Evaluation of biofilm formation on acrylic resin surfaces coated with silicon dioxide – an in situ study

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ABSTRACT

The biofilm on acrylic resin dental prostheses may cause gingival inflammation. This study evaluated the influence of a silicon dioxide coating layer applied onto acrylic resin on the adhesion of microorganisms. Blocks (5x5mm) of acrylic resin were evaluated for surface roughness and divided into 2 groups: control (GCT) and waterproofing with silicon dioxide (GVL). The specimens were evaluated by scanning electron microscopy (n=1) and by contact angle analysis (n=2). For the *in situ* study, 20 volunteers used acrylic palatine devices containing 3 samples of each group (n=60) for 2 days. The biofilm formed was quantified by the metabolic activity and total biomass through crystal violet assay. The results were submitted to the Barllet normality test and Gamma model with random effect for the response variable (α=5%). The mean contact angle of the coated group was significantly lower than that of the non-coated group ($p<0.05$). The metabolic activity produced by the microorganisms in the biofilm on the blocks treated with the coating was significantly lower than that produced on the control blocks ($p=0.02$). Regarding the amount of extracellular matrix produced by the microorganisms, there was no difference between the GCT and the GVL groups ($p=0.05$). The application of a silicon dioxide coating on acrylic resin reduced the activity of the polymicrobial biofilm formed *in situ*. This coating may be advantageous in patients with conventional full dentures or implants made of acrylic resin and who have motor difficulties to carry out hygiene of the prosthesis.

Keywords: silicon dioxide, liquid glass, microorganisms, acrylic resin, cell adhesion

INTRODUCTION

In the last National Oral Health Survey[1] in Brazil carried out in 2010, despite a significant decrease in the rate of caries in different age groups and increase in access to health services, the population continues to lose teeth throughout life[1]. It is estimated that among adults the need for prosthesis occurs in 68.8% of cases. Among the elderly aged 65 to 74 years, only 23.5% did not use maxillary prostheses. Studies carried out in Europe concluded that Finland, Greece, Turkey and Bulgaria have approximately one third of their elderly population as being edentulous. Several other countries on that continent have approximately 20% of their population facing the same problem[2].

Acrylic resin is widely used in dentistry as a base for prostheses since 1937, due to its characteristics, among which stand out their low cost, easy handling and ability to combine colors[3,4]. However, over time, this material retains pigments and

microorganisms organized in biofilm capable of causing bad breath and gingival inflammation. An effective method capable of reducing microbial adhesion and of pigments retained in the surface porosities of acrylic resin has not yet been discovered[3,5]. Various cleaning methods have been used for disinfection[6,7,8] or structural alteration, creating resins with antimicrobial properties[9,10]. Despite helping to decrease microbial biofilm on surfaces, these methods can negatively alter the physical and mechanical properties of the resin [6,9], influencing its aesthetics and durability. In addition, a degree of coordination and manual dexterity is required for the cleaning to be effective. Elderly patients often lose their motor capacity required to perform the procedure[7].

A waterproof and smooth surface is of great importance in preventing biofilm retention[11]. A set of applications of silicon dioxide coating as well as their use had already been established in a German patent filed in 2007[12]. Nano-scale silicon dioxide has been used in hospital environments[13]. Its coating results in a surface with a massively reduced amount of microorganisms and is easy to clean. In addition, silicon dioxide has been shown to be effective in reducing the adhesion of *C. albicans* to acrylic resin surfaces[14, 15, 16] and a recent *in vivo* study demonstrated a high level of tissue biocompatibility of acrylic resin coated with silicon dioxide with low deleterious risk to the individual[17]. Currently, it is possible to find it in the form of a spray, so it can be used easily in clinics.

The null hypothesis was that the use of silicon dioxide as a coating layer on acrylic resin does not act as a retention inhibitor of polymicrobial biofilm *in situ*.

MATERIAL AND METHODS

This project was approved by the Local Ethics Committee (CAAE: 86984317.9.0000.0104) under the reference number 2.698.664.

