Synthesis of new antibacterial composite coating for titanium based on highly ordered nanoporous silica and silver nanoparticles

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A B S T R A C T

Infection is the most common factor that leads to dental titanium implant failure. Antibacterial implant surfaces based on nano-scale modifications of the titanium appear as an attractive strategy for control of peri-implantitis. In the present work, the preparation and antibacterial properties of a novel composite coating for titanium based on nanoporous silica and silver nanoparticles are presented. Starch-capped silver nanoparticles (AgNPs) were synthesized and then incorporated into sol–gel based solution system. The AgNP-doped nanoporous silica coatings were prepared on titanium surface using a combined sol–gel and evaporation-induced self-assembly (EISA) method. The coating nanostructure was characterized by XRD, SEM–EDX, and HR-TEM. Antibacterial activity was evaluated against Aggregatibacter actinomycetemcomitans, a representative pathogen of dental peri-implantitis. Colony-forming units (CFUs) were counted within the biofilm and at the planktonic state. Biofilm development was quantified using crystal violet staining and viability of adherent bacteria was confirmed with the Live/Dead fluorescence assay.

Silica-based composite coating containing AgNPs (AgNP/NSC) was prepared on titanium surface by direct incorporation of AgNP suspension into the sol–gel system. The self-assembly technique enabled the spontaneous formation of a highly ordered nanoporosity in the coating structure, which is a desired property for osseointegration aspects of titanium implant surface. AgNP/NSC coating produces a strong antibacterial effect on titanium surface by not only killing the adherent bacteria but also reducing the extent of biofilm formation. Biofilm survival is reduced by more than 70% on the AgNP/NSC-modified titanium surface, compared to the control. This antibacterial effect was verified for up to 7 days of incubation. The long-term antibacterial activity exhibited by the nanostructured AgNP/NSC-titanium surface against A. actinomycetemcomitans suggests that this type of nano-scale surface modification is a promissory strategy to control infections associated with dental implant rehabilitation.

1. Introduction

Multiple factors affect the clinical success of dental implants, including osseointegration of bone implants and the degree of bacterial colonization surrounding the implants [1]. Peri-implantitis is the inflammatory disease marked by bacterial infection and the destructive process affecting the soft and hard tissues around osseointegrated implants, leading to the loss of supporting bone [2,3]. Although the treatment of the infected implants is a challenge in clinical practice, its consequent loosening is still most common in implanted devices. In recent years, strategies based on the modification of the geometry and physicochemical properties of the titanium implants are being investigated to prevent or reduce their bacterial colonization [4,5]. One of these approaches consists in the fabrication of antibacterial coatings on the titanium implant surface. Antibacterial polymeric coatings loaded with antibiotics such as vancomycin and gentamicin have been prepared on titanium surfaces [6,7]. Coatings loaded with non-antibiotic organic antimicrobials (NOA) such as chlorhexidine, chloroxylenol, and polyhexamethylene biguanide have also been studied [8–10]. However, antibiotics and NOA cannot be incorporated into the traditional hydroxyapatite coatings during their formation because of the extremely high processing temperature. On the other hand, physical adsorption of these antimicrobial agents onto the surface of coatings limits the loaded amount and release characteristics [11]. Antibiotics and NOA also raise the risks by new multidrug-resistant strains (MRS). Precisely, the multidrug-resistance of microorganisms has increased [12,13], and several reports indicate that some antibiotics and NOA agents are harmful to cell functions [14,15].

Recently, studies have introduced silver nanoparticles (AgNPs) as a new antimicrobial generation for biomedical applications with many
advantages over antibiotics and NOA, such as good antibacterial activity, excellent biocompatibility, and satisfactory stability [16–18]. The study of bactericidal metallic nanoparticle is particularly timely considering the recent increase of MRS. Silver nanoparticles present unique physical, chemical and biological properties, better than micrometer size particles. Nanometric silver has a large total surface area available for bacterial interaction and exhibits a stronger antibacterial effect, which affects cellular functions as well as structures [19,20]. Current research is focused on improving the antibacterial properties of biomaterials by using silver nanoparticles. Several antimicrobial dental products based on nanosilver have been studied, such as silver-doped hydroxyapatite [21], dental adhesives [22], primers [23], and mouthwash loaded with AgNPs [24], pointing to increasing interest in the potential applications of nanosilver in the dental area. Regarding the use of silver nanoparticles to produce antimicrobial surfaces on titanium implants, Juan et al. [25] reported the preparation of nanosilver-modified titanium surfaces through a simple coating method. A silane-modified titanium surface was immersed in an aqueous suspension of silver nanoparticles. The titanium surfaces loaded with 4.26% of AgNP killed over 94% of Staphylococcus aureus and Escherichia coli in bacterial suspensions. Although bacterial inhibition of the material surface was not quantified, SEM examination revealed that an important decrease in the bacteria adhesion occurred on the AgNP-modified titanium surface. Despite the promising antibacterial activity, silver nanoparticles were sparsely deposited on the titanium surface, forming aggregates, and probably weakly bonded to the silane-modified titanium surface.

