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

Angelo Taglietti a,***, Carla Renata Arciola b,c,*, Agnese D’Agostino a, Giacomo Dacarro a, Lucio Montanaro b,c, Davide Campoccia b, Lucia Cucca a, Marco Vercellino d,f, Alessandro Poggi e,f, Piervando Pallavicini a, Livia Visi a,***

a Inorganic Nanochemistry Laboratory, Department of Chemistry, University of Pavia, Pavia, Italy
b Research Unit on Implant Infections, Rizzoli Orthopaedic Institute, Bologna, Italy
c DIMES, University of Bologna, Bologna, Italy
d Center for Tissue Engineering (C.T.E.), Department of Molecular Medicine, INSTM UdR of Pavia, University of Pavia, Pavia, Italy
e Pediatrics Unit, Department of Mother and Child, University of Modena and Reggio Emilia, Modena, Italy
f Nanotechnology Laboratory, Department of Occupational Medicine, Ergonomy and Disability, Salvatore Maugeri Foundation, IRCCS, Pavia, Italy

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A B S T R A C T

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 SiO₂ films on their surfaces.

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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].
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 aetiologic 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 control 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–Alrich. 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 –NH₂ 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) AgNO₃ solution, after a minute 1 mL of 1% (w/v) sodium citrate and, after a further minute, 0.75 mL of a solution 0.07235% in NaBH₄ and 15% 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 colloidal for every sample. A similar strategy was applied to the preparation of modified standard glass cuvettes, which were filled with the AgNPs colloidal 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 covette was carried out by filling the covette 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 Jem 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 HNO₃ diluted 1:5 with water (13% was the final concentration) in a vial, and keeping it overnight at RT on a Heidolph Promax 1020...
reciprocating platform shaker. The Ag content in solution was then determined by Inductively Coupled Plasma (ICP) atomic emission spectroscopy. Release of Ag$^+$ versus time was followed on a set of 8 AgNPs-modified glass slides ($21 \times 26$ mm coated on both sides, total coated surface $= 10.92 \text{cm}^2$) prepared as described above. Each slide was then immersed in 3 mL of ultrapure water. Slides were taken off the water after 1, 5, 7, 24, 168, 240, 360 and 456 h, the content of Ag$^+$ in the 3 mL water volume was determined by ICP. Measures were repeated three times, and mean values are given. ICP data were collected with an ICP-OES OPTIMA 3000 Perkin Elmer instrument.

2.3. Bacterial assays

2.3.1. Bacterial strain and culture condition

In this study S. epidermidis RP62A, which is known as a strong biofilm-producing strain, was used. S. epidermidis RP62A was a gift from Tim Foster (Department of Microbiology, Dublin, Ireland) and was routinely grown overnight in tryptic soy broth (TSB, Difco, Detroit, MI, USA) under aerobic conditions at 37 $^\circ$C. The bacterial suspension was adjusted to obtain a final turbidity of 10 nephelometric turbidity units (NTU). The titre of the suspension was further analysed by means of contact angle measure, which showed a significant and reproducible change after silanization. After cleaning and silanol activation performed in piranha solution, static water contact angle of glass samples is less than 10$^\circ$, because of high surface concentration of –OH groups. After silanization with APTES (NH$_2$ SAM) [30], the resulting stacks of images were analysed using Leica confocal software.

3. Results

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

Efficiency and repeatability of the silanization procedure were checked by means of contact angle measure, which showed a significant and reproducible change after silanization. After cleaning and silanol activation performed in piranha solution, static water contact angle of glass samples is less than 10$^\circ$, because of high surface concentration of –OH groups. After silanization with APTES (see experimental details), a sensible increase in hydrophobicity was obtained, showing a mean value of 55$^\circ$, which is in good agreement with data reported in literature for the formation of an –NH$_2$ SAM [30].

