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Self-assembly of flagellin on Au(111) surfaces

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ABSTRACT

The adsorption of flagellin monomers from *Pseudomonas fluorescens* on Au(111) has been studied by Atomic Force Microscopy (AFM), Scanning Tunneling Microscopy (STM), X-ray Photoelectron Spectroscopy (XPS), Surface Plasmon Resonance (SPR), and electrochemical techniques. Results show that flagellin monomers spontaneously self-assemble forming a monolayer thick protein film bounded to the Au surface by the more hydrophobic subunit and exposed to the environment the hydrophilic subunit. The films are conductive and allow allocation of electrochemically active cytochrome C. The self-assembled films could be used as biological platforms to build 3D complex molecular structures on planar metal surfaces and to functionalize metal nanoparticles.

1. Introduction

Protein interaction with metal surfaces raises fundamental questions regarding their binding tendency, their distribution on the surface, as well as the occurrence of adsorption-induced conformational modifications which may change their biological properties; addressing these questions, and being able to tune protein–surface interactions, is of primary importance for the control of biointerfaces. In particular, protein–surface interactions have great significance in many applications ranging from nanotechnology to medicine. In fact, protein immobilization on surfaces has important applications in drug screening, biosensing, bioassaying, and protein characterization.

Fundamental interactions between proteins and solid surfaces include one or a combination of the following processes: physical adsorption, electrostatic forces, specific recognition and covalent binding [1]. These interactions depend sensitively upon the local structures and environment of protein binding sites on surfaces.

Flagellin is a globular protein that assembles itself in forming a hollow cylinder structure that constitutes the bacterial flagella. Flagellin from *Salmonella* has been extensively studied as a prototypical member of this family. This protein contains well-folded protein domains and intrinsically disordered regions [2]. Electron cryomicroscopy and X-ray diffraction studies [3,4] have revealed the complex subunit structure within the filament. Polymeric flagellin consists of four linearly connected domains labeled D0, D1, D2, and D3, which are arranged from the inside to the outside of the filament. While D0 and D1 domains are highly conserved along different bacterial species, D2 and D3 domain show high variability [5]. The disordered terminal regions are involved in D0 and partly in D1, forming long helical bundles, and their direct interaction is responsible for stabilizing the filament structure. The segment involving D2 and D3, is exposed on the surface of flagellar filaments. D3 is a structurally independent part of flagellin; it is not essential for filament formation [3].

The D3 domain has been proposed as a good target for genetic engineering studies. Substituting the central D3 domain by an appropriate artificial peptide sequence can give flagellin analyte affinity sensing or enzymatic properties [4,6]. Recently, the incorporation of green fluorescent protein (GFP) was also demonstrated [7]. The easy formation of compact, stable, well ordered and oriented functional layers can facilitate the development of these directions. Thus, flagellin can have interesting applications in chemical or biological sensing, medical diagnostic, environmental monitoring, as well as in nanobiotechnology and nanomedicine. On this regard, it has been reported that flagellin is able to increase

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the immune response, either *in vitro* or *in vivo*, after systemic administration [8]. For instance, the recombinant flagellin–ovalbumin fusion protein induces both, humoral and cell-based immunity of mice [9]. Thereby, biocompatible calcium phosphate nanoparticles functionalized with flagellin have been tested as adjuvant in vaccination, resulting in an enhanced immunostimulation [10]. Flagellin-coated bioadhesive poly(anhydride) nanoparticles have also been investigated as possible strategy in oral vaccination, resulting in a strong long lasting systemic and mucosal immune responses than the respective non-conjugated vectors [11]. On the other hand, metallic nanoparticles, and particularly gold nanoparticles, are being widely studied in relation to their biological applications, such as cancer diagnostics and therapy, drug delivery and vaccine preparation, among others [12]. Along this line, the possibility of immobilizing flagellin on gold, as well as the elucidation of the spatial structure of the protein layer, opens the opportunity of modifying the surface of metallic nanoparticles and design useful systems for biomedical applications.

Otherwise, the structure of flagellin thin films adsorbed on highly hydrophobic (silanized) SiO_2/TiO_2 planar substrates has been recently elucidated [13]. Thus, the potential applications of flagellin-modified electrical conducting metal surfaces can be extended to electroanalytical techniques on the fields mentioned above.

2. Experimental

2.1. Bacterial culture

P. fluorescens strain, kindly provided by Dr. Christine Gaylarde (Department of Biology and Chemistry, UNIJUI, RS, Brazil) was identified by standard bacteriological tests using selective growth media and biochemical tests. This strain was maintained as inoculated slant in Cetrimide Agar (DIFCO) at 28 °C. The slant was incubated under conditions allowing microbial growth and then stored in a refrigerator at a temperature below the minimum required for culture growth. Passages of the stock were performed every 20 days. The inoculum was prepared by suspending a Cetrimide agar slant (24 h old) in 2 mL sterile Nutrient Broth for microbiology (MERCK). The inoculum was poured into an Erlenmeyer flask containing 300 mL nutrient broth medium and kept on a rotary shaker overnight at 28 °C. Following incubation, the bacterial suspension was adjusted to 10^8 colony-forming units (CFU) mL^{-1} in fresh growth medium which was confirmed by viable count method. It is worth to mention that our studies were conducted without gene modification of the strain.

