNase I was tagged with dockerin from *R. flavefaciens* on its C-terminus. The cellulase Cel9T from *R. cellulolyticum* with its native dockerin was used as positive control to confirm binding of native enzyme-ligand to scaffold receptor [39]. Enzymes were tagged with ligand of different species to ensure that each tagged enzyme had a specific receptor on the scaffold and thus that each enzyme could bind to the scaffold. Far western blots were the first step of our work to evaluate the

ability of the enzymes to bind to the scaffold. Electrophoresis gel with the tagged and non-tagged lysostaphin, lysozyme and DNase I were transferred onto a membrane and biotinylated scaffold was used to probe the membrane. Bands at 36 kDa, 23 kDa and 41 kDa were revealed and attributed to tagged lysostaphin, tagged lysozyme and tagged DNase I, respectively (Figure 1). No bands were observed when the biotinylated scaffold was used to probe non-tagged enzymes, namely lysostaphin (27 kDa), lysozyme (16 kDa) and DNase I (31 kDa). This confirmed that tagged enzymes specifically bound to the scaffold whereas non-tagged ones were unable to bind to it. Based on these results, it was assumed that, all three tagged enzymes bound to the scaffold and that the receptor located on the scaffolds targeted specifically tagged enzymes.

3.2. Enzymes tagged with ligand interacted specifically with scaffold-coated surfaces

Probing the interaction between the enzymes and scaffold-coated surfaces was a crucial step in order to demonstrate that the surface functionalization strategy led to the immobilization of tagged enzymes through specific interactions between the ligands of the enzymes and the receptors located on the scaffolds.

First, scaffolds were covalently grafted on material surfaces and topography AFM images were performed to confirm the grafting. In order to evaluate the thickness of the coatings, a part of each coating was removed by "scratching" a squared area of 1 x 1 μm^2 at high forces (> 10 nN) and high frequencies with AFM tips. A coating was considered present on the surface of the material when a scratched square was visible on the image. The depth of the scratched area was considered an estimation of the coating thickness (Figure 2).

Images of surfaces coated only with SAMs revealed a smooth and flat surface on which the coating was not visible even after using the "scratching" AFM technique (Figure 2a). The relatively small size of the molecules composing the SAMs, the typical thickness of carboxylic acid terminated SAMs (few ångströms [40]) and the covalent nature of the coating made difficult to evidence the SAMs with the "scratching" AFM technique. However, when the scaffold was added on the SAMs-coated surfaces, the

resulting coating was observable on the AFM images and was homogeneous throughout the surface (Figure 2b). Its thickness was evaluated at 1.95 ± 0.24 nm which confirmed that the scaffold bounded with the carboxyl groups of the SAMs. When the tagged-enzymes were added on the surface coated with the SAMs and the scaffold, the thickness of the coating increased and reached 7.80 ± 0.67 nm (Figure 2c). This evidenced the binding of the enzymes' ligands onto the scaffold's receptors as the increased thickness of the coating was attributed to the addition of the tagged enzymes. When scanned in contact mode at small forces, the coatings remained stable and no visible topography alterations were observed indicating a strong anchorage of the coatings on the substrate.

AFM imaging and far western blots showed that the enzymes had the ability to bind to the scaffold but to investigate these bonds further, the specificity of the receptor-ligand interactions was assessed by AFM-based SMFS. For that, each enzyme was grafted one at a time on AFM cantilevers which were then used to probe scaffold-coated surfaces (Figure 3).

For each enzyme-decorated AFM tip, adhesion in presence of calcium was first probed on the scaffold coating by recording adhesion maps. To test the calcium-dependency of the receptor/ligand pairs, a calcium chelating agent (EDTA) was added to the buffer to trap free calcium ions and SMFS measurements were subsequently performed a second time. Lastly, EDTA was removed, the buffer containing calcium was added and adhesion was recorded for the last time.

Figures 4, 5 and 6 show the adhesion frequencies and the rupture lengths histograms as well as representative force-distance (FD) curves for ligand-tagged lysostaphin, lysozyme and DNase I and their corresponding negative controls, *i.e.* non-tagged enzymes. For all enzymes, adhesive pixels on adhesion maps were randomly spread and adhesion maps did not show cluster patterns (data not shown). This suggests that the scaffolds were homogeneously grafted on the surfaces which matched the results observed above with the AFM topography images (Figure 2).

Before adding EDTA, representative FD curves showed adhesion peaks for all ligand-tagged enzymes (Figure 4a, 5a and 6a). In contrast, less adhesion signatures were found on FD curves when EDTA was

added with the ligand-tagged enzymes (Figure 4b, 5b and 6b). However, once EDTA was removed, adhesion signatures were restored (Figure 4c, 5c and 6c). Little to no adhesion signature were observed on FD curves of homologous enzymes lacking ligands (Figure 4d, 5d and 6d). Adhesion frequency in presence of calcium for tagged lysostaphin was high since 86% of the FD curves showed an adhesion signature (Figure 4a). Adhesion frequency then dropped to around 42% when EDTA was added (Figure 4b) and increased again to 87% after EDTA was rinsed and calcium chloride was added (Figure 4c). In contrast, only 8% of the FD curves showed adhesion signature for non-tagged lysostaphin (negative control, Figure 4d) which we attributed to non-specific adhesion events. In presence of calcium, the average adhesion frequency of tagged lysozyme was 42% (Figure 5a); it decreased to 12% with EDTA (Figure 5b) and increased to 33% when EDTA was removed (Figure 5c). Non-tagged lysozyme (negative control) adhesion frequency was 13% which shows that EDTA was able to completely inhibit ligand/receptor recognition in the case of this tagged lysozyme sample (Figure 5d). Adhesion frequencies of tagged DNase I was 61% in presence of calcium (Figure 6a), 4% when EDTA was added (Figure 6b) and 25% when EDTA was removed (Figure 6c). EDTA was also able to totally inhibit interactions for this sample since only 8% FD curves recorded with non-tagged DNase I presented adhesion peaks (Figure 6d). Adhesion forces and rupture lengths of scaffold-enzymes interactions ranged from 100 to 400 pN and 5 to 150 nm, respectively. Tagged lysostaphin, tagged lysozyme and tagged DNase I mean rupture forces, in absence of EDTA and over three independent experiments of 1024 FD curves each, were on average 211±2 pN, 297±4 pN and 246±3 pN, respectively (results are reported as mean ± standard error of the mean).

Figure 7 represents adhesion frequencies for each condition and each enzyme on three independent experiments of 1024 FD curves each. Adhesion frequencies decreased significantly for all enzymes in presence of EDTA which confirmed the calcium-dependent nature of the ligand/receptor interactions. Before EDTA addition, differences were observed in the adhesion frequencies between the tagged-enzymes. Tagged lysostaphin reached an average of 87±4% adhesion frequency whilst tagged lysozyme

and DNase I were around 42%±4% and 61%±17%, respectively (Figure 7). Tagged lysostaphin led to the most adhesion compared to the other tagged enzymes before and after addition of EDTA.

No significant differences were found between the EDTA condition and the negative control (non-tagged enzymes) for tagged lysozyme and tagged DNase I. EDTA was therefore able to entirely inhibit ligand/receptor interactions for these tagged enzymes. As for the interactions between tagged lysostaphin and the scaffold, EDTA was able to inhibit by around half of them. Since tagged lysostaphin resulted in the most adhesion compared to other tagged enzymes, EDTA not being able to inhibit all interactions for tagged lysostaphin was attributed to the high affinity of the ligand/receptor pair and/or to a limited EDTA accessibility to the calcium-binding loop-helix motif of this ligand/receptor.

