

Enhancement of the Photodynamic Therapy Effect on *Streptococcus Mutans* Biofilm

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Received: November 07, 2013 / Accepted: October 14, 2013 / Published: February 15, 2014.

Abstract: Biofilm is a community of bacteria, less susceptible to traditional treatments. Although photodynamic therapy (PDT) is a very effective way to microorganism inactivation, in biofilm it is not as efficient as it is in planktonic bacteria cultures. The increment of an element to increase the effectiveness of PDT was our aim. Therefore, this *in vitro* study evaluates the susceptibility of a biofilm formed by *Streptococcus mutans* on metallic surface of orthodontic accessories under the application of PDT with a surfactant. Samples obtained from blades of orthodontic bands (NiCr), where used as adhesion surface for the biofilm. They were treated with 1 mg/ml of curcumin, with 0.1% of sodium dodecyl sulfate and exposed to 30 J/cm² of light (455 nm). Eight experimental groups were studied, including the positive and negative controls. The results show that the group with PDT and surfactant had a significant decrease ($p < 0.001$) in viability. In this case, the reduction observed was of 5.6log10 (CFU/ml) in comparison to the control group. We have shown that, even though the biofilm is very tough and complex structure, we are able to promote almost the complete inactivation of *S. mutans* in systems similar to an orthodontic treated patient's mouth.

Key words: Photodynamic therapy, light-induced damage, oral biofilm, streptococcus mutans, curcumin, surfactant.

1. Introduction

There are two forms for bacteria to survive in their natural environment. They may be free-floating, as a planktonic cell, or attached to a surface, as in a biofilm. The biofilm is a complex community in which most of bacteria live. It is a well-organized community that adheres to surfaces and is embedded in an extracellular adherent layer. Bacterial resistance increases when they are organized as biofilm. Also, once in a biofilm, the bacteria display different characteristics from those that they had as a free-floating organism. Biofilms can be found in many places, and the human mouth has perfect conditions for the bacteria to grow.

For that reason, biofilms are precursor for most common oral diseases, such as caries and periodontal disease. Prior to the formation of the biofilm, a thin microorganism layer adheres on the surface of the tooth or gum. These microorganisms use salivary proteins as substrate for adhesion. *Streptococcus mutans* produces adhesins that together with these proteins are used to form the substrate of adhesion. The extracellular substrates that they produce allow the accession of other types of microorganism to form the mature biofilm, and finally the dental plaque.

The dental biofilm can be categorized into sub- and supra-gingival. It is formed subsequent to an increase of saccharolytic and acidogenic microorganisms, such as *Streptococcus mutans* and *Lactobacillus*, which occur in most people who eat sucrose-rich food. Hence, these biofilms produce acids that cause tooth

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demineralization and caries. Four components are needed to create the carious lesion [1, 2]: a host, high-carbohydrate diet, microorganisms and time.

The *Streptococcus mutans*, that may be one of the most common bacteria in the oral cavity, produces a polyhedral matrix to structure the biofilm. The microorganisms existent in a biofilm become sheltered by this polyhedral matrix, reducing the action of antimicrobial agents. Compared to planktonic cells, the biofilm structure protects the bacteria, and other microorganisms. The thickness of this structure prevents contact between antimicrobial agents and microorganisms, keeping the chemicals away from the deeper layers of the biofilm. As a result, the biofilm boosts the growth rate of the microorganisms [2-4]. Consequently, microorganisms embedded in biofilms are 10 to 1000 times more resistant to antimicrobial agents [5, 6].

One cause that enhances the biofilm formation in the mouth is the installation of orthodontic accessories [7]. The usage of orthodontic accessories creates new retention surfaces for microorganisms in the oral cavity, increasing their retention. Therefore, it increases biofilm formation and the amount of bacteria in the mouth [7]. The high level of *S. mutans* in the oral cavity of patients with orthodontic accessories, compared to non-orthodontic treatment patients, is caused both by the presence of the accessories and also by the poor hygiene control of it by the patient.

