Bacterial interactions with nanostructured surfaces

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The formation of biofilm on material surfaces due to bacterial adhesion is a serious problem for both the health and economic field. Marine fouling on ship hulls causes tremendous increases in drag and associated fuel consumption, hence economic losses due to the settlement of organisms on not only ship hulls but also power plant cooling systems, aquaculture systems, fishing nets, pipelines, submerged structures, oceanographic research instrumentation is enormous. In food industry, steel, aluminum and titanium are metals widely used which can be affected by the adhesion and colonization of bacteria. Metal surfaces can corrode by way of microbially induced corrosion caused by biofilm formation with sulfide-producing bacteria. In the human body, microbial adhesion and growth can also cause serious health hazards by causing difficult to treat infections, especially on contaminating biomaterial implants and devices.

An approach explored in the prevention of bacterial adhesion and biofilm formation that yet has to find its way to clinical use is to mechanically or chemically engineer specific surface properties that directly repel bacteria, such as through engineered roughness or hydrophobicity. Among the engineered surfaces, nanostructured surfaces are new and their possible merits as infection-resistant implant surfaces, or for that matter anti-adhesive surfaces in general, has never been truly explored.

With the aim of discouraging bacterial adhesion, in **Chapter 2**, nanoporous and nanopillared aluminum surfaces were engineered by anodizing and post-etching processes and made hydrophilic (using the inherent oxide layer) or hydrophobic (applying a Teflon coating). Adhesion of *Staphylococcus aureus* ATCC 12600 (Gram-positive, spherically-shaped) and *Escherichia coli* K-12 (Gram-negative, rod-shaped) was evaluated to the nanoengineered surfaces under both static and flow conditions (fluid shear rate of 37 s^{-1}). Compared to a non-structured electropolished smooth surface, the nanostructured surfaces significantly reduced the number of adhering colony forming units (CFUs) for both species, as measured using agar plating. For the hydrophilic surfaces, this was attributed to a decreased contact area, reducing bacterial adhesion forces on nanoporous and nanopillared surfaces to 4 and 2 nN, respectively, from 8 nN on flat surfaces. Reductions in the numbers of adhering CFUs were more marked on hydrophobic surfaces under flow, amounting to more than 99.9 and 99.4% for *S. aureus* and *E. coli* on nanopillared surfaces, respectively. Scanning electron microscopy revealed the few bacteria found on the hydrophobic nanopillared surfaces adhered predominantly to defective or damaged areas, whereas the intact area preserving the original nanopillared morphology was virtually devoid of adhering bacteria. The greater decrease in bacterial adhesion to hydrophobic nanopillared surfaces than to hydrophilic or nanoporous ones is attributed to effective air entrapment in the three-dimensional pillar morphology, rendering them superhydrophobic and slippery, in addition to providing a minimized contact area for bacteria to adhere to.

Transmission of bacteria from a donor to a receiver surface is an underestimated means of bacterial mass transport preceding the formation of biofilms in many industrial and biomedical

applications. Source of bacteria is often assumed from aqueous suspensions or air, but in real-life bacteria are often transmitted between surfaces. Mechanistically, transmission involves detachment of adhering bacteria from a donor and adhesion to a receiver surface, controlled by the relative values of the adhesion forces exerted by both surfaces. In **Chapter 3**, we relate staphylococcal adhesion, detachment and transmission to, from, and between smooth and nanopillared Si surfaces with staphylococcal adhesion forces. Nanopillared-Si surfaces were prepared with pillar-to-pillar distances of 200, 400 and 800 nm. On smooth surfaces, staphylococcal adhesion forces, measured using bacterial-probe Atomic-Force-Microscopy (AFM), amounted to 4.4–6.8 and 1.8–2.1 nN (depending on the AFM-loading force) for Extracellular-Polymeric-Substances (EPS) producing (*S. aureus* ATCC 12600) and non-EPS producing (*S. epidermidis* ATCC 12228) strains, respectively. Accordingly, the EPS producing strain adhered in higher numbers than the non-EPS producing strain. Fractional adhesion forces on nanopillared-Si surfaces relative to the smooth surface ranged from 0.30-0.95, depending on AFM-loading force, strain and pillar-to-pillar distance. However, for each strain, the number of adhering bacteria remained similar on all nanopillared surfaces. Detachment of adhering staphylococci decreased significantly with increasing adhesion forces, while staphylococcal transmission to a receiver surface also decreased with increasing adhesion force exerted by the donor. In addition, the strain with ability to produce EPS was killed in high percentages and induced to produce EPS during transmission on nanopillared-Si surfaces, presumably by high local cell-wall stresses. This must be accounted for in applications of nanostructured surfaces: whereas killing may be favorable, EPS production may reduce antimicrobial efficacy.