After removal of chelating agent, tagged lysostaphin adhesion frequency was entirely restored as no significant differences were found before adding EDTA and after removal of EDTA (Figure 7). Adhesion frequencies of tagged lysozyme and tagged DNase I were significantly higher before adding EDTA than after which showed that the adhesion was partially restored after the chelating agent was removed. This partial restoration of adhesion was also observed with the positive control achieved with the natural cellulosome cellulase Cel9T from *R. cellulolyticum*. Adhesion of the positive control was inhibited by EDTA and almost half of the adhesion was restored after removal of EDTA. However, for all enzymes, after EDTA was removed, a significant increase in adhesion frequencies was observed. These results confirmed that in absence of free calcium in the buffer (*i.e.,* when EDTA was added), the ligand/receptors interactions between the tagged-enzyme and the scaffolds were inhibited and therefore that these interactions were calcium dependent.

Non-tagged enzymes displayed very low adhesion, compared to tagged enzymes (Figure 7). This showed the high specificity of the scaffold-enzymes interactions and this ensured that only the tagged enzymes were anchored on the scaffold and that substitution with other proteins were highly unlikely. As a result, very low adhesion observed on non-tagged enzymes were attributed to non-specific interactions.

3.3. Multi-enzymatic scaffold coating improved antimicrobial protection of surfaces

To assess surface protection achieved by our system, antibacterial effect was evaluated over multiple waves of contaminations (challenges) with fresh bacteria by standard plating assays. Bacteria were exposed to surfaces coated with the multi-enzymatic scaffolds. To evaluate the relevance of the scaffold in the system, results were compared to samples onto which enzymes were directly grafted without any protein scaffolding. Surfaces only coated with the scaffolds were used as negative controls. Bacterial viability was evaluated after 0h, 2h, 4h, 6h and 24h of exposition. Each 24h, the media was replaced by a fresh suspension of bacteria. The same procedure was repeated over 5 consecutive days (Figure 8).

On the control surface, bacterial viability was around 90 to 100 % after 24h for most challenges (Figure 8). Slight decreases (< 10 %) were observed over time for some samples and were attributed to: i) bacteria adhering to the plastic well plate where the surfaces were immerged and thus not being collected when performing viability assays, and/or ii) possible bacterial aggregation that reduce the number of CFU, *i.e.* one CFU could have been corresponding to aggregated bacteria instead of individual cells growing as a colony, thus artificially reducing the survival rate [41].

The multi-enzymatic coating showed broad spectrum antimicrobial effects as both Gram-negative, namely *E. coli,* and Gram-positive bacteria, *S. aureus* and *M. luteus* were killed. Around 83 % of *S. aureus*, 71 % *E. coli* and 84 % of *M. luteus* cells were lysed after the first 24h when in contact with the multi-enzymatic scaffold (Figure 8a, b, c). In contrast, enzymes directly grafted on surfaces without any scaffold support had a milder effect on bacterial lysis. Around 62 % of *S. aureus* were lysed in contact with enzymes grafted directly on the surface after the first 24h (Figure 8a) and around 52 % and 46 % for *E. coli* and *M. luteus*, respectively (Figure 8b, c). Multi-enzymatic scaffolds exhibited more antimicrobial effects throughout the five consecutive challenges than enzymes grafted directly on the material surface.

368 The antibacterial effects of multi-enzymatic scaffolds remained unchanged after five consecutive
369 challenges for *E. coli* and *M. luteus*. Around 71 % of *E. coli* bacteria were lysed at the end of the first
370 challenge and 73 % at the end of the fifth challenge for the multi-enzymatic scaffold. *M. luteus* lysing
371 rate varied from 84 % at 24h to 82 % at the end of the fifth challenge. A slight decrease in bacterial
372 lysing rate was observed with *S. aureus* with the multi-enzymatic scaffold but the antimicrobial action
373 remained high nonetheless. Around 70% of *S. aureus* were lysed by the multi-enzymatic scaffold after
374 five consecutive challenges whereas the lysed rate was roughly 83% for the first challenge. A similar
375 decrease in lysing rate was also observed for the unsupported enzymes for *S. aureus* dropping from
376 62% after 24h to 45% after the fifth challenge.

377 **4. DISCUSSION**

378 Key features of a successful protective surface reside in a long-lasting and broad-spectrum
379 antibacterial effect. Aside from many crucial advantages in the fight against infections, antibacterial
380 enzymes are known to be long lasting as long as their catalytic site is active in comparisons of other
381 strategies that often relies on a "one-shot" effect [12, 13, 15-20]. However, antibacterial enzymes are specific
382 and target a narrow range of pathogens. This presents the advantage of limiting adverse effects by
383 avoiding the lyse of benign commensal microbiome threatened by broad spectrum antibiotics [42]. But,
384 at the same time, antibacterial surface treatment composed of a single antibacterial enzyme have
385 intrinsically a limited spectrum of action. In order to increase the range of action of the antibacterial
386 surfaces, our bio-inspired strategy in based on the simultaneous grafting of complementary active
387 enzymes. Previous studies showed the relevance of bi- and trifunctional chimeric scaffolds combining
388 different cellulosomal enzymes for the construction of designer cellulosomes [31, 43]. The proposed bio-
389 inspired strategy was based on a similar approach to simultaneously assemble different yet
390 complementary antibacterial enzymes on a recombinant protein scaffold. This allowed a broad-
391 spectrum effect and a controlled positioning of each tagged enzyme on the scaffold [31]. Ligand/receptor

392 pairs, dockerin and cohesin respectively, from different species producing cellulosomes were used to
393 anchor concurrently different ligand-tagged enzymes.

394 Dockerin/cohesin pairs are considered to be one of the most stable ligand/receptor interactions known
395 with rupture forces that occurs *via* a tensile-based mechanism and above 120 pN [32, 44], which is in
396 concordance with our SMFS results. We concluded that unfolding of dockerin and cohesin which
397 typically occurs at forces higher than 300 pN and up to 750 pN depending of the cohesin module [44-46],
398 was unlikely since such high forces were not representative of our results. The SMFS conducted in the
399 present study showed that ligand/receptor pairs used to dock the enzymes to the scaffold were
400 calcium-dependent similarly to ligand/receptor pairs in natural cellulosome [32, 47, 48]. This excluded the
401 possibility of the tagged enzymes to be docked to the scaffold though unspecific interactions or any
402 other interactions than through the binding of cohesin and dockerin. It also showed the highly specific
403 interaction of our ligand/receptor strategy. The chimeric protein scaffold of our system was therefore
404 not restricted to the anchorage of the lysozyme, lysostaphin and DNase I. As long as other enzymes of
405 interest can be tagged with the ligand, the multienzymatic scaffold could be adapted to different
406 applications to face other contaminants.

407 Discrepancies in adhesion frequencies of ligand and receptor interactions were observed. Tagged
408 lysostaphin led to the highest adhesion frequencies in SMFS when in contact to its corresponding
409 receptor on the scaffold compared to tagged lysozyme and tagged DNase I. These enhanced
410 interaction frequencies might be attributed to species-dependent variations as it was shown that the
411 affinity for the ligand and the receptor of *A. thermocellum* (used to bind lysostaphin to the scaffold in
412 the present study) was higher than for ligand/receptor pairs of *R. cellulolyticum* and *R. flavefaciens*
413 (used to dock lysozyme and DNase I, respectively, to the scaffold in our work) [31]. These variations in
414 adhesion frequencies between tagged enzymes might also be explained by differences in accessibility
415 between the ligand-tag and the receptor site and/or by a variation in steric hindrances due to the
416 different positions of the receptors in the amino acid chain of the scaffold. The discrepancies observed

in rupture lengths might also have been originated from species-dependent variations, lengths of the enzymes, steric hindrances and the positioning of the receptors in the amino acid chain of the scaffold.

