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Residence-time dependent cell wall deformation of different *Staphylococcus aureus* strains on gold measured using surface-enhanced-fluorescence – a phenomenological study

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ABSTRACT

Bacterial adhesion to surfaces is ubiquitous in natural and industrial environments and constitutes the first step in the formation of a biofilm. Over 60% of all human infections are biofilm-related. Bacterial adhesion is accompanied by initial bond-maturation processes and cell wall deformation that may extend to the lipid membrane with an impact on antimicrobial susceptibility of the organisms. Cell wall deformation upon adhesion is difficult to measure and has only been described by extrapolation from the deformation behavior of Δpbp4 mutants, deficient in peptidoglycan cross-linking and therefore highly deformable. Here we evaluate surface-enhanced-fluorescence of fluorescently-engineered staphylococci adhering on gold surfaces. Adhesion-related-fluorescence-enhancement depended on the distance of the bacteria to the gold surface and the cell wall deformation. By comparison with the residence-time dependent adhesion-related-fluorescence-enhancement of different strains of staphylococci prior to and after disruption of their extracellular-polymeric-substances (EPS)-layer with the one of green-fluorescent microspheres, it could be concluded that initial bond-maturation, including condensation of the EPS-layer surrounding the bacteria occurred within 60 min. Cell wall deformation was independent of the integrity of the EPS-layer, except for the isogenic Δpbp4 strain, for which cell wall deformation proceeded faster and started earlier after EPS disruption. Since cell wall deformation causes altered membrane stresses and re-arrangement of membrane lipids with an impact on bacterial susceptibility to antimicrobials, quantitative measurements of cell wall deformation are of high importance for the development of new antimicrobials, in an era in which antimicrobial-resistant organisms form an increasing threat.
INTRODUCTION

Bacterial adhesion to substratum surfaces constitutes the first step in the formation of a biofilm. Biofilms can pose considerable problems in many industrial and environmental applications and over 60% of all human bacterial infections are due to biofilms [1, 2]. On the other hand, there are applications where the development of biofilms is beneficiary to processes like bioremediation of soil, or to support host-protection against invading pathogens [3, 4]. The bacterial cell wall consists of a relatively soft outermost layer, crucial for adhesion and biofilm formation, and a more rigid, hard core enveloped by a cross-linked peptidoglycan layer. The peptidoglycan layer is relatively thick in Gram-positive bacteria as compared to Gram-negative ones. The outermost bacterial cell layer can be composed of a variety of different surface appendages and a matrix of “extracellular polymeric substances” (EPS) containing amongst others, polysaccharides, lipids, proteins and eDNA. eDNA is pivotal for the integrity of the EPS layer around a bacterium and serves as a glue holding its various components together [5, 6].

The outermost surface of bacteria behaves differently upon adhesion to a substratum surface than the one of inert, non-biological particles, although similarities exist too. Both bacteria as well as inert particles show initial maturation of an adhesive bond by progressive removal of interfacial water, re-arrangement of surface structures to increase the number of contact points and unfolding of surface-associated macromolecules. Residence-time dependent desorption phenomena in a parallel plate flow chamber, time dependent adhesion force measurements using atomic force microscopy (AFM) and experiments with a quartz-crystal microbalance with dissipation (QCM-D) have all
indicated that this type of physico-chemical bond-maturation proceeds on a time-scale of several minutes [7]. The forces involved in bacterial adhesion to a substratum surface not only affect this initial bond-maturation, but moreover dictate the amount of EPS produced [8] and when exceeding a threshold force lead to so-called “stress de-activation” of an adhering bacterium [9]. Stress de-activation can become so severe as to cause cell death. Nanoscale cell wall deformation upon bacterial adhesion to a substratum surface has been suggested to trigger the bacterial response to an adhering state [10, 11]. Nanoscale bacterial cell wall deformation is extremely difficult to measure due to the rigidity of the peptidoglycan layer. Evidence for bacterial cell wall deformation as a result of adhesion to a surface, stems from work with so-called Δpbp4 isogenic mutants. For Δpbp4 Staphylococcus aureus mutants lacking cross-linking in their peptidoglycan layers [3], relatively large deformations of up to 100-300 nm have been reported, depending upon the method applied [12]. Thus by extrapolation, it can be expected that wild-type strains also deform as a result of their adhesion to a surface, but less than their Δpbp4 isogenic mutants.

