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Antibiofilm activity of a monolayer of silver nanoparticles anchored to an amino-silanized glass surface

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ABSTRACT

Biofilm production is the crucial pathogenic mechanism of the implant-associated infection and a primary target for new anti-infective strategies. Silver nanoparticles (AgNPs) are attracting interest for their multifaceted potential biomedical applications. As endowed with highest surface/mass *ratio* and potent antibacterial activity, they can profitably be applied as monolayers at biomaterial surfaces. Desirably, in order to minimize the risks of toxic effects from freely circulating detached nanoparticles, AgNPs should firmly be anchored to the modified biomaterial surfaces. Here we focus on a newly designed glass surface modified with AgNPs and on its antibiofilm properties. Link of a self-assembled monolayer of AgNPs to glass was obtained through preliminary amino-silanization of the glass followed by immersion in an AgNPs colloidal suspension. Static contact angle measure, AFM, TEM, UV-Vis spectroscopy, ICP atomic emission spectroscopy were used for characterization. Antibiofilm activity against the biofilm-producer *Staphylococcus epidermidis* RP62A was assayed by both CFU method and CLSM. Performances of AgNPs-glasses were: i) excellent stability in aqueous medium; ii) prolonged release and high local concentration of Ag^+ without any detaching of AgNPs; iii) strong antibiofilm activity against *S. epidermidis* RP62A. This AgNPs surface-modification can be applied to a large variety of biomaterials by simply depositing glass-like SiO_2 films on their surfaces.

1. Introduction

Biofilm-associated infections are still the leading cause of implant failure [1,2]. New tactics are being devised and new weapons designed to combat implant infections. Researches are more and more focussing on the development of innovative biomaterials endowed with anti-infective properties, applying ground-breaking technologies to finish biomaterial surfaces with films or layers repellent to bacteria or even bactericidal [3,4].

The use of silver nanoparticles (AgNPs) as antimicrobial agents has aroused lively interest in recent years [5–12]. Although the debate concerning the mechanisms by which AgNPs exert their antibacterial action is still underway, it is generally accepted that their mechanism of action involves a release of Ag^+ ions and the following interaction of Ag^+ with bacteria. Recently, a hypothesis has been formulated, by us and other authors, suggesting that the antibacterial properties of AgNPs can be ascribed to a short-distance nanomechanical action involving their direct interaction with the bacterial cell membrane [13,14].

Anti-infective surfaces based on AgNPs can be achieved by different strategies. The "layer-by-layer" (LbL) approach [15] has been proposed to obtain surfaces on which thin films/layers of AgNPs are deposited or formed as a molecular self-assembled monolayer (SAM) [16,17]. In some cases, an antibacterial activity of this kind of surfaces has been demonstrated [8,18]. Nonetheless, the increase of AgNPs applications has raised concerns for the potential health risks posed by the exposure to nanoparticles [5,19].

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This is also due to the fact that, with a few exceptions [11,19], NPs layers appear to be weakly bound to the surface, with serious risks of a NPs release in the *milieu* of their application, *i.e.*, in perspective, in the fluids and tissues of the human body.

A very recent study on immune-toxicity of AgNPs in an animal model demonstrated that the immune system is highly sensitive to the toxic effects of free AgNPs. After intravenous administration of high doses of AgNPs, the authors observed the almost complete suppression of the natural killer cell activity in the spleen, together with the decrease of various serum cytokines and the increase of neutrophil granulocytes in blood [20].

These observations and considerations are the reason of our efforts in searching for well-grounded (and cost-effective) strategies to prepare a monolayer of AgNPs steadily linked on a suitable surface. The obvious goal should be to keep nanoparticles confined to the surface, obtaining a valid antibacterial activity due to the release of the only Ag^+ ions. Consequently, in the case of implants, catheters, artificial organs, and other medical devices, this kind of surface functionalization should be able to fight in particular bacterial colonization and subsequent biofilm formation, through contact-killing activity.

And indeed, the crucial aspect in the pathogenesis of the implant-associated infection is the ability of bacteria to grow on a biomaterial as a biofilm, "a sessile community of bacterial cells that is embedded in an extracellular polymeric matrix" [21]. Biofilm formation by staphylococci—the first aetiological agents of implant infections [1] - includes several sequential steps in which, first, planktonic bacteria attach by their adhesins to the material surface [22]. Then, they proliferate and produce an extracellular polymeric matrix consisting of various macromolecular components (besides the well-known polysaccharide intercellular adhesin [PIA], also proteins and extracellular DNA [2,23]), where bacteria accumulate encased in multi-layered clusters, thus forming a well-sheltered colony able to resist antibiotics [21,24] and escape immune defences [25]. Once the biofilm structure has developed and matured, some bacteria detach and disperse in the surrounding medium, enabling the biofilm to spread at distance over the biomaterial surface, or within fluids and tissues [2]. Therefore, weapons able to prevent or contrast early adhesion/colonisation by viable bacteria on the material surface may also hamper biofilm formation, finally resulting in a decrease of the risk of infection [26].

In the perspective of a practical application, the formation of a monolayer/film of AgNPs on an appropriate surface could reduce the amount of the expensive material to be used (silver) and, at the same time, of the required amount of NPs to be applied onto the device. It could be underlined again that a permanent binding of NPs to the material surface is required to ensure the proper stability of the antimicrobial coating together with the right level of biological safety. It should also be remarked that Ag^+ ions released by AgNPs in body fluids were usually not enough to exert toxic effects on eukaryotic cells, while nanosilver, acting as a Trojan horse, enters eukaryotic cells and, in the limited and sensitive inside of the cell, becomes a potent source of Ag^+ ions able to interact with cell molecules and damage cell functions [27].

