Chapter 8
Biofilm Adhesion on TiAl6V4 using Laser Spallation Technique

Sahar Afshari and Martha E. Grady

Abstract Biomedical implants such as orthopedic, dental, cardiac devices, and catheters are prone to infections. The primary factor of implant infections is the adhesion of bacteria to the implant surface, leading to colonization and biofilm formation, which could eventually cause implant failure. Thus, quantifying the adhesion strength of biofilms on the implant surfaces is crucial for characterizing and improving surfaces and materials, and preventing biofilm infections. In this study, the laser spallation technique is utilized to measure the adhesion of biofilm to the implant surface. The aim of this study is to evaluate the adhesion strength of *Staphylococcus aureus* biofilms on Ti6Al4V (Ti64) substrates and to qualitatively compare with *Streptococcus mutans* on pure Ti. For this purpose, a substrate assembly suitable for measuring biofilm and cell adhesion on bulk materials such as Ti64, and additively manufactured Ti and Ti alloys, is employed. The results demonstrated that a substrate assembly comprising a layer of implant material as the substrate and a waterglass layer as the confining layer can be successfully utilized to assess biofilm failure across varying laser fluences. The ongoing challenges that need to be addressed to achieve effective adhesion measurement of cells and biofilms are also described.

Keywords Laser spallation · Biofilms · Biomedical implants · Titanium alloy · Ti6Al4V · Adhesion

Introduction

Biomedical devices such as catheters, cardiac devices, dental implants, and artificial joints have significantly altered the lives of patients and health care. However, their use has made the body more vulnerable to infections. Biomedical device-associated infections comprise 25.6% of all healthcare-associated infections in the US [1]. Among these infections, implant-associated infections, primarily caused by biofilm formation and colonization, cause a significant challenge due to the difficulties in diagnosis and treatment [2]. Biofilms with their ability to resist antibiotics and firmly attach to implant surfaces, can lead to persistent infections and even implant loss in severe conditions. Several factors, such as the type of implant and its location, prevalent microorganisms, surgical environment, and host immune system influence implant-associated infections [3]. Predominant microorganisms in biomedical implant infections vary according to the type and location of implants. For instance, staphylococci are the most common pathogens in orthopedic implant infections, with *Staphylococcus aureus* (*S. aureus)* and *Staphylococcus epidermidis* accounting for two-thirds of cases [4]. *S. aureus* is also the most frequent pathogen in cardiac-related implant infections such as pacemakers, stents, and left ventricular assist devices [3]. In dental implants, biofilm communities are more complex and contain multiple species such as *Streptococci* and *Actinomyces* which are prominent early colonizers, providing conditions favorable for late colonizers [5]. Generally, multiple species tend to be more challenging and difficult to treat than single species infections [6]. However, in vitro assessments of oral biofilms have also been conducted using single species such as *Streptococcus mutans*, which has proven to be an early colonizer in dental caries [7, 8].

Ti and its alloys are widely used metals in dental implants, knee and hip replacements, screws for fracture fixation, artificial hearts, pacemaker cases, and prosthetic heart valves [9, 10]. Ti-6Al-4V, the most commonly used Ti alloy in high-strength and load-bearing applications [9], is selected as the implant material for this study. This alloy is favored for its good corrosion resistance, biocompatibility, superior strength-to-weight ratio, and robust fatigue resistance [11, 12].

Sahar Afshari · Martha E. Grady
Department of Mechanical and Aerospace Engineering, University of Kentucky, Lexington, KY, USA 40508
email: s.afshari@uky.edu; m.grady@uky.edu

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The proposed study focuses on utilizing bulk materials such as conventional Ti64 and additively manufactured (AM) Ti64 as the substrate to obtain cell and biofilm adhesion. *S. aureus* on Ti64 substrates and *S. mutans* on pure titanium were evaluated as representatives of biofilm infections in orthopedic and dental implants, respectively. Although conventional Ti64 is utilized in the current study, the substrate assembly procedure is also applicable to AM Ti and AM Ti alloy.

