# Measuring Adhesion Strength of an Improved Dental Biofilm Model on a Titanium Surface

M.N. Hessin, J.D. Boyd, and M.E. Grady

Department of Mechanical Engineering
College of Engineering at the University of Kentucky

Kentucky, Lexington, 20508

**ABSTRACT**
Adhesion of bacteria to oral implant surfaces can lead to oral infections, and the prevention of strong biofilm adherence to implant surfaces can assist in the prevention of these infections like peri-implantitis. In prior studies, single species biofilm adhesion has been quantitatively measured via the laser spallation technique. However, colonizing oral biofilms rarely consist of a single bacteria species. Multiple early colonizer species, including several strains of Streptococci, dominate initial oral biofilm formation. This study aims to characterize the adhesion of a multi-species oral biofilm consisting of *S. oralis*, *S. sanguinis*, and *S. gordonii* on titanium, a common implant material, using the laser spallation technique. Previous work has established these specific Streptococci strains as a multi-species periodontal biofilm model. This study is the first to provide a quantitative adhesion measurement of this multi-species model onto a dental implant surface. First, adhesion strength of the multi-species model is compared to adhesion strength of the single-species streptococci constituents. Fluorescent staining and imaging by fluorescent microscopy are used to identify individual bacteria species within the biofilm. The multi-species biofilm presented in this study provides a more representative model of in vivo early biofilms and provides a more accurate metric for understanding biocompatibility on implant surfaces.

**Keywords:** Laser Spallation, Adhesion, Biofilms, Dental Implants

**INTRODUCTION**
Laser spallation technique, originally used on thin film substrates, has recently been evaluated on its effectiveness to detach cells and biological substances [8]. Boyd et al adopted the laser spallation technique to measure adhesion of single-species biofilms containing *S. mutans* [1,2]. However, oral biofilms contain more than one single species, and initial oral biofilms on teeth are composed of mainly Streptococci species, three of which are *S. oralis, S. sanguinis*, and *S. gordonii* [6]. These three gram-positive streptococci species are common pioneer colonizers since they attach directly to the pellicle surface on the tooth [3,11]. The pellicle is made up of salivary proteins that coat the teeth [6]. After early colonizing bacteria adhere to the pellicle surface, later colonizing bacteria attach to the early colonizing bacteria to form a mature biofilm [7]. Strongly adhered biofilms to dental implants cause infections and can lead to implant failure. Studying the adhesion of bacteria and cells to these surfaces can potentially allow for better implant biomaterial selection that favors the adhesion of cells over the adhesion of bacteria.

Since early colonizing bacteria form the bases of a dental biofilm, they are examined in this study as a multi-species biofilm to represent an initial dental biofilm. A stress-based adhesion measurement using laser spallation is analyzed on the multi-species model. *S. oralis*, *S. sanguinis*, and *S. gordonii* are all facultative anaerobes, signifying they can survive in both an anaerobic and aerobic environment [9]. Oral bacteria can migrate underneath the gum between the gum tissue and dental implant, which is an anaerobic environment, and can lead to implant failure. Hence, the adhesion of these bacteria cultured anaerobically is of interest. Future experimentation with an anaerobic multi-species biofilm will be done using a Sheldon Manufacturing anaerobic chamber to culture biofilms. The intention is to use the laser spallation technique on bacteria cultured anaerobically to simulate an environment close to that underneath the gums.

## METHODS AND MATERIALS

*Culturing Bacteria:* All single species biofilms of *S. oralis* in this study were cultured planktonically for 24 hours in Brain Heart Infusion (BD Difico Bacto Brain Heart Infusion; Fisher Scientific) supplemented with yeast extract (Bacto Yeast Extract; Thermo Fisher Scientific), L-Cysteine (Sigma-Aldrich), Hemin (Pig Hemin; Thermo Fisher Scientific) and Menadione (2-mehtyl-1, 4-naphthoquinone, vitamin K3; Thermo Scientific). Per each biofilm, 1 mL of the planktonic culture was dispensed on a glass bottom petri dish at an $OD_{600}$ of 0.7 along with 3 ml of 75 mM sucrose in BHI culture media broth. Biofilms were cultured for 24 hours in an incubator at 37 C with 5% $CO_2$.

