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Bacteria repellent layer made of flagellin

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ABSTRACT

The development of bacteria repellent surface coatings is critical in various fields ranging from biosensing to health care, biotechnology and food production. In the present study we exploit that the protein flagellin rapidly forms a dense and oriented monolayer on hydrophobic surfaces upon adsorption from aqueous solution. This oriented layer mimics the surface of bacterial flagellar filaments and has excellent bacteria repellent properties. *In situ* OWLS (Optical Waveguide Lightmode Spectroscopy) measurements were used to monitor on-line both the formation of the protein layer on the silanized sensor surface and subsequent bacterial adhesion. The adhered cells were also visualized by fluorescent microscopy and the formed protein film was characterized by AFM (Atomic Force Microscopy). In parallel control experiments, the adherence of bacteria was measured on bare hydrophobic surfaces as well. Both OWLS and microscopy results well confirmed that the flagellin coating drastically reduced the adhesion of *E. coli* cells. Therefore, a novel type of bacteria repellent layer made of flagellin is demonstrated.

1. Introduction

Pathogen colonization is widely studied and has important consequences in food safety and medical system design [1–4]. During infection surface adhesion is crucial for a bacterium [4,5].

Bacterial adhesion is a complicated process, which is influenced by various factors like the material properties of the surface, the type of the bacteria, the environmental conditions and the presence of different chemicals, like proteins and antibiotics. This adhesion process can be described by two phases. *Phase one* is a reversible physical phase, which is directed by physical forces (e.g.: van der Waals attraction forces, electrostatic forces, and hydrophobic interactions). The properties of the bacteria like hydrophobicity and charge distribution, and the features of the surface like chemical composition, roughness and local configuration play an essential role. *Phase two* is an irreversible and time-dependent cellular phase, where the connection pattern between bacterial surface structures and the substratum is evolved. Bacterial surface structures like fimbriae, pili and slime are mainly responsible for the adhesion [5].

There is an intensive research and development worldwide to create cheap, mass producible and easy-to-employ coatings to hinder the adhesion of cells on solid surfaces. As anti-adhesive surface coatings the most commonly used are synthetic polyethylene glycol (PEG)-based polymers [6,7] or copolymers [8]. Surface-immobilized biopolymers are also frequently applied such as carboxymethyl-dextran (CMD) [9,10], alginic acid [11] or hyaluronic acid [12]. Typically, these molecules do not adsorb spontaneously on surfaces with appropriate density to repulse cells. Generation of anti-adhesive surfaces usually requires advanced chemical techniques [13] and complicated surface modification procedures [14]. There are just a few materials which can adsorb spontaneously and form coatings effectively preventing the surface adhesion of cells. A good example is mucin, which is an accessible and effective coating to control adhesion of various cells and it can be combined with patterning techniques [15]. Polymers grafted with antibacterial enzymes [16] or drugs [17,18] were also fabricated and their antibacterial effect was demonstrated.

As an alternative coating material, the bacterial protein flagellin is a promising candidate to fabricate bacteria repellent surfaces. Flagellin monomers are the building blocks of the bacterial flagellar filaments. The monomers have well-separated hydrophobic and hydrophilic surface regions, which feature is necessary for effective

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and fast self-assembly by hydrophobic interactions to form flagellar filaments [19]. Flagellin is composed of 4 domains (D0, D1, D2, D3) where D0 and D1 are partially disordered and control polymerization ability [20]. We demonstrated previously that flagellin quickly adsorbs onto hydrophobic surfaces and forms a dense, stable and oriented monolayer [21]. Based on optical birefringence data, it was concluded that flagellin binds to the hydrophobic surface through the disordered D0 and D1 domains, and exposes the hypervariable D3 domain to the solution. We found previously that this oriented flagellin monolayer prevents surface adhesion of cancer cells [22]. It is also important to emphasize that, flagella are known to serve as adhesins to promote binding to certain plant and animal cell surfaces [23,24]. In addition, the D3 domain of flagellin can be genetically engineered or replaced by other proteins or appropriate binding motifs to furnish the molecule with various functionalities [22,25,26]. For example, the integrin binding RGD motif was inserted into the variable central portion of flagellin, and RGD-displaying flagellin variants were used as biomimetic coatings to create cell-adhesion-regulating surfaces [22].

In the present study we studied the adhesion of *Escherichia coli* cells on oriented flagellin films self-assembled on hydrophobic surfaces, and demonstrated the bacteria repellent properties of the flagellin coating. The surface attachment kinetics of bacterial cells can be monitored by several label-free sensing techniques [3,27,28]. From our previous experiments with OWLS (Optical Waveguide Lightmode Spectroscopy), we concluded that this waveguide based label-free technique is well-suited for monitoring the kinetics of both protein adsorption and bacterial surface adhesion with high resolution [21,29]. Therefore, we used OWLS and parallel microscopic investigations in the present work. The proposed flagellin based coating can be employed on any hydrophobic surface in a straightforward and cost-effective manner to diminish bacterial adhesion.

