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S. sanguinis adhesion on rough titanium surfaces: Effect of culture media

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ABSTRACT

Bacterial colonization plays a key role in dental implant failure, because they attach directly on implant surface upon implantation. Between different types of bacteria associated with the oral environment, *Streptococcus sanguinis* is essential in this process since it is an early colonizer. In this work the relationship between titanium surfaces modified by shot blasting treatment and *S. sanguinis* adhesion; have been studied in approached human mouth environment. Bacteria pre-inoculated with routinary solution were put in contact with titanium samples, shot-blasted with alumina and silicon carbide, and adhesion results were compared with those obtained when bacteria were pre-inoculated with modified artificial saliva medium and on saliva pre-coated titanium samples. Our results showed that bacterial adhesion on titanium samples was influenced by culture conditions. When *S. sanguinis* was inoculated in routinary culture media, colonies forming unities per square millimeter presented an increment correlated with roughness and surface energy, but separated by the type of particle used during shot-blasting treatment; whereas in modified artificial saliva only a relationship between bacteria adhered and the increment in both roughness and surface energy were observed, regardless of the particle type. Finally, on human saliva pre-coated samples no significant differences were observed among roughness, surface energy or particle.

1. Introduction

Titanium and its alloys are nowadays among the most used metals as biomaterials, which have several applications in orthopedics, oral and maxillofacial surgery and implantology. However, problems associated with bacterial colonization on these kinds of devices can result in a localized inflammation, that might cause the loss of integrity of surrounding tissues, leading to treatment failure.

With those precedents, several studies about bacterial adhesion on dental material surfaces have been performed, in order to find the best processing conditions to modify the surface to avoid bacterial colonization, and at the same time improve surrounding tissue regeneration (bone and gingival tissue).

In vivo studies have been performed, either with dental implants in function for more than 1 year [1] or by testing different surfaces, such as Teflon, cellulose or acetate, mounting on a partial fixed bridge in volunteers [2–4]. Similar studies were performed in animal models [5]. However, even their results have shown a correlation between the increment on surface energy and the amount of bacteria adhered, this sort of experiments are not recommendable, because they implied that volunteers had had to avoid oral health, allowing dental plaque development.

On the other hand, *in vitro* studies had been also developed; which can be divided into two groups. The first group has been performed resembling as much as possible human oral conditions, making a pre-coating of samples with human saliva. In these studies, a wide variety of materials had been evaluated: titanium [6,7], titanium alloys [8], dental enamel, gold, ceramics and composites [9]. However, human saliva pre-coating samples present unrepeatable conditions, because of the variability in saliva composition (nutrients, ion concentrations and pH) even in the same person, which can present variations even daily. The second group of *in vitro* studies consists of adhesion bacterial experiments using routinary bacteria culture media. Studies on rough titanium samples [10,11] have permitted the analysis of initial bacterial attachment on surfaces with different kinds of surface modification treatments, in the best conditions of culture. These studies have established a direct correlation between surface characteristics (physico-chemical properties, roughness, surface energy,) and bacterial adhesion. However, experiments resembling oral conditions are necessary.

Therefore, the objective of this work was to study the relationship between surface modified titanium by shot blasting treatment and

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Streptococcus sanguinis adhesion, but in approached human mouth environment. For that, experiments in bacterial attachment on modified artificial saliva medium and human saliva pre-coated samples were performed and compared with bacterial adhesion results obtained when bacteria was pre-inoculated in routinary culture media (Todd-Hewitt broth).

2. Materials and methods

2.1. Titanium samples and surface modification

Samples were obtained by cutting commercially pure titanium grade 3 rods, into disks of 5 mm diameter and 2 mm thickness. These samples were divided into two sets, the first one was shot-blasted with aluminum oxide (Al_2O_3) with three increasing particle sizes (Table 1), the samples obtained were labeled Al2, Al6 and Al9. The second set was subject to the same shot-blasting treatment of previous group, but using silicon carbide (SiC) particles, which were labeled SI2, SI6, and SI9 given a total of six different rough surfaces. Shot blasting treatment was carried out at 0.25 MPa using a laboratory blasting machine (Model RB4 from Raytech Industries®). These treatment conditions (particle size, hardness and composition), were selected from a previous work [11] due to these particles ability to obtain roughness in the range of 1–10 μm, which maximizes bone–implant surface interlocking [12] and their excellent results in biocompatibility [13,14]. Prior to use, titanium disks were washed with distilled water, ethanol and acetone (15 min each), dried at room temperature and autoclaved.

2.2. Surface characterization

Roughness analysis was performed with Optic Profiling System WYKO NT1100 and software Vision 32 coupled to a microscope, from Veeco Metrology Group®. A surface area of 600 μm × 460 μm was analyzed in triplicate in three samples of each kind of surface treatment (given 9 measurements for each condition). From the image analysis, were obtained the average roughness (R_a) and the index area or roughness factor (r), which correspond to the real/geometric area ratio.

