Effect of Host Surface Factors on Biocompatible Adhesion Index

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Abstract
Biofilm formation is a significant problem in America, accounting for 17 million infections, and causing 550,000 deaths annually. An understanding of factors that contribute to strong biofilm surface adhesion at implant interfaces can guide the development of surfaces that prevent deleterious biofilms and promote osseointegration. The aim of this research is to develop a metric that quantifies the adhesion strength differential between a bacterial biofilm and an osteoblast-like cell monolayer to a medical implant-simulant surface. This metric will be used to quantify the biocompatible effect of implant surfaces on bacterial and cell adhesion. The laser spallation technique employs a high-amplitude short-duration stress wave to initiate spallation of biological films. Attenuation of laser energy results in failure statistics across increasing fluence values, which are calibrated via interferometry to obtain interface stress values. Several metrology challenges were overcome including how membrane tension may influence laser spallation testing and how to determine stress wave characteristics when surface roughness precludes in situ displacement measurements via interferometry. Experiments relating loading region within biofilm to centroid of biofilm revealed that location played no role in failure rate. A reflective panel was implemented to measure stress wave characteristics on smooth and rough titanium, which showed no difference in peak compressive wave amplitude. After overcoming these metrology challenges, the adhesion strength of *Streptococcus mutans* biofilms and MG 63 monolayers on smooth and rough titanium substrates is measured. An Adhesion Index is developed by obtaining the ratio of cell adhesion to biofilm adhesion. This nondimensionalized parameter represents the effect of surface modifications on increases or decreases in biocompatibility. An increase in Adhesion Index value is calculated for roughened titanium compared to smooth titanium. The increase in Adhesion Index values indicates that the increase in surface roughness has a more positive biological response from MG 63 than does *S. mutans*. In this work further experiments quantifying impact of various surface coating including blood plasma, and adhesion proteins found within the extracellular matrix to expand the Adhesion Index.

Keywords Biofilms, Laser spallation, Adhesion, *Streptococcus Mutans*, MG 63, Blood plasma, Surface treatment

The focus of this study is to examine the effect of improved *in vivo* conditions experienced during the medical implantation process. Specifically, *in vivo* conditions experienced by dental implant devices. During implantation the first thing to reach the surface is blood which permeates the active wound site during coagulation and hemostasis [1]. Proteins found within the blood will coat the newly implanted dental device before future osteoblastic cells adhere [2]. In order to model the competition more accurately between cellular and bacterial adhesion and the implantation an inclusion of the blood plasma proteins associated with wound healing are critical. During this competition the early colonizing bacteria are the foundational microbes which promote the adhesion and growth of more pathogenic bacteria onto the newly implanted surfaces [3]. Preventing the initial adhesion of these early colonizer can drastically diminish infection rates. These early colonizing bacteria are primarily dominated by oral *Streptococcus*, making up nearly 80% of the early biofilm constituents [4]. As such to model these typical early colonizing bacteria, *Streptococcus mutans* was selected in this study. *S. mutans* is associated with dental caries and promotes the adhesion of other bacteria [3]. Dental implants are susceptible to adherent bacteria simultaneous to the natural wound healing process that begins when the titanium implant is placed in bone, i.e., as osteoblasts contribute to osseointegration [5]. The bodies natural cellular response is also modeled to accurately assess the competition between invasive bacterial adhesion and host osteoblast cells, bone cells. MG 63 osteosarcoma cells were selected to model the behavior of immature osteoblast cells, for their excellent immature osteoblastic traits [6]. The scaffold for these adhesion studies was chosen to be commercially pure titanium, as titanium is the most commonly used dental implant material [7]. Dental implants contain both smooth and roughened titanium surfaces, roughened titanium is shown to increase cellular adhesion, thus both roughened and smooth titanium substrates were examined in order to mimic the varying surface roughnesses found on dental implants [8]. The dental implant mimicking substrate is first coated with a physiosorbed layer of blood plasma before inoculated with either the *S. mutans* or MG 63 cells. *S. mutans* bacteria are cultured in Todd Hewitt Yeast with sucrose, while MG 63 is cultured in Eagle's Minimum Essential Medium, until confluent biofilms and monolayers are produced, respectively. The dental implant mimicking assemblies, cultured with appropriate films, undergo laser spallation experiments to determine the sample-biofilm interfacial adhesion strength. The effect of surface morphology on adhesion has previously been examined using the laser

spallation technique [9], but the inclusion of blood plasma coating provides an improved biometric for biocompatibility of these smooth and roughened surfaces. The adhesion strength values for both films on the smooth and rough surfaces are directly comparable values, additionally the adhesion strengths with and without plasma coating can be compared. The uncoated smooth surface titanium is used as a baseline and the effect of surface roughness with blood plasma coating is examined. Optimal implantology designs would look for surfaces which promote host cellular adhesion while deterring invasive bacterial adhesion. A previously developed metric known as the Adhesion Index examines the ratio of bacterial and cellular adhesion to determine the biocompatibility of various surface morphologies [9]. The impact blood plasma protein coatings have on existing Adhesion Index metrics are examined in this study in order to determine the role they play on both bacterial and cellular adhesion. The goal being to improve this existing metric to allow for better predictive *in vitro* metrics in implantology, so that *in vivo* results can be predicted and improved.

