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Antimicrobial surfaces containing cationic nanoparticles: How immobilized, clustered, and protruding cationic charge presentation affects killing activity and kinetics

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ABSTRACT

This work examines how the antimicrobial (killing) activity of net-negative surfaces depends on the presentation of antimicrobial cationic functionality: distributed versus clustered, and flat clusters versus raised clusters. Specifically, the ability to kill *Staphylococcus aureus* by sparsely distributed 10 nm cationic nanoparticles, immobilized on a negative surface and backfilled with a PEG (polyethylene glycol) brush, was compared with that for a dense layer of the same immobilized nanoparticles. Additionally, sparsely distributed 10 nm poly-L-lysine (PLL) coils, adsorbed to a surface to produce flat cationic "patches" and backfilled with a PEG brush were compared to a saturated adsorbed layer of PLL. The latter resembled classical uniformly cationic antimicrobial surfaces. The protrusion of the cationic clusters substantially influenced killing but the surface concentration of the clusters had minor impact, as long as bacteria adhered. When surfaces were functionalized at the minimum nanoparticle and patch densities needed for bacterial adhesion, killing activity was substantial within 30 min and nearly complete within 2 h. Essentially identical killing was observed on more densely functionalized surfaces. Surfaces containing protruding (by about 8 nm) nanoparticles accomplished rapid killing (at 30 min) compared with surfaces containing similarly cationic but flat features (PLL patches). Importantly, the overall surface density of cationic functionality within the clusters was lower than reported thresholds for antimicrobial action. Also surprising, the nanoparticles were far more deadly when surface-immobilized compared with free in solution. These findings support a killing mechanism involving interfacial stress.

1. Introduction

The quest for antimicrobial materials has diverged into two strategies: Antimicrobial compounds are either leached from a material or their surfaces are rendered bacteriocidal. Indiscriminate leaching of biocides into large volumes of fluid is wasteful and lowers overall efficiency [1,2]. Conversely, antimicrobial surfaces kill only the bacteria which come into intimate contact. For surfaces with an established correlation between adhesion and killing activity [3,4], fouling reduces their effectiveness [5,6]. Cationic surfaces exemplify this behavior, possessing contact-killing properties [5,7–11] but tending to retain, through electrostatic attractions, adherent bacteria [5]. A general design goal for contact-kill surfaces is the facile release of dead bacteria so that the active surface is constantly accessible to additional live bacteria. Deadly surfaces of low bacteria adhesion are therefore a design target.

Since cationic functionality is responsible for both bacterial killing and adhesive fouling [4], we pursued surface presentations of positive charge that minimize adhesion and maintain killing activity. The appropriate surface design is not obvious in the absence of universally accepted contact-kill mechanisms. One school of thought tethers killing moieties on polymer chains attached to a surface, to facilitate penetration of the bacterial membrane [10,12,13], similar to the solution-based mechanism. Membrane insertion on biocidal surfaces has not, however, been proven. In fact, there is mounting evidence that contact-killing can occur on surfaces whose functional groups cannot access the bacterial membrane (buried 50 nm or more within the bacterial envelope) [14–17]. These surfaces include amine-functionalized self-assembled monolayers [18] and layer-by-layer structures [19,20]. Efficient killing by high molecular weight polycationic brushes allows for polycation chains insertion into the

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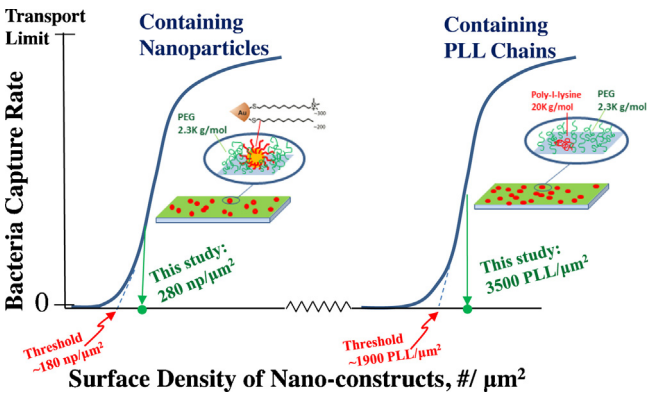

Fig. 1. General schematic of the surface structure and the influence of surface composition on the capture rates of flowing *S. aureus* cells onto PEG brush surfaces containing adhesive nanoconstructs. Thresholds are indicated quantitatively for the particular types of PEG brush and nanoconstructs in the current work. These threshold surface loadings for each type of nanoconstruct motivate the choice of the surface loadings of nanoconstructs in this study of antimicrobial activity. Notably, at the surface loadings of nanoparticles and PLL studied here, the bacterial capture rates are well below the transport-limit.

bacterial membrane [5,21]; however, the killing with lower molecular weight polycation brushes argues against the necessity of membrane penetration [5,21]. An alternate hypothesis involves ion exchange and release of multivalent cations from the bacterial membrane in the region where the bacterial cell contacts a cationic surface [22]. More recently, mechanisms involving deadly interfacial forces have been proposed [4,23,24]. Despite the lack of clarity on the mechanism, it is generally accepted that killing requires (cationic) surface charge densities exceeding a threshold of $1–5 \times 10^{15}$ [21] or $10^{12}–10^{16}$ [22] amines/cm^2 depending on the bacteria.

