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Evidence of extensive diversity in bacterial adherence mechanisms that exploit unanticipated stainless steel surface structural complexity for biofilm formation

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ABSTRACT

Three protease-resistant bioorganic 304 stainless steel surfaces were created through the reaction of synthetic peptides consisting of the D-enantiomeric isomer (D-K122-4), the retro-inverso D-enantiomeric isomer (RI-K122-4), and a combination of the two peptides (D + RI) of the *Pseudomonas aeruginosa* PilA receptor binding domain with steel surfaces. The peptides used to produce the new materials differ only in handedness of their three-dimensional structure, but they reacted with the steel to yield materials that differed in their surface electron work function (EWF) while displaying an identical chemical composition and equivalent surface adhesive force properties. These surfaces allowed for an assessment of the relative role of surface EWF in initial biofilm formation. We examined the ability of various bacteria (selected strains of *Listeria monocytogenes*, *L. innocua*, *Staphylococcus aureus* and *S. epidermidis*) to initiate biofilm formation. The D-K1224 generated surface displayed the lowest EWF (classically associated with greater molecular interactions and more extensive biofilm formation) but was observed to be least effectively colonized by bacteria (>50% decrease in bacterial adherence of all strains). The highest surface EWF with the lowest surface free energy (RI-K122-4 generated) was more extensively colonized by bacteria, with the binding of some strains being equivalent to unmodified steel. The D + RI generated surface was least effective in minimizing biofilm formation, where some strains displayed enhanced bacterial colonization. Fluorescent microscopy revealed that the D and RI peptides displayed similar but clearly different binding patterns, suggesting that the peptides recognized different sites on the steel, and that differential binding of the peptides to the steel surfaces influences the binding of different bacterial strains and species. We have demonstrated that stainless steel surfaces can be easily modified by peptides to generate surfaces with new physiochemical properties. The D-K122-4-modified surface substantially decreases biofilm formation compared to the RI-K122-4 and D + RI surfaces.

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1. Introduction

Bacterial biofilms form rapidly on virtually every surface exposed to a bacterial population. It has proven exceedingly difficult to control, minimize or remove bacterial biofilms from surfaces despite a vast number of approaches. The extensive and pervasive role of biofilms in the environment, industry and medicine is well established thanks to the pioneering efforts of Zobell [1], Marshall et al. [2,3], Fletcher et al. [4,5] and Costerton et al. [6–8], among a host of others, yet our understanding of the molecular basis for biofilm formation remains limited. A number of studies have demonstrated that biofilm formation depends on the deposition of a conditioning layer which adsorbs onto the surface [9,10], rather than through a direct interaction with the surface, and that one needs to modulate conditioning film formation and/or bacterial binding to adsorbed organic material to control biofilm formation [10]. However, we have documented that *Pseudomonas aeruginosa*, the classic model organism for biofilm and quorum sensing studies [11–13], directly binds to stainless steel surfaces through molecular interactions of the type IV pilus through the pilin or PilA protein receptor binding domain (RBD), and that this binding occurs in the absence of any condition films [14–16].

Interestingly, synthetically synthesized peptides constituting the self-organizing or folding PilA RBD (a synthetic peptide consisting of residues 128–144 with a formed disulfide bridge, containing only L-amino acid residues, a native RBD) competitively inhibited

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whole cell binding to stainless steel surfaces and displayed a remarkably high apparent binding affinity for steel that could not be readily understood given the conformational flexibility of synthetic receptor binding domains [17]. Subsequent investigation established that the extraordinary binding affinity of the RBD was due to the formation of a covalent or semi-formal covalent bond with the stainless steel surface that generated a new material, which we have termed bioorganic stainless steel (borg-SS) (16). The characterization of borg-SS revealed that the surface electron work function (EWF), described as the amount of energy in electron volts that is required to pull an electron from inside a metal at the Fermi level to a point just beyond its surface, was significantly increased relative to unmodified stainless steel. The adhesive force, a surface property reflecting the force required to pull a standard atomic force microscope tip from the surface once it interacts with the surface that essentially described the surface stickiness or the tendency of the surface electrons to interact with a different material, of the borg-SS was significantly decreased relative to unmodified stainless steel [16]. The altered borg-SS surface suggested that bacteria, proteins and other materials would be less likely to interact with and adhere to borg-SS due to the decrease in surface reactivity and stickiness. One would anticipate that conditioning films would not be as readily established and that, given that bacterial binding usually occurs in the presence of a conditioning film, in general, bacterial biofilm formation would be reduced. However, the native RBD, which is composed entirely of L-amino acids, is readily degraded by proteases, even when bound to surfaces [16], and bacteria produce a number of extracellular proteases that could degrade the bound RBD, thereby compromising the utility of the native RBD in controlling biofilm—experimentally the native RBD is ineffective in inhibiting biofilm formation.

