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Plasma-deposited nanocomposite polymer-silver coating against *Escherichia coli* and *Staphylococcus aureus*: Antibacterial properties and ageing

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ABSTRACT

A plasma-deposited coating, containing silver nanoparticles embedded in an organosilicon matrix, was synthesized, using AISI 316L stainless steel as the underlying substrate. The coating antibacterial property was evaluated on the Gram-negative *Escherichia coli* K12 MG1655 and the Gram-positive *Staphylococcus aureus* MW2 strains, by combining indirect (plate counting) and in situ (fluorescent bacteria labelling) methods. Both approaches were shown to be highly complementary and converged on a maximal antibacterial efficacy against *E. coli*, as plate counts showed a decrease of 6 and 1 Log and dead bacteria represented 25% and 2% of the total adhering bacteria for *E. coli* and *S. aureus*, respectively. The coating antibacterial potential was then determined over time on *E. coli* biofilm. Whatever the biofilm age, growth inhibition was observed due to silver-mediated bacteriostatic effect. The coating bactericidal activity was initially strong. However, differences between coated and bare stainless steel surfaces tended to collapse above a 2-day contact time. A thorough characterization of the film properties after ageing in biological suspension or saline solution (short and longer-term exposures) revealed an oxidation of both the organosilicon matrix and the silver nanoparticles, accompanied by silver release at the extreme surface. However, a silver reservoir was still present and potentially active in the deep layers of the coating.

1. Introduction

Biofilms can be defined as a complex and dynamic ecosystem, constituted by a community of microorganisms adhering to a substrate and often embedded within a self- produced extracellular polymeric matrix. Biofilm formation can lead to harmful effects, including medical device-related infections, food spoilage, spread of foodborne diseases and biofouling of materials [1–3]. Surface engineering for preventing microbial adhesion and biofilm formation is thus a challenging question, which has fuelled an explosion of research in surface science for the development of antimicrobial and/or anti-adhesive materials by physical or chemical modifications. Much attention has been directed towards silver-based coatings [4–8], due to the broad-spectrum biocide activity of silver towards many bacterial, fungal and viral agents at low concentrations [9]. In particular, plasma-assisted nanosilver technology was shown to achieve the desired structural and functional film properties for limiting bacterial adhesion and biofilm formation [10–12]. Indeed, in previous studies [13–15], a plasma-based synthesis process was developed, associating silver sputtering and simultaneous Plasma Enhanced Chemical Vapour Deposition (PE-CVD), in an argon-hexamethyldisiloxane (HMDSO) plasma, using an asymmetrical radio-frequency discharge at 13.56 MHz. A series of plasma-deposited thin films (~175-nm thickness), containing metallic silver nanoparticles embedded in an organosilicon matrix and showing controllable properties (silver content, nanoparticle size, matrix composition) were synthesized, considering AISI 316L stainless steel as the underlying substrate. The coating antimicrobial action was established against the target eukaryotic microorganism *Saccharomyces cerevisiae* [15]. Based on

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NanoSIMS50 elemental mapping, a quite homogeneous distribution of released silver all over the yeast cell was observed after exposure, overlapping with sulfur and phosphorous signals. This was consistent with synchrotron Fourier transform infrared (sFTIR) microspectroscopy, showing an alteration of the cell proteinaceous structure (i.e. loss in α-helix structures of peptide bonds) [14]. Using a shear stress flow chamber under well-controlled hydrodynamics, Saulou et al. [15] further demonstrated the coating anti-adhesive properties towards *S. cerevisiae*, with a maximal efficacy in cell detachment achieved for the matrix without embedded silver nanoparticles. Such properties were confirmed on the Gram-negative rod-shaped *Escherichia coli* bacterium, albeit to a lesser extent [16]. Furthermore, different coating-associating subpopulations under shear flow were characterized (tethered, lying flat, laterally moving and rotating bacterial cells), with contributions closely related to the coating properties and detachment profiles [16].

Despite these consistent studies, the antimicrobial activity of the plasma-deposited nanocomposite polymer-silver on both adhering and biofilm-forming bacterial cells, in relation with the evolution of its structural properties with time, remained to date unknown.

Within this framework, the present work aimed at evaluating and comparing the antimicrobial efficacy of the coating towards Gram-negative (*E. coli*) and Gram-positive (*Staphylococcus aureus*) bacteria, together with characterizing its surface properties in native and aged forms. First, the coating antibacterial property was evaluated on adhering *E. coli* and *S. aureus* bacterial cells, using both indirect (plate counting) and in situ (fluorescent bacteria labelling) methods. The film efficacy on the inhibition of *E. coli* biofilm growth was then evaluated, by combining fluorescent bacteria labelling and Confocal Laser Scanning Microscopy. In both approaches, bare stainless steel was considered as the control. To support the temporal evolution of its anti-fouling properties, a thorough characterization of the coating was performed before and after ageing by combining a set of analytical techniques. To this end, different ageing conditions were tested: (i) after the antimicrobial test (i.e. in the biological suspension) and (ii) after short (few hours) or longer-term (two weeks) exposure in saline solution (NaCl 150 mM).

2. Material and methods

2.1. Synthesis of the plasma-deposited coating

Austenitic AISI 316L stainless steel with a mirror polished finish (APERAM, France) was used as the substrate in the form of square coupons (2×2 cm^2) for plasma-deposited coating.