Photodynamic therapy (PDT) is a well-known medical therapy for cell inactivation and microorganism control [8, 9]. This technique is based on the combined use of light and some types of drugs, called photosensitizers (PS), which produce an oxidation reaction. This oxidative reaction can then cause the death or inactivation of selected cells. PDT can also be used as a therapy with a potent antimicrobial effect [10]. Inactivation of microorganisms by PDT can ensure several advantages over the traditional use of antimicrobials. First, bacterial killing is rapid, reducing the need to maintain

high concentrations of chemicals for long periods, as in the case of antibiotics and antiseptics usage [11]. Secondly, bacterial killing is not linked to the intervention of chemicals, so, development of resistance is improbable to happen [12]. Finally, since neither the PS, nor the light, are bactericides by themselves, the destruction of bacteria can be controlled by restricting the irradiated region, preventing the destruction of the normal micro-biota elsewhere.

PDT is an alternative therapy for preventing and treating dental caries and periodontal diseases. It is an innovative way to control the formation of the bacterial biofilm, controlling the incidence of these pathogens. Most studies present high efficiency of PDT to planktonic bacteria. On the other hand, since the biofilm protects its microorganisms, the results over clinical trials are not as efficient [13]. Therefore, researches over systems that emulate the patients' mouth are so important to find the "perfect therapy". Thus, the purpose of this paper is to evaluate the effect of the antimicrobial activity of PDT on biofilms formed on orthodontic accessories. This *in vitro* study evaluates the use of PDT and a surfactant on metal surfaces, observing the susceptibility of biofilms formed by *S. mutans*. Our objective is to increase the performance of PDT, adding one element to the treatment, in a system that emulates the mouth real environment.

2. Material and Method

For this study 24 metal samples measuring 5 × 5 mm were used, obtained from orthodontic band blades (NiCr). On the metal surface, *S. mutans* biofilms were grown and treated with PDT and a surfactant. The PDT was induced by curcumin and light emitting diode (LED) in the blue range, and the surfactant used was the sodium dodecyl sulfate (SDS). There were tested eight experimental groups, each test repeated three times and each solution measured twice.

2.1 Biofilm Growth and CFU Preparation

For *Streptococcus mutans* biofilm, an inoculum from stock culture (ATCC 25175) was grown in Brain Heart Infusion (BHI) broth (Difco, Detroit, USA) and incubated at 37 °C for 24 h. The suspensions were centrifuged, 3,000 rpm per 15 min (Excelsa II centrifuge, model 206-BL, FANEM), and the bacterial pellet was dispersed into BHI broth with 20% sucrose. To archive the desired population density, the culture was adjusted to obtain standardized suspension containing 10^6 cells/ml, the optical density of the final suspensions was verified using a digital spectrophotometer (FEMTO 600).

The biofilm was grown in orthodontic band metal blades (orthodontic appliance, Tecnident, São Carlos, Brazil), cut into 5 × 5 mm squares, and sterilized in autoclave. Each sample was placed inside individuals wells of a 24-well microtiter plate. Then, aliquots of 1 mL of the *S. mutans* were transferred to the plates. The biofilms were incubated at 37 °C up to 7 days, with the growth medium changed every two days. All the samples were washed in PBS solution and placed in a new well into the 24 microtiter plate to be prepared for experimental treatment. They were separated between eighth groups and treated accordingly.

After treatment, the samples and theirs solutions were transferred to eppendorfs, 1ml of PBS was added, and they were homogenized, to break and loosen the biofilm into the solution, during 8 min in an ultrasound shaker (150 Watts, 50 Hz), before the metal squares were removed. The solutions were diluted up to 10^{-5} and uniformly spread to petri dishes containing culture medium BHI AGAR (Difco, Detroit, USA) plus 20% sucrose. They were aerobically cultured at 37 °C for 48 h prior to the colony-forming unit (CFU) count.