The prerequisite for long-lasting antibacterial coatings is the constant degradation of bacteria and the elimination of contaminants that can accumulate and promote biofilm formation over time. One of the major advantages of using enzymes as antimicrobial agents is that they are "reusable" and they continuously degrade bacteria as long as their catalytic site remains active. The proposed strategy combined cell wall degrading enzymes as well as DNase I which degraded DNA, an important cohesive component of the adhesion of microbial colonies and biofilm and the major polymer that would be released during cell lysis after cell wall degradation. *S. aureus* (Gram-positive), *E. coli* (Gram-negative) and *M. luteus* (Gram-positive) were chosen to assess the antimicrobial properties of the surfaces coated with the multi-enzymatic scaffolds. *S. aureus* and *E. coli* are some of the most etiologic agents in nosocomial infections [2, 49, 50]. Despite its typical low virulence, *M. luteus* can increase *S. aureus* pathogenesis by acting as a "proinfectious agent" and present risks of contaminations [51-55]. Previous studies evidenced that grafting enzymes directly on surfaces resulted in a non-oriented distribution of the enzymes [29, 56-60]. Supported enzymes were shown to have optimized antimicrobial activity since the orientation of their catalytic sites is improved as well as their 3D conformations [30]. As expected in our system, when enzymes were not supported by a scaffold, antimicrobial properties were evidenced to some extend but they were enhanced when enzymes were anchored on the supporting scaffold. The reduced efficacy of the unsupported enzymes was attributed to the randomness of their orientation on the substrate and the possible modification of their conformation leading to some of them being denatured and inactivated. The fact that consecutive challenges did not appear to alter significantly the enzymes ability to lyse bacteria whether supported or unsupported confirmed (i) the long-term "re-usable" antibacterial effect of the enzymes and (ii) the long-term properties of DNase I preventing the accumulation of lysed bacteria that are known to promote bacterial adhesion which can later lead to biofilm formation. The relevance of the use of a multi-enzymatic scaffold was confirmed throughout all the consecutive challenges since enzymes organized on the protein scaffold exhibited a higher

antimicrobial response compared to the enzymes grafted directly on the surfaces. The enzymes organization and immobilization on a molecular scaffold allowed a controlled orientation and better accessibility to their target and resulted in an improved protection.

5. **CONCLUSIONS**

Cellulosomes rely on a supramolecular organization to enhance enzymatic efficiency and degrade recalcitrant biopolymers. Our bio-inspired multi-enzymatic scaffold relied on a similar organization to lyse bacteria in order to protect surfaces from bacterial colony formation. Single-molecule force spectroscopy evidenced that enzymes were anchored on the scaffold through highly specific ligand/receptor interactions to from a supramolecular structure analogous to natural cellulosomes. The simultaneous use of complementary enzymes on the scaffold provided broad-spectrum protection concurrently against Gram + and Gram - bacteria. Our system exhibited substantial antimicrobial activity against planktonic biofilm forming bacteria, namely *S. aureus*, *E. coli* and *M. luteus*, by lysing 71 % to 84 % of bacteria within the first 24 hours. The “re-usable” nature of the lysozyme and lysostaphin as well as the properties of DNase I ensured long term antimicrobial effects after 5 consecutive 24h challenges. The scaffold improved the protective antimicrobial activity compared to unsupported enzymes. This was attributed to an optimized orientation and spatial conformation of enzymes attained with the multi-enzymatic scaffold. Enzymes were anchored on the scaffold through highly specific ligand/receptor interactions that enabled a controlled organization and positioning of the enzymes. Our system had great adaptability potential as it offered the possibility to tailor enzymes cocktails to the contamination risk of specific applications. Furthermore, considering the rapidly expanding repertoire of specific cohesin/dockerin devices described in the literature, gathering a larger number of different antimicrobial enzymes/proteins should be straightforward, whereas elongation or reduction of the inter-cohesins linkers in the scaffold could help gaining efficiency.

CONFLICTS OF INTEREST

Conflicts of interest: none.

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AUTHOR CONTRIBUTIONS

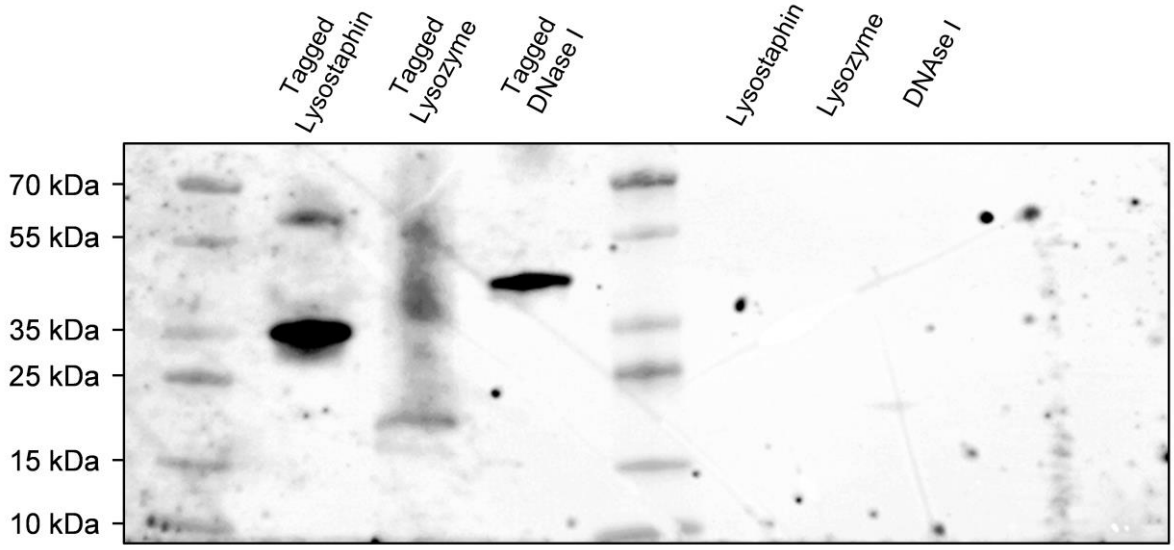
BA, FQ, GF, HPF and SEKC designed the research and wrote the manuscript; BA, CR, FQ, HPF and SEKC contributed to the acquisition, analysis, and interpretation of the data. BA, FQ, HPF and SEKC revised the paper and all authors approved the final version.