3.2. AgNPs preparation

AgNPs typically display an LSPR (Localized Surface Plasmon Resonance) band, observed in absorption spectroscopy both for colloidal suspensions and for nanoparticles monolayers [31,32]. Bandwidth, intensity and position of the LSPR bands are related with the size and shape of the nanoparticles [33], the stabilizing agents covering the NP surface, and the dielectric constant of the
surrounding medium [34]. In the case of spherical silver nanoparticles with size < 50 nm of diameter, this band is around 400 nm [34].

In the preparations used in this work, the spectra of citrate-stabilized silver NPs showed an LSPR peak centred at \( \lambda = 392 \) nm in aqueous solutions. Preparations were repeated several times, giving reproducible results. TEM images were collected and sizes of AgNPs were calculated for four different images, coming from two preparations, giving a mean value of 9 ± 4 nm diameter. Fig. 2 shows representative TEM images of AgNPs colloid used for glass coating, with details of particles count. AgNPs colloids can be stored in glass flasks. Stability of NPs was monitored recording several spectra during 4 weeks, showing no detectable changes of the LSPR peak.

3.3. Silver NPs SAM

After immersion of amine-modified glasses into the colloidal suspension of AgNPs, and after washing and sonication in order to remove unlinked AgNPs, the slides were dried with a nitrogen stream. Spectra of silver nanoparticles linked on APTES-modified glass slides were then measured in air. In this case the LSPR peak was found to be at the average \( \lambda_{\text{max}} = 396 \) nm (\( \sigma = 4 \) nm).

As an example, in Fig. 3 is reported the result (as UV-Vis spectra) of a typical synthesis involving the functionalization of 8 glass slides obtained simultaneously from the same freshly prepared silver NP solution: as can be clearly seen, no differences in intensity (or position) of the LSPR peak can be observed among the samples. Other three set of UV-Vis spectra were obtained from other 3 identical preparations (see Fig. 4), demonstrating the reproducibility of the method. And indeed we obtained reproducible \( \lambda_{\text{max}} \) values and we did not observed significantly different intensities of the LSPR band between different glass samples deriving from the same preparation, and just very small differences between samples coming from different preparations could be noted. Samples with an LSPR-band intensity outside of the standard deviation of the average absorbance were discarded. For example, in a set of 4 preparations of 8 samples each (shown in Figs. 3 and 4) a total of 3 samples over 32 were discarded.

Moreover, contact angle measure was performed for two samples taken from every eight-sample preparations, showing a strong decrease in hydrophobic character, which should produce favourable electrostatic interactions between bacteria and functionalized surface.

AgNPs-modified glass slides were stable in air, i.e. their spectra did not change significantly in a 3-week period. Atomic force microscopy images were taken from a freshly prepared AgNPs-modified glass, after drying (see Fig. 5A).

Although average dimensions and polydispersity of NPs cannot be calculated precisely by the software because of the high density of objects, on these AFM images the dimensions of NPs on the surface appear larger than those observed in the parent colloidal solution (as deduced from TEM data). The analysis of the profiles on
500 × 500 nm images yielded a statistic value of the average diameter of 28 ± 6 nm. This size increase of AgNPs in comparison with those obtained from TEM images is only apparent. In tapping-mode AFM convolution of the finite tip size (10 nm curvature radius in our case) with true surface morphology is known to result in overestimated in-plane dimensions of an object, if its width is comparable with the tip curvature (apparent > 3 × real dimensions) [35]; thus, object with 9 nm diameter, should have an apparent diameter of at least 27 nm. On the other hand, as expected [35,36], the measure of average particle height from line profile analysis gives an average value $h = 7.3$ nm ($±1.6$ nm), consistent with the mean diameter value determined by TEM ($9 ± 4$ nm), considering that the AgNPs are partially “immersed” in the organic monolayer.

**Fig. 4.** a–c. Sets of UV–Vis Spectra of modified glasses obtained in three different 8-piece preparations.

**Fig. 5.** (A) AFM image taken from a freshly prepared glass slide grafted with AgNPs after silanization with APTES and (B) AFM image of the same glass slide kept in bidistilled water for 20 days.
Count of the objects on a 250 × 250 nm image (see Fig. 6) shows the presence of about 130 objects, allowing the calculation of a concentration of 2.1 × 10^11 NPs/cm².

