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Non-proteinaceous bacterial adhesins challenge the antifouling properties of polymer brush coatings

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ABSTRACT

Polymer brushes of poly(ethylene glycol) have long been considered the gold standard for antifouling surfaces that resist adsorption of biomolecules and attachment of microorganisms. However, despite displaying excellent resistance to protein adsorption, the polymer brush coatings cannot entirely avoid colonization by bacteria. Here we investigate and identify which non-proteinaceous bacterial adhesins challenge the antifouling properties of polymer brush coatings and how these challenges might be overcome. We quantified biofilm formation on a well-known polymer brush coating of poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) on titanium. The coating successfully resisted colonization by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, but not *Staphylococcus epidermidis*. This colonization pattern was also reflected on the adhesion forces measured on single bacterial cells. The biofilm produced from *S. epidermidis* on PLL-g-PEG were found to be rich in polysaccharides and extracellular DNA, and quantification of DNA, polysaccharides and proteins on PLL-g-PEG surfaces revealed that although the coating almost fully resisted protein adsorption, polysaccharides could adsorb, and exposure to DNA led to desorption of the polymer from the titanium surface. We hypothesized that this problem could be overcome by increasing the polymer brush density to better resist the penetration of DNA and polysaccharides into the polymer layer. Indeed, high density PLL-g-PEG brushes prepared by the recently discovered temperature-induced polyelectrolyte (TIP) grafting method resisted the interaction with DNA and polysaccharides, and therefore also the colonization by *S. epidermidis*. The TIP grafting is a simple improvement of PLL-g-PEG brush formation, and our results suggest that it provides an important advancement to the bacterial resistance by polymer brush coatings.

Statement of Significance

The antifouling properties of poly(ethylene glycol) brush coatings against protein adsorption are well documented, but it is not well understood why these coatings do not perform as well against bacterial colonization when tested against a wide range of species and over periods of days. Here we investigated bacterial colonization on poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) grafted on Ti, and revealed that bacteria relying mostly on polysaccharides and extracellular DNA for adhesion and biofilm formation could successfully colonize PLL-g-PEG coated surfaces. The coatings could not resist adsorption of polysaccharides, and DNA could even desorb the coatings from the Ti surface. Fortunately, the shortcomings of conventional PLL-g-PEG could be overcome by increasing the graft density, using the recently discovered and very simple grafting method, 'temperature-induced polyelectrolyte (TIP) grafting'. Our study highlights that it is of utmost importance to develop coatings which resist adsoprtion of non-proteinaceous bacterial adhesins such as polysaccharides and DNA, and we demonstrated that TIP grafted high density PLL-g-PEG coatings are promising materials to achieve diverse bacterial resistance.

1. Introduction

Biofilms are consortia of bacterial cells encased in a matrix of self-produced extracellular polymeric substances (EPS) comprised

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of polysaccharides, proteins, and extracellular DNA (eDNA), protecting them from the host immune system and antibiotic treatment [1–4]. They are therefore the cause of persistent infections associated with medical devices and implants [4]. The ineffective treatment of biofilm infections is driving an enormous effort to develop materials that prevent bacterial colonization. Biocides and antibiotics have either been embedded in the bulk material or been coated on the surface of biomaterials to create antibacterial surfaces which kill bacteria in the vicinity. However, the selection for antibiotic resistance has discouraged this approach [5–7]. Anti-adhesive coatings have therefore attracted a lot of attention, as they can prevent bacterial attachment and hence biofilm formation.

Anti-adhesive coatings against bacteria have been developed from the common assumption that surfaces which resist protein adsorption would also prevent bacterial adhesion. To achieve protein resistance, anti-adhesive coatings commonly possess chemical structures that are hydrophilic, neutral, and highly hydrated, as they can bind water molecules strongly, reducing interactions with proteins and causing osmotic penalty when proteins come in contact with such surface [8]. Materials that fit this guideline include the extensively studied poly(ethylene glycol) (PEG) [9–12] and other alternatives including dextran [13,14], mannitol [15], glycine peptoid [16], polyglycerol dendrons [17,18], and zwitterionic compounds with strong water-binding abilities [19–21].

Developing anti-adhesive coatings against bacteria remains a challenging task, because protein resistance does not necessarily translate into bacterial resistance [22–25]. While PEG coatings on stainless steel rejected protein adsorption completely, they failed to reduce attachment of either Pseudomonas sp. or *Listeria monocytogenes* [22]. Compared to proteins, bacterial cells are much bigger, and they utilize more complex and diverse mechanisms to attach to the surface, involving both non-specific and specific interactions through proteins, polysaccharides, eDNA, and surface appendages such as pili and flagella [2,26,27]. A recent report revealed that *Pseudomonas aeruginosa* was able to adhere to protein-resistant surfaces via the selective interactions between alginate and zwitterionic surfaces [25]. Biosurfactants such as rhamnolipid may also be involved in bacterial adhesion to PEG coatings [28]. Furthermore, once attached to the surface, bacteria produce polysaccharides, enzymes, and biosurfactants to further challenge the underlying coating [2]. It is possible that it only takes a few adhered cells to serve as anchoring points for further attachment and biofilm growth. As a result, although various PEG coatings have been shown to reduce the initial adhesion of bacteria [28–38], biofilms eventually developed when surfaces were incubated longer (1 day) [39]. It is therefore critical to look into non-proteinaceous bacterial adhesins to understand why surfaces that are resistant to protein adsorption fail to prevent bacterial colonization.