REFERENCES

1. K. S. Ikuta, L. R. Swetschinski, G. R. Aguilar, F. Sharara, T. Mestrovic, A. P. Gray, N. D. Weaver, E. E. Wool, C. Han and A. G. Hayoon, *The Lancet*, 2022, **400**, 2221-2248.
2. M. Shirtliff, J. G. Leid and M. Shirtliff, *The role of biofilms in device-related infections*, Springer, 2009.
3. S. Percival, *Journal of British Surgery*, 2017, **104**, e85-e94.
4. E. M. Padegimas, M. Maltenfort, M. L. Ramsey, G. R. Williams, J. Parvizi and S. Namdari, *Journal of shoulder and elbow surgery*, 2015, **24**, 741-746.
5. A. Premkumar, D. A. Kolin, K. X. Farley, J. M. Wilson, A. S. McLawhorn, M. B. Cross and P. K. Sculco, *The Journal of Arthroplasty*, 2021, **36**, 1484-1489. e1483.
6. M. Assefa and A. Amare, *Infect Drug Resist*, 2022, **15**, 5061-5068.
7. H.-C. Flemming and J. Wingender, *Nature reviews microbiology*, 2010, **8**, 623-633.
8. M. Otto, *Bacterial biofilms*, 2008, DOI: https://doi.org/10.1007/978-3-540-75418-3, 207-228.
9. K. Sauer, P. Stoodley, D. M. Goeres, L. Hall-Stoodley, M. Burmølle, P. S. Stewart and T. Bjarnsholt, *Nature Reviews Microbiology*, 2022, **20**, 608-620.
10. H.-C. Flemming, *Water Res.*, 2020, **173**, 115576.
11. D. Campoccia, L. Montanaro and C. R. Arciola, *Biomaterials*, 2013, **34**, 8533-8554.
12. I. Francolini and G. Donelli, *FEMS Immunology & Medical Microbiology*, 2010, **59**, 227-238.
13. K. Glinel, P. Thebault, V. Humblot, C.-M. Pradier and T. Jouenne, *Acta Biomater.*, 2012, **8**, 1670-1684.
14. S. Tambe, L. Sampath and S. Modak, *J. Antimicrob. Chemother.*, 2001, **47**, 589-598.
15. D. Alves and M. Olivia Pereira, *Biofouling*, 2014, **30**, 483-499.
16. A. K. Muszanska, H. J. Busscher, A. Herrmann, H. C. van der Mei and W. Norde, *Biomaterials*, 2011, **32**, 6333-6341.
17. A. Shah, J. Mond and S. Walsh, *Antimicrob Agents Chemother*, 2004, **48**, 2704-2707.
18. J. J. Swartjes, P. K. Sharma, T. G. van Kooten, H. C. van der Mei, M. Mahmoudi, H. J. Busscher and E. T. Rochford, *Curr Med Chem*, 2015, **22**, 2116-2129.
19. C. Von Eiff, B. Jansen, W. Kohnen and K. Becker, *Drugs*, 2005, **65**, 179-214.
20. X. Wu, K. Fraser, J. Zha and J. S. Dordick, *ACS Appl Mater Interfaces*, 2018, **10**, 36746-36756.
21. R. H. Doi and A. Kosugi, *Nature reviews microbiology*, 2004, **2**, 541-551.
22. E. A. Bayer, L. J. Shimon, Y. Shoham and R. Lamed, *Journal of structural biology*, 1998, **124**, 221-234.
23. V. M. Dillon, 2014.
24. M. Derde, V. Vié, A. Walrant, S. Sagan, V. Lechevalier, C. Guérin-Dubiard, S. Pezennec, M. F. Cochet, G. Paboeuf and M. Pasco, *Biopolymers*, 2017, **107**, e23040.
25. M. Derde, V. r. Lechevalier, C. Guérin-Dubiard, M.-F. o. Cochet, S. Jan, F. Baron, M. Gautier, V. r. Vié and F. o. Nau, *J. Agric. Food Chem.*, 2013, **61**, 9922-9929.
26. A. Pellegrini, U. Thomas, R. von Fellenberg and P. Wild, *J Appl Bacteriol*, 1992, **72**, 180-187.
27. A. Pellegrini, U. Thomas, P. Wild, E. Schraner and R. von Fellenberg, *Microbiol. Res.*, 2000, **155**, 69-77.
28. J. K. Kumar, *Appl. Microbiol. Biotechnol.*, 2008, **80**, 555-561.
29. J. J. Swartjes, T. Das, S. Sharifi, G. Subbiahdoss, P. K. Sharma, B. P. Krom, H. J. Busscher and H. C. van der Mei, *Advanced Functional Materials*, 2013, **23**, 2843-2849.
30. A. Beaussart, C. Retourney, F. Quilès, R. D. S. Morais, C. Gaiani, H.-P. Fiérobe and S. El-Kirat-Chatel, *J. Colloid Interface Sci.*, 2021, **582**, 764-772.
31. H.-P. Fierobe, F. Mingardon, A. Mechaly, A. Bélaïch, M. T. Rincon, S. Pages, R. Lamed, C. Tardif, J.-P. Bélaïch and E. A. Bayer, *J. Biol. Chem.*, 2005, **280**, 16325-16334.
32. S. W. Stahl, M. A. Nash, D. B. Fried, M. Slutzki, Y. Barak, E. A. Bayer and H. E. Gaub, *Proceedings of the National Academy of Sciences*, 2012, **109**, 20431-20436.

33. S. D. Jeon, J. E. Lee, S. J. Kim, S. W. Kim and S. O. Han, *Biosensors Bioelectron.*, 2012, **35**, 382-389.
34. K. Sakka, Y. Sugihara, S. Jindou, M. Sakka, M. Inagaki, K. Sakka and T. Kimura, *FEMS Microbiol. Lett.*, 2011, **314**, 75-80.
35. M. A. Jobst, L. F. Milles, C. Schoeler, W. Ott, D. B. Fried, E. A. Bayer, H. E. Gaub and M. A. Nash, *Elife*, 2015, **4**, e10319.
36. A. L. Carvalho, F. M. Dias, T. Nagy, J. A. Prates, M. R. Proctor, N. Smith, E. A. Bayer, G. J. Davies, L. M. Ferreira and M. J. Romão, *Proceedings of the National Academy of Sciences*, 2007, **104**, 3089-3094.
37. J. Ravachol, R. Borne, I. Meynial-Salles, P. Soucaille, S. Pages, C. Tardif and H. P. Fierobe, *Biotechnol Biofuels*, 2015, **8**, 114.
38. J. L. Hutter and J. Bechhoefer, *Review of scientific instruments*, 1993, **64**, 1868-1873.
39. J. Ravachol, R. Borne, C. Tardif, P. de Philip and H.-P. Fierobe, *J. Biol. Chem.*, 2014, **289**, 7335-7348.
40. M. Carot, V. Macagno, P. Paredes-Olivera and E. Patrito, *The Journal of Physical Chemistry C*, 2007, **111**, 4294-4304.
41. P. Herman-Bausier, S. El-Kirat-Chatel, T. J. Foster, J. A. Geoghegan and Y. F. Dufrêne, *MBio*, 2015, **6**, e00413-00415.
42. A. Langdon, N. Crook and G. Dantas, *Genome medicine*, 2016, **8**, 1-16.
43. H. P. Fierobe, E. A. Bayer, C. Tardif, M. Czjzek, A. Mechaly, A. Belaich, R. Lamed, Y. Shoham and J. P. Belaich, *J. Biol. Chem.*, 2002, **277**, 49621-49630.
44. M. Gunnoo, P. A. Cazade, A. Galera-Prat, M. A. Nash, M. Czjzek, M. Cieplak, B. Alvarez, M. Aguilar, A. Karpol and H. Gaub, *Advanced materials*, 2016, **28**, 5619-5647.
45. A. Galera-Prat, S. Moraïs, Y. Vazana, E. A. Bayer and M. Carrión-Vázquez, *J. Biol. Chem.*, 2018, **293**, 7139-7147.
46. A. Valbuena, J. Oroz, R. Hervás, A. M. Vera, D. Rodríguez, M. Menéndez, J. I. Sulkowska, M. Cieplak and M. Carrión-Vázquez, *Proceedings of the National Academy of Sciences*, 2009, **106**, 13791-13796.
47. S. Pagès, A. Bélaïch, J. P. Bélaïch, E. Morag, R. Lamed, Y. Shoham and E. A. Bayer, *Proteins: Structure, Function, and Bioinformatics*, 1997, **29**, 517-527.
48. M. T. Rincón, J. C. Martin, V. Aurilia, S. I. McCrae, G. J. Rucklidge, M. D. Reid, E. A. Bayer, R. Lamed and H. J. Flint, *J. Bacteriol.*, 2004, **186**, 2576-2585.
49. G. Sharma, S. Sharma, P. Sharma, D. Chandola, S. Dang, S. Gupta and R. Gabrani, *J. Appl. Microbiol.*, 2016, **121**, 309-319.
50. A. Reisner, M. Maierl, M. Jörger, R. Krause, D. Berger, A. Haid, D. Tesic and E. L. Zechner, *J. Bacteriol.*, 2014, **196**, 931-939.
51. E. Boldock, B. G. Surewaard, D. Shamarina, M. Na, Y. Fei, A. Ali, A. Williams, E. J. Pollitt, P. Szkuta and P. Morris, *Nature microbiology*, 2018, **3**, 881-890.
52. R. Peces, E. Gago, F. Tejada, A. Laures and J. Alvarez-Grande, *Nephrology, dialysis, transplantation: official publication of the European Dialysis and Transplant Association-European Renal Association*, 1997, **12**, 2428-2429.
53. U. Dürst, E. Bruder, L. Egloff, J. Wüst, J. Schneider and H. Hirzel, *Zeitschrift fur Kardiologie*, 1991, **80**, 294-298.
54. G. Rodriguez-Nava, A. Mohamed, M. A. Yanez-Bello and D. P. Trelles-Garcia, *IDCases*, 2020, **20**, e00743.
55. H. Seifert, M. Kaltheuner and F. Perdreau-Remington, *Zentralblatt für Bakteriologie*, 1995, **282**, 431-435.
56. D. Alves, A. Magalhães, D. Grzywacz, D. Neubauer, W. Kamysz and M. O. Pereira, *Acta Biomater.*, 2016, **44**, 313-322.
57. G. Yeroslavsky, O. Girshevitz, J. Foster-Frey, D. M. Donovan and S. Rahimipour, *Langmuir*, 2015, **31**, 1064-1073.

