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Real-time Assessment of *Streptococcus mutans* Biofilm Metabolism on Resin Composite

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Abstract

Objectives—The release of unpolymerized monomers and by-products of resin composites influences biofilm growth and confounds the measurement of metabolic activity. Current assays to measure biofilm viability have critical limitations and are typically not performed on relevant substrates. The objective of the present study was to determine the utility of firefly luciferase assay for quantification of the viability of intact biofilms on a resin composite substrate, and correlate the results with a standard method (viable colony counts).

Methods—Disk-shaped specimens of a dental resin composite were fabricated, wet-polished, UV-sterilized, and stored in water. Biofilms of *S. mutans* (strain UA159 modified by insertion of constitutively expressed firefly *luc* gene) were grown (1:500 dilution; anaerobic conditions, 24h, 37°C) in two media concentrations (0.35x and 0.65x THY medium supplemented with 0.1% sucrose; n=15/group). An additional group of specimens with biofilms grown in 0.65x + sucrose media was treated with chlorhexidine gluconate solution to serve as the control group. Bioluminescence measurements of non-disrupted biofilms were obtained after addition of D-luciferin substrate. The adherent biofilms were removed by sonication, and bioluminescence of sonicated bacteria was then measured. Viable colony counts were performed after plating sonicated bacteria on THY agar plates supplemented with spectinomycin. Bioluminescence values and cell counts were correlated using Spearman Correlation tests ($\alpha=0.05$).

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Results—Strong positive correlations between viable colony counts and bioluminescence values, both before- and after-sonication, validated the utility of this assay.

Significance—A novel non-disruptive, real-time bioluminescence assay is presented for quantification of intact *S. mutans* biofilms grown on a resin composite, and potentially on antibacterial materials and other types of dental biomaterials.

Keywords (MeSH)

Biofilms; *Streptococcus mutans*; Composite Resins; Bioluminescent Assays; Colony Count; Microbial; Dental Materials

1. Introduction

The oral cavity is the habitat of a broad variety of microorganisms [1]. Bacteria are the most common type of microorganism present in the oral milieu. Over 700 bacterial species have been detected in the oral microflora using various cultural and molecular methods [2–5]. *Streptococcus mutans* has been implicated as the main causative agent of both primary and secondary caries [6, 7]. However, since *S. mutans* has been shown to account for only 1.6% of the total cariogenic biomass in active carious lesions [8], its role as the primary cause of tooth decay has been questioned [9]. An uncontested attribute of active carious lesions is the presence of polymicrobial biofilms and particularly the acid-producing microorganisms within them. *S. mutans* has been investigated for many years as a model cariogenic organism for its ability to metabolize sugars to acid and because it forms biofilms by the deposition of water-insoluble glucans and other extracellular polymeric substances (EPS).

The viability of bacterial cells within biofilms is most commonly determined using the viable colony count method [10]. The major advantage of this approach is the ability to quantify only the number of viable bacteria [11]. This method's main disadvantage is the requirement that bacteria be separated from the EPS by vortexing, sonication or matrix-dissolving enzymes. Significant errors could be introduced during that step because manipulation of the biofilms may impact the cells' viability and does not guarantee complete or reproducible removal [12]. In addition, microbial aggregation of bacteria like *S. mutans* that typically grow in dense microcolonies could lead to inaccurate counts of viable cells [11]. Furthermore, resin composites release by-products and unpolymerized monomers that could influence biofilm growth [13, 14]. In our experience, biofilms are harder to remove from polymer-based materials as compared to other dental materials, thereby potentially limiting the accuracy of colony counts of biofilms on resin-based dental biomaterials.