2.2 Experimental Treatment Procedure

The experimental groups included: control group (P-L-S-); light (P-L+S-), surfactant (P-L-S+) and photosensitizer (P+L-S-) groups; surfactant-light

(P-L+S+), photosensitizer-surfactant (P+L-S+) and PDT (P+L+S-) groups; and the PDT-surfactant (P+L+S+) group. Each group was named accordingly to the addition of the photosensitizer (P+ or P-), surfactant (S+ or S-) and application of light (L+ or L-).

All the samples were placed in a new well into the 24 microtiter plate and received the correct solution necessary for treatment. To the control (P-L-S-) and light (P-L+S-) groups, there was added 1 ml of a solution of PBS with 5% of DMSO. There was added 1 ml of surfactant solution to the surfactant (P-L-S+) and surfactant-light (P-L+S+) groups. The surfactant solution was made in PSB with 0.1% of SDS and 5% of DMSO. To the photosensitizer (P+L-S-) and PDT (P+L+S-) groups it was added 1 mL of a photosensitizer solution. This solution had 1 mg/ml of curcumin, and was made in PBS with 5% of DMSO. And to the photosensitizer-surfactant (P+L-S+) and PDT-surfactant (P+L+S+) groups, there was added 1 mL of a photosensitizer-surfactant solution, which had 1 mg/mL of curcumin, 0.1% of surfactant, and 5% of DMSO in PBS.

All the experimental groups were kept in the dark for 5 min, incubation of the PS, before the application of light in the designated groups. The experimental groups with light (L+) were then irradiated in a home-made blue LED device (high power royal blue LEDs, 355mW, centered at 455 nm with 20 nm bandwidth), for 10 min and 54 s, ensuring a 30 J/cm² dose at all the wells. The LED device (Fig. 1), called Biotable (LAT-IFSC-USP), was made to guarantee uniform distribution of light (46 mW/cm² intensity) in the plane where the samples were placed. The experimental groups (L-) where no light was applied were kept in the dark at room temperature, for the same amount of time.

2.3 Data Analysis

The effect of the photodynamic therapy and surfactant on the biofilms was evaluated by counting colony-forming units. The results were statistically evaluated by ANOVA, differences were considered

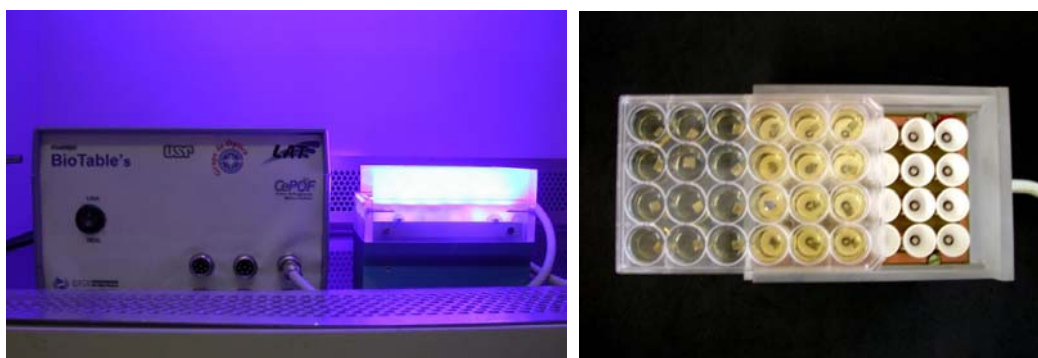


Fig. 1 Photography of the 24-well blue Biotable and details of the metal plate samples, placed in a 24-well microtiter plate, being put in place on the LED device.

when $p < 0.05$. The difference between treated groups with the control group (P-L-S-) was made with the OneWay ANOVA with post hoc Turkey.

3. Results and Discussion

To ensure biofilm formation, selected metallic samples were imaged with confocal microscopy (Fig. 2). The images showed the formation of *S. mutans* biofilm after 7 days of cultivation on the metallic samples of orthodontic appliance (NiCr). The photodynamic inactivation was also observed, by means of a live-dead biological marker, imaging plate before and after PDT treatment. The confocal reflection images show the metal surface, indicating the presence of colonies of *S. mutans*. The confocal fluorescence images show the fluorescence of the propidium iodide (PI) dye and

curcumin in the bacteria cells. The colonies can be highlighted when both images are superposed, showing the correspondence between the colonies seen by the reflection and fluorescence images.