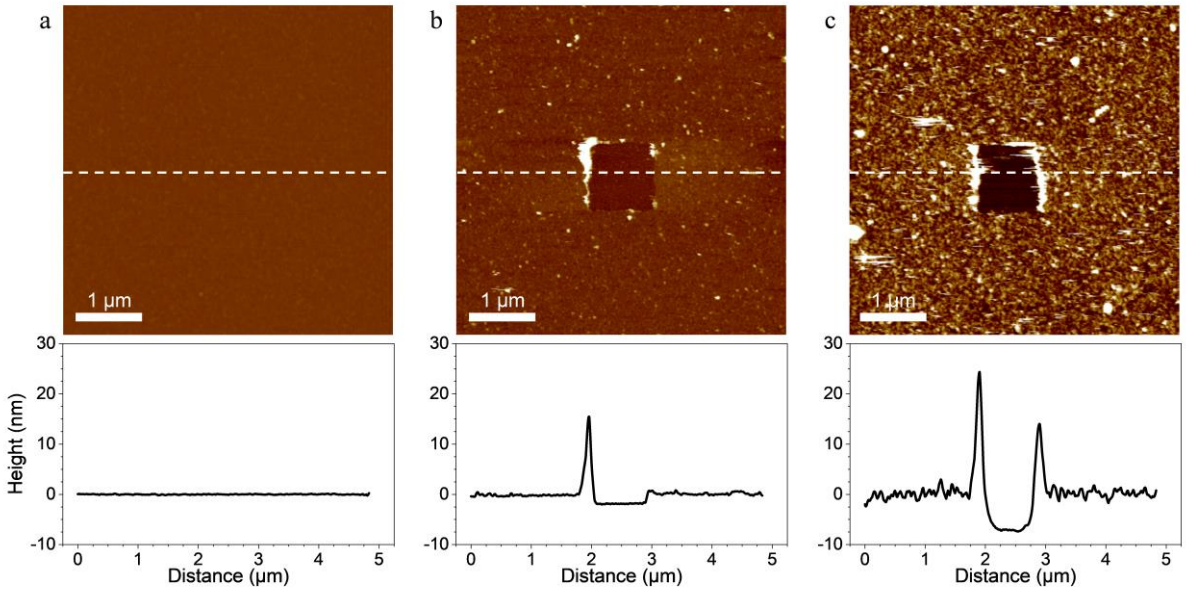
58. A. Caro, V. Humblot, C. Méthivier, M. Minier, L. Barbes, J. Li, M. Salmain and C.-M. Pradier, *J. Colloid Interface Sci.*, 2010, **349**, 13-18.
59. A. Caro, V. Humblot, C. Méthivier, M. Minier, M. Salmain and C.-M. Pradier, *The journal of physical chemistry B*, 2009, **113**, 2101-2109.
60. M. Minier, M. Salmain, N. Yacoubi, L. Barbes, C. Méthivier, S. Zanna and C.-M. Pradier, *Langmuir*, 2005, **21**, 5957-5965.



589

590 **Figure 1.** Far western blot using biotinylated scaffold (and Streptavidin-POD) to probe the membrane.
591 Tagged lysostaphin, tagged lysozyme, tagged DNase I (left channels) were identified at 36 kDa, 23 kDa
592 and 41 kDa, respectively. Non-tagged lysostaphin, lysozyme and DNase I (right channels) did not lead
593 to any interaction with biotinylated scaffold as no bands were identified.

594



595

596 **Figure 2.** Topography of surfaces coated with (a) self-assembled monolayers (SAMs) only (b) SAMs and
597 scaffold and (c) SAMs, scaffold and the three tagged enzymes. AFM height images (5 x 5 µm²; z values
598 represented by the LUT with a difference of 40 nm between the maximum and the minimum values).
599 A square of 1 x 1 µm² was first scanned at high forces (> 10 nN), then, the same area was imaged (5 x
600 5 µm²) with smaller forces. No scratched area was observed with the SAMs-coated surface. The AFM
601 image revealed smooth and homogenous coatings of the surface with the scaffold and the scaffold
602 coupled with the tagged enzymes. Scratched areas were observed and the thickness of the scaffold
603 coating and scaffold+enzymes coating were evaluated at 1.95 ± 0.24 nm and 7.80 ± 0.67 nm,
604 respectively by taking a vertical section (showed below the AFM images) along the dashed line drawn
605 on the AFM image.