In this study we investigate the adhesion and biofilm colonization on PEG coatings from bacteria that employ different adhesion mechanisms to understand which types of biomolecules are responsible for the bacteria to achieve successful colonization onto coatings that are otherwise considered protein repellent. The three model organisms were chosen based on their clinical relevance, ability to form biofilm, and involvement of different cell surface biomolecules in the initial adhesion: *P. aeruginosa* is Gram negative, motile, and is the only of our three model species to use Type IV pili in attachment and biofilm formation [39]; *Staphylococcus aureus* is Gram positive and relies mostly on cell wall bound proteins [40]; and *Staphylococcus epidermidis* relies mostly on polysaccharides [40]. They all use eDNA to stabilize the biofilm, but it's role in initial adhesion has only been confirmed for *S. epidermidis* [41].

In order to investigate of roles of non-proteinaceous adhesins, we challenge PLL-g-PEG brushes with not only proteins, but also model compounds of polysaccharides and DNA, revealing distinctive modes of interactions with the protein-resistant brushes. We further demonstrate that these challenges can be overcome if the density of the polymer brushes is sufficiently high. Using the recently developed temperature-induced polyelectrolyte (TIP) grafting at elevated temperature [42], we obtained high-density PLL-g-PEG brushes which show superior antifouling performance against non-proteinaceous adhesins. Our results highlight the necessity of including not only proteins, but also polysaccharides and DNA for testing antifouling performance of coatings. The performance of high-density PLL-g-PEG is very encouraging for the future development of antifouling coatings against bacterial biofilm.

2. Experimental section

2.1. Materials and surface modification

Si wafers (Si-Mat GmbH, Kaufering Germany) were pre-sputtered with Ti (100 nm) using a standard PVD chamber equipped with a RF sputtering system at an Ar pressure of typically 2×10^{-3} mbar from Ti targets of 10 cm diameter at 200 W. The Ti surfaces were subsequently cut into wafers of 2 cm^2 and cleaned by sonication in ethanol and MilliQ water for 15 min each, followed by 20 min treatment using UV/Ozone. The PEG brushes were prepared by incubating Ti surfaces in PLL-g-PEG (Susos AG, Dubendorf, Switzerland, PLL (20 kDa)-g(4.0)-PEG (5 kDa)) in 10 mM HEPES buffer at a concentration of 100 μg/mL at 20 °C (conventional) and 80 °C (high-density) for 24 h and thoroughly washed in MilliQ water and dried in N_2.

2.2. Surface characterization by X-ray photoelectron spectroscopy (XPS)

XPS data acquisitions were performed using a Kratos Axis UltraDLD instrument (Kratos Analytical Ltd., Telford, UK), using a monochromated Al kα X-ray source (hν = 1486.6 eV) operating at 10 kV and 15 mA (150 W). Survey spectra (binding energy (BE) range of 0–1100 eV with a pass energy of 160 eV) and high resolution spectra (with pass energy of 20 eV) of C 1s, O 1s, and N 1s were obtained to determine chemical state information of the surfaces. All data acquisition were performed over three areas per sample and repeated at least once. High resolution spectra were normalized by the total counts in order to make a direct comparison between spectra. The acquired data were converted to VAMAS format and analyzed using CASAXPS software (CASA XPS Ltd., UK). The BE scales for the high resolution spectra were calibrated by setting the BE of the O 1s Ti—O—Ti component to 530.0 eV [43]. Peak fittings were conducted with Gaussian/Lorenzian functions of 30/70% using Shirley background and chemical species were identified by referring to database [44]. The overlayer thickness was calculated from XPS data as described previously [42].

2.3. Preparation of bacterial cultures

S. aureus DSM20231, *S. epidermidis* 1457 wild type, 1457 icaADBC::dhfr, 1457 ΔatlE were grown in 3% tryptic soy broth (TSB) or on tryptic soy agar, and *P. aeruginosa* PAO1 was grown in 2.5% Luria broth (LB) or LB agar. Bacterial strains were first grown for 24 h on agar and stored at 4 °C. A single colony was used to inoculate 10 mL broth and incubated overnight at 37 °C.

2.4. AFM imaging of PEG brushes

A NanoWizard II atomic force microscope (AFM) (JPK Instruments, Germany) combined with an inverted optical

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