Preparation of the specimens

Eighty block specimens of thermosetting acrylic resin were produced (VipiCril Plus, VIPI Indústria - Pirassununga, SP, Brazil) in dimensions of 5mm x 5mm. For this, the thermopolymerizable acrylic liquid (PALATON, Dencril Odontological Products, Pirassununga, SP, Brazil) together with acrylic resin powder (VipiCril Plus, VIPI Indústria - Pirassununga, SP, Brazil), were mixed in a container, respecting the proportions indicated by the manufacturer (6.5 ml of liquid for 14 g of powder). After

entering the plastic phase, the material was pressed and placed in a thermo pneumatic polymerizer at a temperature of 80°C and 100 pounds of pressure for 10 minutes. The finishing was done with drills and cutters, leaving the specimens with standardized dimensions.

Surface roughness

Considering the need for standardization of the surface roughness of the specimens, the acrylic resin blocks were subjected to sequential polishing, initially with # 320, 400 and 600 granulation silicon carbide discs in a polishing machine (Aropol -2V, Arotec SA Ind. and Com., Cotia, SP, Brazil) for 15 seconds and then with felt (TOP, RAM and ABOVE-Arotec), and diamond pastes (6 µm, 3 µm-RAM, 1 µm- SUPRA-Arotec). Between each step and after finishing and polishing, the samples were washed for 2 minutes, with distilled water in an ultrasonic bath (Biowash STD-Bioart, São Paulo, Brazil) to remove debris from the surface.

The surface roughness (µm) of the specimens was then measured using a profilometer (Surfcorder SE 1700; Kosaka Laboratory Ltd., Kosaka, Japan), with an accuracy of 0.01 mm, calibrated with a sample length of 0.8 mm, 2.4 mm and 0.5 mm/s percussion. Three readings were taken on each specimen and the mean value was obtained.

Preparation of samples and specimens

The specimens were randomly divided into two groups (N = 60: GCT (control), GVL (experimental), waterproofed with Liquid Glass Shield silicon dioxide (TOPTEK Equipamentos Ltda, Belo Horizonte, Mg, Brazil). Each specimen was coated with silicon dioxide according to the manufacturer's instructions. Initially, a vial of silicon dioxide was shaken before each application, and sprayed on sterile gauze. This gauze was used to apply the product to the resin surface by rubbing[16]. The blocks were stored for 24 hours at room temperature to remove any solvent interferences and to wait for the curing time.

Scanning Electron Microscopy (SEM)

To evaluate the surface characteristics of the specimens with or without the application of silicon dioxide coating, one specimen from each group was fixed to a support, coated with gold in an argon atmosphere, using a gold module on a vacuum evaporator, according to Jarros et al.[18]. The samples were observed using a Quanta 250

™ SEM scanning electron microscope (ThermoFisher, Waltham, Massachusetts, U.S) at 1000 × magnification.

Contact angle

Two randomly selected specimens from each group had their surfaces evaluated according to the degree of hydrophobicity. The degree of hydrophobicity (ΔGsws) and surface energy were evaluated using the approach of van Oss et al.[19] where drops of a standardized liquid (water, glycerol and bromonaphthalene) are placed on the resin surface. The drop generates a surface tension on the resin, and these results in a variable angle of the drop itself, according to the hydrophobicity of the surface. The average angulation of each liquid was analyzed, and the surface energy was calculated. This angle was measured with an optical tensiometer (model OCA 15 PLUS, DATAPHYSICS) equipped with image analysis software (Attension Theta).

The contact angles with water were used as a qualitative indication of the hydrophobicity of the surface, with an angle less than 65°, indicating a more hydrophilic surface[20]. Each test was performed in triplicate and at least 20 contact angles per sample were measured.

***In situ* exposure**

For the *in situ* study, 20 volunteers were selected following the inclusion criteria: normal salivary flow, absence of caries and/or periodontal disease. The exclusion criteria included patients who were smokers, patients using orthodontic devices, fixed or removable prostheses. The volunteers' upper arch was molded with alginate (Jeltrate Dustless, Dentsply - Rio de Janeiro, Brazil) and the plaster models were made of special plaster (Durone IV, Dentsply Indústria e Comércio. Petrópolis - RJ - Brazil).