Surface enhanced fluorescence (SEF) is a relatively newly discovered phenomenon that was first described for fluorescent proteins and later also for fluorescently-engineered bacteria. It involves enhanced emission of fluorescent light when fluorophores come close to a reflecting metal surface [13, 14]. SEF on average extends over a distance of around 100 nm and decreases exponentially with the distance of the fluorophore from a reflecting surface, as demonstrated by measuring SEF of proteins adsorbed to reflecting surfaces with polymeric spacers of different lengths in between [15, 16]. In principle, bacterial cell wall deformation brings the intracellular content closer to a substratum surface, and
hence it can be expected that SEF will enable quantitative evaluation of cell wall deformation of fluorescent bacteria upon their adhesion to a reflecting substratum.

The aim of this study is to measure SEF of three green-fluorescent S. aureus strains upon adhesion to gold surfaces as a function of their residence-time. Secondly, a model is proposed to describe the decrease of SEF with distance between green-fluorescent microspheres and a reflecting gold surface, based on the measurement of SEF of green-fluorescent microspheres adhering to gold-coated quartz surfaces with adsorbed poly(ethylene glycol) methyl ether thiol (PEG-thiols) layers of different thickness. Further elaboration of the model enables to quantitatively evaluate bacterial cell wall deformation from SEF. Two S. aureus strains with differential expression of EPS were employed, as well as a Δpbp4 mutant, expected to yield more extensive deformation than its parent strain. All strains were evaluated prior to and after treatment with DNase I to disrupt the integrity of their EPS [17], therewith enabling separation of effects of initial physico-chemical processes, condensation of EPS and cell wall deformation. S. aureus was chosen as it represents a major pathogen in human health and disease, with especially pathogenic trades when involved in biomaterial-associated infections.
RESULTS

Fluorescence enhancement during deposition of staphylococci and microspheres

Figure 1. Total fluorescence enhancement, $TFE(t)$, and percentage staphylococci and microspheres deposited to a gold-coated surface as a function of deposition time for three, green-fluorescent $S. aureus$ strains. (a) $S. aureus$ ATCC 12600$^{GFP}$, (b) $S. aureus$ RN4220$^{GFP}$, (c) $S. aureus$ ATCC 12600 $\Delta pbp4^{GFP}$ and (d) green-fluorescent microspheres. TFE is due to planktonic and adhering bacteria and microspheres, while deposition is expressed as a percentage of the number of adhering bacteria or microspheres, $n_a$ with respect to their total numbers in the system, $n_{tot}$. Error bars represent standard errors over four separate experiments with different bacterial cultures and microsphere suspensions. Open symbols represent data for staphylococci treated with DNase I.

Deposition of $S. aureus$ ATCC 12600$^{GFP}$ increased relatively fast towards a stationary level within 2 h, while its $\Delta pbp4^{GFP}$ isogenic mutant exhibited a slightly slower increase towards stationary levels, on a comparable time-scale as of $S.$
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*aureus* RN4220GFP (Figs. 1a-1c). Green-fluorescent microspheres deposited most slowly (Fig. 1d). Concurrent with increasing numbers of staphylococci or microspheres adhering, the total fluorescence enhancement increased as well, but stationary levels of total fluorescence enhancement were only obtained for fluorescent microspheres and not for staphylococci within the time-scale of the experiment. Treatment of the staphylococci with DNase I hardly affected their deposition, while yielding a small increase in total fluorescence enhancement that is consistently present over time (see Figs. 1a-1c).

**Adhesion-related fluorescence enhancement as a function of residence-time**

Fluorescent enhancement will increase over time due to increasing numbers of adhering staphylococci or microspheres on the gold surface and time dependent deformation of the bacterial cell wall. Using a finite summation procedure, we were able to calculate the adhesion-related fluorescence enhancement, \( \alpha(\tau) \), as a function of residence-times, \( \tau \), of adhering fluorescent bacteria and microspheres. Both bacteria as well as inert particles showed a rapid, initial increase of adhesion-related fluorescence enhancement (Fig. 2), followed by a slow, but continuous increase for the staphylococci (Figs. 2a-2c). For the fluorescent microspheres, however, a stationary level was obtained within 10 min (Fig. 2d). Also for *S. aureus* RN4220GFP stationary levels of adhesion-related fluorescence enhancement were observed albeit that a stationary level was only reached after a residence-time of 2 h. Neither *S. aureus* ATCC 12600GFP nor its isogenic mutant *S. aureus* ATCC 12600 \( \Delta pbp4 \) reached stationary levels of adhesion-related fluorescence enhancement, suggesting ongoing deformation processes for both strains up to 5 h of residence on a surface. This suggests that the rapid, initial increase is due to physico-
chemical processes and EPS-condensation, while the slower, continued increase results from cell wall deformation. Importantly, the rate of continued increase is slightly higher for the $\Delta pbp4^{\text{GFP}}$ mutant (0.11 h$^{-1}$) than for its parent strain (0.08 h$^{-1}$).
for residence-times exceeding 1 h. For shorter residence-times, this effect was less consistent (Figs. 2a-2c).