In a previous work, we reported the synthesis and bioactive properties of a nanostructured porous silica coating on titanium surfaces by a combined sol–gel and evaporation-induced self-assembly process [26]. This silica coating with highly ordered sub-10 nm porosity improves titanium osseointegration, speeding up the osteoblasts’ adhesive response and promoting the osteogenic differentiation of stem cells; probably due to mechanical stimulus from the nanostructured topography. We hypothesize that an adequate incorporation of AgNPs into this nanoporous silica coating could generate a surface with antimicrobial properties oriented at preventing peri-implant infection. Sol–gel technique offers finer control of the nanostructure [27], as compared to previously reported methods for incorporating AgNPs on titanium surface. Sol–gel coatings enable a more homogeneous distribution and better attachment of AgNPs on the titanium surface. In addition, future studies could lead to the development of a bifunctional coating for dental titanium implants combining both osseointegration and antimicrobial properties.

We have now produced a nanostructured silica coating loaded with AgNPs on titanium surfaces by incorporating the metallic nanoparticles during a combined sol–gel and evaporation-induced self-assembly process. The antibacterial properties of titanium surface modified with the nanostructured coating are evaluated against a representative pathogen of dental peri-implantitis.

2. Materials and methods

2.1. Synthesis of silver nanoparticles

In order to obtain a more biocompatible nanomaterial, silver nanoparticles were synthesized using a green chemistry approach. In this case soluble starch was used as a biocompatible reducing and stabilizing agent. In a typical synthesis, 1 g of soluble starch was added to 100 mL of distilled water and heated in a microwave oven. After complete dissolution, 1 mL of a 2 M aq solution of silver nitrate was added with stirring (2000 ppm AgNP suspension). The reaction mixture was stirred for 2 min and then heated at 70 °C for 55 min in an oil bath.

2.2. Synthesis of silica coatings on a titanium surface

Silica coatings were prepared on sheets of Ti6Al4V titanium alloy (Zimmer Dental Inc.) using the evaporation induced self-assembly (EISA) sol–gel technique [28]. Titanium sheets (15 × 15 × 1 mm) were sanded with silicon carbide paper (800 grit) and cleaned ultrasonically with acetone and ethanol before use. The sol–gel precursor solutions were prepared using both the amphiphilic triblock copolymer Pluronic P123 (P123) (EO20P070EO20, MW = 5800, Aldrich) and poly(ethylene glycol) (MW = 600, PEG) as pore structure directing agents (SDA). Briefly, 3.7 g of tetraethyl orthosilicate (TEOS 98%, Aldrich) was prehydrolyzed in a solution containing 20 mL of ethanol (95%) acidified with 0.5 mL of 0.5 N HCl (pH 2.0) with vigorous stirring at room temperature for 20 min. After that, 5 mL or 2.5 mL of 2000 ppm AgNP suspension was added to the TEOS solution to obtain silica films with 5 wt.% or 2.5 wt.% AgNP content, respectively. This prehydrolyzed silica/AgNP solution was added to a solution containing 2 g of the SDA dissolved in 20 mL of ethanol. The resulting solution was then submitted to an aging period at room temperature for 24 h with stirring, and films were prepared by slip-coating on the titanium sheets. Pure nanoporous silica coatings (NSC) were prepared as control, using the same procedure described above, but without adding the AgNP suspension to the TEOS solution.