On the isolated plaster models (Cel-Lac, SS White Goods Dental Ltda., Rio de Janeiro, Brazil) were glued six cubes of heavy addition silicone (Elite HD +, Zhermack Dental, Rome, Italy) in the dimensions of 5 mm x 5 mm x 3 mm with cyanoacrylate glue (Super bonder: Loctite, Henkel Ltda, São Paulo, Brazil) in order to form spaces for the subsequent insertion of the specimens, facing the oral cavity.

The model set + silicone blocks were isolated for the manufacture of intraoral palatal devices in self-curing acrylic resin (JET - Clássico Artigos Odontológicas Ltda., Campo Limpo Paulista, SP, Brazil).

The acrylic resin palatine devices containing 6 samples, 3 of each group (N = 60) were used by the volunteers for 2 days. They were instructed to use the device throughout the day, except during meals, drinking fluids (except water) and cleaning. During these periods, the devices were stored in a container with gauze soaked in distilled water. The volunteers were instructed not to subject them to fluoride-containing solutions, and the device was to be cleaned only on the inside. On the morning of the 2nd day, the devices were delivered to the researchers for the removal of specimens and quantification of biofilm.

Quantification of Polymicrobial Biofilm

The biofilm formed on acrylic resin blocks by the classic methods of assessing the cell viability[21] and total biomass[22] was quantified.

Metabolic activity

The reagent 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl) -5- (phenylamino) -carbonyl-2H-tetrazolium-hydroxide was used to evaluate the metabolic activity (quantification and viable cells in the biofilm) (XTT; Sigma-Aldrich, USA) according to Negri et al.[23]and Jarros et al.[24]. Each specimen was removed from the palatal device and placed individually in a well of a 96-well plate. 200 µl of final XTT solution was added to each well and incubated for 3 h at 37ºC with shaking at 110 rpm. The final XTT solution was prepared with 10 µg/µl of phenazine methoesulfate (PMS) (Sigma-Aldrich, USA). Then, absorbance was measured on a spectrophotometer (SpectraMax Plus 384, USA) at 490 nm. The absorbance values were standardized per unit area of the well (absorbance / cm2). As a negative control, a specimen that did not come into contact with the patient was used. The absorbance values of the negative control wells were subtracted from the values of the test wells to account for any background absorbance.

Total biomass

To assess the total biomass composed by extracellular matrix and cells, the biofilm was fixed with 200 µl of 100% (v / v) methanol, for 15 minutes. The specimens were stained with a 0.1% v/v crystal violet solution (CV; Sigma-Aldrich, USA) for 5 min. Subsequently, the specimens were washed twice with sterile Milli-Q water and bleached with acetic acid (33% v/v). Finally, 100 µl of the decolorization solution for each sample was transferred to a new plate and measured with a spectrophotometer plate reader

(SpectraMax Plus 384 microplate reader, USA) at 620 nm. Specimens that did not come into contact with patients were used as negative control. The absorbance values of the negative controls were subtracted from the values of the test wells to minimize background interference. The final absorbance values were standardized according to the area (absorbance / cm^2).

Statistical analysis

To calculate the sample size, preliminary data from the pilot sample was used. The Cohen distance was used to evaluate the effect size. Consequently, a test power of 85% was obtained, an n=19, considering a significance level of 5% and effect size 0.73. The data were submitted to the Barllet normality test and did not present a normal distribution. The fact that each individual received 3 replicates of blocks in the mouth, suggests that there is an inherent variability in each patient that possibly interferes with the response variable. To accommodate these characteristics, the methodology of mixed generalized models was adopted, and since the data presented a positive asymmetry, a mixed Gamma model was adopted. This means that the variability of treatments was considered as fixed effects and the inherent or latent variability of each individual as a random effect. A significance level of 5% was established. The analyses were performed using the R i386 3.0.2 software. The contact angle results were analyzed using the SPSS 23.0 version (Statistical Package for the Social Sciences) program. A significance level of $p \leq 0.05$ was adopted. The statistical significance of the contact angle treated with the silica coating agent was determined using a Student's t-test.