The confocal reflectance images present the surface of the metal sample. It was expected that the orthodontic appliance squares show some roughness, and appears in the image as a random pattern. It is also noticed a pattern similar to a bunch of grapes, and the superposition of the reflectance and fluorescence images confirm that those patterns correspond to the biofilm. Meanwhile, the fluorescence image show red and yellow-green dots. The yellow-green dots are the fluorescence of the curcumin within the bacteria cells. The red dots are the fluorescence if the propidium iodide (PI) dye, which appear in the dead cells.

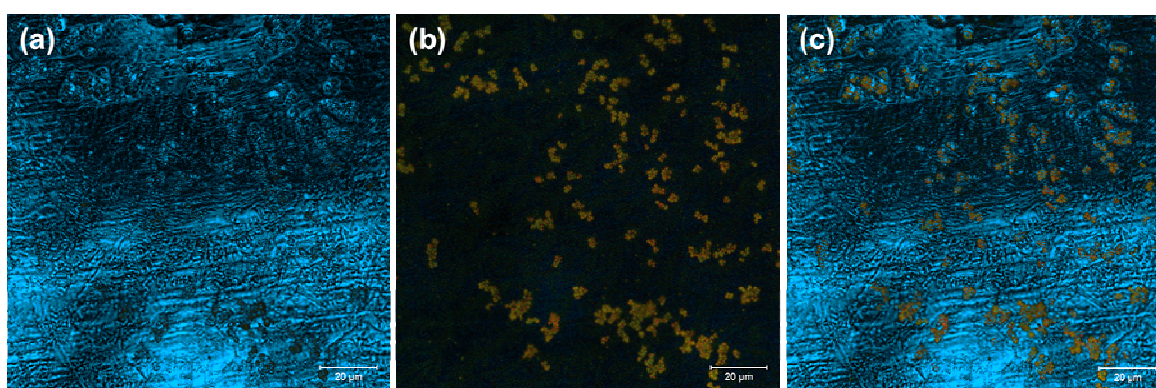


Fig. 2 Biofilms of *S. mutans* on metallic sample with 7 days, after PDT treatment: (a) Confocal reflection imaging, the image show the appearance of the metal surface, indicating the presence of *S. mutans* colonies; (b) confocal fluorescence image, showing the fluorescence of the propidium iodide (PI) dye and curcumin. The colonies appear as bright spots in the image, red indicates dead cells and yellowish-green show the living microorganisms and (c) reflection and fluorescence images superposed (a + b). The image shows the correspondence among the colonies seen by reflection and fluorescence images. The scale bar corresponds to 20 μm .

The bacterial effects of PDT and the surfactant were evaluated by the cell viability (CFU/ml). The efficacy of the treatment differs depending on the association of curcumin and surfactant (Fig. 3). They showed significant reductions in the viability of the *S. mutans* (over 1 log₁₀) for all groups, except for the experimental conditions (P-L-S+) and (P+L-S-). The highest reduction in the cell viability of the biofilms was equivalent to 5.6 log₁₀ (CFU/ml) and correspond to the association of PDT and surfactant.

The results show remarkable behavior of the bacteria population depending on the association of curcumin, surfactant and the application of light (Table 1). In some cases the illumination can promote biostimulation, but none of the studied cases presented a relatively relevant

increase on the bacterial population. Applying just PDT (P+S-L+) or surfactant (P-S+L-), it was observed small decreases on the *S. mutans* population. The same occur when light was applied mutually with surfactant (P-S+L+), but in this case the antibacterial effect was somehow smaller. The test group where only the photosensitizer was used (P+S-L-) was statistically similar to the control group, confirming that curcumin, by itself, is not capable to a significant bacteria inactivation. On the other hand, the association of curcumin and SDS (P+S+L-) upheld an even smaller decrease in the population. However, when the three were used together (P+S+L+), it was observed a huge antibacterial effect (-5.6 log₁₀). In this case, only one in a thousand part of the population survived.