*Staining Bacteria:* Each bacterial species was stained with a different nucleic acid stain to distinguish each species in the multi-species biofilm. The stains were chosen based on excitation and emission wavelengths corresponding to DAPI, FITC, and mCHERRY filter cubes. SYTO-41, SYTO-21, and SYTO-64 were chosen as the DAPI, FITC, and mCherry stains respectively. The stains were chosen so that each stain would only be detected in its corresponding filter cube on the fluorescent microscope. Three single-species biofilms were cultured on glass petri dishes for 24 hours and then stained with either SYTO-41, SYTO-21, or SYTO-64. Each SYTO stain was mixed with PBS to a final concentration of 1 μM and dispensed over its respective biofilm. Each biofilm was then washed with 1mL of PBS to remove excess stain. Another single-species biofilm of *S. oralis* was stained planktonically before biofilm formation. The stain was added to the planktonic culture to a final concentration of 1 μM. Once stained, the bacteria were centrifuged and resuspended in media to omit excess stain. After resuspension, the bacteria were plated on glass petri dishes and cultured for 24 hours.

*Imaging:* Images of each biofilm were taken on a fluorescent microscope at 10x magnification to validate that each stain has an emission spectrum that can only be shown in its respective filter cube. After culturing, each biofilm was aspirated to remove excess media before being analyzed under DAPI, FITC, and mCherry filter cubes on the Nikon fluorescent microscope. The brightness and exposure were kept constant when switching from one filter to the next.

*Preparing Substrates for Laser Spallation:* Future experimentation will include biofilms cultured on titanium surfaces. The substrate will include a glass layer sandwiched between one thin layer of titanium and one thin layer of aluminum. The glass layer will provide a medium for the laser shock wave to travel, while the thin aluminum layer will absorb the energy of the laser. Waterglass will be coated onto the aluminum side of the substrate to enable the propagation of the shock wave into the substrate towards the side of the titanium layer.

## PRELIMINARY RESULTS

Three biofilms of *S. oralis* were cultured for 24 hours, then stained after biofilm formation (Figure 1). The first biofilm was stained with SYTO-41 blue stain and imaged with the DAPI (1a) FITC (1b) and mCHERRY (1c) filter cubes. Only in the DAPI filter cube was STYO-41 stain detected. Detection of the stain was determined based on whether the fluorescent microscope received enough signal from the fluorophore to be seen in color on the Nikon microscope software. *S. oralis* was stained with SYTO-64 red stain and imaged with the DAPI (1d) FITC (1e) and mCHERRY (1f) filter cubes. The presence of the STYO-64 stain was only detected in the mCHERRY filter cube. Another *S. oralis* biofilm was stained with SYTO-21 green stain in the DAPI (1g) FITC (1h) and mCHERRY (1i) filter cubes. The presence of SYTO-21 was only detected in the FITC filter cube. Due to the presence of fluorophore signal of each stain only being detected in their respective filters, Figure 1 validates that the emission spectra of the stains do not overlap. After the bacteria species are stained with their respective SYTO dye and combined into a multi-species biofilm, each bacteria fluorophore will be distinguishable in their respective filter on the fluorescent microscope.

Figure 2 shows a biofilm of *S. oralis* stained planktonically, before biofilm formation. The key difference between Figure 1 and Figure 2 is the staining procedure. Figure 1 shows biofilms of *S. oralis* stained after biofilm formation, whereas Figure 2 shows biofilms of *S. oralis* stained planktonically before the biofilm formed. Because the multi-species biofilm will include multiple stains, the bacteria species must be stained before being combined and plated. The fluorescence of the biofilm in Figure 2, stained with SYTO-41 prior to biofilm formation, was only detected in the DAPI filter cube. This is congruent with the finding in Figure 1 showing the fluorescence of SYTO-41 only being detected at wavelengths corresponding to the DAPI filter cube and not the FITC or mCHERRY filter cubes. Hence, each species in the multi-species biofilm will be stained with a corresponding SYTO stain, and images of each species will be visible in the fluorescent microscope when the corresponding filter cube is used. Preliminary images shown in Figures 1 and 2 are of aerobic biofilms. Future work will focus on culturing the multi-species biofilm anaerobically in an anaerobic chamber shown in Figure 3a. The petri dishes will be placed in a 37 C