For the slip-coating procedure [26], the titanium sheet was suspended in an inverted position from tweezers attached to a clamp fixed loosely enough to a stand to allow rotation of the tweezers. The polished side was brought in contact with the silica sol. The titanium sheet was kept in this half-immersed position for 20 s, slipped away horizontally by rotating the tweezers, and then dried in a vertical position for 40 s. The silica coatings were kept for 24 h at 35 °C, and then calcined by heating at a rate of 0.5 °C/min to 400 °C, holding that temperature for 4 h to remove the SDA.

2.3. Material characterization

AgNPs were examined by transmission electron microscopy (TEM) in a Philips Tecnai 12 Bio Twin microscope. Specimens were prepared by transferring a small drop of synthesized suspension to carbon-film coated copper grids. Particle size distribution of the silver nanoparticles was obtained using dynamic light scattering (DLS) with a ZetaPALS instrument (Brookhaven Instruments).

The unmodified and coated titanium surfaces were examined by scanning electron microscopy (SEM) in a JEOl JSM 5410 microscope equipped with energy-dispersive X-ray spectroscopy (EDX). The structural order of the porous silica coatings was analyzed by low angle X-ray diffraction (XRD) within a 2θ range of 0.5–5°. XRD patterns were collected on a Siemens D 5000 diffractometer using CuKα radiation at a scanning speed of 0.2°/min. The porous nanostructure was examined by high resolution transmission electron microscopy (HRTEM) on a FEI-Tecnai G2 F20 S-Twin HRTEM microscope equipped with a Field Emission Gun (FEG) operating at an accelerating voltage of 120 kV. Plan-view film specimens were prepared by removing the silica films from a titanium sheet and suspending them in ethanol. This suspension was then dispersed on a holey carbon film supported by a copper grid.

2.4. Antibacterial activity

The antimicrobial activity of the AgNP/NSC-modified titanium surfaces was tested against Aggregatibacter actinomycetemcomitans (serotype b). The strain was grown in BHI broth or agar (Brain Heart Infusion, Oxoid, Wesel, Germany) and incubated in a 5% CO2 atmosphere at 37 °C for 48 h. From a grown plate, bacteria were transferred to fresh BHI medium to a density equivalent to McFarland 2 standard. Each sterilized titanium disk was placed in 12-well plates, with the coated titanium side facing up. Then, 990 µL of fresh BHI medium and 10 µL of the inoculum were added to each well, and incubated for 1, 2, 4 and 6 days in a 5% CO2 atmosphere at 37 °C. After the incubation period, antibacterial activity was evaluated by total viable counts of each well. To remove bacteria from the disks we used the protocol reported elsewhere [29] with some modifications. Briefly, after the incubation period
the disks were transferred to a new 12-well plate, washed 3 times with PBS, and then the samples were thoroughly washed with a 0.88 wt.% NaCl solution and 1% Tween 80 to remove the bacteria from the surface of the disk. Samples of 100 μL were taken from the original plate (planktonic) and the bacterial suspension (biofilm), diluted and plated in BHI agar. After 48 h of incubation at 37 °C in a 5% CO₂ atmosphere, the colonies were counted and the colony forming units per mL (CFUs) were calculated for each disk in the biofilm and in the planktonic state.

The bacteria viability on the titanium surfaces was also analyzed with the BacLight live/dead bacterial viability kit (Invitrogen). After 24 h of incubation, non-adherent cells were removed by washing with distilled water. Live bacteria were stained with Syto 9 to produce green fluorescence, and bacteria with compromised membranes were stained with propidium iodide to produce red fluorescence. The stained surfaces were imaged using an epifluorescence Nikon Eclipse 50i microscopy.

The amount of biofilm formed on the material surfaces was quantified using crystal violet (CV) staining. In this case, after completed the bacterial incubation period, the samples were removed from medium and washed with distilled water in order to remove non-adherent cells. Then, 200 μL of 0.01% (w/v) CV solution was added to the wells for 15 min. The stained biofilms were rinsed with distilled water and extracted with 200 μL of 95% ethanol. The amount of biofilm was quantified by measuring the OD₅₉₅ of dissolved CV using a plate reader (Metertech 960). Each material surface was assayed in quintuplicate. Uninoculated medium controls were also included.

2.5. Ag release measurement

The release of Ag ions from the AgNP/NSC-modified titanium surfaces was measured up to 45 days of incubation in distilled water at 37 °C. Each titanium sheet was immersed in 20 mL of distilled water with the coated titanium side facing up. The Ag ion concentration in the aqueous phase was analyzed at different time intervals with a Silver/Sulfide Solid-State Combination Ion Selective Electrode (Hanna HI4115). The amount of silver released was calculated as silver mass per material surface (μg/cm²).