We completed the characterization of the linked NPs monolayer by means of quantitative oxidation of the AgNPs and analysis of the obtained Ag⁺ solution by means of ICP. From 7 experiments coming from 3 different preparations we found an average of 7.3 × 10⁻⁷ g/cm² (σ = 1.2 × 10⁻⁷) of silver. From this data, considering AgNPs of 9 nm in diameter (volume = 381.51 nm³, mass = 4 × 10⁻¹⁸ g, using dAg = 10.49 g/cm³), a surface concentration of 1.83 × 10¹¹ nanoparticles/cm² (corresponding to 114 NPs in an area of 250 × 250 nm) can be calculated, nicely fitting with the number derived from counts on AFM images (2.1 × 10¹¹ NPs/cm²). Thus, ICP data, together with the observation of AFM images, clearly indicate that a single layer of AgNPs is obtained on our modified glass surfaces, and that the procedure allows to link to glassy surfaces a controllable and reproducible amount of AgNPs.

3.4. Stability of the NPs SAM and Ag⁺ release

The quantity of released Ag⁺ in water as a function of time was determined by an experiment on a set of 8 AgNPs-modified glass slides (see materials and methods). The 21 × 26 mm glass slides were immersed in 3 mL of pure water. The solutions obtained after glass slide immersions were analysed with ICP. The data of silver concentrations found in these solutions were expressed as µg of released Ag⁺/cm² of exposed surface as a function of time (Fig. 7), and showed a well-defined trend. It is important to state that these experiments were performed in ultrapure water and not in physiological buffer (e.g. PBS), as the ICP analysis cannot be performed in such kind of media with strong salinity. Anyway, even if the results indicated by the ion-release curves that are typically measured in pure water cannot be immediately transferred to more complex or biological environments, as silver ions can give poorly soluble salts or complexation with biomolecules when they are released in "real" media [37], we can observe that the values reported allow to state resolutely that: i) silver ions are progressively released in water as a function of immersion time, reaching a stationary concentration after approximately 3 days; ii) 80% of dissolved silver ions are released in the first day of immersion; iii) the maximum amount of silver ions released in these conditions is about 16% (1.2 × 10⁻⁷ g/cm²) of the total quantity of silver initially bound on the surface (7.3 × 10⁻⁷ g/cm²) as determined by quantitative oxidation and ICP analysis. To further study the behaviour of AgNPs monolayers when exposed to water as a function of time, we used a standard glass cuvette functionalized on the internal walls with AgNPs using the same procedure described for glass slides, and examined the spectroscopic behaviour of the monolayer in contact with water for days. The cuvette was filled with bidistilled water, closed with a Teflon stopper and stored at RT for a period of 19 days. During this time, UV-Vis spectra were taken at intervals similar to those used for the Ag⁺ release measure performed with ICP. It is important to notice that their LSPR peak is more intense as compared to spectra of the same samples in air, and shifted to approximately 412 nm, as a consequence of the change of the effective refractive index of the medium surrounding the AgNPs.

It can be observed (Fig. 8) that only a small variation (about 12% in intensity of LSPR band after 19 days, no changes in LSPR peak position) in spectra features is produced in the investigated time, taking place mainly in the first days, with a behaviour which seems related to the release silver ions. It has also to be noted that a final spectra taken on the solution extracted from the coated cuvette after the 19-day water contact did not show any presence of LSPR absorption, ruling out AgNPs measurable detachment from cuvette walls. The spectra observation immediately suggests: i) the absence of detachment of AgNPs from glass cuvette walls; ii) the absence of changes in morphology or dimensions of AgNPs.

These observations well fit to the AFM images obtained on AgNPs-functionalized glass slides kept in bidistilled water for 19 days, under the same conditions (Fig. 5B). The AFM images show an AgNPs layer approximately identical to those obtained for a modified glass before immersion in water (Fig. 5A), both in the morphological and dimensional features of the AgNPs-modified surface.