incubator with 5% $CO_2$ located inside the anaerobic chamber for 24 hours to allow biofilm formation. Microscope imaging and laser spallation will be done aerobically. Future studies will use laser spallation to quantify the stressed-based adhesion of this multi-species biofilm cultured anaerobically.

The laser spallation schematic is shown in Figure 3b. A high powered, 1064 nm laser passes through a focusing lens to decrease the spot size of the laser, then is directed by a mirror optic towards the sample. The sample consists of a titanium surface with a biofilm cultured on top of the surface. The laser impinges upon the titanium-coated substrate, which sends a compressive shock wave directed through the substrate. After reflection at the free surface, the wave then loads the biofilm-substrate interface in tension, which causes spalling of the biofilm from the substrate. Laser-induced shock waves are used in prior research studies to measure interfacial stress of aluminum test films [8]. Similarly, this study will capture interfacial stress between a thin film substrate and biofilm. However, due to the requirement of a vertical standing biofilm to negate effects of gravity, the interfacial stress is captured in a calibration experiment to correlate fluence with substrate stress. The fluence that is required to spall the biofilm is measured, and the stress at the titanium-biofilm interface corresponding to that fluence is recorded as the stress-based adhesion for the biofilm. A calibration experiment is performed to determine the interface stress that corresponds to each fluence. In a calibration experiment, the titanium substrate will be impinged upon by the 1064 nm laser at a known fluence. A 532 nm laser will be interferometrically set up to measure the displacement at the spot where the substrate was impinged. A photodetector will be placed in front of the fringe patterns that are created from the interferometry setup, and an oscilloscope will output voltage readings in terms of light intensity. The number of fringes can be found by unwrapping the oscilloscope voltage data [5]. The substrate stress can be determined by using the doppler effect and 1D wave mechanics [8,10].

## CONCLUSION

Stress-based adhesion of a multi-species dental biofilm on a dental implant surface can be obtained through laser spallation. Adhesion of single species has been measured via laser spallation, but biofilms contain more than one single species. Streptococci are common early colonizers, and because early colonizers form the basis of initial biofilms, they are examined in this study. Preliminary data shows that SYTO-41, SYTO-21, and SYTO-64 are stains without overlapping emission spectra. Incorporating the three stains will enable the distinction of each species in the multi-species biofilm. Distinguishing constituent species in the biofilm verifies that the biofilm contains all three strains. Adhesion of a multi-species biofilm is examined in this study as a representative model of an initial dental biofilm.

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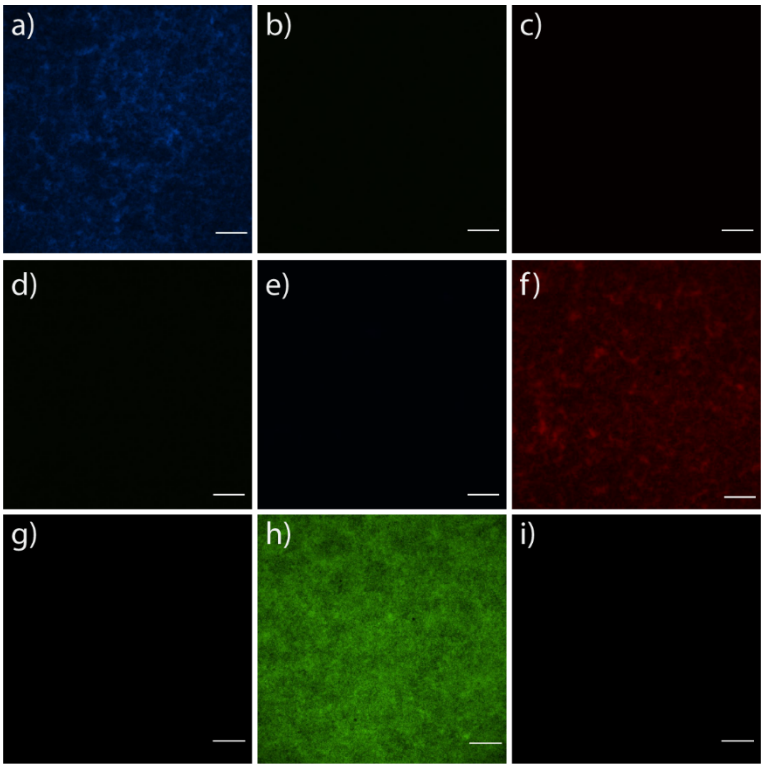