**Modeling the distance-dependence of adhesion-related fluorescence enhancement of fluorescent microspheres on PEG-thiol layers**

SEF of fluorescent proteins as a function of distance has been determined on reflecting surfaces with polymeric spacers of different lengths in between [15, 16]. The task at hand in this manuscript however, is more difficult and challenging, as we want to determine not only the effects of bringing an undeformed, fluorescent bacterium closer to a reflecting substratum surface as a result of physico-chemical processes and EPS-condensation under the influence of the adhesion forces, but we also want to quantify further deformation of the bacterial cell wall. Therefore, we first studied the time-dependence of the total fluorescence enhancement of undeformable, fluorescent microspheres adhering on gold surfaces with polymeric spacers of different molecular weights in between and determined their thickness using QCM-D (Fig. 3a).
Figure 3. Analysis of the fluorescence enhancement of green-fluorescent microspheres adhering to a gold-coated surface. (a) Total fluorescence enhancement, $TFE(t)$ as a function of time to gold-coated surfaces with adsorbed PEG-thiol layers of different molecular weight. (b) Adhesion-related fluorescence enhancement, $\alpha(\delta)$, for green-fluorescent microspheres adhering to a gold-coated surface as a function of the adsorbed layer thickness of PEG-thiols. Fluorescent enhancement values are taken in the stationary phase of the deposition process (see Fig. 3a) and are independent of residence-time (see also Fig. 2d). The dotted line represents fluorescence enhancement as function of distance according to the model presented for undeformed fluorescent microspheres on a reflecting metal surface, using the model parameters $r=10^6 \text{s}^{-1}$, $k_{nr}=4\times10^8 \text{s}^{-1}$, $N_{0nr}=38000$, $dn=8.5 \text{Å}$, $N_{r}=186$, $dr=300 \text{Å}$, $N_{0ex}=70$ and $de=350 \text{Å}$ to provide an optimal fit of the calculated versus the measured fluorescence enhancement. Error bars represent standard errors over four separate experiments with different suspensions of microspheres. (c) Calculated fluorescence enhancement, $\alpha(d)$, of green fluorescent molecules as a function of distance, $d$, to the substratum as calculated on basis of model parameters obtained from the fitting procedure.
Fig. 3b presents the adhesion-related fluorescence enhancement of green-fluorescent microspheres of similar size as our staphylococci on gold surfaces, coated with PEG-thiol layers as a function of the coating thickness. Adhesion-related fluorescence enhancement for microspheres decreased with increasing thickness, i.e. the distance between the microspheres and the reflecting gold surface. Since adhesion-related fluorescence enhancement of microspheres was immediate and not increasing over time (see Fig. 1d), it can be assumed that the surfaces of the microspheres were in direct contact with the PEG-thiol coating within the 10 min time-resolution of our measurements. SEF of single fluorophores can be described [15] as the combined result of metal-induced increases in the rate of (1) fluorescence quenching or non-radiative decay \( (k_{nr}) \) by a factor \( N_{nr} \), (2) fluorescence emission or radiative decay \( (\Gamma) \) by a factor \( N_r \) and (3) excitation of fluorophores by a factor \( N_{ex} \). The distance-dependent fluorescence enhancement of a single fluorophore, \( \alpha(d) \), on a reflecting metal surface can be described by the relative increase of the quantum yield \( Q(d) \) as related to the quantum yield far away from the substratum, \( Q_\infty \), multiplied by the increase in the excitation rate

\[
\alpha(d) = \frac{Q(d)}{Q_\infty} \times N_{ex}(d)
\]

(1)

The quantum yield, \( Q(d) \), decays according to the ratio of radiative decay relative to the total decay [13], i.e. the sum of the radiative and non-radiative decays

\[
Q(d) = \frac{N_r(d)\Gamma}{N_r(d)\Gamma + N_{nr}(d)k_{nr}}
\]