This behaviour can be explained with the formation, in the initial 3-day period, of an Ag₂O layer on the water-exposed part of the surface of AgNPs linked to glass and placed in an aqueous environment, reaching a steady-state in which the slowly released Ag⁺ ions are replaced by Ag oxidized from the bulk.

3.5. Antibiofilm activity

Antibiofilm activity of control materials and of AgNPs-glass specimens was assayed with S. epidermidis RP62A, a strain capable

Fig. 6. AFM image taken from a freshly prepared glass slide covered with AgNPs after silanization with APTES: count of objects on a 250 × 250 nm area, showing the presence of 131 objects.

Fig. 7. Silver ion released (µg/cm²) of exposed surface versus time in 3 mL water for AgNPs coated glass slides (21 × 26 mm).
of producing a PIA-dependent biofilm (Fig. 9). As clearly reported in Fig. 9A, significantly lower value of cell survivability was obtained from the AgNPs-glasses when compared with the control glasses: cell survival on the AgNPs-glasses was $10^2$ times lower than that on the control glasses. This finding corresponds to the results of ICP analysis, showing that AgNPs coatings are readily available to react with water and to release the Ag⁺ ions. Therefore, we deduced that Ag⁺ interactions either with DNA or with the proteins of the bacterial cell-wall [38,39] led to cell death, as emerged from cell viability assay. To confirm the effect of AgNPs on bacterial viability, staphylococcal biofilms of both controls and Ag-modified materials were studied by CLSM (Fig. 9B and C). The robust S. epidermidis biofilms grown on the control materials presented a green (in the web version) fluorescence and appeared organized as multilayered aggregates (Fig. 9B).

Bacteria close to Ag-glass mostly showed red (in the web version) fluorescent (dead) cells and only a few green (viable) cells (Fig. 9C), and appeared much more dispersed than those on the control material (Fig. 9B). In particular, staphylococcal cells more in proximity to the surface of the AgNPs-modified glass were all dead (red), as clearly shown by the sagittal projections.

4. Discussion

Owing to its known ability to express bactericidal/bacteriostatic activity even at very low concentrations, silver is certainly the metal most used to confer anti-infective properties to biomedical devices. The use of silver as a bulk material has been dropped over time, while its utilisation for coating or doping solid or hydrogel materials, or for alloying with other metals, has been more and more thrived. Nowadays, silver has become one of the most largely used anti-infective materials.

Silver is considered able to exert its antimicrobial activity by several mechanisms, of which those till now recognized have been surveyed in a recent review [40]. Briefly, Ag⁺ ions interact with sulphur- or phosphorus-containing groups belonging to proteins of bacterial cell wall or plasma membrane, create membrane holes by which cytoplasmic content flows out of the cell, and then cause bacterial cell death. Inside the microbial cell, Ag⁺ ions inhibit cytochromes of the electron transport chain, bind to and damage DNA, RNA and ribosomes, and also lead to the formation of reactive oxygen species (ROS), which are toxic to both bacterial cells and eukaryotic host cells.

Interestingly, bacterial resistance to silver is rare and develops slowly in comparison to the resistance to antibiotics. This is presumably due to the multiple antimicrobial mechanisms mediating silver bactericidal activity, whereas antibiotics have usually only one mechanism of action.

AgNPs have been designed and introduced as a new generation of antimicrobials, especially devoted to be used as coatings [41]. Nanosilver is presumed to be more active than bulk silver materials as it should be able to reach bacteria in tightest proximity at a highest surface/mass ratio, thus producing higher local concentrations of Ag⁺ and, consistently, higher bactericidal effects [42].

A major criticism related to the use of AgNPs in preventing/combating biomaterial-associated infections is the potential risk of cytotoxic effects on eukaryotic cells. Intriguingly, in a quite different context, beneficial effects related to the cytotoxicity of AgNPs on eukaryotic cells have recently emerged. Both generation of ROS and release of silver ions by free AgNPs have been demonstrated to be exploitable against human acute myeloid leukaemia (AML) cells. An anti-leukaemia effect against multiple cell lines and primary isolates from AML patients has been shown [43].

