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Analysis of the oral microbiome on the surface of modified dental polymers

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ABSTRACT

Objectives: This study characterized the microbial diversity of formed biofilm on the surface of acrylic resins modified with nanostructured silver vanadate decorated with silver nanoparticles ($AgVO_3$) after incubation in human saliva.

Design: Resin specimens prepared with $AgVO_3$ at concentrations 0%, 1%, 2.5%, and 5% by either vacuum mixing or polymer solubilization were characterized by scanning electron microscopy (SEM) and X-ray diffraction (XRD). After 24 h and 7 days of saliva incubation, biofilm samples were collected from the surface of the specimens. The 16S rDNA genes were amplified, sequenced with the 454-Roche next-generation sequencing platform and analyzed to identify the Operational Taxonomic Units at the genus or higher level.

Results: Significant differences in the dispersion pattern of the nanoparticles were observed among the two different methods of $AgVO_3$ incorporation. In the microbiological analysis, a total of 103 genera and 7 more inclusive taxa, representing the phyla *Bacteroidetes, Firmicutes* and *Proteobacteria* were identified colonizing resin surfaces. The incorporation method of the $AgVO_3$ had little to no significant effect on the microbiota of samples. Significant time and concentration-dependent responses to $AgVO_3$ caused changes in the taxonomic profile at the phylum and genus level.

Conclusions: The results show differences in relation to the microbial diversity of modified resins during the initial phase of biofilm maturation. The incorporation of $AgVO_3$ seems to significantly affect the colonizing microbiota.

1. Introduction

Prosthetic stomatitis affects 30–70% of denture wearers, and *Candida albicans*is considered the main causative agent (Gendreau & Loewy, 2011). However, recent evidence also lists a panel of bacteria as putative pathogens, with the association of several species commonly found in the oral cavity (O'Donnell et al., 2015; Shi et al., 2016). In addition, the proliferation of some oral bacteria related to poor hygiene has been associated with several systemic diseases such as bacterial endocartitis, aspiration pneumonia, chronic obstructive pulmonary disease, generalized infections of the respiratory tract, mainly in dependent elderly people (Coulthwaite & Verran, 2007; O'Donnell et al., 2016).

Studies have proposed methods to promote antimicrobial activity to acrylic resins in order to avoid or reduce the colonization and proliferation of microorganisms related to the oral prostheses, mainly those focused on the antimicrobial properties of the silver ions (Ficklin, Kunkel, Suber, Gerard, & Kowaleski, 2016; Kurt et al., 2017). Silver vanadate nanowires decorated with silver nanoparticles ($AgVO_3$) was recently described as a promising antimicrobial agent (Holtz et al., 2010; Holtz, Lima, Souza Filho, Brocchi, & Alves, 2012) and has been demonstrated to be effective against *Staphylococcus aureus*, *Streptococcus mutans*, *Pseudomonas aeruginosa* and *Candida albicans* when combined with acrylic resins (de Castro, Valente, Agnelli et al., 2016;

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de Castro, Valente, da Silva et al., 2016). The probable intrinsic antimicrobial activity promoted by the nanowires results from the interaction of vanadium and the thiol groups located on the bacterial cell surface. The $AgVO_3$ acts as support for the silver nanoparticles (AgNPs), leading to a continuous release of silver ions, which can promote changes in the morphology of bacterial membranes, generate reactive oxygen species (ROS) and/or interfere in the DNA replication (Holtz et al., 2012).

Most studies evaluating the effectiveness of antimicrobial agents incorporated into dental materials have used conventional culture techniques to detect and quantify microbial colonization. The main limitation of these techniques is that they are selective or even restricted to specific and classified microbial species. According to the current literature, there are more than 700 prokaryotic taxa colonizing the oral cavity and many of them cannot be conventionally isolated (Kilian et al., 2016; Nascimento et al., 2016).

The sequencing technology has revolutionized our ability to understand the diversity of microbial communities in the human body, and amplification techniques based on the 16S rDNA gene have been widely used for profiling oral bacterial communities (Grossner-Schreiber et al., 2009; Kumar, Mason, Brooker, & O'Brien, 2012; Dabdoub, Tsigarida, & Kumar, 2013). This gene is present in all prokaryotes and contains unique hypervariable regions that demonstrate considerable sequence diversity among different bacteria. Species-specific sequences from a given hypervariable region constitute useful targets for diagnostic assays. The advent of next-generation sequencing, as for instance the 454 platform, has allowed millions of sequences to be generated in a single run (Kilian et al., 2016).

The aim of this investigation was to characterize, by Roche's 454 pyrosequencing platform, the profile of early and mature microbial communities colonizing the surface of two different $AgVO_3$ modified acrylic resins after exposure to human saliva. The hypothesis is that there are significant differences in the microbial profile of biofilm over time depending on the $AgVO_3$ concentration, irrespective of the incorporation method.