RESULTS

The values of the surface roughness of the specimens (0.47 + 0.06µm) were presented as suggested in the literature by Lima et al.[25]. The photomicrographs obtained by SEM from the surface of the acrylic resin in the control group (GCT) and treated with liquid glass (GVL) are shown in Figure 1.

Although the acrylic resin surface in Figure 1b received the application of liquid glass, this was not noticeable in the SEM as the silicon dioxide forms a homogeneous coverage, of approximately 100 nm.

The calculated values for the evaluation of the contact angles (degree) formed between a drop of liquid and the surface of the acrylic resin blocks in both tested groups are shown in Table 1.

The hydrophobicity calculation showed a total ΔGsws of -9.5 in the control group (GCT), and was lower (-54.4) in the group with silicon dioxide (GVL), thus demonstrating that the GVL group was more hydrophobic ($p<0.05$). Regarding the ability to donate and receive electrons (γ- (mJm-2) and γ + (mJm-2), there was a change in polarity, in which the control group (GCT) had less γ + (12.0) and greater γ - (14.4) and the treated group (GVL) presented a greater γ + (2.1) and a lower γ- (1.6), thus changing the surface from polar to non-polar.

The results of the analysis of XTT and the CV produced by the microorganisms for the control groups (GCT) and treated with silicon dioxide (CVL) are shown in table 2.

The metabolic activity produced by microorganisms in the biofilm on the blocks treated with silicon dioxide (GVL) was significantly lower than that produced on the control (GCT) blocks ($p = 0.02$). However, in relation to total biomass there was no significant difference ($p = 0.05$).

DISCUSSION

As far as we know, this is the first study evaluating the effect of silicon dioxide as a coating layer on acrylic resin surfaces on biofilm adhesion, in an *in situ* design. It was possible due to the good results of a recent *in vivo* study, which demonstrate the highest level of tissue biocompatibility of acrylic resin coated with NP-Liquid Glass[17].

The null hypothesis was rejected once the use of silicon dioxide as a coating layer on acrylic resin demonstrated acting as a retention inhibitor of polymicrobial biofilm *in situ*. The results demonstrated that the blocks of acrylic resin coated with silicon dioxide presented less free energy (GCT vs GVL, -9.5 vs -54.4) and greater contact angle (GCT vs GVL, 104.1° vs 114,3°). In addition, the metabolic activity produced by microorganisms in the biofilm (analysis XTT) on the blocks treated with silicon dioxide (GVL) was significantly lower than that produced on the control blocks (GCT), however

in relation to total biomass (analysis of CV) there was no significant differences between the GVL and GCT groups.

The adopted statistical methodology was a Gamma model, as the data do not follow a Normal distribution. Furthermore, as each patient received three blocks of each group in his/her mouth, it is reasonable to assume that there is an inherent correlation between them. To accommodate this characteristic, a Gamma model with random effects on the individual was adopted.

Previous studies indicate the need to standardize the surface roughness, with averages greater than 0.2µm, in order to allow the assessment of the accumulation of microorganisms[25,26]. The surfaces analyzed in this study had an average of 0.47µm. This data is extremely important, as it demonstrates the standardization of surfaces, thus enabling the adhesion of microorganisms uniformly, reducing the variables that could affect the results.

The surface topography, analyzed by SEM, was similar in both groups, even with 1000× magnifications. The silicon dioxide forms a thin and homogeneous layer on the entire surface, without changing the visual characteristics of the acrylic resin, which represents a great advantage of the product applied. This may have happened because in the present study, only one thin layer of silicon dioxide was applied on the acrylic specimens, as suggested by the manufacturer. In the SEM images of coated specimens presented by Azuma et al.[14] (500× magnification), several different particles were detected and the surface roughness decreased compared to the non-coated specimens. Furthermore, in the coated group, the surface roughness decreased in SEM images and Si was consistently detected in EDS analysis. In their study the coating agent was applied three times on the acrylic resin blocks' surface, which differs from the present study in which it was done only once.