Conversely, when, as in the present case, NPs are strongly anchored to the material surface, cytotoxic effects on peri- and para-prosthesis cells and tissues can be expected to be negligible, or nearly absent for the tissues non in direct contact/distant from the surface of the biomaterial.

Mechanisms by which AgNPs exert their antibacterial effect have been for long debated, dealing with the question whether the AgNPs themselves can directly damage bacteria, by a “particle-specific” action, besides silver ions. Xiu et al. showed that AgNPs are devoid of bactericidal effects when synthesized and tested under strictly anaerobic conditions precluding Ag⁰ oxidation and Ag⁺ release. They concluded that the antibacterial activity of AgNPs has
to be ascribed solely to the Ag⁺ release, which could be modulated through manipulation of oxygen availability, particle size, shape, and/or type of coating [44].

As far as prostheses are concerned, AgNPs have been added to various types of biomaterials [11,13,45–50], to realize composite materials endowed with bactericidal abilities. For example, AgNPs have been mixed with chitosan NPs in hybrid nanocomposite particles, as “raisins” (silver nanoparticles) in a “pudding” (chitosan) [49]. However, just the chitosan, which should both add its antimicrobial activity to that of AgNPs and, moreover, be useful in the prevention of AgNPs agglomeration [51], has been imputed to alter the AgNPs properties [41].

In regard to the AgNPs embedded in bone substitute materials, such as the AgNPs generated in situ within polymethyl methacrylate [50], or the AgNPs incorporated with calcium phosphate cements, although they are opening interesting perspectives for the surgical orthopaedic field, they have shown till now only a weak ability to release Ag⁺ ions. And in fact, plunged as they are in a matrix material, they are prevented from exposing their surfaces to the bone–biomaterial interface, i.e., after all, the sites where infection actually starts. But, during the degradation of the composite material, AgNPs are released and come into close contact with blood cells, penetrate them, and form aggregates into cytoplasm [52].

To decrease AgNPs toxicity, peptides have been proposed as capping agents. However, undesirably, peptide-coated AgNPs undergo agglomeration in response to changes in pH, as those occurring in hypoxic or inflamed tissues [53]. Agglomeration poses serious limits in medical applications of AgNPs. Moreover, it has been pointed out that capping peptides could even turn out anti-genic and trigger an (unwanted) immune response [41].

Monolayers of AgNPs on glass via the interaction with thiolos through the formation of a SAM on (3-mercaptopropyl)trimethoxyxysilane have been achieved [45]. Similar layers have been obtained starting from amino-terminated silanes [11,46–48]. These and other pioneering applications of AgNPs-coatings have encountered a favourable consideration, although weak points have been sometimes recognized in the transience and rapid lowering of the antibacterial activity of silver released in physiological fluids and, once again, in the supposed cytotoxic effects that free nanoparticles detached from the anchor material surfaces could induce. In fact, the grafting of AgNPs on glass via thiols involves a covalent S–Ag bond, which, although strong in pure water, could be dissociated in the physiological reducing environment, with detaching of free NPs. On the contrary, when the link of AgNPs to glass is established via amino-silane, the NH₂–Ag bond, based on the donation of an amine ion pair, cannot be dissociated in reducing conditions. Therefore, with the link to the amino-silane, detaching of free AgNPs is averted.

The characterization of the NPs-coating here presented demonstrates that a monolayer of silver nanoparticles can be successfully immobilized on glass by the LbL technique. A satisfactory coverage of the surface (about $7.3 \times 10^{-12}$ g of Ag per cm², corresponding to about $2 \times 10^{-11}$ nanoparticles/cm²) was reached. This procedure allows to link to glass surfaces (or, in perspective, to glassy/glazed surfaces) a controlled, well-distributed, reproducible amount of silver nanoparticles.

With respect to the feared event of cytotoxic effects by free NPs, this study shows, by both physical and microscopic analyses, the stability of the AgNPs monolayer, seen in the lack of detachment of AgNPs from their anchor glass during the investigated period, as well as in the absence of variations in shape or size of the AgNPs.