(2)
The non-radiative, radiative and excitation rates occurring in Eqs. 1 and 2 decrease exponentially as a function of the distance to the reflecting metal surface according to

\[ N_{nr}(d) = N_{0}^{nr} \exp(-d / dn) + 1 \]
\[ N_{r}(d) = N_{0}^{r} \exp(-d / dr) + 1 \]
\[ N_{ex}(d) = N_{0}^{ex} \exp(-d / de) + 1 \]  

(3)

where \( dn, dr, \) and \( de \) are the characteristic distances over which these effects decrease. In our case of macroscopic particles, model parameter values have been obtained by least-square fitting calculated fluorescent enhancements against experimental adhesion-related fluorescence enhancements as a function of distance, \( \delta \), between microspheres and the gold surface (Fig. 3b). To this end, it is assumed that fluorophores distribute homogeneously within the microspheres, while we describe their volume as a stack of 100 cylindrical disks. Eqs. 1 to 3 subsequently allow calculation of the fluorescent enhancement by each disk and summation values can be compared with experimental data. Least-square fitting was done for values of \( N_{0}^{ex} \), \( dr \) and \( de \), resulting in a high quality of the fit \( (R^2 = 0.99; \text{see Fig. 3b}) \). Values for the decay rates in the absence of a metal, \( \Gamma \) \( (10^{9} \text{ s}^{-1}) \) and \( k_{nr} \) \( (4 \times 10^{8} \text{ s}^{-1}) \) and enhancement factors \( N_{0}^{nr} \) \( (38000) \) and \( N_{0}^{r} \) \( (186) \) and \( dn \) \( (8.5 \text{ Å}) \) were taken identical to values derived for surface enhanced fluorescence of Cy3-labeled oligonucleotides on silver particles [15], because they were independent of SEF \( (\Gamma \text{ and } k_{nr}) \) or had the lowest impact on adhesion-related fluorescence enhancement of microspheres calculated. Based on these parameter values the distance dependency of fluorescence enhancement of a single fluorophore, \( a(d) \) on gold, could be obtained as shown in Fig. 3c. The range of
around 100 nm over which SEF extends was very similar to the distance dependency of single molecule fluorescence enhancement on gold described earlier [18], whereas the maximum enhancement of 79, at $d=6.6$ nm was within the range of the maximum theoretical effect predicted to be 140 [19].

**Residence-time dependent adhesion-related fluorescence enhancement and staphylococcal cell wall deformation**

Adhesion-related fluorescence enhancement of undeformable microspheres immediately reached a stationary value of around 1.6, within the time-resolution of our fluorescence measurements. Adhering staphylococci however, did not reach that level of fluorescence enhancement, which indicates that they kept a larger distance between the cell wall through the presence of the EPS layer around them that could be calculated using the model for the distance-dependence of adhesion-related fluorescence enhancement forwarded above. If we assume that cell wall deformation only occurs when a bacterium has approached the gold surface to the closest possible distance, we can first derive the residence-time dependent distance between the staphylococci and the surface. The initial distance varied between 25 and 45 nm, depending on the strain considered and decreased within an hour (Fig. 4). Interestingly, DNase I treated staphylococci with a disrupted EPS layer approached the surface faster than strains with an intact EPS layer to a distance of 18.5 nm. Adapting this as the closest possible distance to which bacteria can approach the substratum surface, further interpretation of adhesion-related fluorescence enhancement was done in analogy to the model outlined above for fluorescent microspheres, but now allowing cell wall deformation. Cell wall deformation brings a larger fluorescent
volume of the adhering staphylococcus closer to the surface and accordingly adhering staphylococci were assumed to deform from an initial sphere with radius $R_0$ to an oblate ellipsoid, with a short, polar radius, $b$ and a circular equatorial plane with radius, $a$. Requiring constant volume $V$, i.e.

$$V = \frac{4\pi}{3}a^2b = \frac{4\pi}{3}R_0^3$$  \hspace{1cm} (4)

The ellipsoids could also be divided in stacks of discs and using the model proposed above and the parameters presented in Fig. 3, cell wall deformation could be quantitated and expressed as the difference between the radius of the undeformed staphylococcus, $R_0$ and the short, polar radius of the ellipsoidally deformed bacterium. All three staphylococcal strains deformed between 1 and 5 h after deposition on the gold surface. It should be noted that deformation was calculated up to 5 h for demonstration of the principle, while under more physiologically relevant conditions adhering bacteria may well have divided by then. $S.\ aureus$ ATCC 12600 deformed more extensively than $S.\ aureus$ RN4220, but both parent strains with cross-linked peptidoglycan layers demonstrated similar cell wall deformations irrespective of DNase I treatment. $S.\ aureus$ ATCC 12600 Δpbp4GFP, deficient in peptidoglycan cross-linking showed the most extensive deformation of its cell wall (Fig. 4), that initially seemed dampened by the presence of an intact EPS layer compared to the deformation observed for the DNase I treated Δpbp4GFP mutant.