2. Material and methods

2.1. Synthesis and characterization of nanomaterial

The nanomaterial was synthesized by precipitation reaction between silver nitrate ($AgNO_3$) and ammonium vanadate (NH_4VO_3). Initially, 0.9736 g of NH_4VO_3 and 1.3569 g of $AgNO_3$were each solubilized in 200 mL of distilled water. After, $AgNO_3$ solution was added dropwise into the NH_4VO_3 solution under constant stirring at 65 °C. A precipitate was obtained, which was then washed with distilled water and absolute alcohol, filtered and dried in a vacuum line for 10 h (Holtz et al., 2010, 2012). A JEOL JEM-100CX II Scanning Transmission electron microscope (STEM) was used to observe the morphology and presence of silver nanoparticles on the surface of the crystals.

2.2. Preparation of specimens

Cylindrical metallic matrices (Ø9 mm × 2 mm) were embedded with type III dental stone (Gesso Rio, Rio Claro, Brasil) and polyvinyl siloxane (Zetalabor, Zermack, Badia Polesine, Italy) into two-part metallic flasks in order to create molds (Jon, São Paulo, Brazil). After dental stone crystallization, the flasks were opened and the matrices were removed. Two methods were used to modify the heat cure acrylic resin (Clássico Artigos Odontológicos, Brazil) with $AgVO_3$. In the first, the acrylic resin was manipulated according to manufacturer's recommendations, using the powder/liquid ratio of 3:1. During initial manipulation, the $AgVO_3$ was incorporated into the resin at concentrations of 1%, 2.5% or 5%. Next, vacuum mixing was performed using the Turbomix equipment (EDG, São Carlos, SP, Brazil) for 60 s. In the second method, the polymer was first solubilized in a concentrated chloroform solution prior to addition of the $AgVO_3$ fractions. The solubility parameter (δH) of polymethyl methacrylate (PMMA) is of around 18.8 MPa1/2, close to that of chloroform (δH = 19.0). The polymer and $AgVO_3$ were weighed to obtain a polymeric film. For the groups containing $AgVO_3$, 10 g of polymer were solubilized in 100 mL of chloroform followed by resuspension in 5 mL of solvent. After, the suspension was added to the polymer solution and the mixture was stirred for 15 min on a magnetic stir plate. The solution was poured into a glass petri dish, which remained in the fume hood until the complete evaporation of the solvent. The films were removed and subjected to cryogenic grinding in a Mikro-Bantam mill, model CF (Micron Powder Systems, New Jersey). The obtained powder was manipulated by manual mixing using a 3:1 powder/liquid ratio. In both methods, a control group was constituted without addition of $AgVO_3$. The acrylic resins were manipulated and packed into the mold of metallic flasks.

The samples were polymerized by conventional heating (immersion in water at 73 °C for 90 min and boiling for 30 min), by using an electric thermocycler (Thermocycler T100, Ribeirão Preto, Brazil). For microbiological analysis, specimens were finished with 180 and 400-grit sandpapers (Norton, Guarulhos, Brazil) aiming to standardize a surface roughness ranging from 2.7 to 3.7 µm, in order to simulate the internal surface of removable dental prosthesis (Zissis, Polyzois, Yannikakis, & Harrison, 2000).

2.3. Characterization of specimens

Specimens were submitted to microscopy analyses to characterize the load dispersion of incorporated nanoparticles. Three specimens of each group were analyzed by scanning electron microscopy (SEM) using a Zeiss Evo 50 microscope (Carl Zeiss, Peabody, MA) and one specimen was analyzed by X-ray diffraction (XRD), at room temperature, using a XRD-7000 diffractometer (Shimadzu, Kyoto, Japan) operating with Cu Kα radiation (λ = 1.5406 Å), 40 kV, and 30 mA. The data were collected continuously between $10° < 2\theta < 70°$ at a scan speed of 2θ per minute.

2.4. Participants and saliva collection

Non-stimulated whole saliva collected from participants wearing removable complete dentures was used as contaminant medium for the incubation of the specimens. Five milliliter of saliva were collected from 30 healthy individuals (15 women and 15 men) aged between 39 and 84 years (mean age 65 years) and mixed together into the same tube. The participants were selected in the Clinic of the School of Dentistry of Ribeirão Preto – University of São Paulo, and received guidelines regarding the research procedures and were given the right to decide whether or not to participate in the experimental phase of the study that was approved by the Ethics Committee (CAAE: 53668016.5.0000.5419). Potential participants were invited to participate in the study if: (A) had upper and lower total prosthesis; (B) did not exhibit oral manifestations of systemic diseases or acute infectious processes in the oral cavity; (C) had not used antibiotics in the previous 3 months.

2.5. Microbiological analysis

The specimens were sterilized with ethylene oxide prior to incubation. Twenty specimens of each group were distributed in 24-well plates. Saliva was deposited in each well and the plates were incubated at 37 °C for either 24 h-early biofilm (n = 10) or 7 days-mature biofilm (n = 10). After incubation, the biofilms formed on specimen's surface were collected with sterile microbrushes.

After harvesting, the samples were transferred to individual microtubes containing 300 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for further extraction of the genetic material and 16S rDNA PCR amplification. All tubes were stored at −20 °C prior to sequencing.