These limitations have precipitated the development of simpler and less sensitive quantification methods, such as metabolic assays. Their mechanisms of action are based on either the quantification of extracellular metabolites (e.g., lactic acid) or on the cells' capacity to reduce organic dyes (e.g., resazurin[15] [15]). However, existing metabolic assays require the use of calibration curves derived from planktonic bacteria to quantify bacteria in biofilms, which introduces large errors in the assessment of cell viability due to the distinct metabolic rates between biofilms and their planktonic counterparts [12]. In addition, metabolic assays typically do not permit evaluation of other parameters present in

vivo, such as the effect of physiochemical characteristics of the substrate materials on which biofilms grow [16].

One of the major limitations of the resazurin (Alamar Blue) metabolic assay is the need for large numbers of cells (greater than 5- or 6-log CFU) to obtain results reasonably quickly (1–5 hours) [15–18]. In addition, Erb and Ehlers [19] demonstrated that reduction of the fluorescent product (resorufin) into the nonfluorescent product (hydroresorufin) could lead to inaccurate results.

In light of the above limitations, a method is needed to quantify the viability of bacterial cells in biofilms in a non-disruptive, minimally invasive and real-time manner. One possible alternative is the utilization of bioluminescence assays based on the use of the North American firefly luciferase (*Photinus pyralis*; Fan and Wood [20]). These assays are considered highly efficient because nearly all of the ATP pool is converted into light (yield of 0.88 [21, 22]). The equation below by Shama and Malik [23] describes the basic mechanism.

where, *ATP* stands for adenosine triphosphate, *AMP* stands for adenosine monophosphate, *PPi* for Pyrophosphate, and *hν* indicates light emission.

In 1998, Loimaranta et al. [24] introduced a bioluminescent strain of *S. mutans* that was used to screen the *in vitro* efficacy of antimicrobial agents. The *luc* gene was placed under the control of a phage promoter and introduced into *S. mutans* via shuttle plasmid. The placement of the *luc* gene, as described, presents important drawbacks because the stability and the number of copies of the shuttle plasmid cannot be predicted during biofilm growth, which may affect the assay's accuracy.

Merritt et al. [25] introduced a new construct in which the *luc* gene was placed under the control of the *S. mutans* lactate dehydrogenase (*ldh*) gene promoter. The system *ldh-luc* was shown to be advantageous over previous constructs because lactate dehydrogenase is an essential enzyme for *S. mutans* carbohydrate fermentation as well as the primary source of *S. mutans* cariogenicity via lactic acid production [6, 26, 27]. Therefore, any strategy that alters this enzyme's activity or the intracellular energy balance can be directly quantified. Other important benefits of the *ldh-luc* reporter system are the ability to non-destructively assess the metabolic status of *S. mutans* in real time and the potential use of this method in a high-throughput format to screen the efficacy of antibacterial agents on oral pathogens. However, the biofilms in the study by Merritt et al. [25] were grown for 16 hours in microcentrifuge tubes and in the wells of 96-well plates, which are not substrates of relevance to the clinical practice of dentistry.

With this in mind, we present a non-disruptive bioluminescence assay that is optimized to quantify the viability of *S. mutans* biofilms grown for 24 hours on a resin composite. The assay proposed has been validated using the standard viable colony counts method. We also

investigated the ability of the proposed assay to differentiate between viable and non-viable cells, as well as its utility as a screening method for antibacterial strategies focused on the control of oral pathogens.

2. Materials and Methods

2.1 Specimen fabrication

Specimens (diameter 6.0mm, height 1.1mm) of Point 4TM microhybrid resin composite (shade A2; Kerr Corp., USA) were fabricated in a single increment using a custom-made mold. Specimens were photopolymerized against glass slides (40sec) using a LED light-curing unit (Ultra-Lume LED 5, Ultradent Products, Inc., USA). Then, specimens were subjected to a sequential wet-polishing procedure (180–1,200 grit SiC disks; final polish with 0.5µm diamond suspension) using a semi-automated grinder-polisher (MultiPrepTM, Allied High Tech Products, Inc., USA). The specimens were UV-sterilized (254nm, 800,000µJ/cm², model CL-1000 UVP Crosslinker, UVP, LLC, USA) and stored in sterile ultra-pure water at 37°C for 72 hours to extract unreacted monomers. Specimens were then distributed randomly among the experimental groups.