A large number of potentially useful synthetic peptides for therapeutic applications have displayed similar protease susceptibility issues that have prevented their direct use in the clinic. Proteins consist entirely of amino acids consisting of the L-enantiomeric chiral form and proteases only degrade proteins with L-amino acid residues. Two approaches, both employing the alternative chiral form of amino acids (the D-enantiomeric form), have been used to overcome protease sensitivity of peptides such that they can be used for clinical applications. D-amino acid residues can be incorporated into the peptide at protease cleavage sites to prevent the recognition of the peptide by proteases. Alternatively, peptides termed retro-inverso peptides can be created where D-amino acid residues are used to synthesize the peptide but the amino acid sequence is reversed. The retro-inverso peptide positions the side chains of the amino acid residues in the same general positions as would be found in the native L-amino acid residue containing peptide, creating a peptide with a three-dimensional (3-D) structure which is very similar to the native L-peptide structure and which frequently functions as effectively as the native peptide, except that it is protease resistant [18–20]. As the synthesis of proteins that contain only D-amino acid residues results in proteins having the mirror image of the native L-amino acid containing proteins [21,22], we chose to synthesize an all-D-amino acid residue synthetic RBD (D peptide) to avoid potential structural alterations of the peptide that would prevent peptide–steel interaction through potential disruption of the RBD structure if one or a few residues were chosen to be of the D-configuration and found no chemical basis for stainless steel to display chiral features (and thus anticipated that the mirror image of the native RBD would react equivalently to the native RBD form). We also synthesized a retro-inverso synthetic RBD (RI peptide) to create a peptide with a 3-D structure that would be similar, but not identical, to the native L-amino RBD peptide while still retaining the protease resistance supplied by the D-amino acid residues as a more classical peptide chemistry mimetic approach. The D and RI peptides are chiral to each other and their structures cannot be superimposed. We thus utilized these peptides with altered protease susceptibility to determine whether they would interact with stainless steel and whether these peptides could be utilized to modulate biofilm formation. Davis et al. [16] established that both the D-K122-4 peptide and the RI-K122-4 peptide reacted with stainless steel and were protease resistant, but that the resulting surfaces differed in their observed EWF.

We thus sought to confirm that both the D-peptide and the RI-peptide significantly altered the surface properties of the stainless steel and to determine whether the different forms of protease-resistant borg-SS surfaces were less susceptible to biofilm formation or not. We hypothesized that by creating protease-resistant peptides that alter the surface reactivity and stickiness we could generate stainless steel surfaces that had greater resistance to bacterial colonization. While we had hypothesized that the D-K122-4 peptide and the RI-K122-4 peptide would react equivalently with the stainless steel surface, our observations indicate the substantial structural complexity of stainless steel surfaces results in differential binding of the D-K122-4 and RI-K122-4 peptides. This results in surfaces that are differentially colonized by different bacterial species and even by different strains within a particular species. We also observed that adherence of some bacterial species was more effectively inhibited by D-peptide modification of the surface while other species were more effectively inhibited by the RI-peptide, with bacterial adherence being reduced by ^{3}50% compared to unmodified surfaces. Further, we observed that modification of the surface by both the D-K122-4 peptide and the RI-K122-4 peptide did not minimize bacterial binding beyond what was observed for either the D-K122-4 or the RI-K122-4 peptide, and for a number of strains actually enhanced bacterial binding significantly above that which was observed with unmodified stainless steel surfaces. We conclude that the surface of stainless steel is much more complex than anticipated, and that chiral peptides will interact differentially with the surface to generate new borg-SS surfaces that differ in their susceptibility to bacterial colonization.

2. Materials and methods

2.1. Peptide synthesis

All peptides (see Fig. 1 for peptide sequences) were prepared by solid-phase peptide synthesis as the N-terminal acetylated form with a C-terminal amide, and purified by reversed-phase high-performance liquid chromatography (HPLC) as described by Wong et al. [23,24]. The disulphide bridge form of the peptide used in this study was generated by air oxidization, as described previously [25]. Appropriate biotinylated peptides (biotin was coupled to the N-terminus of the peptides possessing a triglycine linker during synthesis) were also synthesized as previously described [26]. Purity of all peptides was verified by HPLC analysis and mass spectroscopy, all peptides utilized were >95% pure. Peptides were synthesized by the Peptide and Protein Chemistry Core Facility of the University of Colorado Health Sciences Center at Fitzsimons on a fee-for-service basis.

2.2. Generation of bioorganic stainless steel

Coupons (~1 mm thick and approximately 1 cm × 1 cm) of 304 grade stainless steel were annealed at 1040 °C for 1 h to release stress and then cooled at room temperature before further preparation. The stainless steel surface was polished to uniformity by sequentially employing sandpaper from 120# to 1200# grit size (MetTech Inc, Calgary) and then polished with an aqueous slurry of 0.05 μm colloidal silica. Coupons were then manually washed

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