Even though there were no observed differences between GCT and GLV in the SEM images, it was possible to verify by means of the contact angle test, that the surfaces covered with silicon dioxide showed greater hydrophobicity than the non-coated ones. These results were also demonstrated in previous studies[14,15].

Van Oss[19] methodology was used in this research, with the purpose of evaluating the contact angle, or humectance angle of each surface and, as a consequence, to present results that would allow the analysis of the wettability of each surface. In this

methodology, drops of different liquids, previously standardized, were inserted on the analyzed surfaces. The average of each liquid was calculated and, based on this information, the surface energy was defined. It can be said that in both groups the surfaces appeared to be hydrophobic when the drops of water and glycerol were analyzed, which are between 90° <θ <180°. In the bromonaphthalene drop, the surfaces showed greater wettability with an average angle of 48.3 ° in the GCT group and 64.7° in the GVL group.

The surface with a smaller contact angle has more free energy, being more hydrophobic. On surfaces with high free energy, the liquid interacts with it through chemical bonds, while on surfaces with low free energy the substrates bond by means of forces. Chemical bonds are stronger than forces, which makes more free energy more hydrophilic than low free energy[27]. On surfaces with high contact energy, the liquid spreads more, creating a smaller contact angle. The calculation of free energy showed the GVL group with less free energy ($\Delta G_{|w|=}$ = -54.4) than the GCT group ($\Delta G_{|w|=}$ =-9.5). These results are extremely important, as they make it possible to verify that silicon dioxide is on the surface and causes changes in it, as these changes were not noticeable in the photomicrographs, and indicate that the surfaces treated with the product were more hydrophobic than the control group, which may disadvantage microbial adhesion. As for the change in polarity, which is a relationship between the ability to donate and receive electrons (γ- (mJm-2) and γ + (mJm-2)) from the surface, the results presented in this study are directly related to the low formation of biofilm on the surface. The adhesion of microorganisms can be considered the first stage for the formation of biofilms on abiotic surfaces, and the change of free energy, which altered the polarity of the evaluated surface making it repulsive, can have direct results in the formation of polymicrobial biofilm[28,29].

The resin treated with silicon dioxide in the *in situ* experiments showed significantly less cell activity, that is, less number of adhered microorganisms, compared to the control group (untreated). The principle of the XTT reduction technique lies in the ability of active cells to metabolize the tetrazolium salt, making them colored compounds indicating the viability of microbial biofilm metabolism[30, 31]. For the analysis of total biomass with CV dye, the GCT group showed a slightly lower average than the GVL group, but without a statistically significant difference, that is, there was no difference in total biomass between the materials (treated and untreated). The CV method is widely used to detect total biomass of mono and polymicrobial biofilms, quantifying all the structures that make up the biofilm (polysaccharide matrix and microorganisms).

Accordingly, it can be inferred that on the treated surface there is a smaller number of microorganisms attached (by the technique of XTT), but the same amount of total biomass, specifically the extracellular matrix (the CV technique). It is important to note that both tests used in this research are widely used to evaluate different microbial biofilms[32]. However, one bias that should be mentioned in the CV technique, is the inability to define the biofilm mass of dead or living cells, since the CV is able to stain both[33, 34]. Therefore, any results must be correlable with XTT. In addition, it was not possible to specify which microorganisms were present in the biofilm and its structure.

It can be noted that the limitations of the techniques were minimized with correlations between the methods that complemented each other and allowed us to make a critical and effective analysis on the analyzed groups. Although *in situ* studies often have a greater number of variables, in this research it was possible to reduce them with the careful selection of volunteers, the standardization of resins, with the analysis carried out in triplicate and the Gamma statistical model, which was adopted to accommodate the random characteristics, inherent to a study carried out with volunteers.

Wady et al.[35] used an XTT assay to assess *Candida albicans* adhesion and biofilm formation in dentures made with acrylic resin incorporated with silver nanoparticles. In this *in vitro* study, the hydrophobicity of the surface was evaluated using the contact angle technique, and the formation and adherence of microorganisms by the XTT test. The researchers were able to notice a reduction in hydrophobicity and no effect on fungal and microbial formation.