2.2 Bacterial strain and growth conditions

Bioluminescent *S. mutans* strain JM10 [25], a derivative of wild type UA159 was used. Details about the strain construction were reported by Merritt et al. [25]. Briefly, the strain was constructed by transforming plasmid pJM-1 ($\Phi::(ldh-luc)$, Spc^R) into UA159 selecting for chromosomal integration of the plasmid via its spectinomycin resistance, which is only expressed when the plasmid integrates into the chromosomal region via homologous recombination at the *ldh* locus. The presence of the reporter fusion was confirmed by selection of antibiotic-resistant colonies on TH (Todd-Hewitt, BD Difco, USA) plates supplemented with 0.3% yeast extract (EMD Millipore, USA) and 800µg/mL of spectinomycin (MP Biomedicals, USA). Colonies were cultivated under anaerobic conditions at 37°C for 48 hours. It is important to note that the manipulation of recombinant strains requires trained researchers and certified laboratory facilities.

2.3 In vitro growth of biofilms

Planktonic cultures of JM10 were grown in TH culture medium supplemented with 0.3% yeast extract (THY) and spectinomycin (32µL) for 16 hours (static cultures, anaerobic conditions, 37°C). Planktonic cultures having optical densities (OD_{600}) 0.900 were used as inocula for biofilm growth. A 1:500 dilution of the inoculum was added to either 0.35x or 0.65x THY biofilm growth medium supplemented with 0.1%(w/v) sucrose. Two concentrations of biofilm growth media (0.35x and 0.65x) were chosen to produce biofilms having different levels of viability. Aliquots (2.5mL) of each dilution were dispensed into the wells of sterile 12-well microtiter plates (Falcon, Corning, USA) containing the polished, sterile specimens (n=15/group). Specimens in media without inoculum served as the sterility control. All biofilms were grown under the same conditions (static cultures, anaerobic conditions, 37°C, 24 hours), unless otherwise specified. The specimens were transferred to microcentrifuge tubes containing 200µL of fresh 1x THY + 1%(w/v) glucose culture medium (recharge medium) one hour before the measurement of bioluminescence.

2.4 Treatment with chlorhexidine

Since chlorhexidine gluconate (CHX) has been shown to be a potent antibacterial agent against *S. mutans* [28], an additional group of specimens (n=15) on which biofilms were grown in 0.65x THY medium supplemented with 0.1%(w/v) sucrose for 24 hours was treated with 2% CHX solution (CHX-Plus™, Inter-Med, Inc., USA) to serve as the control group. Specimens in that group were subjected to the same fabrication, polishing, sterilization, monomer extraction and biofilm growth protocols described in Sections 2.1 – 2.3 above. Next, the media was carefully aspirated from the microcentrifuge tubes containing the specimens, and then 1mL of 2% CHX solution was added to the tubes for 2 minutes. Then, the CHX solution was carefully aspirated, and replenished with recharge medium.

2.5 Bioluminescence assay

To determine the proposed method's utility for the assessment of *S. mutans* metabolic status in biofilms grown on the surfaces of resin composite specimens, and to be able to correlate these results with standard viable colony counts, bioluminescence measurements were obtained before and after biofilm sonication for specimens in all groups. D-luciferin aqueous solution (100mM) suspended in 0.1 M citrate buffer (pH 6.0) was added (40µL) to microcentrifuge tubes containing the specimens and 200µL of recharge medium. Bioluminescence was measured using a luminometer (TD 20/20n, Turner BioSystems, USA). The temporal assessment of luciferase activity in non-disrupted *S. mutans* biofilms was evaluated in 2-minute increments (6 minutes total) after the addition of D-luciferin substrate. Next, the media was carefully aspirated from the microcentrifuge tubes, and then replenished with recharge medium. The replenished biofilms were sonicated to facilitate removal of the adherent biomass using a sonicator (Q700 sonicator, QSonica, LLC, USA) connected to a water bath (4°C; 4 cycles of 1 minute, 15 seconds interval between cycles; power 230±10W). The specimens were then incubated at 37°C (1 hour). The second bioluminescence measurement was obtained in an identical manner to the first measurement.