In this work, the laser spallation technique is implemented as a non-contact method that generates high stress levels. This technique allows the precise macroscale quantification of cell and biofilm adhesion on substrates as well as localized impingement and multiple adhesion tests on a single substrate assembly. The laser spallation technique employs a high-energy YAG laser pulse that impinges on a substrate and generates a compressive stress wave. The generated stress wave propagates through the substrate, transmitting to the film and eventually reflecting off from the free sample surface [13]. However, due to the unreflective nature of biological films, in situ measurement of cells is not possible, and accurate calibration experiments on the substrates are required. A typical laser spallation specimen consists of a confining layer, an absorbing layer, a substrate, and a thin film. The laser spallation technique has been successfully adopted to measure the interfacial adhesion of cells and biofilm as a thin film on different substrates in numerous studies [14–16].

Experimental Procedures and Results

Fig. 1 (a) illustrates the experimental setup of the laser spallation technique for the biofilm and cell adhesion measurement. A Nd:YAG laser pulse is controlled using an attenuator and focused in a desired spot size using a focusing lens. The controlled and focused YAG laser pulse is then directed upward toward the substrate assembly using a 45° angled mirror.

A 1 mm thick Ti64 sheet purchased from American Metal Xchange, Inc. was cut into 1-inch by 1-inch square samples. The surface roughness, Ra, of Ti64 samples was measured using a Sensofar S neox optical profilometer. at 0.26 ± 0.02 μm from three samples, with measurements taken in three scanned lengths for each sample. One side was coated with a 12 μm thick layer of waterglass (Ricca Chemical sodium silicate) using the Specialty System G3P-8. The substrate assemblies consisted of Ti64 as the substrate and waterglass as the confining layer, as shown in Fig. 1 (b). The waterglass thickness was measured using a Bruker Dektak XT Surface Profiler. The substrates were then adhered to the bottom of a petri dish containing a 13/16-inch hole using the biocompatible NOA86H adhesive and cured under a Lesco CUREMAX Chamber Lamp (Fig. 1 (b)).

In order to culture bacteria, 1 μL of *S. aureus* (ATCC 6538) was taken from frozen stock using an inoculation loop and transferred to a 15 mL centrifuge tube containing 5 mL of Todd Hewitt Yeast (THY) broth. Bacteria were grown in a bead bath at 37 °C for 24 hours. The following day, prepared substrates were sprayed with 70% ethanol and sterilized under UV light for 30 minutes. The grown bacteria were diluted with THY to achieve an OD of 0.7 ± 0.05, which was measured using a GE Ultrospec 8000 spectrophotometer. The substrate was placed in a second petri dish, wrapped with parafilm to protect the waterglass layer from the heat in the incubator, and 1 mL of the 0.7 OD *S. aureus* and THY solution, combined with 3 mL of a 75 mM glucose-THY solution, was added to the substrate assembly. These substrate assemblies were then cultured at 37 °C with 5% CO2 for 24 hours. The media were then removed from the dishes, and the biofilms on the substrates were gently rinsed with phosphate-buffered saline (PBS) to remove any non-colonized bacteria. *S. mutans* (Wild type Xc) was also cultured using the same protocol, except on the second day, a 75 mM sucrose-THY solution was used.

Fig. 1 (c) illustrates *S. aureus* before and after loading at a high iso-fluence experiment of 96 mJ/mm^2 on a Ti64 sample, where the loaded area demonstrated complete biofilm detachment. The substrate assemblies for the *S. aureus* experiment consisted of a 1 mm Ti64 substrate and a 12 μm thick waterglass confining layer. As depicted in Fig. 1 (c), the substrate assemblies successfully led to full spallation at the highest fluence, indicating that the Ti64 substrate was able to absorb the YAG laser energy and generate high compressive stress through the substrate, delaminating the biofilm from the substrates without the need for any additional absorbing layer. However, the thickness of the waterglass layer plays a significant role in the magnitude of the stress wave generated by the YAG laser, with an increase in waterglass thickness significantly increasing the amplitude of the generated stress wave.