It was possible to observe that the acrylic resins coated with silicon dioxide showed significant advantages over the resins with conventional surface, in relation to the activity of the polymicrobial biofilm present on them. These results may have implications of great clinical importance, since silicon dioxide is inexpensive and easy to apply. The present study, based on 20 volunteers, is just a first step to assess the possible clinical differences in patients using conventional prostheses and the resins proposed in this work, but it is an important study, considering the clinical potential of its applications.

Future studies are recommended to evaluate the duration of the silicon dioxide coating on the acrylic resin surface, and its resistance to brushing, thus presenting an average time for reapplication of the product.

CONCLUSIONS

The application of a coating layer based on silicon dioxide on thermally activated acrylic resin has been shown to reduce the activity of the polymicrobial biofilm formed *in situ*. This procedure can be advantageous in patients with conventional full dentures or on protocols for implants made of acrylic resin and who have motor difficulties to perform their cleaning.

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Declaration of interest statement:

The authors declare that they have no conflicts of interest.

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Caption List:

Figure 1 – SEM images of acrylic resin block surfaces at 1000 × magnification: (a) non-coated specimen; (b) silicon dioxide coating specimen.

Table 1 - Water contact angle (θ), surface tension parameters (γ +, γ-) and degree of hydrophobicity (ΔGsws) of the acrylic resin blocks in the control group (GCT) and treated with silicon dioxide (GVL). The values are represented by the means ± standard deviations of three independent experiments for each condition.

Table 2 - Evaluation of metabolic activity and total biomass of biofilms in the control (GCT) and experimental (GVL) groups.

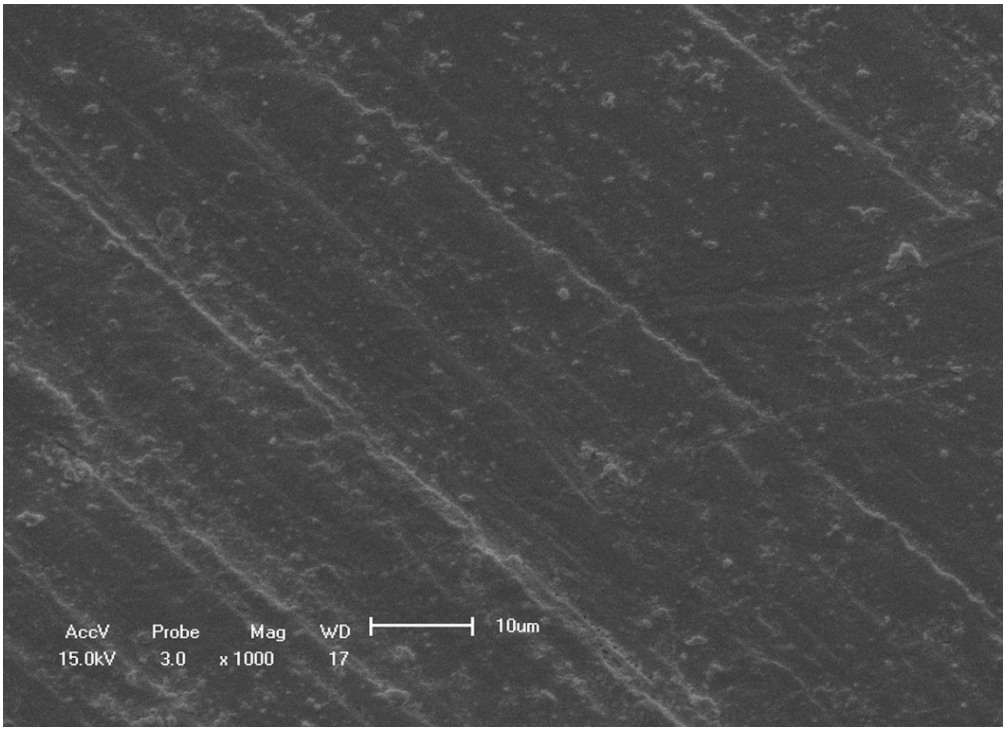

Figure 1 – SEM images of acrylic resin block surfaces at 1000 × magnification: (a) non-coated specimen