**DISCUSSION**

The biofilm-mode of growth is a ubiquitously occurring, but unique form of bacterial growth during which the organisms experience forces from the surfaces...
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to which they adhere, i.e. either substratum surfaces or surfaces of neighboring bacteria. This is unlike the situation during planktonic growth, where they are freely suspended in an aqueous phase. The forces experienced by bacteria in a biofilm-mode of growth have been demonstrated to have severe impact on their susceptibility to antimicrobials and general viability [9, 20]. The response of bacteria to these adhesion forces has been suggested to be due to cell wall deformation, causing altered membrane stresses [9], and re-arrangement of membrane lipids [21]. AFM has demonstrated that the bacterial cell wall can indeed be deformed up to the level of its rigid peptidoglycan layer, but these experiments have all been carried out by wrenching bacteria between a substratum surface and an AFM-cantilever [22] or tip [23, 24] under the influence of an applied loading force, rather than under the influence of the naturally-occurring adhesion force arising from a substratum surface. Besides AFM-imaging of bacteria artificially immobilized on positively charged surfaces [25], not naturally-occurring, bacterial cell wall deformation under the influence of naturally-occurring adhesion forces has never been demonstrated nor reliably quantified. In this study, we used recently described surface enhanced fluorescence of adhering bacteria [14, 26] to assess bond-maturation processes and cell wall deformation of staphylococci adhering to gold surfaces.

To this end, we have developed a new model to describe the distance dependence of SEF for undeformable microspheres and bacteria, that we extrapolate to deformation of the rigid, bacterial core of adhering bacteria containing the fluorophores. The initial bond-maturation process includes physico-chemical changes that have been described to occur within several minutes [7] and that by consequence of the 10 min time-resolution of our
experiments cannot be separated from effects of EPS-condensation. In this initial bond-maturation process, significant effects of DNase I treatment of staphylococci are seen for \textit{S. aureus} ATCC 12600\textsuperscript{GFP} and \textit{S. aureus} RN4220\textsuperscript{GFP}. Although this initial bond-maturation is more extensive for \textit{S. aureus} ATCC 12600\textsuperscript{GFP} than for its isogenic mutant \textit{S. aureus} ATCC 12600 \textit{Δpbp4}\textsuperscript{GFP} (Fig. 4), this difference disappears after DNase I treatment. \textit{S. aureus} RN4220\textsuperscript{GFP} differs from \textit{S. aureus} ATCC 12600\textsuperscript{GFP} in the sense that DNase I treatment of \textit{S. aureus} ATCC 12600\textsuperscript{GFP} removes virtually all stainable EPS, while stainable EPS clearly remains behind after DNase I treatment in case of \textit{S. aureus} RN4220\textsuperscript{GFP} (Supplementary Fig. S1). Thus, whereas DNase I treated \textit{S. aureus} ATCC 12600\textsuperscript{GFP} immediately reaches the distance of closest possible approach to the gold surface, this requires more time for \textit{S. aureus} RN4220\textsuperscript{GFP} (see Fig. 4).

The bacterial core of adhering staphylococci enveloped by peptidoglycan, deforms more readily in case of \textit{S. aureus} ATCC 12600 \textit{Δpbp4}\textsuperscript{GFP}, deficient in peptidoglycan cross-linking than observed for both wild-type strains, which supports the validity of our model. Nevertheless also the staphylococcal cores enveloped by cross-linked peptidoglycan deform. DNase I treatment to disrupt the integrity of the EPS layer, destabilizes the cell wall of the \textit{Δpbp4}\textsuperscript{GFP} mutant, resulting in an almost instantaneous cell wall deformation right after adhesion. This confirms a recently proposed new role for EPS as a stress-absorber [25], hampering cell wall deformation and the associated development of membrane stresses that may increase bacterial susceptibility to antimicrobials [9].
Bacterial deformation on gold surface measured using SEF

Figure 4. Bacterium-substratum distance, $\delta$, and bacterial cell wall deformation, ($R_0-b$), as a function of the residence-time of staphylococci adhering to gold surfaces. Left axis - Bacterium-substratum distance, $\delta$, (circles) as a function of residence-time. Right axis - Bacterial cell wall deformation, ($R_0-b$), (squares) as a function of residence-time. Open symbols refer to staphylococci treated with DNase I. Error bars represent standard errors calculated from adhesion-related fluorescence enhancement data from four different bacterial cultures.