**Figure 1.** Fluorescent microscopy images (10x magnification) of single-species aerobic biofilms of *S. oralis* stained with (a-c) SYTO-41 (d-f) SYTO-64 and (g-i) SYTO-21 under DAPI filter (a,d,g) FITC filter (b,e,h) and mCHERRY filter (c,f,i). Bacteria were stained after biofilm formation. Scale bar is 200 µm.

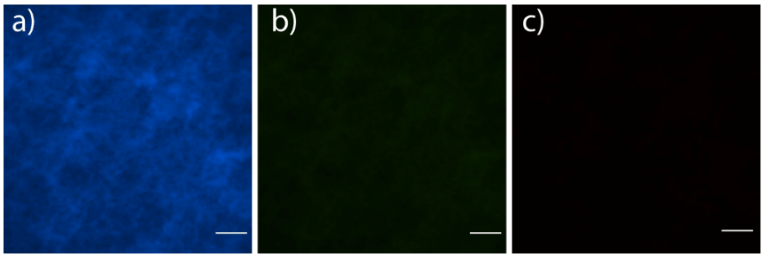


**Figure 2.** Fluorescent microscopy images (10x magnification) of single-species aerobic biofilm of *S. oralis* under the a) DAPI b) FITC and c) mCHERRY filter. *S. oralis* was stained with SYTO-41. Bacteria were stained before biofilm formation. Scale bar is 200 µm.

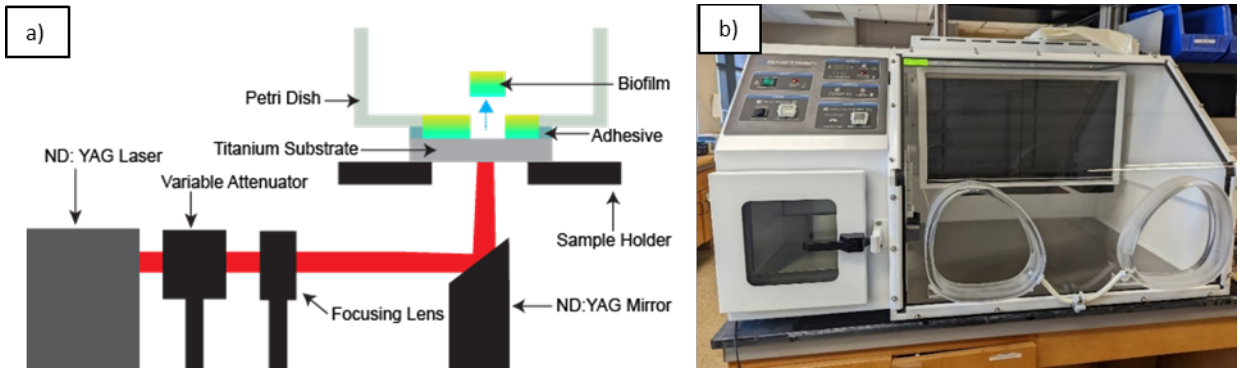


**Figure 3.** a) Laser spallation schematic and b) Sheldon Manufacturing anaerobic chamber.

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