2.6. Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by multiple comparison Tukey’s test. Statistical significance was set at p < 0.05. Each standard deviation (SD) serves as the estimate for the standard uncertainty associated with a particular measurement.
3. Results

3.1. Fabrication of AgNP-silica coating

Fig. 1A–B shows TEM images and particle size distribution determined by DLS analysis of AgNPs synthesized using starch as reducing and capping agent. The mean particle size of the AgNPs was 8 nm, a value consistent with that estimated from TEM observations. The starch-capped nanoparticles exhibited long-term colloidal stability in aqueous solution and were successfully incorporated into the silica sol-gel system for coating fabrication. Fig. 1C–F shows SEM images of the unmodified titanium surface and of that coated with the AgNP-loaded silica films. The coatings were uniform on the metal surface and only scant microstructural defects were detected. The BSE-SEM image of the 5.0AgNP/NSC-coated surface revealed a high dispersion of the metal nanoparticles in the silica matrix (Fig. 1F). The silver content determined by EDX was 6.1 wt.%, confirming the presence of AgNPs in the silica coating matrix. The self-organized porous nanostructure of the AgNP-loaded silica coatings was analyzed by XRD and HRTEM (Fig. 2). The XRD patterns present the characteristic Bragg reflections corresponding to a highly ordered mesoporous silica structure, indicating that the incorporation of metal nanoparticles did not alter the ordered nanostructure of the silica coating. The low-angle XRD reflections are the result of an ordered hexagonal array of the cylindrical pores, which is indexed assuming a hexagonal unit cell (space group p6mm) [28]. HRTEM images (Fig. 2B) show that these self-ordered films present a honeycomb-like structure, consisting of a hexagonal close-pack array of nanopores. The diameter of the nanopores estimated from HRTEM images is around 4 nm.

3.2. Antibacterial activity

Antibacterial activity of AgNP/NSC-modified titanium surfaces was evaluated by their ability to inhibit both the planktonic growth and biofilm formation of *A. actinomycetemcomitans*. Fig. 3A shows the bacterial survival in the supernatant placed in contact with the titanium surfaces for different incubation periods. It is seen that the AgNP/NSC-modified surfaces significantly reduce bacterial survival with respect to the control surface. The coatings loaded with 5 wt.% and 2.5 wt.% AgNP decreased bacterial survival by approximately 60 and 40%, respectively. Surprisingly, the neat silica coating (NSC) also presented antibacterial activity by reducing bacterial planktonic growth by around 10%. The effect of AgNP-doped surfaces on bacterial growth in the supernatant was maintained for extended incubation periods. Fig. 3B shows the survival of biofilm formed on the different titanium surfaces. The results show that biofilm survival is reduced by up to around 70% on the AgNP/NSC-modified titanium surfaces. This bactericidal action was verified over an extended period of time.

Live and dead bacterial cells on the titanium surfaces were visualized by staining with Live/Dead fluorescent reagent. Fluorescence micrographs (Fig. 4) confirm that the bacterial viability is notably reduced on the AgNP/NSC-modified surfaces, and that this bactericidal effect is increased with the AgNP content in the NSC. Thus, the biofilm-forming bacteria are effectively killed in contact with the AgNP-loaded silica surfaces. Furthermore, the bactericidal effect of the neat silica coating is also visualized in the fluorescence images.

The extent of biofilm formation on the surfaces was measured using crystal violet. Fig. 5 shows that the presence of AgNP on the titanium surface decreases the biofilm coverage by more than half compared to the control.

3.3. In vitro Ag release study

In order to investigate the possible antibacterial principle of the AgNP/NSC coating, Ag release from the AgNP/NSC-modified titanium surfaces in water was also evaluated. Fig. 6 shows the amount of total Ag released per material surface area over a prolonged period of time. The material surfaces presented relatively low levels of Ag release in water, with a significant increase in Ag release up to approximately 15 days of immersion. As expected the amount of Ag released increases with AgNP content in the nanostructured coating. Ag release became stabilized after 24 days at around 0.022 and 0.011 μg/cm² for Ag contents of 5 and 2.5 wt.%, respectively, showing a sustained release rate of the metal.