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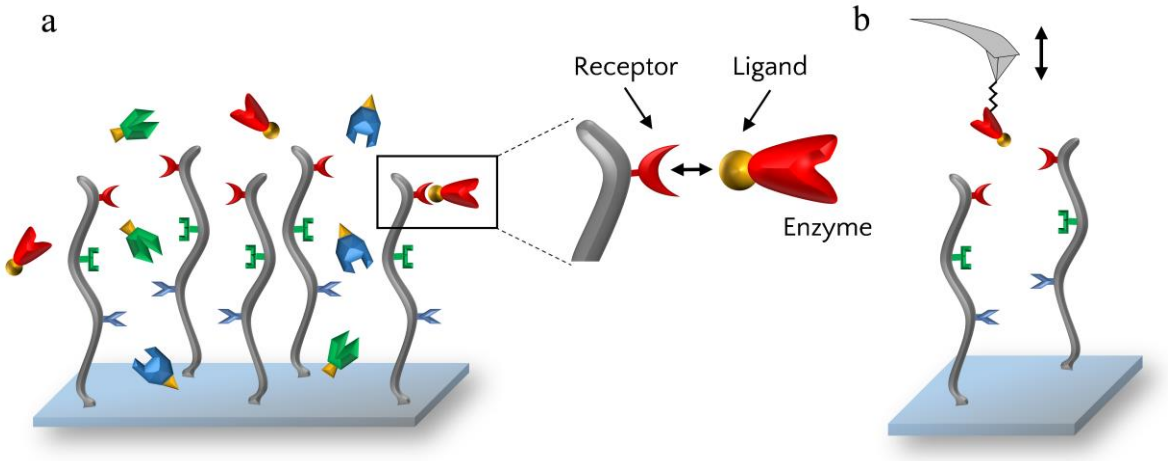
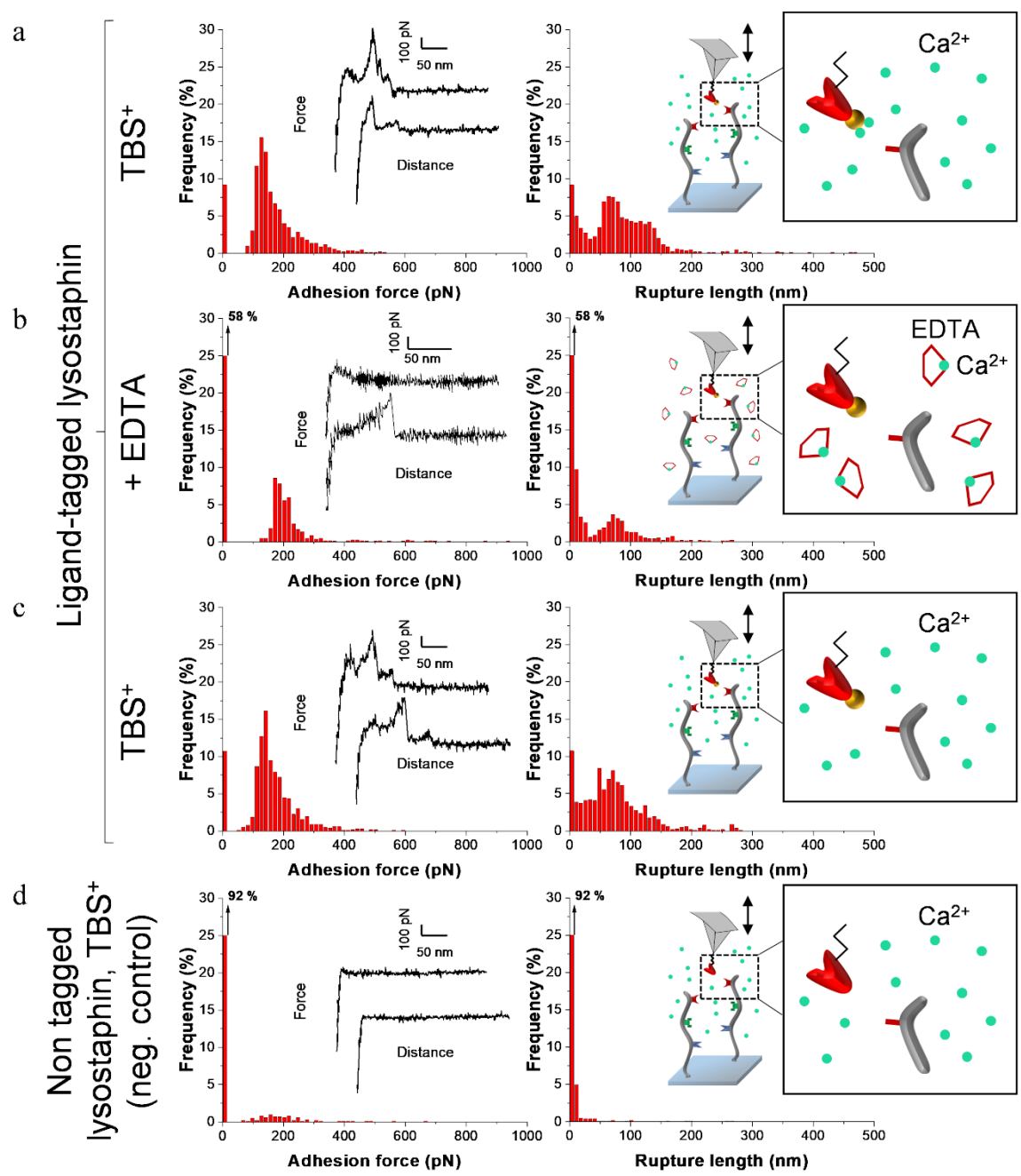


607

608 **Figure 3.** (a) Principle of enzyme grafting on scaffold-coated surfaces via the ligand-receptor
609 interaction. (b) Principle of single-molecule force spectroscopy to probe the interaction and specificity
610 between ligand-enzymes and receptors.