Bacterial transmission from biofilm on one surface to another is an entirely different process than growing biofilm, as it involves compression of two surfaces including the biofilm, followed by a tensile force leading to bacterial detachment from the donor or biofilm and subsequent adhesion to the receiver. Therewith transmission depends on a tedious balance between the surface properties of the donor, the biofilm and the receiver surfaces. In **Chapter 4**, we compare bacterial transmission from biofilms of an EPS producing and a non-EPS producing strain on smooth Si donor surfaces to nanopillared Si receiver surfaces with pillar-to-pillar distance of 200, 400 or 800 nm. After transmission, biofilms were found on both donor and receiver surfaces, suggesting that transmission occurred for both strains through adhesive failure at the interface between the biofilm and the smooth Si surface and cohesive failure in the biofilm.

Bacteria in their biofilm mode of growth are relatively insensitive to antibiotic treatment, also caused by the growing antibiotic resistance among many bacterial strains. Gentamicin is a commonly used antibiotic in local delivery systems, typically applied in porous beads or biodegradable coatings. Such coatings generally release their gentamicin content ad libitum, making a high burst release followed by a low level tail-release that can continue for years. The issue is not that there are not enough drugs, but every time we use them, they become slightly less effective. Therefore, it is important to increase efficacy of the antibiotics. In **Chapter 5**, we

show that hierarchical nanostructuring of titanium and the subsequent coating of resulting topographical features with a self-defensive, antibacterial layer-by-layer (LbL) film enables a synergistic action of hierarchical nanotopography and localized, bacteria-triggered antibiotic release to dramatically enhance the antibacterial efficiency of surfaces. Although sole nanostructuring of titanium substrates did not significantly affect adhesion and growth of *S. aureus*, the coating of 3D-nanopillared substrates with an ultrathin tannic acid / gentamicin LbL film resulted in a 10-fold reduction of the number of surface-attached bacteria. This effect is attributed to the enlarged surface area of the nanostructured coating available for localized bacteria-triggered release of antibiotics, as well as to the lower bacterial adhesion forces resulting in subsided activation of bacterial antibiotic-defense mechanisms when bacteria land on nanopillar tips. The result shows that a combination of 3D nanostructuring with a bacteria-triggered antibiotic-releasing coating presents a unique way to dramatically enhance antibacterial efficacy of biomaterial implants.

In the general discussion (**Chapter 6**), we first highlight the two main outcomes of this thesis:

1- a way to produce large scale micro-nanoscaled surfaces with an impact on bacterial adhesion, detachment and transmission.

2- a new phenomenon named "pressure-induced EPS" production and associated cell death which should be taken into account for future studies on "nanostructured" surface designs for reducing biofilm formation.

It is argued that the term "nanostructured surfaces" used in literature has been abused too often and the true "control" of bacterial adhesion on nanostructured surfaces has never been carefully investigated. Experimental mishandling the surfaces (e.g. passing an air layer before enumeration) can create enormous detachment force and inevitable results of "reduced number of adhering bacteria", also equally so on smooth as on nanostructured surfaces. More carefully designed experiments are often necessary to substantiate the conclusions drawn in the literature. Also from the experiments described in this thesis, an outlook on future prevention of biomaterial-associated biofilms using nanostructured surfaces is given in the general discussion.