The substrate assemblies for *S. mutans* consisted of 9 μm thick waterglass as the confining layer, 100 nm Al absorbing layer, 1 mm thick glass substrate, and 100 nm pure Ti film. *S. mutans* were grown on the Ti surface to mimic a dental implant surface. The grown *S. mutans* were subjected to varying laser fluences which resulted in biofilm ejection at the loaded areas. Figure 1 (d) demonstrates the *S. mutans* biofilm grown on smooth titanium before and after loading. Figure 1 (e) represents the fluorescence microscopy images of the loaded *S. mutans* on smooth titanium. The applied fluence increased from 37.5 to 75.7 mJ/mm^2, leading to a significant increase in the ejection area at higher fluences compared to lower ones. At the highest laser fluence of 75.7 mJ/mm^2, complete ejection of *S. mutans* was observed from the pure Ti substrates across all loaded areas.

Results revealed that the adoption of the substrate assembly for bulk materials such as Ti64 and AM Ti64 has successfully generated a high compressive stress wave for cell and biofilm adhesion measurement studies. This effective implementation of the substrate assembly was achieved by applying a controlled confining layer on the Ti64 substrate to generate sufficient interface stress, compared to the common multilayer substrate assembly that includes a confining layer, an absorbing layer, a substrate, and a thin film. Both the loaded *S. mutans* and *S. aureus* on different substrates exhibited a similar failure area. However, to accurately measure the interfacial adhesion of *S. aureus* on Ti64, further experiments under varying laser fluences should be conducted.

In the preliminary applications of the laser spallation technique for the biological cell adhesion measurement, studies quantified the interfacial adhesion strength of cells and biofilms on substrates by identifying the onset of detachment as the critical threshold, where the minimum laser fluence required to detach cells from the substrate is determined as the critical laser fluence [15, 17, 18]. More recently, the 50% adhesion strength has been reported as the interfacial adhesion strength of the cells and biofilms by researchers [16, 19]. Because of the non-uniform nature of the biological film, instead of a particular laser fluence, a range of fluences is contributed to the onset of failure adhesion [19]. Thus, this 50% adhesion failure represents the adhesion failure of cells and biofilm more effectively. Although the appearance of a dark spallation area has been identified as the failed test in other studies [16, 19], similar to the failed biofilm depicted in Fig. 1 (d), results indicated that the appearance of the bare implant surface may provide a more accurate determination of failure, which may appear lighter in color depending on the tilt of the sample when viewing, as shown in Fig. 1 (c).