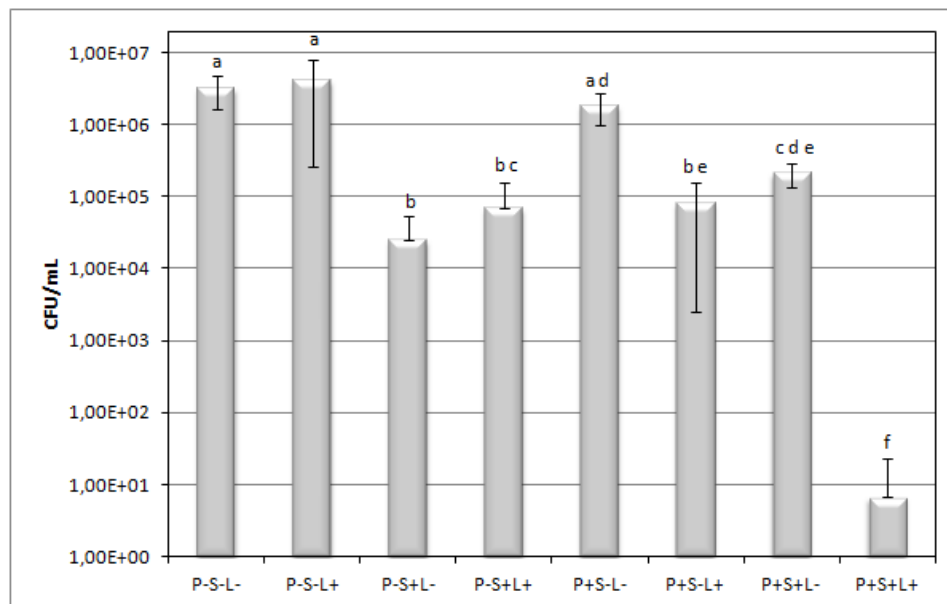


Fig. 3 Reduction of CFU for *S. mutans* biofilms. Individual difference made by ANOVA One Way: post hoc Turkey. Groups with the same letter are not significantly different ($p > 0.005$).

Table 1 Expression of cell viability (CFU/ml), related to the control group data, for all experimental groups. Groups with the same letter are not significantly different ($p > 0.005$).

Experimental groups	CFU percentage difference	Expression (log ₁₀)
(P-S-L-) ^a	0	
(P-S-L+) ^a	+29.17	+0.1
(P-S+L-) ^b	-99.22	-2.1
(P-S+L+) ^{bc}	-97.84	-1.6
(P+S-L-) ^{ad}	-42.91	-0.2
(P+S-L+) ^{be}	-97.46	-1.5
(P+S+L-) ^{cde}	-93.37	-1.1
(P+S+L+) ^f	-99.999792	-5.6

The oral microbiota is composed of more than 500 types of microorganisms, even though *S. mutans* is the etiologic agent of caries and demineralization. It is also responsible for the adhesion, colonization and dental biofilm formation [14]. This study evaluated particularly the susceptibility of *S. mutans* biofilm on the metal surface (NiCr) of orthodontic blade band by means of photodynamic therapy with curcumin (1 mg/mL), 0.1% of SDS and LED illumination. As expected, we have shown that photodynamic therapy reduces the number of living cells of *S. mutans* in a biofilm model created on metallic surface [15-17]. The presence of photosensitizer, without irradiation, was not able to achieve an effective antibacterial outcome. This confirms the essential rule of light to obtain an anti-microbial effect [18, 19] by means of PDT.