2. Materials and methods

2.1. Bacterial samples

For the surface adhesion experiments BL21 C + RIL *Escherichia coli* cells were used, which contained the pVJsGFPa plasmid encoding the GFP protein [30]. This bacteria overexpressed the GFP protein which resulted in the green color of the cells facilitating observation of the cells by fluorescent microscopy. The bacterial cells were stored in 10% glycerol containing PBS (phosphate buffered saline) buffer (pH 7.4, Sigma-Aldrich) at −20 °C. After thawing the cells were centrifuged at 855*g* for 5 min and resuspended in PBS at a final concentration of approximately 3×10^7 cell/mL, determined using a haemocytometer.

2.2. Surface modification

Surface modification of the sol–gel SiO_2–TiO_2 planar optical waveguides (MicroVacuum Ltd., Hungary) started with a standard cleaning protocol: the chips were soaked for 3 min in chromosulfuric acid (VWR International), rinsed in Milli-Q ultrapure water, immersed into 1 M KOH solution, and then rinsed in copious amounts of ultrapure water. This procedure yielded a hydrophilic surface. For AFM measurement we used Au patterned Si/SiO_2 substrates (Si/SiO_2 samples half covered with ca. 60 nm Au layer) which were oxygen plasma cleaned. Highly hydrophobic surfaces were created by hexamethyldisilazane (HMDS, Alfa Aesar) treatment using xylene reflux. The employed protocol resulted in water contact angles of 90–95°.

2.3. Contact angle measurement

Water contact angle measurements were carried out at room temperature. Water drops of ~5 μl were deposited on the surface and left to stabilize in a closed chamber saturated with water vapor before the image was taken. The DropSnake method was used to calculate the contact angle [31].

2.4. Flagellin coating

In this study the flagellin monomers of *Salmonella typhimurium* were used. Bacterial flagellar filaments are built up from thousands of flagellin units. Filament samples from *Salmonella* cells were prepared as described earlier [32]. Heat treatment (15 min incubation at 70 °C) of the filament induced the depolymerization of the fiber and produced monomeric flagellin units. The solution was centrifuged through a 100 kDa filter (Millipore, UFC510008) to remove fragments of filaments still remaining in the sample. Flagellin was dissolved in PBS to a final concentration of 1 mg/mL. It was demonstrated previously that flagellin readily formed oriented monolayer on hydrophobic surfaces [21].

2.5. Atomic force microscopy

The Si/SiO_2 samples were half covered with ca. 60 nm Au layer using e-beam evaporation before surface functionalization to preserve the unmodified surface for the AFM (Atomic Force Microscopy) layer thickness measurement. After silanization and subsequent flagellin deposition, the Au layer was mechanically removed, providing the original surface next to the silanized and protein covered area. This technique enables the direct thickness measurement of the assembled protein layer. The AFM images were collected using a SmartSPM 1000 system (AIST-NT) with silicon cantilevers (curvature radius less than 10 nm) in tapping mode. The optimal tapping mode frequency was set around 320 kHz and the typical scan rates were between 0.5 and 1 Hz depending on the size of area scanned. AFM data were evaluated by using Gwyddion software [33].

2.6. Optical waveguide lightmode spectroscopy (OWLS)

OWLS 210 (MicroVacuum Ltd., Hungary) temperature controlled instrument was applied to monitor the surface adsorption of the proteins and subsequent bacterial adhesion. The sensing principle of the OWLS is based on the evanescent waves of the guided waveguide modes. The evanescent field extends a few hundred nanometers above the waveguide surface. The sensor is capable to probe the change in local refractive index in the proximity of the sensor surface [34]. The OWLS instrument records the incoupled light intensity while the illumination angle of the exciting laser beam is varied. In the incoupled intensity-illumination angle function, two sharp resonant peaks appear, corresponding to the TE_0 and TM_0 waveguide modes [35]. From the position of the peaks the effective refractive indices of the waveguide modes (N_{TE} and N_{TM}) can be calculated using the grating equation [34,35]. Both modes were used during the protein and bacteria adsorption measurements. Taking the effective refractive indices of the modes, the adsorbed surface mass density can be calculated in a straightforward manner using the mode equations of planar optical waveguides [34–36]. The refractive indices of the employed solutions were measured with a Rudolf refractometer and inserted into the mode equations during the calculations. For the refractive index increment of protein and bacterial solutions a value of $dn/dc = 0.189\ cm^3/g$ was employed [35,36].

The experiments were carried out at 25 °C on pre-treated sensor chips. Both the buffer and sample solutions were injected into