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Role of protein environment and bioactive polymer grafting in the *S. epidermidis* response to titanium alloy for biomedical applications

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ABSTRACT

Joint implant-related infections, namely by *Staphylococci*, are a worldwide problem, whose consequences are dramatic. Various methods are studied to fight against these infections. Here, the proposed solution consists in grafting a bioactive polymer on joint implant surfaces in order to allow the control of the interactions with the living system. In this study, sodium styrene sulfonate, bearing sulfonate groups, was grafted on the surface of titanium alloys. Scanning Electron Microscopy, colorimetric method, Fourier-transformed infrared spectroscopy and contact angle measurements were applied to characterize the surfaces. Bacterial adhesion studies were studied on poly(sodium styrene sulfonate) grafted Ti_6Al_4V and Ti_6Al_4V surfaces previously adsorbed by proteins involved in the bacteria adhesion process. Fibrinogen and fibronectin were demonstrated to increase staphylococcal adhesion on Ti_6Al_4V surfaces. Ti_6Al_4V grafted sodium styrene sulfonate surfaces inhibited the adhesion of *Staphylococcus epidermidis* in 37% and 13% on pre-adsorbed surfaces with fibrinogen and fibronectin, respectively. The mechanism of the observed inhibiting bacteria adhesion properties is related to the differences of proteic conformations induced by poly(sodium styrene sulfonate) grafting.

1. Introduction

The growing human life span and the continuous demand for a better quality of life are the main motivations behind all the developments in biomaterials industry and research. Despite all the advances in the quality of healthcare, the probability of infection during a surgical procedure is still high. In the orthopedics field, where biomaterials find a high variety and number of applications, the risk of infection is of about 2–5% [1]. Currently implanted medical devices are still unable to actively resist bacterial adhesion, colonization, and biofilm formation. Ideally, it would be possible to eradicate implant-related infections with effective antibiotics, but this strategy faces two major problems: the bacterial biofilm resistance to the antibiotic penetration and the development of microbial resistance [2,3].

When a biomaterial is introduced in the body its surface is instantaneously covered by plasma proteins, thus becoming the interface that will contact with the surrounding biological environment [4]. Bacteria, namely *Staphylococcus aureus* and *Staphylococcus epidermidis*, the most important microorganisms causing implant-associated infections, present, on their surface, receptors to domains present in the adhesive proteins, such as fibrinogen (Fg) and fibronectin (Fn), called adhesins, which can quickly promote their adhesion [5,6]. After adhering, bacteria may colonize and change their phenotype in order to start the biofilm production. Inside the polymeric matrix of the biofilm, a bacterial community is developed and its elimination either by the host immune system or by the usage of antibiotics becomes extremely difficult [7].

With the aim of avoiding bacterial adhesion and the consequent biofilm formation, biomaterial surface properties can be improved [8,9]. In addition, the protein adsorption process as well as the composition of the adsorbed protein layer and the conformation of adsorbed proteins are also dependent on the surface properties, namely chemistry, hydrophilicity and roughness by allowing the exposure or not of protein domains involved in bacterial adhesion [9,10]. Covalent grafting is one type of surface modification that offers a strongest link between biomaterial implant and its coating, producing a more durable interface [11]. Grafting of poly(sodium styrene sulfonate) (poly(NaSS)) on titanium surfaces has been investigated and shown to enhance bone cells' adhesion and also has antibacterial properties to some extent [9,12–15].

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This work aims to study the potential application of a poly(NaSS) grafting process of medical titanium alloys to prevent bacterial colonization.

2. Materials and methods

2.1. Materials

13 mm diameter Ti_6Al_4V discs (CERAVER, Roissy France) were used as substrates. The grafting process was conducted on Ti_6Al_4V surfaces by CERAVER company at Plailly, France. The details of the process have been described [16,17]. Prior to antibacterial adhesion test, all discs were extensively washed: 1.5 M, 0.15 M NaCl water solutions (sodium chloride, Fischer), distilled water, phosphate buffer saline solution (PBS, Gibco). This procedure was repeated 3 times. The samples were then sterilized by using ultraviolet light (UV, 30 W) for 15 min, each side. For bacterial adhesion assays, bovine serum albumin (BSA; Sigma, ref. A4503) and bovine plasma fibrinogen — fraction I, type I-S (Fg; Sigma, ref. F8630) solutions were prepared in Dulbecco's phosphate-buffered saline (DPBS) at concentrations of 400 and 4000 μg/mL for BSA, and at 300 μg/mL for Fg. Human plasma fibronectin (Fn; Chemicon International, ref. FC010) was dissolved in a 1% (w/v) BSA solution at a concentration of 20 μg/mL, in order to stabilize the structure of this high molecular weight protein. The concentrations of the prepared protein solutions were 10% of their serum concentration.

