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Enhanced antifungal activity by disinfected titanium dioxide nanotubes via reduced nano-adhesion bonds

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ABSTRACT

We have provided evidence that the beneficial effect of super-oxidized water (SOW) disinfected Ti6Al4V-TiO_2 nanotubes (NTs) can reduce bacterial adhesion and biofilm formation. However, the need of antifungal nanostructured surfaces with osteoactive capabilities is an important goal that has been arising for dental implants (DI) applications. Thus, in the present study we isolated and tested the effects of *Candida albicans* (*C. albicans*) on disinfected, wetter and nanoroughness NTs compared to a non-modified control. Moreover, we elucidated part of the fungal adhesion mechanism by studying and relating the mycotic adhesion kinetics and the formation of fungal nanoadhesion bonds among the experimental materials, to gain new insight of the fungal-material-interface. Similarly, the initial behavior of human alveolar bone osteoblasts (HAOb) was microscopically evaluated. NTs significantly reduced the yeasts adhesion and viability with non-outcomes of biofilm than the non-modified surface. Cross-sectioning of the fungal cells revealed promoted nano-contact bonds with superior fungal spread on the control alloy interface; meanwhile NTs evidenced decreased tendency along time; suggesting, down-regulation by the nanostructured morphology and the SOW treatment. Importantly, the initial performance of HAOb demonstrated strikingly promoted anchorage with effects of filopodia formation and increased vital cell on NTs with SOW. The present study proposes SOW treatment as an active protocol for synthesis and disinfection of NTs with potent antifungal capability, acting in part by the reduction of nano-adhesion bonds at the surface-fungal interface; opening up a novel route for the investigation of mycotic-adhesion processes at the nanoscale for bone implants applications.

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1. Introduction

Oral candidiasis (OC) is the most common and dangerous fungal infection among the oral cavity. This disease has high incidence in diabetic, cancer, malnourished, advanced aged, renal, oral-prosthetic and immunosuppressed patients [1,2]. If is not correctly managed, it could trigger a systemic mycosis (i.e. dermatologic complications) [2] which in turn can also improve the resistance to antifungals, malignant changes (e.g. hyperplastic candidiasis) or in worst case the patient's death [3]. These manifestations mainly start by an uncontrolled colonization of *C. albicans*, the main specie present in the mouth [4]. On the other hand, Titanium (Ti) and Ti-based alloys are the principal options selected for DI manufacturing and for the clinical treatment of edentulous patients. Unfortunately, *C. albicans* is commonly isolated from peri-implantitis lesions (which lead to DI loosening) [5], suggesting to play an important role in DI failures; possibly by the virulence gained by its rapid colonization on the implant surface; and/or by synergistically interacting with other bacteria such as *Staphylococcus aureus* (*S. aureus*) resulting in a polymicrobial biofilm [6,7]. Moreover, a prerequisite for the initial material-bacteria interactions may involve the formation of close-contact adhesion bonds at the nanoscale between the biofilm and the rough implant surface [8,9], information that suggests the need for analytic studies characterizing those phenomena.

SOW, is an electrolyzed water that has been widely used for the disinfection of an extensive variety of medical instruments and as a root canal irrigant for endodontic procedures [10], due to its potent antibacterial effect possibly by the action of its oxidizing radicals (i.e. hypochlorous species and chlorine molecules) [11]. Recently, we explored the antibacterial efficacy of anodized NTs synthesized and disinfected by exploiting the use of SOW against *S. aureus*, which showed striking significant reduced bacterial adhesion and biofilm

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formation without altering the osteoblasts behavior [12]. Moreover, the use of NTs have shown improved osteogenic activity on a wide variety of dental implants alloys; nonetheless, the presence of different NTs arrays and/or diameters can outset a promoted bacterial activity [13–15]. Thus, taking into account the above mentioned information, and the need of antifungal surfaces for DI applications; we hypothesized that a Ti6Al4V dental alloy with NTs synthesized and disinfected by SOW will promote antifungal capability (which is completely different to the antibacterial actions) to the NTs; in part via reduction of nano-adhesion bonds (a rigorous prerequisite for bacterial/fungal adhesion and biofilm formation) at the fungal-material interface (which has never been described); without affecting the osteoblast adhesion and viability, which in turn will generate a novel, easy and economic strategy for dental implants cleaning and disinfection.

The objective of the present in vitro study was to test for the first time the anti-fungal activity of NTs synthesized and cleaned by SOW; extending our previous report regarding the antibacterial capability of NTs with SOW, and realize a postulate for the role of nano-bonds between *C. albicans* and disinfected NTs (exploiting the FIB's technology as a novel tool) as a part of a possible mechanism involved in the disruption of *C. albicans* adhesion and strongly required for DI success. This is the first study focusing on the antifungal capability of Ti6Al4V-NTs (of importance for DI applications) instead of only bactericidal activity. Moreover, we were interested in to test the initial HAOb (a proper model of oral osteoblasts) behavior of our disinfected nanostructured materials for DI applications.