Zydrko et al. [25] employed surface brushes containing both cationic and sterically repulsive PEG (polyethylene glycol) chains to tune bacterial capture. Li et al. [26] demonstrated that surfaces with nanoscale roughness captured bacteria more efficiently than flat surfaces of similar chemistry. Neither study investigated killing.

To facilitate tunable bacterial adhesion, our lab developed PEG brush surfaces containing embedded cationic nanoparticles [27] or cationic polyelectrolyte chains [28], which we refer to here as cationic "nanoconstructs". The cationic nanoparticles and poly-L-lysine (PLL) chains contained similar cationic content, 200 amines/nanoparticle and 130 amines/PLL chain; however, immobilized nanoparticles protruded on the order of 10 nm from the substrate while adsorbed PLL coils lay relatively flat (within 1–2 nm) to the substrate, detailed below. Our PEG brush surface architectures, in the absence of embedded cationic nanoconstructs, resisted protein adsorption [29,30] and were non-adhesive for *Staphylococcus aureus* on timescales of interest [27,31]. This ensured that, on the test surfaces containing both the nanoconstructs and the PEG brush, bacteria were retained only through adhesion to the nanoconstructs. Indeed, *S. aureus* adhered only on surfaces whose densities of nanoconstructs exceeded a distinct threshold, shown schematically in Fig. 1 [27,28]. With bacterial adhesion well characterized on these surfaces, the current paper examines their bacteriocidal activity. The current study focuses on the least adhesive surfaces still able to capture and hold substantial numbers of bacteria in gentle flow. Worth noting, partial to near-complete bacterial release from these surfaces was previously documented [32].

The current study focuses on two test surface designs from previous libraries: A test surface with a low cationic nanoparticle density (280 nanoparticles/μm^2) and a test surface with a low PLL patch density (3500 patches/μm^2), both backfilled with a non-adhesive PEG brush. Both test surfaces are benchmarked against control surfaces containing dense loadings of the corresponding cationic nanoconstructs. The two sparsely-loaded test surfaces each contain the minimum respective nanoconstruct densities needed to capture and retain substantial numbers of flowing *S. aureus*, indicated in Fig. 1. (Fig. 1 is a schematic representation of prior results for bacterial capture [27,28], and motivates the specific surface compositions employed here.) The large differences in the adhesion thresholds in Fig. 1 resulted from the differences in the nanoparticle protrusion relative to the brush. Thus the two test surfaces in this study have very different nanoconstruct densities. Prior work, using shear flow chambers, established relatively weak bacterial adhesion on these test surfaces. For instance, 90% of captured *S. aureus* could be rinsed from the sparse nanoparticle surface with 13 pN shearing force, even 30 min after bacterial capture [32].

In addition to comparing the killing activity of different surfaces, this paper also compares the surface activity of immobilized cationic nanoparticles to that in free solution. Immobilized nanoparticles were fundamentally more deadly than free nanoparticles in buffer or growth medium.

2. Materials and methods

2.1. Nanoparticles and polymers

Cationically functionalized gold nanoparticles were synthesized according to standard methods [33]. Characterization by TEM indicated a 7 nm core, and an overall diameter of 11 nm. The ligand shell contained ~200 cationic ligands (N,N-trimethyl(11 mercaptoundecyl) ammonium chloride) and 300 hydrophobic (1-mercaptoundane) ligands.

Poly-L-lysine (PLL), of nominal molecular weight 20,000 g/mol, was purchased from Sigma–Aldrich (catalog number P7890, M_v in the range 15,000–30,000 g/mol) and used directly to create cationic surface regions. The same PLL was modified by the attachment of 2300 g/mol-molecular weight polyethylene glycol (PEG) chains to produce a PLL-PEG graft copolymer for the surface brush. We targeted functionalization of about one third of the amines on the PLL, based on reports [29,31] and our own confirmation [32] that a copolymer of this composition, when adsorbed on negative surfaces, prevents bacteria and protein adsorption by formation of a PEG brush. Copolymer synthesis followed published methods [29,30]. The composition of the graft copolymer was assessed by ^{1}H NMR in D_2O using a Bruker 400 mHz instrument. Comparison of the lysine side chain peak at 2.909 ppm and the PEG peak at 3.615 ppm revealed functionalization of 34% of the PLL amines.

2.2. Surface fabrication

Four surfaces, in Table 1, were studied. All were based on acid-etched microscope slides, soaked overnight in concentrated sulfuric acid and then rinsed in DI water. Onto this were deposited polymers and/or nanoparticles that were irreversibly physically bound (beyond the relevant timescales). Effectively permanent attachment of nanoparticles was confirmed [27,32] in a variety of conditions: rinsing in water and buffers between pH 5 and 8, drying, passing of an air bubble (3-phase contact line), addition of up to 5 M NaCl, organic solvents, sonication, and protein and sodium dodecylsulfate adsorption challenge. PLL retention was established at ionic strengths up to 1 M NaCl, and exposure to proteins and polymers [34].

Surfaces were prepared by placing each slide in a laminar flow chamber. DI water or pH 7.4 phosphate buffer flowed continuously at a wall shear rate of 10 s^{-1} over each surface and different nanoparticle and polymer solutions were introduced. A saturated PLL control surface was created by flowing a 5 ppm solution of PLL in phosphate buffer for 10 min followed by reinjection of the