2.2. Surface characterization

2.2.1. Surface topography by Scanning Electron Microscopy (SEM)

Titanium alloy surfaces were analyzed with support of an environmental Scanning Electron Microscope (SEM, Hitachi TM-3000) in secondary electron mode. The microscope was operated at 15 kV under 10^{-5} Torr vacuum. The working distance between the microscope sensor and the samples ranged between 8.7 and 11.2 mm. The observation was performed at ×100 and ×1000 magnifications. The ungrafted and grafted samples that were not inoculated with bacteria were dried and were not subjected to any pre-treatment before SEM observation. After 1 h of incubation with the bacteria, the cells were fixed on ungrafted and grafted samples overnight with 4% formaldehyde solution at 4 °C. Before SEM observation, samples were rinsed twice with DPBS.

2.2.2. Contact angle measurements

The contact angle formed between a 2 μL drop of distilled water and the samples was measured by KRUSS DSA10 measuring system (Germany). For each sample 6 contact angle measurements were performed to determine the mean value.

2.2.3. Toluidine blue colorimetric assay

Grafted Ti_6Al_4V samples were individually immersed in a toluidine blue (TB) aqueous solution (5×10^{-4} M) for 6 h at 30 °C to induce complexation of TB with sulfonate groups. After that period, the samples were rinsed with 1×10^{-3} M sodium hydroxide aqueous solution until the excess dye was completely removed. The stained surfaces were then immersed in a 50% acetic acid solution for 24 h, to induce decomplexation. The decomplexed TB solutions were measured by visible spectroscopy using a Perkin-Elmer spectrometer lambda 25 at 633 nm [12].

2.2.4. Fourier-transformed infrared spectroscopy/Attenuated Total Reflectance (FTIR/ATR)

FTIR spectra, recorded in an attenuated total reflection (ATR) mode, were obtained using a Thermo Nicolet Avatar 370 Spectrometer. The presented data comes from 128 spectra, which were obtained with a 4 cm^{-1} resolution, using a 45° Ge crystal, and a wavenumber from 600 cm^{-1} to 4000 cm^{-1}.

2.3. Bacterial adhesion studies

2.3.1. Bacterial adhesion

S. epidermidis RP62A (ATTC 35984) was cultured in trypticase soy broth (TSB) (Bio-Rad, ref. 64144), at 100 rpm and 37 °C, for 12–16 h (overnight culture) and suspended in DPBS to 3×10^5, 3×10^6 or 3×10^7 bacteria/mL after quantification by Multisizer III-Coulter counter (Beckman).

Prior to bacterial inoculation, the sterilized Ti_6Al_4V discs were immersed in a 400 μg/mL BSA solution for 30 min, in a 24-well tissue plate. Then, the discs were immersed in a protein–PBS solution (Fg or Fn) at 10% its plasmatic concentration, for 1 h at 37 °C and under agitation (100 rpm) to promote their adsorption to the surface. Before the bacteria inoculation, the protein solution was removed and the discs were washed three times with 400 μg/mL BSA solution. Afterwards, 1 mL of the bacterial suspension at the desirable concentration was added to each well, and incubated at 37 °C for 1 h, under agitation.

2.3.2. Quantification by the detachment method

After 1 h of incubation with bacteria suspensions, the excess of bacteria was removed by washing the discs three times with DPBS. Afterwards, a trypsin–EDTA solution at 0.05% (w/v) (Gibco, ref. 25300) was added to detach the bacteria adhered to the surface. When trypsin solution was added, 40 aspirations were made with the micropipette, before it was removed, and a short wash with DPBS was performed. The adhered bacteria (now in the trypsin–DPBS solution) were then quantified using the Coulter counter. Triplicates were used to evaluate each condition.

2.3.3. Quantification by the imaging-based method

Acridin orange (AO), as used by Levon et al. [18], was used to stain adhered *S. epidermidis* on Ti_6Al_4V disc surfaces. Briefly, after the incubation with bacteria and they being rinsed twice with DPBS, the Ti_6Al_4V discs were immersed for 20 min in 1:10,000 (w/v) AO stain (Sigma A-6014) in 0.2 M acetate buffer (pH 3.8), in the dark at room temperature. Afterwards, the samples were rinsed twice with distilled water. The observation of the stained bacteria was carried out using a fluorescence microscope (Carl Zeiss Axiolab E re).

Using the staining method previously described, nine photos of each disc ($n = 2$) were taken using a digital camera (1×HRD 100-NIK, Diagnostic Instruments Inc.) mounted on a fluorescence microscope (Carl Zeiss Axiolab E re) at ×100 magnification. From the digital images obtained, and using Matlab, it was possible to create a color filter to quantify the number of adhered bacteria and determine the average percentage of surface colonized by the bacteria. The threshold values that characterize the orange filter were empirically determined, and defined by the 3 conditions in RGB color space (Fig. 1). Briefly, in the RGB color space each pixel has intensity in red, green and blue components. Combining the intensity and the ratio of the intensity values in each component, it was possible to define the orange color. All the pixels that fulfill the stated conditions were counted and used to calculate the percentage of colonized surface.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). *T* test was used to assess the difference between contact angles. One-way analysis of variance (ANOVA), followed by Tukey's post hoc test was performed on bacterial adhesion studies. Differences were considered statistically significant at $p \leq 0.05$. Results were expressed as mean ± standard deviation (error bars).