611



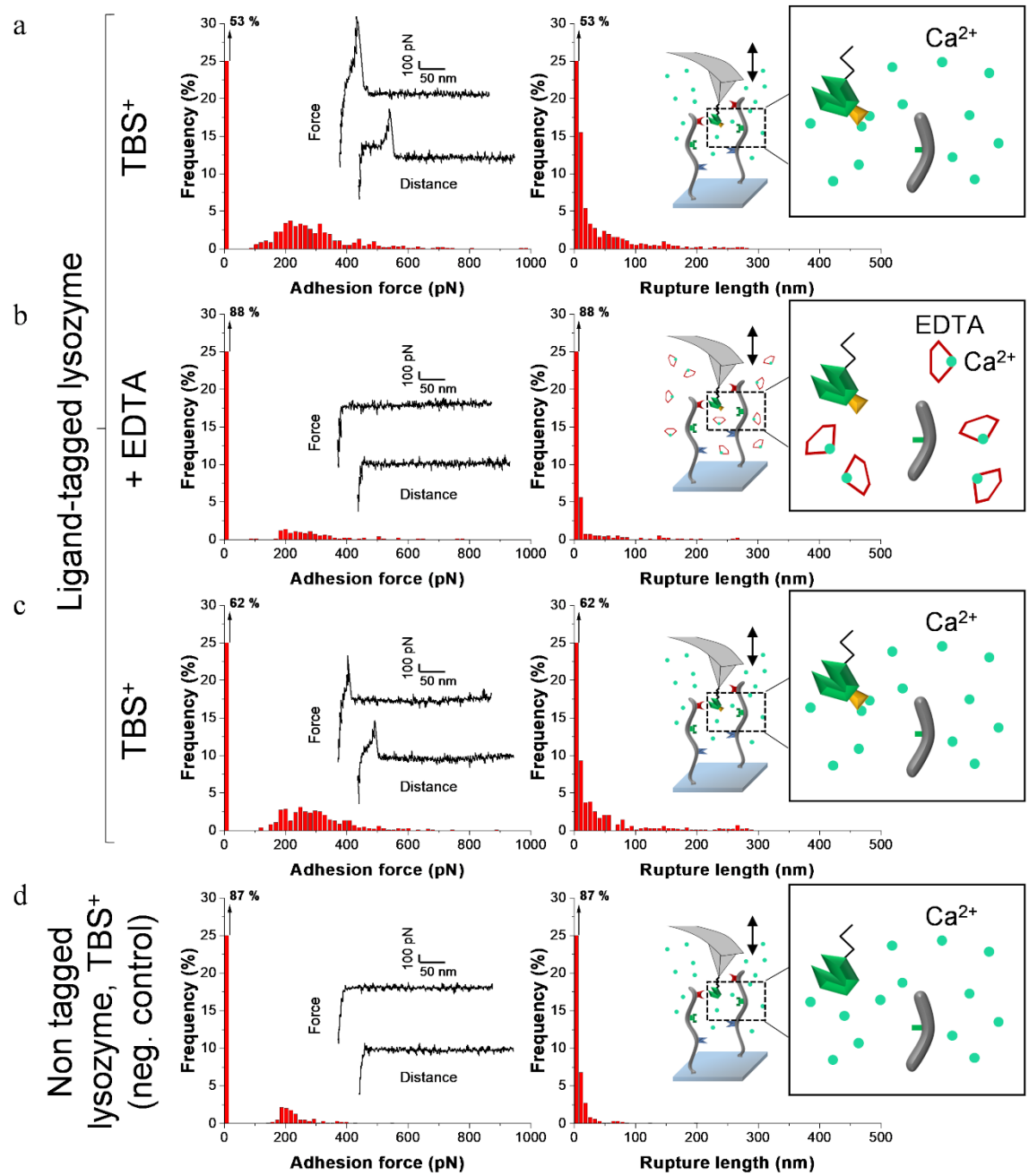
612

613 **Figure 4.** Adhesion frequency histograms with representative force curves (left) and corresponding
614 rupture length histograms (right) extracted from adhesion maps (5x5 µm²) recorded on scaffold-
615 coated surfaces with AFM cantilever decorated with tagged lysostaphin. Schematics on the right
616 represent the different conditions: adhesion was consecutively recorded on one sample (a) in a calcium
617 rich buffer before adding EDTA, (b) in presence of EDTA and (c) after removal of EDTA and addition of

618 TBS^+ containing calcium. (d) Negative control adhesion was recorded in TBS^+ with tips decorated with

619 lysostaphin lacking dockerin ligand.

620



621

622 **Figure 5.** Adhesion frequency histograms with representative force curves (left) and corresponding
623 rupture length histograms (right) extracted from adhesion maps (5x5 µm²) recorded on scaffold-
624 coated surfaces with AFM cantilever decorated with tagged lysozyme. Schematics on the right
625 represent the different conditions: adhesion was consecutively recorded on one sample (a) in a calcium
626 rich buffer before adding EDTA, (b) in presence of EDTA and (c) after removal of EDTA and addition of

627 TBS^+ containing calcium. (d) Negative control adhesion was recorded in TBS^+ with tips decorated with

628 lysozyme lacking dockerin ligand.

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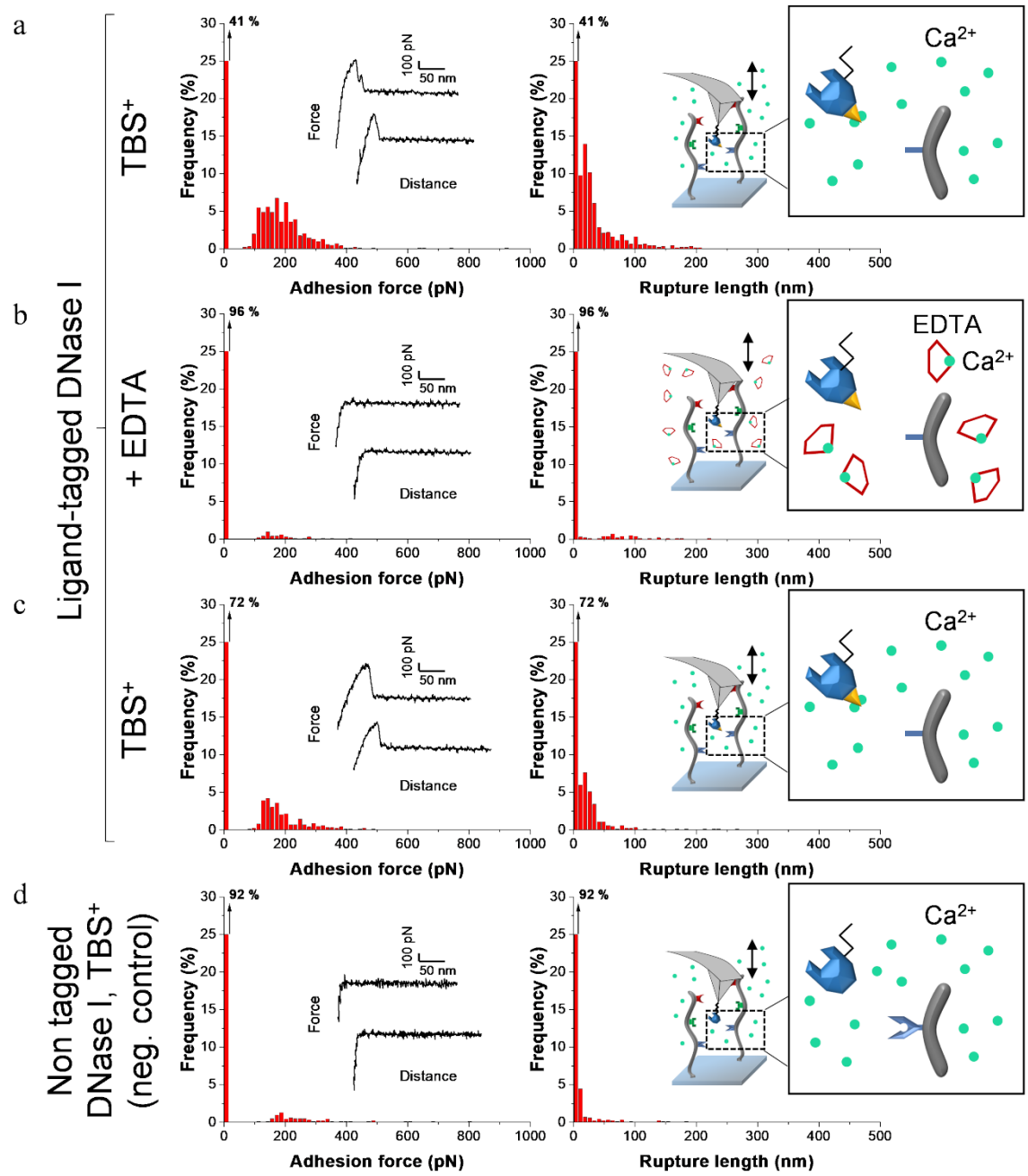