Cell wall deformations, obtained using AFM-imaging measured within approximately 1 h of contact for a similar collection of staphylococcal strains immobilized on a positively charged, $\alpha$-poly-L-lysine coated surface are within the range of the deformations observed here after 5 h of bond-maturation on a negatively charged gold surface [12]. This attests to a major influence of the substratum surface charge on final cell wall deformation. Moreover, it demonstrates that SEF is the only method to quantitatively derive cell wall deformation, with an accuracy that exceeds AFM-imaging by far. Wrenched between V-shaped and colloidal-probe AFM tips, deformations of Gram-negative *Pseudomonas aeruginosa* PAO1 under an applied force of 10 nN, exerted during a time-period of 10 s, amounted 200 nm, while similar conditions for Gram-positive...
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*Bacillus subtilis* 168 strain yielded 80 nm deformation [23, 27]. Considering the extremely short time-periods involved in these studies while yielding deformation in the same order of magnitude as found in our studies after 5 h, it can be concluded that experiments in which bacteria are wrenched between a substratum and an AFM cantilever overestimate bacterial cell wall deformation as occurring under the influence of naturally-occurring adhesion forces. The relevance of such experiments can be increased by matching the applied force to the naturally-occurring adhesion force, but the highly localized force by the AFM tip or colloidal-probe attached to a cantilever will always yield a stress concentration alien to the natural situation of bacterial adhesion to a substratum surface.

Summarizing, we have forwarded a new method to determine residence-time dependent adhesion-related fluorescence enhancement, and developed a model through which bond-maturation of bacteria adhering on reflective metal surfaces can be analyzed in terms of initial bond-maturation processes, including EPS condensation and cell wall deformation. Cell wall deformation plays an important role in understanding bacterial susceptibility to antimicrobials and with the era of current antimicrobials approaching its end [28], accurate measurement of cell wall deformation as a result of bacterial adhesion to surfaces, irrespective of whether of synthetic or biological origin, is important to develop alternatives for current antimicrobials.
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METHODS

Bacterial strains and culture conditions

Three different S. aureus strains were involved in this study, i.e. S. aureus RN4220, S. aureus ATCC 12600 and its isogenic \(\Delta pbp4\) mutant differing in the degree of cross-linking of their peptidoglycan layer [3]. To generate GFP expressing bacteria, the plasmid pMV158 GFP containing optimized GFP under control of the constitutively expressed MalP promoter [29], was introduced into these S. aureus strains by electroporation [26]. Bacteria were routinely cultured aerobically at 37°C on a Tryptone Soya Broth (TSB; OXOID, Basingstoke, England) agar plate supplemented with 10 µg cm\(^{-3}\) tetracycline. One colony was used to inoculate 10 cm\(^3\) TSB also supplemented with 10 µg cm\(^{-3}\) tetracycline and this pre-culture was grown for 24 h at 37°C. The pre-culture was diluted 1:20 in 200 cm\(^3\) TSB and grown for 16 h at 37°C. Cultures were harvested by centrifugation (Beckman J2-MC centrifuge, Beckman Coulter, Inc., CA, USA) for 5 min at 4000 g, and washed twice with 10 cm\(^3\) phosphate buffered saline (PBS; 5 mM \(K_2HPO_4\), 5 mM \(KH_2PO_4\), 0.15 M NaCl, pH 7.0). To break staphylococcal aggregates, sonication at 30 W (Vibra Cell Model 375, Sonics and Materials Inc., Danbury, CT, USA) was applied (3 times 10 s), while cooling in an ice/water bath. Finally, bacteria were resuspended in PBS to a concentration of \(3 \times 10^8\) cm\(^{-3}\) as determined in a Bürker-Türk counting chamber.

DNase I treatment

All three S. aureus strains produced EPS, as they grew black colonies on Congo Red agar plates (data not shown). To address the contribution of the EPS-matrix on cell wall deformation, bacterial pellets harvested from 200 cm\(^3\) TSB culture
were suspended in 10 cm$^3$ PBS solution with 100 $\mu$g cm$^{-3}$ DNase I (Fermentas Life Sciences, Roosendaal, The Netherlands) for 1 h at 37°C, after which sonication at 30 W was applied (3 times 10 s) to remove naturally present endogenous eDNA and therewith disrupting the EPS matrix on the bacterial cell surfaces. Subsequently, bacteria were harvested, washed and sonicated to break staphylococcal aggregates, as described above. Finally, bacteria were resuspended in PBS to a concentration of $3 \times 10^8$ cm$^{-3}$, as described above.