4. Discussion

The direct incorporation of starch-capped AgNP into the silica sol–gel system was made possible through the procedure developed in this work. Silver has been commonly incorporated into silica matrices.
in its ionic form [30–32], or allowing the formation of metallic nanoclusters only under special conditions [33,34]. Unlike a previous study, in which AgNPs were sparsely deposited on a silanized titanium surface [24], our sol–gel based method enables a more homogeneous distribution of the nanoparticles over the whole titanium surface and a more precise control of surface silver content. In addition, the AgNP-doped sol–gel solutions prepared in the present work make possible the fabrication of nanoporous silica coatings with a highly ordered nanostructure. This sub-10 nm ordered porosity has been found to be an important property for improving the osseointegration process of implants. We have recently reported that the nanoporous silica coating produced on the surface of titanium is able to stimulate the adhesion and osteogenic differentiation of stem cells, thereby improving the osseointegration properties of titanium [26]. The current results demonstrate that the addition of AgNPs into the nanoporous silica matrix generates a composite material with antibacterial properties. Although the AgNP/NSC nanocomposite coating exhibits antibacterial activity against both planktonic and biofilm bacteria, the antibacterial effect is more marked on the surface-adherent bacteria. The AgNP/NSC surface not only effectively kills the bacteria adhered to the surface, but it also has an anti-adhesive effect by reducing the extent of biofilm formation. Since the AgNP/NSC coating presents relatively low levels of silver release in aqueous solution, the bactericidal mechanism can be explained by bacterial contact with or in the close vicinity of the AgNP-doped nanoporous silica surface. Erosion of the silica coating matrix to the nanometer level can occur in an aqueous medium [35], causing exposure and release of the metal nanoparticles at the material/aqueous solution interface. Although the antibacterial mechanisms of AgNPs have not been fully elucidated, the prevailing paradigm suggests various combinations of silver ion release followed by cellular uptake and a cascade of intracellular reactions, generation of reactive oxygen species (ROS) and cell membrane damage, and direct interaction between nanoscale silver and cell membranes [36]. In addition to the antibacterial effect of the metal nanoparticles, the silica coating matrix also exhibited bactericidal activity. Silica has silanol functional groups on its surface which have shown stronger antimicrobial activity than that of analogous alcohols due to their physicochemical properties, in particular higher hydrophobicity and H-bond acidity compared to alcohols [37]. Thus, the antibacterial activity of the AgNP/NSC nanocomposite coating may be attributed to a combined bactericidal effect of AgNPs and the surface chemistry of the nanoporous silica matrix.

Previous works on titanium surface modifications with AgNPs report antibacterial activity after 18 [38] and 24 h [25] of incubation with the bacterial suspension. The results of our study demonstrate that the antibacterial activity of the AgNP/NSC-modified titanium surface is maintained for up to 7 days of incubation, confirming a sustained antibacterial effect over a prolonged period of time. This effect can be related with the sustained release rate of silver exhibited by the nanostructured coating, and constitutes an important aspect for the long-term control of peri-implant infection. A. actinomycetemcomitans and P. gingivalis are the predominant pathogens implicated in dental peri-implant destruction [39], however the antimicrobial activity of AgNPs on these periodontal bacteria has been only scantily explored [40], particularly in order to produce antibacterial surfaces for dental implant devices. In the current work, the AgNP/NSC-modified titanium surface was particularly effective under anaerobic conditions against A. actinomycetemcomitans, therefore showing potential properties for antibacterial control of pathogens commonly associated with peri-implantitis.

5. Conclusion

Preparation of highly ordered nanoporous silica coatings loaded with AgNPs on the surface of titanium is feasible by direct incorporation of the metal nanoparticles into the sol–gel system. AgNP/NSC coating produces a strong antibacterial effect on the titanium surface by killing the adherent bacteria and inhibiting biofilm formation. The long-term antibacterial activity exhibited by the nanostructured AgNP/NSC-titanium surface against A. actinomycetemcomitans indicates that this type of nanoscale surface modification is a promising strategy to control infections associated with dental implant rehabilitation.

Acknowledgments

The authors acknowledge the financial support of National Commission for Scientific and Technological Research (CONICYT) of the Government of Chile through FONDECYT Project 11100495.

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