In the present work, we aim at preparing antibacterial surfaces bearing small and well-defined quantities of well-bound AgNPs. We present a very simple, quick and cost-effective technique to link nanoparticles on glass, involving the preliminary amino-silanization of the glass surface followed by the link of a SAM of AgNPs.

We also aim at characterising the total quantity of silver brought on the glass surface and the stability of the SAM of AgNPs in aqueous environment. Finally, we aim at testing the *in vitro* influence of these newly synthesized surfaces on the biofilm formation by a reference biofilm-producer *Staphylococcus epidermidis* bacterial strain.

2. Materials and methods

2.1. Materials

Silver nitrate (>99.8%), sodium borohydride (≥99.0%), sodium citrate (>99.0%), (3-aminopropyl)triethoxysilane (>99%, APTES) and PBS were purchased from Sigma–Aldrich. Reagents were used as received. Microscopy cover glass slides (21 × 26 mm), and round glass slides (10 mm diameter) were purchased from Forlab (Carlo Erba). Glass cuvettes were standard optical glass cuvettes purchased from Hellma. Water was bidistilled, prepared from deionized samples.

2.2. Syntheses

Fig. 1 reports schematically the procedure followed to obtain the preliminary silanization of glass surface with (3-aminopropyl)triethoxysilane (APTES) and the subsequent link of the SAM of AgNPs by means of non-covalent interactions between $-NH_2$ groups and silver atoms. Steps are analytically described in the following paragraphs.

2.2.1. Nanoparticles preparation

Silver nanoparticles were prepared essentially as described in [28]. To 100 mL of ice cooled water the following ice-cooled solutions were added in sequence under vigorous stirring: 1 mL of 1% (w/v) $AgNO_3$ solution, after a minute 1 mL of 1% (w/v) sodium citrate and, after a further minute, 0.75 mL of a solution 0.075%w in $NaBH_4$ and 1%w in sodium citrate. After the last addition, stirring was immediately stopped, in order to avoid coagulation. The colloidal suspensions were stored in the preparation flask, maintained in the dark and used within 2 days from preparation.

2.2.2. Preparation of a (3-aminopropyl)triethoxysilane SAM on glass surface

Cover glass slides were cleaned with piranha solution and then washed three times with ultrapure water under sonication. Glasses were then immersed for 5 min in a 5% (v/v) solution of (3-aminopropyl)triethoxysilane in ethanol and kept thermostatted at 60 °C. In a typical preparation with rectangular (26 × 21 mm) samples used for contact angle, AFM, UV-Vis and ICP-OES characterizations, 8 glass slides were prepared at the same time, *i.e.* reacting in the same APTES solution inside an 8-place glass slides holder (where the slides were kept in a vertical position), thermostated and gently shaken on a Heidolph Promax 1020 reciprocating platform shaker. After this, the amino-modified glasses were washed three times under sonication with ethanol. Finally, they were dried under a nitrogen stream. For the round glass samples used for biofilm evaluations, the same procedure was used, using a single glass test tube filled with 2 mL of the APTES ethanol solution for each sample. A similar strategy was applied to the preparation of modified standard glass cuvettes, which were filled with the described solutions and treated in the same way.

2.2.3. Silver nanoparticle monolayer preparation

Amine-modified glasses were immersed into the colloidal suspension of AgNPs and kept at 30 °C for 15 min. In a typical preparation, 8 glass slides were prepared at the same time, *i.e.* they reacted in the same AgNPs suspension solution, inside an 8-place glass slides holder (where the slides were kept in a vertical position), thermostatted and gently shaken on a Heidolph Promax 1020 reciprocating platform shaker. After this, the obtained yellow glasses were placed in water and sonicated for 5 min. This procedure was repeated twice, and then the glasses were dried under a nitrogen stream and stored in air. For the round cover glasses used for biofilm evaluations (10 mm diameter), the same procedure was used, using a single glass test tube filled with 2 mL of AgNPs colloid for every sample. A similar strategy was applied to the preparation of modified standard glass cuvettes, which were filled with the AgNPs colloid and treated in the same way.

2.2.4. Characterization of the samples

Absorbance spectra of colloidal suspensions were taken with a Varian Cary 100 spectrophotometer in the 200–900 nm range. Spectra of NPs-functionalized glasses were obtained placing the glasses on the same apparatus equipped with a dedicated Varian solid sample holder. Measure of absorbance *versus* time on modified cuvette was carried out by filling the cuvette with bidistilled water and keeping it stoppered and in the dark between successive spectra.

Transmission electron microscopy (TEM) images were obtained on colloidal solutions of AgNPs prepared as described and diluted 1:10 with bidistilled water, deposited on Nickel grids (300 mesh) covered with a Parlodion membrane and observed with a Jeol JEM-1200 EX II instrument.

Atomic Force Microscopy (AFM) images were taken from an Auto Probe CP Research Thermomicroscopes scanning system in tapping mode with Au coated Si probe with a typical spring constant $k = 1.74\ Nm^{-1}$ (NSG03 probes from NT-MDT). Images were analysed using Image Processing 2.1 provided by Thermomicroscopes.

Static contact angle determinations were made with a KSV CAM200 instrument, with the water sessile drop method.

The total Ag content on glasses with NPs monolayers was determined by quantitatively oxidizing the silver NP link on a single slide (21 × 26 mm) by dipping it in 3 mL ultrapure concentrated HNO_3 diluted 1:5 with water (13% was the final concentration) in a vial, and keeping it overnight at RT on a Heidolph Promax 1020