The most significant finding was the lack of viability of *S. mutans* on the surfaces of the samples treated with curcumin combining PDT with surfactant. The result for this group (P+S+L+) is above the minimal bactericidal concentration, which is the minimum concentration able to reduce 4 log₁₀ on the survival of a microorganism [20]. Significant decrease on the viability of microorganisms was also observed when biofilms were exposed to the surfactant alone (P-S+L-), surfactant with light (P-S+L+), photosensitizer with light (P+S-L+), or the combined use of surfactant and curcumin without light (P+S+L-), compared to the control group (P-S-L-). For these groups, the reduction on the bacteria viability was above 90%. Depending on the application, 90% of reduction on a microorganism population might be sufficient. Although the results show that the bacterial inactivation can be almost complete when the surfactant was used together with PDT.

The surfactants are amphipathic molecules composed of a hydrophobic portion and a hydrophilic portion. The first is often a nonpolar hydrocarbon chain, while the second can be ionic (cationic or anionic), nonionic or amphoteric. The surfactants, for their biochemical characteristics, are highly potent and act

as emulsifiers, reducing interfacial and superficial tension [21]. They are responsible for modifications on the ion channels of the bacterial membrane via trans-membranes proteins, similar to the porins. This allows the passage of solutes through the membrane, increasing the permeability of these membranes [21-24]. Surfactants also work as: emulsifiers, dispersing in water hydrocarbons or other insoluble compounds; reducing the adhesion and releasing surfaces cell; and presenting antibiotic activity.

The mechanism of action of PDT is not connected to the mediation of chemical radicals acting in a single target, which is the case of the antibacterial products. Photosensitizers act producing reactive oxygen species (ROS) which have no specific site of action. This avoids the formation of strains resistant to antibiotics [20, 21, 25, 26]. Photodynamic therapy applied as antimicrobial therapy can be also advantageous over mechanical removal methods, since it can reach places such as recesses and protrusions of the orthodontic accessories [27]. It may also enable safe treatment of patients with special needs or difficulty in oral hygiene during orthodontic treatment, avoiding oral infections, such as dental caries, gingivitis and periodontal pockets [13, 27].

Several hypotheses could explain the synergism of the PDT action with surfactant as antimicrobial therapy in *S. mutans* biofilm. The surfactant could be increasing the number of working cycles of PS, protecting it from oxidation, and increasing the efficacy of PDT. It could also be acting increasing the permeability of the curcumin through the cell membrane, increasing the concentration of PS molecules in the bacteria. One other hypothesis is that the surfactant is breaking the biofilm structure, and this allows the PDT to be more effective. Although the surfactant, SDS, can be used as antimicrobial by itself, the combined effects of it with PDT cannot explain such efficacy of the treatment. The reduction on bacteria viability was larger than one could expect from using both treatments separately. Therefore more

studies must be done in order to thoroughly understand this mechanism.

4. Conclusions

The result of this study shows higher reduction in the *S. mutans* population, compared to previous investigations [15, 19]. We demonstrate significant removal of *S. mutans* biofilm after application of PDT with SDS as antimicrobial therapy. PDT associated to surfactants promoted a high inactivation of *S. mutans* biofilm (5.6log10) on the surface of metallic orthodontic accessories. We have shown that, even though the biofilm is very tough and complex structure, we are able to promote almost the complete inactivation of *S. mutans* in a system similar to an orthodontic treated patient's mouth. And this inactivation was achieved with a protocol that does not promote bacterial resistance that applied PDT and SDS.

Given the findings, our results suggest that the surfactant acts on the microorganisms' membrane making them permeable to solutes, and breaking the biofilm structure. The surfactant leads to higher the input of oxygen molecules and curcumin from the extracellular medium into the microorganism. Therefore, the synergistic action of the SDS and PS produces higher amounts of reactive oxygen species (ROS) upon irradiation, optimizing the antimicrobial effect of PDT. At the same time, protecting the PS molecules from oxidation and increasing its lifetime of the PDT cycle. These hypotheses should be put to test in supplementary investigations.

Aknowledgments

The authors would like to acknowledge the support of the Brazilian agencies FAPESP, CNPQ and CAPES, through founding and scholarships. V. H. Panhóca would like to acknowledge scientific contributions and helpful advice from Clóvis Grecco and Fernanda Rossi Paolillo.

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