To determine titanium surface energy a modified contact angle method, proposed previously [11], was used. Here, the advancing contact angle from three different liquids: water (Milli-Q), formamide (Sigma Aldrich®) and diiodomethane (Sigma Aldrich®) was measured using a Contact Angle System OCA from Dataphysics Instruments with CCD coupled and software (SCA20®). Once the measurements were obtained, the apparent contact angle ($\cos\theta_a$) for each value was calculated applying Wenzel correction where roughness factor (r) has been included (Eq. (1)).

$$\cos\theta_a = r\cos\theta. \tag{1}$$

Then, surface energy was calculated solving OWRK equation (Eq. (2)) [11,15].

$$\sigma_{sl} = \sigma_s + \sigma_l - 2\left(\sqrt{\sigma_s^d \cdot \sigma_l^d} + \sqrt{\sigma_s^p \cdot \sigma_l^p}\right) \tag{2}$$

Table 1
Size and composition of particles used for shot-blasting process.

Nomenclature	Shot-blasting particle size (μm)
Flat	
Al2	Al_2O_3 (212–300)
Al6	Al_2O_3 (425–600)
Al9	Al_2O_3 (1000–1400)
SI2	SiC (212–300)
SI6	SiC (425–600)
SI9	SiC (1000–1400)

where the surface energy (σ_{sl}) is given by the contributions to the dispersive (σ_l^d) and polar (σ_l^p) components of the liquid; and the dispersive (σ_s^d) and polar (σ_s^p) components of the solid.

2.3. Evaluation of bacterial attachment to rough titanium surfaces

S. sanguinis strain CECT 480, supplied by Colección Española de Cultivos Tipo (CECT) was used in this work. Cells were routinely grown and maintained in Todd-Hewitt broth from Scharlau® (viscosity 0.77 MPa/s at 37 °C). Furthermore, in order to approach human oral conditions, modified artificial saliva medium (MASM) (Table 2) was also used for bacteria growth (viscosity 23.7 MPa/s at 37 °C), which was monitored by viable cell counting and optical density of bacterial solution (600 nm).

Three kinds of experiments in bacterial attachment on titanium surfaces were performed: a) Todd-Hewitt broth, b) MASM medium and c) human saliva pre-coated samples with *S. sanguinis* grown in Todd-Hewitt broth (Scharlua®). For each sort of experiment a sample set was evaluated (in triplicate), consisting of the six rough surfaces and one flat surface obtained directly from the cutting of process and which was used as negative control of bacterial adhesion.

Previous to bacterial adhesion experiment, *S. sanguinis* was grown in Todd Hewitt broth and in MASM medium for 24 h (final bacterial concentration, about 10^7 CFU/ml). Then, titanium samples were added individually to Eppendorf® tubes, containing 1 ml aliquots of *S. sanguinis* (Todd-Hewitt broth and MASM) and incubated for 2 h at 37 °C. Disks were then washed twice with PBS 1X and introduced in Eppendorf® tubes containing 1 ml Ringer solution (Scharlau®). Adhered cells were released from the disks by continued vortexing (1 min). Finally, viable cell counts in the supernatant were determined seeding in solid agar and incubating for 48 h. Detached CFU's were quantified, and normalized as CFU's/mm^2.

The pre-coated titanium samples were prepared by adding human saliva which was previously centrifuged (4000 rpm 5 min at 4 °C) and filtered (0.22 μm pore size). One set of samples was coated and incubated for 30 min at 37 °C, finally samples were dried at room temperature for 10 min. These pre-coated samples were then used in the bacterial adhesion experiment, following the procedure described in the above paragraph.

Finally, in order to check presence of bacteria adhered on titanium, one set of samples was prepared and observed by Scanning Electron Microscope (JEOL JSM-6400), previously dehydrated (alcohol dilutions 20–100%) and covered with a gold layer by sputtering.

2.4. Statistical analysis

Results about surface characterization and bacterial assays have been expressed as mean and standard deviation in three independent experiments performed in triplicate (resulting in 9 samples of each

Table 2
Modified artificial saliva medium composition.

Modified artificial saliva medium	
Potassium chloride	1.200 g
Sodium chloride	0.840 g
Magnesium chloride	0.050 g
Calcium chloride	0.150 g
Potassium biphosphate	0.340 g
Sodium carboxymetilcelullose	10 g
Distilled water	1 l
Glucose[a]	10 g
Yeast extract[a]	1 g
Casamino acids[a]	1 g

[a] Supplements were added in order to permit *S. sanguinis* growth.