2.6 Viable colony counts

Immediately after the second bioluminescence measurement, aliquots (10µL) of sonicated bacteria were diluted in 90µL of recharge medium (10^{-1}) after the second bioluminescence measurement. Five ten-fold serial dilutions (10^{-6}) were carried out in recharge medium for all samples. Aliquots (10µL) of each dilution were then plated in triplicate (total: 30µL/specimen/dilution) using THY plates supplemented with spectinomycin (800µg), as described before. The counting of viable cells was carried out using the method reported by Miles et al. [29]. Optical microscopy was used to confirm removal of biofilms from surfaces.

2.7 Staining and confocal microscopy

A separate set of specimens was subjected to the procedures described in sections 2.1 – 2.4 above in preparation for staining and confocal microscopy. Biofilms on all specimens were stained using a LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, USA; 1.67µM each of Syto® 9 and Propidium Iodide to stain live and dead/damaged bacteria, respectively). Biofilms were kept hydrated in sterile ultrapure water prior to confocal

microscopy. The confocal microscopy procedure has been described in detail in our previous publication [30], and will be summarized here. Images of the full thickness biofilms were acquired at three random locations per specimen using a confocal laser scanning microscope (TCS SP2 MP, Leica Microsystems, Inc., USA) with Ar (488 nm) and He/Ne (543 nm) lasers for excitation of the fluorescent stains. A 63x water immersion microscope objective lens was used. Representative 3-D reconstructions of live and dead/damaged cells in the biofilms were generated using Volocity software (PerkinElmer, USA) to facilitate visualization of their distribution in all groups.

2.8 Statistical Analysis

Summary statistics were calculated using SAS software (version 9.2; SAS Institute, USA). The bioluminescence values and viable colony counts were tested for normality ($\alpha=0.05$). Since the Shapiro-Wilk normality test indicated that data were not normally distributed ($p<0.0002$), correlation was determined using a Spearman Correlation test ($\alpha=0.05$). Viable colony count values of the various groups were analyzed using one-factor ANOVA and *post hoc* Student-Newman-Keuls tests ($\alpha=0.05$).

3. Results

The experimental design presented had the following objectives: i) determination of bioluminescent activity over time, ii) validation of the bioluminescence assay versus a standard assay and, iii) determination of the bioluminescence assay as an effective method for assessment of *S. mutans* biofilm growth on resin composite. To this end, luciferase activity levels were assessed via two independent and sequential bioluminescence measurements. The first measurement was performed on non-disrupted biofilms and served as the baseline. The second measurement was performed after the sonication of biofilms and permitted correlation between the assays.

Figure 1 demonstrates the evolution of luciferase activity over time. Time points BR0 to BR6 indicate time in 2-minute intervals after the addition of D-luciferin substrate to non-disrupted biofilms. The 0.65x and CHX (control) groups, which were both grown in 0.65x THY biofilm growth medium with 0.1%(w/v) sucrose under the same conditions displayed similar levels ($p>0.05$) of luciferase activity from BR0 to BR6. Luciferase activity levels were significantly lower in the 0.35x media group than the remaining groups ($p<0.05$), which is expected because biofilms received less nutrition from the more diluted media in the former. In addition, both media groups showed bioluminescence versus time curves with similar slopes. These findings demonstrate that the kinetics of the luciferase reaction followed a pattern, regardless of media concentration and biomass amount, indicating the level of consistency and robustness of the method proposed. Another common characteristic of the curves was a rapid increase in luciferase activity which takes place between time points BR0 and BR2. From time point BR2 until the end of the first luciferase measurement (time point BR6), the results show contiguous bioluminescence levels for all groups. This finding indicates that the cells assessed in the biofilms had constant levels of intracellular ATP at all time points.