2.2. Flagella and flagellin isolation

Flagella and flagellin isolation was adapted from the purification protocol described by Hiriart et al. [14]. Briefly, 1 mL of bacteria suspension (10^8 CFU mL^{-1}) was used to inoculate 1 L of nutrient broth. This culture was aerated in a glass laboratory bioreactor for 24 h at 30 °C, with an air flow rate of 10 L/min without agitation to avoid mechanical deflagellation.

One litre of bacterial culture prepared in the bioreactor was centrifuged (Biofuge 22R, Heraeus Sepatech, Germany) at 6500 rev/min for 30 min at 4 °C. The cell pellet was suspended in 18 mL of phosphate buffer saline dissolution (PBS) (10 mM, pH 7.0) and submitted to flagellin extraction by shear using vortex agitation for 10 min at 40 Hz (VELP Scientifica, Europe). After shearing, bacterial pellet was obtained by centrifugation at 14,000 rev/min for 15 min. The collected supernatant was then submitted to ultracentrifugation (35,000 rev/min for 2 h at 4 °C, in a Beckman ultracentrifuge) to obtain the flagellar pellet. Finally the flagellar pellet was resuspended in 2 mL of saline solution and then incubated in a 70 °C water bath for 20 min to obtain flagellin in monomeric form. The final concentration of the dissolution was 0.05 mg/mL, determined by the bicinchoninic acid (BCA) method using albumin as standard, following manufacturer's indications (Pierce, CA, USA).

2.3. Sample preparation

Gold substrates consisted in a thin layer of gold deposited on glass and were purchased from Arrandee® (Germany). The substrates were annealed in butane flame in order to obtain (111) terraces and then immersed in the flagellin-containing dissolution diluted 1:10 with phosphate buffer dissolution (PB) 10 mM, pH 7.3 at 4 °C. After 24 h the samples were gently rinsed with PB and dried for 48 h at 4 °C on a desiccant cushion. This procedure allowed us to obtain a thin layer of protein. Thick layers of flagellin on gold were obtained by drop casting 40 μL of the flagellin-containing dissolution (in such a way that the entire electrode was covered) and dried at 4 °C. Horse heart cytochrome C (Cyt C) (Aldrich) 99.9% was used as received. Cyt C adsorption on flagellin was carried out by immersing the flagellin-modified gold substrate in a 1 mg/mL of Cyt C in 1 mM (pH = 7.3) PB dissolution for 30 min. The samples were rinsed with the 1 mM PB dissolution and dried in air. Occasionally, the flagellin self-assembly was performed on nanometer-sized polycrystalline Au. The roughness (measured as root mean square, rms) of the polycrystalline substrate used for AFM imaging is 2–3 nm, similar to those measured for the SPR substrates, which resulted in 2.5 nm.

2.4. AFM and STM imaging

AFM imaging was made in air with a Nanoscope V microscope from Digital Instruments operating in tapping mode. Images were taken at a scanning rate of 1 Hz with etched silicon tips (RTESP, 215–254 kHz and 20–80 N/m). AFM was also used to estimate the protein film thickness by repetitive scanning the sample with the AFM tip. In this case a silicon nitride probe with a spring constant of 0.4950 N/m was used applying 134 nN load in air. This load was sufficient to open a window by removing the protein from the surface without damage of the underlying substrate. The cross section analysis of the window allows an estimation of the film thickness.

Structural data and electronic properties of the flagellin films were also determined by Scanning Tunneling Microscopy (STM) and scanning tunneling spectroscopy (STS), respectively, using a Nanoscope E STM (Digital Instruments) operating in air at room temperature. Pt–Ir tips were used for these measurements. The tunneling current vs bias voltage (I vs V) curves were obtained by averaging over at least five different regions of the samples. I/E data shown in this paper are the averaged result of around 400 I/E curves collected in each zone.

2.5. Electrochemical measurements

Electrochemical measurements were performed in a three-electrode electrochemical cell; substrates prepared as described above were used as working electrode (counterelectrode: platinum foil, reference electrode: saturated calomel electrode, SCE). The electrolyte dissolution was PB 10 mM (pH = 7.3). The sweep rate was 100 mV/s. Voltammograms were recorded by using a Voltalab or Teq galvanostat-potentiostat.

2.6. X-ray Photoelectron Spectroscopy (XPS)

XPS measurements were performed using a Mg Kα source (XR50, Specs GmbH) and a hemispherical electron energy analyzer