From the modified glass surfaces exposed to water, about 16% of the total amount of silver loaded on the monolayer was released in solution as Ag⁺ ions. Ag⁺ release persisted for about twenty days, just the period corresponding to that in which bacterial adhesion and biofilm onset could more probably intervene after an implantation. This is also the period more suitable to face the bacteria attempts at colonizing the material surfaces, when deep-prostheses are going to integrate in the host tissues, and catheters, or other medical devices destined to be removed, perform and then complete their task. Obviously the righter the anti-infective action the lower will be the risk of an infection. Thus, by timely thwarting bacterial adhesion/colonisation, the cause of the implant septic mobilization and failure is removed, as well as catheters are prevented from becoming the source of a haemogenous spreading of bacteria deriving from biofilm dissemination [54]. There are, however, other ways in which the value of an anti-infective material can display. For instance, as above reminded, bacterial resistance to silver has been rarely seen. This is an attractive feature of silver as an anti-infective material, since the decreasing concentrations of Ag⁺ in the days after the twentieth is not expected to evoke resistance phenomena, differently from the circumstances determined when small doses of the conventional antibiotics are leached from antibiotic-loaded materials, known to potentially rouse resistance in exposed bacteria [55].

As far as the antimicrobial activity is concerned, survival of bacteria in contact with Ag-glass was found five log values lower than that in contact with the unmodified glass, thus corroborating the idea that the AgNPs coating was readily available to react with water and to release silver ions, as shown by ICP analysis. Actually, at CLSM analysis, the staphylococcal cells close to the surface of the AgNPs-modified glass appeared dead, and the poor biofilm-like substance in the proximity to Ag-glass exhibited only a scattered, extremely rarefied, and vanishing appearance, well different from the thick and dense biofilm clung to the unmodified glass. Zhao et al. have recently observed by CLSM that S. epidermidis biofilm growing on a biomaterial surface is a dynamic process able to reach a maximal thickness at 12–18 h and maturation by 24 h [56]. And indeed, in the in vitro studies of new anti-biofilm surfaces, a 24-h culture is the most frequently adopted condition [57]. In the present experimental study, density and thickness of the biofilm observed under CLSM on the unmodified glass after 24-h confirm that this culture period is suitable for the development of a mature biofilm by S. epidermidis RP62A and thus appropriate for the assessment of the modified glass anti-biofilm activity. Obviously in this context the ascertained anti-biofilm activity has to be intended sensu lato as the ability to prevent biofilm establishment, and not sensu stricto as the ability to interfere with specific pathogenic mechanisms implicated in the biofilm production [4].

The powerful and, for some aspects, unprecedented antibiofilm activity observed on AgNPs-modified glass surfaces indicates that the new approach to achieve anti-infective surfaces has considerable potential.

Noticeably, glass-like SiO₂ films can be easily deposited by gelification of a siloxane sol on a large variety of materials, to which the LbL procedure here described could be accordingly and conveniently applied. Thus, by glazing the material surface and then immobilizing AgNPs on the glazed surface, antimicrobial properties can be easily conferred also to a bulk material not pristinely glassy.

The coating strategy here described could be turn out useful to cover the majority of medical devices, as they mainly require a short-range action near to their surface to contrast the first adhesion of bacteria on contact and the starting biofilm.

5. Conclusions

Evidences of an antibacterial/antibiofilm ability of the new AgNPs-modified glass have clearly emerged. The findings gathered
on this new AgNPs-modified material indicate that, besides exhibiting good biological performances in terms of anti-infective properties, it is endowed with valuable structural and physicochemical characteristics, i.e. a well-formed monolayer of AgNPs, an opportune stability in aqueous medium, convenient release and release-tempo of the Ag⁺ ions, and, importantly, a tenacious link of AgNPs to the glass.

Seen on the whole, the new approach can be envisaged useful to produce materials intrinsically protected from biofilm growth. Further studies are however required to confirm an adequate performance in terms of biocompatibility and to evaluate the stability of the AgNPs coating also in more complex and reactive pathophysiological environments, as those around an implant.

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