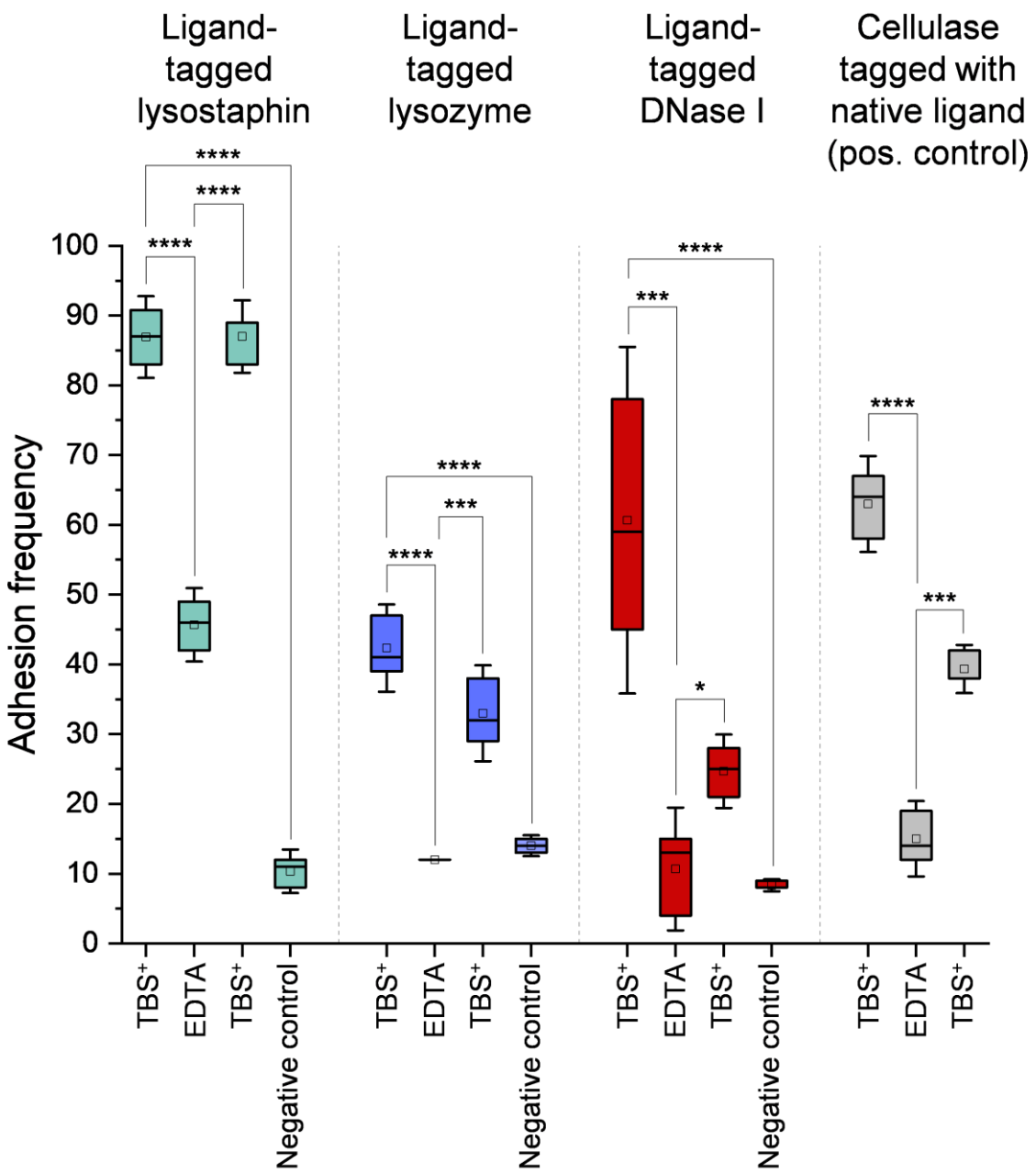
630

631 **Figure 6.** Adhesion frequency histograms with representative force curves (left) and corresponding
632 rupture length histograms (right) extracted from adhesion maps (5x5 µm²) recorded on scaffold-
633 coated surfaces with AFM cantilever decorated with tagged DNase I. Schematics on the right represent
634 the different conditions: adhesion was consecutively recorded on one sample (a) in a calcium rich
635 buffer before adding EDTA, (b) in presence of EDTA and (c) after removal of EDTA and addition of TBS^+

636 containing calcium. (d) Negative control adhesion was recorded in TBS^{+} with tips decorated with DNase

637 I lacking dockerin ligand.

638



639

640 **Figure 7.** Adhesion frequency for each enzyme obtained by scanning the scaffold by SMFS with
641 enzyme-decorated AFM tips. Boxes represent the 25%-75% percentiles, error bars represent mean ±
642 1.5 SD and horizontal bars represent the median of each group. Each box represents 3072 force-
643 distance curves (n=3). Statistical analysis was performed with a one-way ANOVA followed by a Tukey's
644 multicomparison test for pair-wise comparison. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p <$
645 0.0001.

646

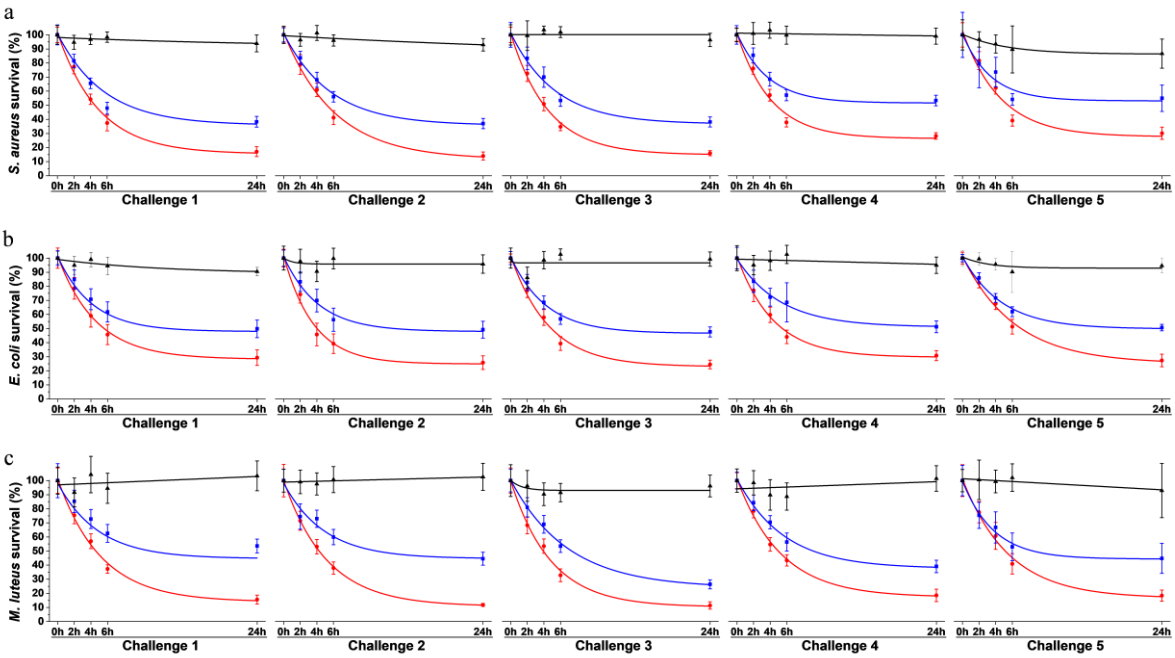


Figure 8. Viability of (a) *S. aureus*, (b) *E. coli* and (c) *M. luteus* exposed to surfaces coated with the scaffold alone (in black), with the three enzymes directly on the surface without any scaffolds (in blue) and with the multi-enzymatic scaffolds *i.e.* the three enzymes docked on the scaffolds (in red). Challenge represent consecutive waves of bacterial contamination. Each challenge started with the replacement of the media with fresh bacterial suspensions. Data was fitted with exponential decays.