2. Materials and methods

2.1. Synthesis and disinfection of NTs

The fabrication and cleaning of NTs was carried out as previously described with slight modifications [12]. Flat disks of Ti6Al4V (ASTM F-136; Supra Alloys Inc., Camarillo, CA, USA) with 150 mm diameter and 5 mm thickness were mirror finished by means of SiC emery paper (100 to 2000 grift) and 1-μm alumina. Next, the samples were mounted on a special flat 125 mL cell and anodized using Microdacyn 60® super-oxidized water (Oculus Technologies, Guadalajara, JAL, Mexico) at pH 6.8, containing 10 mg/L of NH_4F (Sigma-Aldrich, USA) and 100 mg/L NaCl (Sigma-Aldrich, USA). Afterwards, a potential of 20 V was applied using a DC power supply for 5 min with a platinum mesh as counter electrode. The reaction was performed at room temperature (RT). In order to remove fluoride residues, the materials were sonicated for 5 min in double distilled water, rinsed with isopropyl alcohol and dried in a desiccator for 12 h. All experimental materials were sterilized by UV irradiation (285 nm UVB light source) for 30 min on each side, inside of a biosecurity cabinet. In contemplation to disinfect the nanostructured surface, the NTs were immersed in 20 mL of SOW for all night, dried at RT and sterilized again. We do not use a non-disinfected NTs surface as negative control, due to the strikingly potent antibacterial efficiency that was formerly reported [12].

2.2. Surface characterization

In order to reveal the surface morphology of the nanostructured surface and non-modified control, field-emission scanning electron microscopy (FE-SEM; Tescan LYRA 3, Brno Czech Republic) was applied, taking images at 20 kV accelerating voltage on different random fields. For the chemical characterization, an energy dispersive X-ray spectrometric (EDX; Tescan LYRA 3, Brno Czech Republic) analysis was executed using a silicon drift detector coupled to the FE-SEM. For a larger surface area characterization an X-ray fluorescence (XRF; Shimadzu EDX-7000, Japan) evaluation was performed. Surface roughness is a critical parameter for biological control of biomaterials surfaces [12, 16], thus it was explored by atomic force microscopy (AFM; Quesant Q-Scope 350, AMBIOS, Agura Hills, CA, USA), at RT using an anti-acoustic box to prevent noise, which can affect the measurements. The operation scan rate was 1 Hz by contact mode. A 40-μm X-Y and 4-μm Z scanner equipped with a silicon tip and 10 nm tip curvature was used. The experiment scan surface area was 1 $μm^2$. For quantitative roughness comparison between the surfaces, the arithmetic average (Ra) is provided. Wettability is a strict parameter required for the control and study of biomaterial's surface biological behavior, hence the static sessile drop method was conducted at RT to obtain the water contact angle following the procedure reported elsewhere [17,18]. A 15 μL droplet of double-distilled water was dripped onto each material surface using the tip of a syringe, and the shape of the droplet was evaluated taking images using a professional digital camera (Nikon 7200, NY, USA).

2.3. Candida albicans behavior on the surfaces

A pathogenic *C. albicans* strain isolated before the antifungal treatment from a female patient of 62 years old that was diagnosed with chronic atrophic oral denture candidiasis was used. The isolation and macroscopic characterization of *C. albicans* was done by CHROagar Candida (Beckton Dickinson, USA) method as described elsewhere [19,20]. For the preparation of the inoculums, the isolated and purified strain was freshly grown overnight on Sabouraud dextrose agar (SDA) plates (Beckton Dickinson, USA). Discrete colonies were obtained from SDA and suspended in SD broth (SDB) overnight. For the viability analysis on the materials, 50 μL of *C. albicans* suspension containing approximately 2×10^4 CFU/mL (O.D. 0.034) plus 100 μL of fresh SDB was used to cover the surface and prevent samples dryness. The inoculum was incubated on the specimens for 4, 12 and 24 h at 37 °C in a static model. After that, the materials were rinsed three times with 1× PBS to remove any unbounded cells. Each substrate was transferred into an individual well of sterile 24-well polystyrene plate (Corning, USA) with 2 mL of fresh SDB. The plate was placed in an ultrasonic bath (Branson, USA) and sonicated at 120 W for 1 min by 12 periods of 5 s each to avoid cellular lyses, and each surface was scraped off by means of a surgical blade; in order to completely detach any bounded cell. The materials were removed and the remaining suspensions were diluted with PBS and cultured at 37 °C for 24 h in SDA and counted for viability assessment. We performed SEM in order to analyze the density and morphological analysis of *C. albicans* at every incubation time [12,21]. Briefly, each disc was rinsed with warm PBS three times for 5 min respectively, fixed with 2.5% glutaraldehyde for 2 h at RT and dehydrated in graded series of ethanol solutions (30 min each). In order to directly visualize the fungal-surface interface we used FE-SEM as described elsewhere [8]. At 4 h (defined as initial adhesion) and 12 h (late adhesion) of cultivation we used a focused ion beam (FIB; coupled to the FE-SEM) in order to cross-sectioning the adhered fungal cells. Fixed cells were not sputter-coated in regard to evade the coverage of the nanostructured interface at a very high magnification scale. The magnification applied was 50,000× operating at 20 kV and a working distance of 5.92 mm. The FIB operation parameters were at 100 pA using a beam diameter of 3 nm at 17 kV.

2.4. Osteoblasts adhesion and viability

HAOb were isolated from human alveolar bone, immunostained to osteocalcin and cultured for the biocompatibility analysis [12,22]. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA) and 1% antibiotic-antifungal solution (PS; Invitrogen, USA) at 37 °C in 5% CO_2. Each sample was placed in a 12-well polystyrene plate. The cells were seeded using 1 mL of medium containing a concentration of 2×10^4 cells per mL onto the materials and stored in a CO_2 chamber for 24 h. HAOb viability assays were evaluated by means of a live/dead viability/cytotoxicity assay kit (Invitrogen, USA), with a mixture of 1 mM calcein-AM and 2 mg/mL ethidium

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