**Fluorescent microspheres**

Green-fluorescent polystyrene microspheres with a diameter of 1.1 $\mu$m (Molecular Probes, Invitrogen Life Technology, Grand Island, NY, USA) were used as a model system. As received suspensions were diluted in PBS to a concentration of $1 \times 10^7$ cm$^{-3}$ as determined in a Bürker-Türk counting chamber.

**Gold-coated surfaces, coupling of PEG-thiols and their layer thickness using QCM-D**

Gold-coated quartz-crystal sensors (Jiaxing JingKong Electonic Co. Ltd., Jiaxing, China) were used as a reflecting substratum for staphylococcal adhesion and adhesion of green-fluorescent microspheres. Before each experiment, gold-coatings were cleaned by immersion in a 3:1:1 mixture of ultrapure water, NH$_3$·H$_2$O and H$_2$O$_2$ (Merck, Darmstadt, Germany) at 70°C for 10 min. After cleaning, gold-coated crystals were mounted in the chamber of a QCM-D (Q-Sense AB, Gothenburg, Sweden) to allow deposition of staphylococci and microspheres. The QCM-D chamber is disc-shaped with a diameter of 14 mm, and a height of 0.66 mm [30].
In order to establish a relation between SEF with the distance of fluorescent microspheres, the gold surfaces were coated with a self-assembled monolayer with variable thickness. To this end, the gold-coated crystals were placed in the QCM-D chamber and the system was perfused with water at a flow rate of 0.144 cm$^3$ min$^{-1}$ until stable baseline values were obtained with the QCM-D. Subsequently, the chamber was filled with 0.2 mM PEG-thiols (molecular weight of 2000, 5000, and 10000; Sigma-Aldrich, St. Louis, MO, USA) solution in water for 30 min at room temperature after which the chamber was perfused again with water and the resulting changes in frequency and dissipation were used to calculate the adsorbed layer thickness of the PEG-thiols with the QCM-D accompanying software package (Q-Sense, Sweden) [31, 32].

**Deposition of staphylococci and microspheres and fluorescence imaging**

Next, a suspension of fluorescent staphylococci or microspheres was flown into the QCM-D chamber and flow was arrested to allow measurement of deposition using a metallurgical microscope. For deposition measurements, the microscope was equipped with a 40× objective (ULWD, CDPlan, 40PL, Olympus Co, Tokyo, Japan), connected to a CCD camera (Basler A101F, Basler AG, Germany). Staphylococci or microspheres were allowed to sediment under the influence of gravity and the number of bacteria or particles adhering per unit area was expressed as a fraction of the numbers of bacteria or particles adhering to the coatings in a stationary phase, i.e. when all staphylococci present in the chamber had deposited.

For fluorescence imaging, the entire QCM-D chamber was placed on a sample stage inside a bio-optical imaging system (IVIS Lumina II, PerkinElmer, Inc., Hopkinton, MA, USA), and the above described deposition experiments repeated.
The IVIS was kept at 20°C and provided a field of view of 7.5 x 7.5 cm, to encompass the diameter of the crystal surfaces. Excitation and emission wavelengths for detection of both GFP staphylococci and microspheres were 465 nm and 515-575 nm, respectively. An exposure time of 5 s was employed and images were taken every 10 min over the entire period of 5 h. Average fluorescence radiances, \( R \) (p s\(^{-1}\) cm\(^{-2}\) sr\(^{-1}\)) over a 1 cm\(^2\) user-defined region of interest were determined for each image with the Living Image software package 3.1 (PerkinElmer Inc., USA) which transforms electron counts on the CCD camera to an average fluorescence radiance, taking into account the current optical parameters (area of the region of interest, magnification, binning, diaphragm, exposure time and light collecting ability of the camera as calibrated with standard light sources). The total number of staphylococci or microspheres, \( n_{\text{tot}} \) contributing to the fluorescent radiance captured within the region of interest was around \( 2.0 \times 10^7 \) and \( 6.6 \times 10^5 \), respectively. Fluorescence radiance \( R(t) \) was monitored as a function of time during deposition.