Time points AR0 to AR6 in Figure 1 indicate time in 2-minute intervals after the addition of D-luciferin aqueous solution to sonicated bacteria. The 0.65x and 0.35x media groups had luciferase activity levels that were detectable using bioluminescence. The CHX group had luciferase activity levels that were comparable with the sterility control specimens (media without inoculum), which confirms that no viable cells were detected in that group after a 2-minute treatment with 2% chlorhexidine gluconate solution.

Figure 2 displays the results of viable colony counts (CFU/mL) of sonicated bacteria in the 0.65x and 0.35x media groups as well as the CHX (control) group. The results of the one-factor ANOVA and *post hoc* Student-Newman-Keuls (SNK) analyses demonstrated statistically significant differences ($p < 0.05$) among the groups tested. The colony counts of the 0.65x media group were higher than those of the 0.35x group, as would be expected because biofilms in the latter received less nutrition from the more diluted media. The mean value of colony counts of the CHX (control) group, treated with 2% CHX before sonication, was zero, and thereby confirmed killing within the biofilms in that group.

The results of the Spearman Correlation test (Table 1) demonstrated strong correlations between after-sonication colony counts and RLU values of both non-disrupted biofilms and sonicated bacteria. All correlations were statistically significant ($p < 0.0001$), thereby validating the ability of the bioluminescence assay to quantify the viability of *S. mutans* biofilms grown on resin composite.

A visual analysis of the 3-D renderings of living and dead/damaged cells within the biofilms in each group (Figure 3) confirmed that the largest biovolume of living cells was observed in the 0.65x media group, with the 0.35x media group having a smaller biovolume of living cells. The largest biovolume of dead/damaged bacteria was observed in the CHX group. Therefore, the results of the visual analysis confirmed the outcomes of both assays.

4. Discussion

Rapid bioluminescence methods have been used to determine the cellular viability of oral microorganisms since 1970s [31–33]. The use of these assays established a strong and positive correlation between the adenylate energy charge [34], which is a composite measure of bacteria's intracellular energy potential, and the total amount of viable cells within oral biofilms. Fazilat et al. (2010) reported the chairside use of a bioluminescence assay for the rapid screening of oral bacterial load. The authors presented Pearson correlation values for total bacteria, oral streptococci, and *S. mutans* of 0.854, 0.840, and 0.796, respectively. In the present study, we found Spearman correlation values of 0.766 ($p < 0.0001$) for non-disrupted biofilms and 0.910 ($p < 0.0001$) for sonicated bacteria.

However, previously reported methods for the measurement of intracellular ATP require an intensive manipulation of biofilms to obtain adequate ATP extraction [31, 33, 35]. The destructive manipulation of biofilms hindered the ability to track inhibitory kinetics of antibacterial agents and the recovery assessment of antibiotic-treated cells.

In the present study, we have described a simple method to quantify the viability of non-disrupted *S. mutans* biofilms grown on relevant substrates that does not depend on the use of

highly sophisticated equipment, and allows a direct comparison of selected antimicrobial agents against biofilms. In addition, several studies have indicated that bioluminescence assays have 10–1,000x higher sensitivity levels than do fluorescence assays like green fluorescent protein/GFP [36]. Furthermore, the bioluminescence assay provides a quantitative method for the assessment of bacterial viability when compared to the use of confocal laser scanning microscopy with fluorescent stains like the LIVE/DEAD® BacLight™ Bacterial Viability Kit, because LIVE/DEAD® staining is mostly a qualitative assessment method except when 3-D structural parameters are used [30]. The results of this study confirm that bioluminescence could be used to precisely quantify the viability of cells within complex biological systems, such as oral biofilms.