101x73mm (300 x 300 DPI)

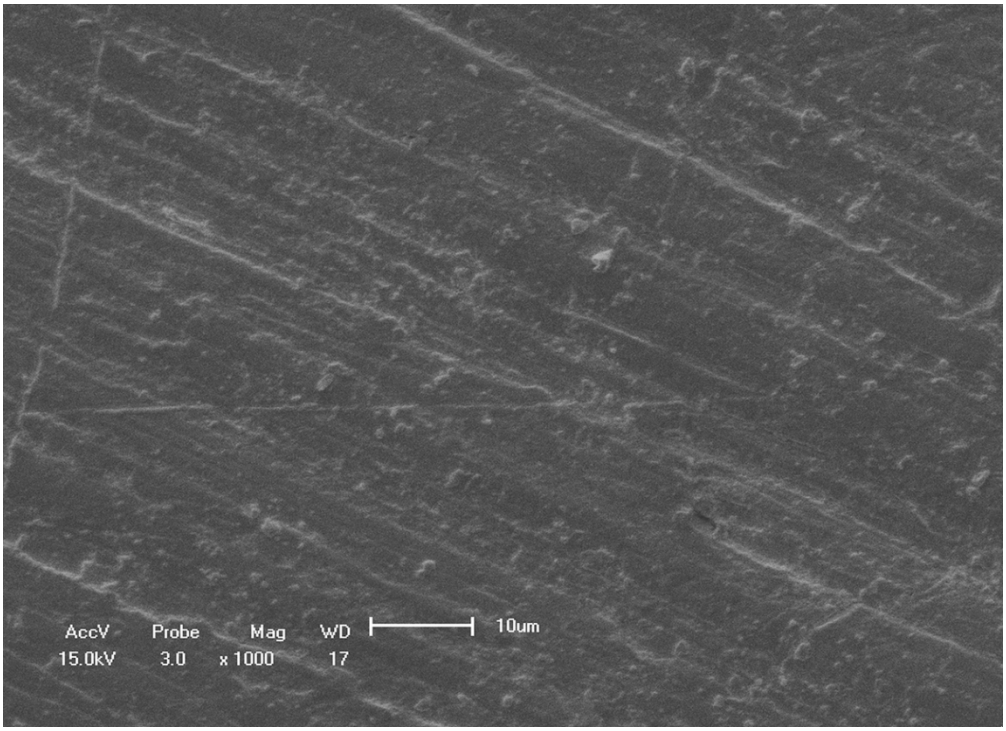

Figure 1 – SEM images of acrylic resin block surfaces at 1000 × magnification: (b) silicon dioxide coating specimen.

101x73mm (300 x 300 DPI)

Table 1 - Water contact angle (θ), surface tension parameters (γ +, γ-) and degree of hydrophobicity (ΔGsws) of the acrylic resin blocks in the control group (GCT) and treated with silicon dioxide (GVL). The values are represented by the means ± standard deviations of three independent experiments for each condition.

Groups	Surface parameters			
	θ (°)	γ^+ (mJm^{-2})	γ^- (mJm^{-2})	ΔG_{sws} (mJm^{-2})
GCT	104.1± 2.8	12.0	14.4	-9.5
GVL	114.3 ± 4.4	2.1	1.6	-54.4

T-test revealed that the coated group (GVL) had a significantly ($p<0.05$) lower hydrophobicity than non-coated group (GCT).

Table 2 - Evaluation of metabolic activity and total biomass of biofilms in the control (GCT) and experimental (GVL) groups.

Test	Group	Mean ± SD	p-value
Metabolic activity	GCT	0.44±0.44	0.02
	GVL	0.34±0.38	
Total biomass	GCT	0.73±0.44	0.05
	GVL	0.79±0.45	

p-valor: considered significance $p < 0.05$