**Calculation of residence-time dependent, adhesion-related fluorescence enhancement**

The increase of the fluorescence radiance due to adhesion of fluorescent staphylococci or microspheres was measured relative to the fluorescence of planktonic ones and expressed as a total fluorescence enhancement, \( TFE(t) \), according to

\[
TFE(t) = \frac{R(t) - R_0}{R(0) - R_0}
\]  

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in which $R(t)$ denotes the fluorescence radiance at time $t$, while $R_0$ and $R(0)$ indicate the fluorescence radiance before and after the introduction of staphylococci or microsphere suspension into the flow chamber, respectively. $TFE(t)$ comprises the fluorescence contribution from adhering bacteria or microspheres and those still in the suspension. Note that for staphylococci, demonstrating a residence-time dependent fluorescent enhancement, $TFE(t)$ comprises the fluorescence contribution from adhering bacteria with various residence-times and the ones still in the suspension. Accordingly,

$$TFE(t) = \frac{\varphi_0 \left[ \int_0^t \alpha(\tau) j(t - \tau) + \left( n_{\text{tot}} - \int_0^t j(t) dt \right) \right]}{\varphi_0 n_{\text{tot}}}$$

(6)

in which $\varphi_0$ is the fluorescence from staphylococci in suspension, $\alpha(\tau)$ is the adhesion-related residence-time dependent fluorescence enhancement, $\tau$ is the residence-time of adhering staphylococci, $j(t)$ is the deposition rate at time $t$ and $n_{\text{tot}}$ is the total number of bacteria or microspheres, both in suspension and attached, contributing to the fluorescent radiance captured within the region of interest.

In order to assess $\alpha(\tau)$, Eq. 6 has been transformed to a finite summation according to

$$TFE_m = \left[ \Delta t \sum_{i=1}^{m} \tilde{j}_i (\alpha_{m+1-i} - 1) \right] + 1$$

(7)

in which $\tilde{j}_i$ is the deposition rate at time $i \times \Delta t$ divided by $n_{\text{tot}}$. Subsequently $\alpha_{\text{min}}$, the adhesion-related fluorescence enhancement for the shortest residence-time
\( \Delta t \), is obtained from the first measurement after the start of an experiment at \( t = \Delta t \)

\[
\alpha_i = \frac{TFE_i - 1}{\Delta t j_1} + 1 \tag{8}
\]

In line, \( \alpha_m \), the adhesion-related fluorescence enhancement for residence-time \( m \times \Delta t \), can be calculated after \( m \) consecutive steps according to

\[
\alpha_m = \frac{TFE_m + \Delta t \left[ \sum_{i=1}^{m} j_i - \sum_{i=1}^{m-1} \alpha_{m-i} \bar{j}_{i+1} \right] - 1}{\Delta t j_1} \quad \text{(for } m > 2) \tag{9}\]

Statistics

Data were statistically analyzed using paired, two tailed Student t-tests. Significance was established at \( p < 0.05 \).
REFERENCES


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Figure S1. Stainable EPS expression in planktonic cultures of *S. aureus* ATCC 12600<sup>GFP</sup> and *S. aureus* RN4220<sup>GFP</sup>.

Staphylococci, grown and harvested as described in the main text, were suspended in 10 cm<sup>3</sup> PBS to obtain an optical density at 578 nm of 1. Subsequently, 1.5 cm<sup>3</sup> of this cell suspension was pelleted at 5000 g for 5 min at 10°C, after which EPS was extracted by re-suspending the pellet in 0.05 cm<sup>3</sup> of 0.5 M EDTA (pH 8.0) for 5 min at 100°C. Concentrated EPS was incubated at 37°C with 0.01 cm<sup>3</sup> of 20 μg cm<sup>-3</sup> proteinase K for 30 min and diluted 1: 100 in water and 0.04 cm<sup>3</sup> was blotted on a nitrocellulose membrane. The membrane was then blocked using 1% bovine serum albumin-Tris Buffered Saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween20) for 1 h under mild shaking at room temperature. The membrane was subsequently incubated with a 1: 10,000 dilution of Wheat germ agglutinin (Sigma-Aldrich) for 1.5 h with mild shaking at room temperature. Wheat germ agglutinin is a biotin labelled antibody specific for poly-n-acetylglucosamine (PNAG), a major constituent of staphylococcal EPS. Finally,
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streptavidin IRDye (LI-COR Biosciences, Lincoln, USA) was added in 1:10,000 dilution for 30 min under similar conditions and the membrane was washed 3 times, 5 min each, with Tween20-Tris buffered saline. The membrane was imaged using an Odyssey Infrared Imaging System (LI-COR), yielding dark spots on the blot indicative of the amount of PNAG.