Robrish et al. [37] studied the effect of the sonication treatment on the viability of cells. Their findings demonstrated that bacteria vary in sensitivity to sonic treatment, with Gram-negative rods being the most sensitive and Gram-positive cocci being the most resistant. They further demonstrated that 28,400 W/s sonication energy was needed to promote the killing of 50% of a *S. mutans* population. The low sonication levels applied in the present study (230 ± 10 W/s) suggests that our removal protocol did not significantly alter cell viability. In addition, the correlation values for sonicated bacteria indicate that most of the biomass was efficiently removed from the adherent surfaces without impacting their viability.

Our results are supported by previous findings [24, 25] that demonstrated a linear and directly proportional relationship between the cells' metabolic status and their viability. Robrish et al. [31] highlighted that bacteria present in oral biofilms have uniform cytoplasmic volumes, which allows the intracellular ATP content of any given organism to be used as a good indicator of biomass.

The present study has demonstrated the utility of the proposed assay to quantify *S. mutans* metabolic activity in a non-disruptive and real-time manner. This bioluminescence assay also has the advantage of eliminating the need to manipulate biofilms, which saves time and decreases data variability, and could therefore be useful when developing new antimicrobial approaches. We propose that this bioluminescence assay is a preferable approach for the quantification of oral biofilms when compared to the metabolic assays previously described in the literature. In addition, we believe that the present assay can be optimized to include saliva pre-conditioning and can be scaled up for high-throughput studies focused specifically on the control of oral pathogens. Further optimization of the present methodology may be required in order to screen different antimicrobial approaches against multi-species biofilms grown on the surfaces of a variety of restorative dental materials and tooth substrates.

5. Conclusions

This study reported the development of a non-disruptive, real-time bioluminescence assay to quantify the metabolic status of intact *S. mutans* biofilms grown on relevant substrates, such as resin composites, in a minimally invasive and real-time manner. Furthermore, we demonstrated strong statistical correlations between viable colony counts and

bioluminescence values, both before and after sonication of biofilms, to validate the utility of this novel assay.

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Highlights

- We propose a bioluminescence assay to quantify biofilm metabolism on resin composite
- Positive correlations with viable colony counts validated the bioluminescence assay
- Antibacterial dental materials could be tested with this non-disruptive, real-time assay

Figure 1.

Expression of luciferase activity (n=15/group) was quantified in terms of bioluminescence. Biofilms grown in 0.65x and 0.35x THY media concentrations had quantifiable luciferase activity after sonication, whereas biofilms in the CHX (Control) group showed metabolic activity comparable to the sterility control (specimens in media without inoculum) after treatment with 2% chlorhexidine gluconate solution and subsequent sonication.

Figure 2.

Mean viable colony counts of sonicated bacteria. Capital letters denote mean viable colony values that were statistically different based on Student-Newman-Keuls rankings (SNK; $p < 0.05$). The mean viable colony count of sonicated bacteria in the CHX (Control) group confirmed the antibacterial behavior of 2% chlorhexidine gluconate solution.

Figure 3. (A–C). Representative 3-D reconstructions of living and dead/damaged bacteria within the biofilms grown in 0.65x (A) and 0.35x (B) media concentrations, and biofilms in the control group that were treated with CHX (C) are shown in this figure. Concurrent overlay of the fluorescent stains permitted the simultaneous visualization of living (green stain) and dead/damaged cells (red stain) within *S. mutans* biofilms. (1 Unit = 24 μm).

Table 1

Correlation between after-sonication viable colony counts and bioluminescence values of non-disrupted biofilms and sonicated bacteria.

Spearman Correlation Coefficients (p-value)	Time after addition of D-luciferin substrate			
	0min	2min	4min	6min
Non-disrupted biofilm (BR)	0.766 (p<0.0001)	0.719 (p <0.0001)	0.731 (p <0.0001)	0.731 (p <0.0001)
Sonicated bacteria (AR)	0.910 (p <0.0001)	0.905 (p <0.0001)	0.905 (p <0.0001)	0.905 (p <0.0001)