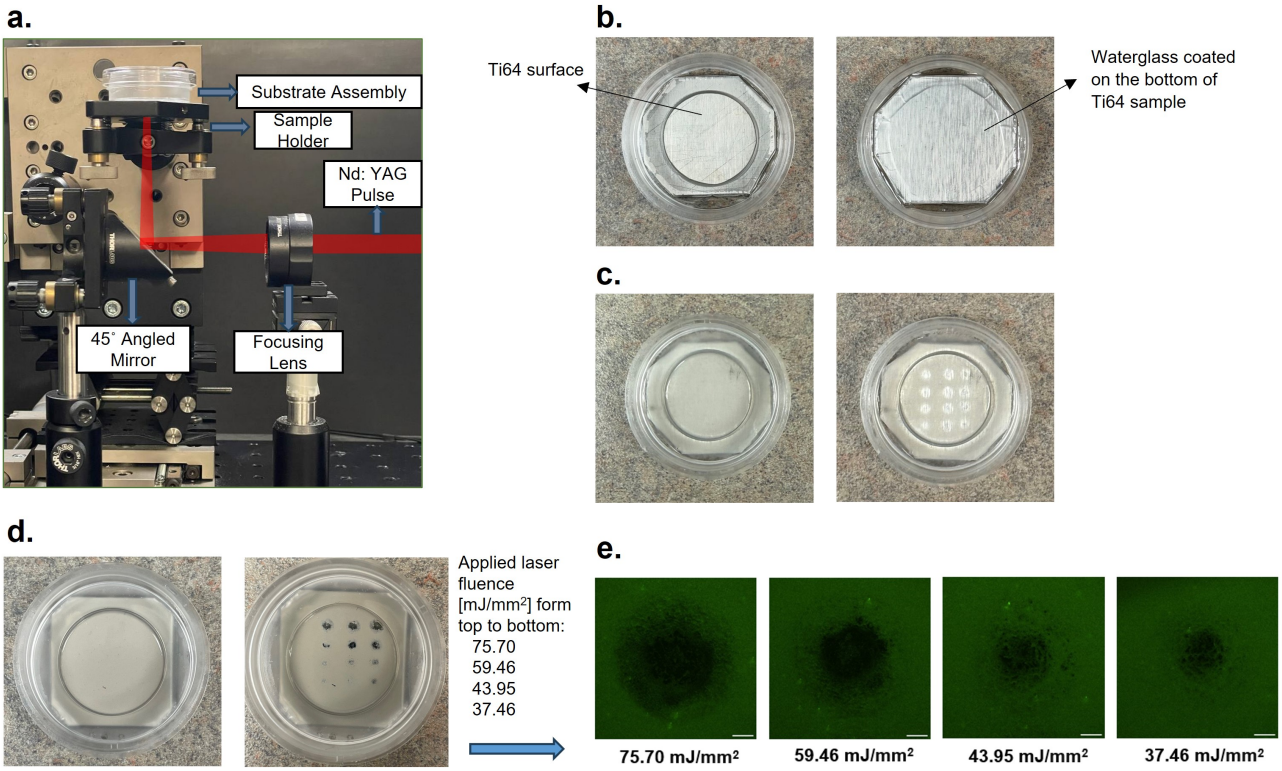

Fig. 1 (**a**) Experimental setup of laser spallation technique for cell and biofilm adhesion measurement. (**b**) Cell and biofilm culture substrate assembly consisted of Ti64 substrate and a waterglass layer as the confining layer. (**c**) *S. aureus* grown on Ti64 before and after loading at an iso-high fluence experiment, substrate assembly consisted of 1 mm Ti64 and 12 μm thick waterglass (**d**) *S. mutans* grown on smooth titanium before and after loading across varying laser fluences. (**d**) Fluorescence microscopy images of spalled biofilms at increasing laser fluence from 37.46 to 75.7 mJ/mm^2, captured using a 4$\times$ objective. *S. mutans* biofilm was stained with Syto 9 before loading. The scale bar is 0.5 mm, and the applied laser spot size is 2 mm

Challenges

Several challenges have emerged while progressing towards the goals. Initially, substrates were attached to the bottom of a Petri dish using Dowsil 732 silicone sealant, which caused a chemical reaction with the waterglass layer, resulting in a waterglass dissolution. To address the waterglass dissolution issue, a biocompatible UV-curing adhesive, NOA86H, was applied to uniformly attach the substrate to the Petri dish, which preserved the integrity of the waterglass layer for further experiments as well as reducing the thickness of the interlayer optical adhesive compared to the silicone sealant. One current issue is the degradation of the waterglass layer in the incubator due to exposure to humidity and heat. To preserve the waterglass layer in the incubator, exploring different types of waterglass, varying application methods, or alternative confining layers might be utilized. For calibration experiments, the free surface of the substrates must be reflective enough to produce a distinct fringe pattern and accurately capture the displacement movement. The reflectivity of the Ti64 samples was first examined using a Michelson interferometric setup, where the Ti64 sample failed to generate any reflective fringes. Although hand-polished Ti64 samples showed improved reflectivity, it was still inadequate for capturing in situ data measurements. Consequently, a major ongoing challenge is achieving mirror-like reflectivity in Ti64 for laser interferometric measurements.

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