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Salivary pellicle composition and multispecies biofilm developed on titanium nitrided by cold plasma

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ABSTRACT

Objective: The aim of this study was to evaluate the composition of the salivary pellicle (SP) and multispecies biofilm developed on titanium nitrided by cold plasma.

Methods: Titanium discs were allocated into a control group (Ti) and an experimental group (TiN – titanium-nitrided by cold plasma). The disc surface topography was characterized by scanning electron microscopy (SEM) and atomic force microscopy (AFM). The chemical composition of the disc surface was determined by X-ray photoelectron spectroscopy (XPS). Stimulated, clarified, and filtered saliva was used to form pellicles on the discs. Proteome analysis of the adsorbed SP proteins was performed by liquid chromatography–mass spectrometry (LC–MS). The surface free energy (SFE) was evaluated before and after SP formation. A multispecies biofilm composed of *Actinomyces naeslundii*, *Streptococcus oralis*, *Streptococcus mutans*, *Fusobacterium nucleatum*, *Veillonella dispar*, and *Candida albicans* was developed on the SP-coated discs. Viable microorganism counts were determined. The biomass and average thickness of biofilms were analyzed by confocal laser-scanning microscopy (CLSM) with COMSTAT software. The biofilm organization was visualized by SEM.

Results: The surface topography was similar in both groups. The SFE of the TiN group did not differ from that of the Ti group ($p > 0.05$), although the adsorption of pellicle proteins increased the SFE in both pellicle-coated groups ($p < 0.001$). Different proteins were identified on the Ti and TiN surfaces. The amount of biofilm was similar for both groups ($p = 0.416$), but the counts of *F. nucleatum* and *S. oralis* were higher in the TiN group ($p < 0.001$). Similar biofilms were characterized by the COMSTAT data, CLSM images, and SEM images.

Conclusion: The titanium nitrided by cold plasma exhibited differences in SP composition and multispecies microbial biofilm population compared to the control titanium surface.

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1. Introduction

Since the advent of osseointegrated implants, titanium has been the preferred material for screws and prosthetic components in oral rehabilitation, due to its biocompatibility with oral tissues and surface properties (*e.g.*, roughness, chemical composition, and topography).[1,2] These properties can be modified by treating the titanium surface to promote protein adsorption, cell signalling, and new bone formation. Treatments include turning/machine-etching, oxidization, and adding/implanting ions.[3] An example of such a treatment is to nitride the titanium surface by cold plasma.[4,5]

Upon exposure to the oral environment, all surfaces are immediately coated by saliva, forming a salivary pellicle (SP). Pellicle formation involves non-covalent interactions, such as hydrogen bonding, electrostatic, and Van der Waals forces, between the surface and salivary proteins. These interactions result in the selective adsorption of the salivary proteins.[6,7] The surface roughness (Ra) and surface free energy (SFE) are two of the most important properties that influence SP formation.[8] Protein adsorption is facilitated when the surface area is increased (*e.g.*, by roughening the surface).[9–12] The SFE enhances the interaction between titanium and the biologic fluids.[11] In situations of poor tissue recovery and bone destruction, saliva-coated surfaces present sites for microorganisms attachment.[13]

As a result of surface treatments that facilitate protein adsorption, the surface may attract microorganisms that can colonize the surface and form biofilms.[13] Oral biofilms are structured communities of bacteria and fungi that develop on different substrata.[14] Biofilm formation on titanium is similar to biofilm formation on enamel.[15] It is generally initiated by species of bacteria, known as first colonizers, which are in direct contact with the coated surfaces. An example of a first colonizer is *Streptococcus oralis*. First colonizers interact by coaggregating with microorganisms known as second colonizers. For example, coaggregation of *Fusobacterium nucleatum* and *Candida albicans* forms a mature multispecies biofilm[15] that can lead to peri-implantitis.[16,17]

Previous studies have shown that biofilm formation is increased on surfaces that have been turned, machine or acid-etched,[19] with the largest increase occurring when two methods of surface treatment are combined.[18,19] However, studies regarding biofilm formation on nitrided surfaces are still scarce. Thus, the aim of this study was to evaluate the composition of the SP and multispecies biofilm developed on titanium nitrided by cold plasma.

2. Materials and methods

2.1. Experimental design

This *in vitro* study had a randomized and blinded design. Titanium discs (Ti – control) and titanium discs nitrided by cold plasma (TiN – experimental group) were used as substrata for SP and multispecies biofilm analyses. The surface topography was characterized by scanning electron microscopy (SEM) and atomic force microscopy (AFM). The surface chemistry was analyzed by X-ray photoelectron spectroscopy (XPS).

SPs were formed on discs by immersion in stimulated and centrifuged-filtered saliva for 2 h. The surface free energy (SFE) was measured before and after SP formation. Adsorbed proteins from the SP were analyzed by liquid chromatography–mass spectrometry (LC–MS/MS) analysis. A multispecies biofilm comprising five types of bacteria and one type of yeast was developed for 64.5 h on pellicle-coated discs. The biofilm was removed from the surface by sonication. The resultant suspension of cells was serially diluted and plated on agar media to count the viable cells (in colony forming units [CFU]/mL). The biomass and average thickness of biofilms were analyzed by confocal laser-scanning microscopy (CLSM) with the COMSTAT software package. The biofilm organization was analyzed by SEM.

2.2. Preparation and surface treatment of titanium discs

Titanium grade IV discs (12.5 mm × 2 mm) (Sandinox; Sorocaba, São Paulo, Brazil) were ground with progressively smoother aluminium oxide papers (200, 320, 400, 600, and 1200 μm grid) (Carbimet; Buehler, Lake Bluff, IL, USA) in a horizontal polisher (model APL-4; Arotec, Cotia, São Paulo, Brazil). To remove surface contaminants, the discs were rinsed and ultrasonically cleaned (Thornton T740; Thornton-Inpec Electronica Ltda., Vinhedo, São Paulo, Brazil) for 10 min twice, in each of the following liquids: 98% acetone, 100% ethanol, and distilled water.[5] The discs were dried in a flow chamber under aseptic conditions and randomly allocated to Ti and TiN groups.

TiN discs were nitrided by cold plasma as previously described.[4] Briefly, the discs were sputtered with argon plasma to remove any residual air contaminants. Then, an ionic plasma containing air, noble gases, and nitrogen was applied for 150 s to one face of the disc under high vacuum. The machine was fabricated by the Physical Institute of University of Campinas (UNICAMP).[4,5] The discs were sterilized by gamma radiation (25 Gy) (Embrarad; Cotia, São Paulo, Brazil).

2.3. Surface analyses

The surface topography of the Ti and TiN discs was characterized by SEM and AFM. The surface roughness of the discs was also analyzed by AFM. The atomic force microscope (Nanosurf EasyScan2 FlexAFM, Liestal, Switzerland) was set at 0.5 s for reading at 256 points. Four images of each set of two discs (experimental and control) were obtained and evaluated by SPIP 5.1.8 software (Image Metrology, Hørsholm, Denmark).[18] The surface topography parameters were as follows: arithmetic mean deviation (*Sa*, arithmetic mean of the roughness area from the mean plane), density of summit (*Sds*, number of peaks per area unit), and developed interfacial area ratio (*Sdr*, ratio between the developed surface area and a flat reference area).

The chemical composition of the surface was determined by XPS. The spectrometer (Vacuum Scientific Workshop, VSW HA100, Manchester, UK) with a hemispherical analyzer was operated in constant transmission mode, resulting in a line