The plasma deposition process for organic Ag–SiCO:H films, described in detail elsewhere [13,15], uses a dual strategy based on sputtering of silver target and simultaneous PE-CVD in an argon-HMDSO plasma, using HMDSO-pulsed injection in an asymmetrical radiofrequency (RF) discharge at 13.56 MHz. Briefly, a RF power of 85 W, corresponding to a self-bias voltage (V_{bias}) of −791 V, was applied through an impedance-matching network. The substrate holder electrode was driven by a RF power input of 3 W, leading to a self-bias voltage of −40 V, whereas the reactor walls were grounded. Pure argon, used as a vector gas, was injected in the reactor at a flow rate of 3.1 sccm and a constant partial pressure of 5.33 Pa measured using an MKS baratron gauge. The balance between silver sputtering and plasma polymerization was monitored through a pulsed HMDSO mass flow rate fixed at 0.4 sccm, within a period T of 5 s (with $T = T_{ON} + T_{OFF}$). The T_{ON} parameter, which is the duration of HMDSO injection (here 1.6 s), favours polymerization whereas T_{OFF} enables target sputtering [13]. The maximal total pressure during HMDSO injection was 5.74 Pa. The duration for plasma deposition was set to 10 min. Under these operating conditions, the nanocomposite coating was constituted of spherical metal nanoparticles (average diameter of 7 nm), homogeneously embedded within the firmly adherent, inert and sufficiently hard organosilicon matrix [13,15]. It exhibited a thickness of about 175 nm and a silver atomic concentration close to 20 at.%, as assessed by XPS [15].

Prior to any testing, bare and plasma-coated stainless steel samples were chemically cleaned as follows: surfaces were first degreased in an ethanol/acetone (50/50 volumic ratio) (Prolabo, Rectapur®) bath for 5 min. The samples were then rinsed three times in distilled water at 50 °C for 1 min and 5 times in distilled water at room temperature for 1 min. Coupons were dried and stored in sterile Petri dishes until use.

2.2. Analytical characterization of the plasma-deposited coating before and after ageing

A series of analyses was performed on the plasma-deposited coating in its native form and after the antimicrobial test (see Sections 2.4.1. and 2.4.2.). Additional experiments on film ageing were also carried out by immersing plasma-coated samples in saline solution (NaCl 150 mM) for short (6 h) or longer-term (2 weeks) exposure.

The experimental procedures have been described in details elsewhere [15]. Briefly, Scanning Electron Microscopy (SEM) was carried out by a SEM-FEG JEOL JSM 6700 F microscope on the coating deposited on intrinsic silicon substrate. Transmission electron micrographs (TEM) were also obtained with a TEM-FEG JEOL JEM 2100 F, equipped with EDS Quantax microanalysis system (BRUCKER) for Energy Dispersive X-ray Spectroscopy (EDS) analysis. For X-ray Photoelectron Spectroscopy (XPS), a Thermo Electron Escalab 250 spectrometer with a monochromated AlK_α radiation (1486.6 eV) was used. The silver atomic percentage in the layer was determined by Rutherford Backscattering Spectrometry (RBS). Surface wettability of the coating was determined by the sessile drop technique, employing a Digidrop goniometer (Contact Angle Meter — GBX Scientific Instruments), coupled with the WinDrop^{++} software to capture and analyse images. The results given are the average of at least five water contact angle measurements per sample.

2.3. Bacterial strains, growth conditions and preparation of suspensions

The Gram-negative *E. coli* K12 MG1655 strain and the Gram-positive *S. aureus* MW2 strain were used in this work. *E. coli* and *S. aureus* bacterial cells were grown at 37 °C, under agitation (100 rpm) in Luria-Bertani (LB) and Tryptic Soy Broth (TSB) media, respectively. Bacteria were harvested at the stationary growth phase by centrifugation (5 min, 6000 g, 4 °C) and finally washed twice in saline solution (NaCl 150 mM). Bacterial suspensions were immediately used for antimicrobial tests, comparing bare and plasma-coated stainless steel (see below).

2.4. Evaluation of antimicrobial property of the native plasma-deposited coating towards adhering E. coli and S. aureus bacterial cells

2.4.1. Adhesion experiments

The Japanese Industrial Standard JIS Z 2801:2000 (identical to ISO 12296:2007) [17] specifies the testing method to evaluate the antimicrobial activity of a given substrate against bacteria in contact with it. According to this method, bare and plasma-coated stainless steel samples in their native form were placed in sterile Petri dishes, then inoculated with 100 μL of the bacterial suspension, adjusted at a cell concentration comprised between 2.5 and 5.0×10^8 colony-forming units per millilitre (cfu/mL). The preparation was covered with a piece of polyethylene film to spread the inoculum over the surface and to avoid drop evaporation. The Petri dishes containing inoculated samples were incubated for 4 h, at a temperature of 35 °C ± 1 °C and a relative humidity of at least 90%.

2.4.2. Plate counting of bacteria: indirect method

According to the JIS Z 2801:2000 standard, immediately after inoculation and at the end of the 4-h incubation period, control samples (bare stainless steel), antimicrobial samples (plasma-coated stainless steel)