The most commonly used adhesion technique used in bacterial studies are counting methods [10]. These counting methods do not give any information on mechanical adhesion of biological films and direct comparison of these results is difficult because of the size scale associated bacteria vs cells. Jet impingement and shear flow studies also have been performed to quantify the adhesion of bacterial and cellular films, however these tests rely on contact methods, fluid flow, to induce stresses and can often lead to deformation of the films before accurate measurements can be obtained [11]. The low cohesiveness of these films can cause them to fail cohesively during testing before adhesion can be measured accurately [12]. Laser spallation is a thin film adhesion test that previously has been used to quantify the adhesion of metallic films onto substrates [13, 14]. More recently the technique has been employed to quantitatively measure the adhesion strength of several biological films [9, 15-17]. The laser spallation setup used is illustrated in Fig. 1a. Laser spallation operates by delivering a photoacoustic shockwave to the biofilm which, with sufficient energy, will spall the films from the interface surface. Calibration experiments are performed following spallation experiments in order to obtain stress wave generation at the interface for interface strength values. Interferometry is applied to measure the free surface displacement during loading to quantify and observe the substrate stress magnitude and profile. Wave transmission and reflection equations are applied to determine the final adhesion strength for films. This protocol is highlighted in greater detail in Boyd *et al.* [9]. The rapid non-contact stress applied by the laser spallation method is superior to other film adhesion testing methods because of the preclusion of biological film deformation over time from the low cohesiveness of the films. Additionally multiple loaded regions can be applied over a single biofilm to increase results from a single biofilm [18].

Dental implants include smooth and rough titanium surfaces, the threads of dental implants are roughened to increase osseointegration for years, while the gingival portion of the implant is often smooth to prevent irritation and oral bacteria adhesion. To mimic these surface characteristics glass slides are purchased with e-beam evaporated 100 nm coatings of titanium. The opposite side of the slide is coated in 300 nm of aluminum which acts as the energy absorbing layer for the laser used. A separate set of slides are first sandblasted with large grit to obtain a surface roughness between 1-1.5 μm. The glass slides are cut into 1”x1” and the titanium surface is adhered to the bottom of a 35 mm petri dish with a 13/16” hole, using biologically inert silicone [9, 15]. In order to obtain powerful enough stress waves to initiate delamination, a waterglass layer was added to the back side of the samples, over the aluminum layer. Samples are then adhered to the bottom of a 35 mm diameter petri dish using a biologically inert silicone. Human blood plasma is then diluted down to 55% by volume in phosphate-buffered saline, to achieve the concentration found within the human body, and 1 ml is added on top of the titanium surface for one hour. The solution of plasma is aspirated out of the dish leaving only the physiosorbed proteins on the titanium surface, Fig. 1a. Bacteria or cells are then inoculated inside of the substrate assembly until confluent films are formed.

Multiple substrate assemblies of biofilms constructed and tested, each film consisting of multiple loading locations. Onset of failure of the bacterial biomaterial-titanium interface is marked by spallation of the biofilm from the surface. For example, Fig. 1c depicts the adhesion failure progression for *S. mutans* on plasma coated roughened titanium substrates. As fluence, energy per area, values increase the spallation increases for the films tested. During testing the failure statistics for each film at each fluence are recorded in order to determine the minimal energy needed to initiate spallation. Uncultured smooth substrates are used as calibration specimen and are loaded with identical fluences used in the spallation study, and the free surface displacement is measured using a Michelson interferometer. The substrate stress profile can be measured using the free surface velocity and converted into interface strength using previously accepted methods [19]. Smooth substrates of this type have previously been shown to be viable substitutes for the roughened sample as well due to the negligible effects of substrate stress propagation from the roughened surface [20]. Weibull analysis [21, 22], common in macroscopic adhesion analyses, calculates the half-life from a Weibull distribution, which is used as the adhesion strength. A decrease in adhesion strength is initially noted for the *S. mutans* films cultured on top of the plasma protein layer. Adhesion strength for *S. mutans* on smooth plasma

coated titanium weas measured as 148 MPa, and the adhesion strength measured on rough plasma coated titanium was 257 MPa. When we compare these values to previously uncoated smooth and rough titanium adhesion strength values, we can see that the plasma coating resulted in a much less strongly adherent biological film. Previous values for *S. mutans* on smooth and rough titanium are 320 MPa, with a 95% C.I. (304, 333), and 332 MPa, with a 95% C.I. (324, 343) respectively. There is a two-fold difference in adhesion strength between MG 63 monolayers and *S. mutans* biofilms on smooth titanium. Adhesion strengths values are shown in Fig. 1d. The adhesion strength for bacterial biofilms is greater than that of the cell monolayers. Future studies aim to examine the effect of plasma coated surfaces on cellular adhesion. Thus, resulting in a modified Adhesion Index. If cell adhesion remains constant or increases, then the resulting Adhesion Index will also increase. Meaning that in a more accurate *in vivo* model that surface roughness has a much more positive impact on adhesion strength for host cells than the bacterial adhesion.

This study directly compares the adhesion for *S. mutans* biofilms onto both smooth and roughened titanium dental implant mimicking surfaces, with and without a plasma pretreatment. Laser spallation was used in order to quantify the adhesion strength for the biological films. The adhesion values were directly compared to determine the effect of surface modifications and plasma protein coating on their respective adhesion. The inclusion of the plasma protein physiosorbed layer resulted in a decrease in adhesion for *S. mutans* on both the smooth and roughened titanium surfaces. Future work intends to further model *in vivo* conditions by understanding the effect of the plasma coated surface on MG 63 adhesion. Additionally, understanding the specific plasma proteins and their impact on adhesion is of interest. Fibronectin shall be applied to the titanium surfaces and more laser spallation experiments will be performed to understand its effect on adhesion. Furthermore, studies involving other implantable devices, like orthopedic implants, will be examined by varying the bacterial model to include invasive *Staphylococcus aureus*. The goal of this research is to expand upon an adhesion testing metric, the Adhesion Index. This Index will be used to aid in the design of medical devices, and ultimately be used to reduce biofilm-related infections while promoting the successful integration of these medical devices.

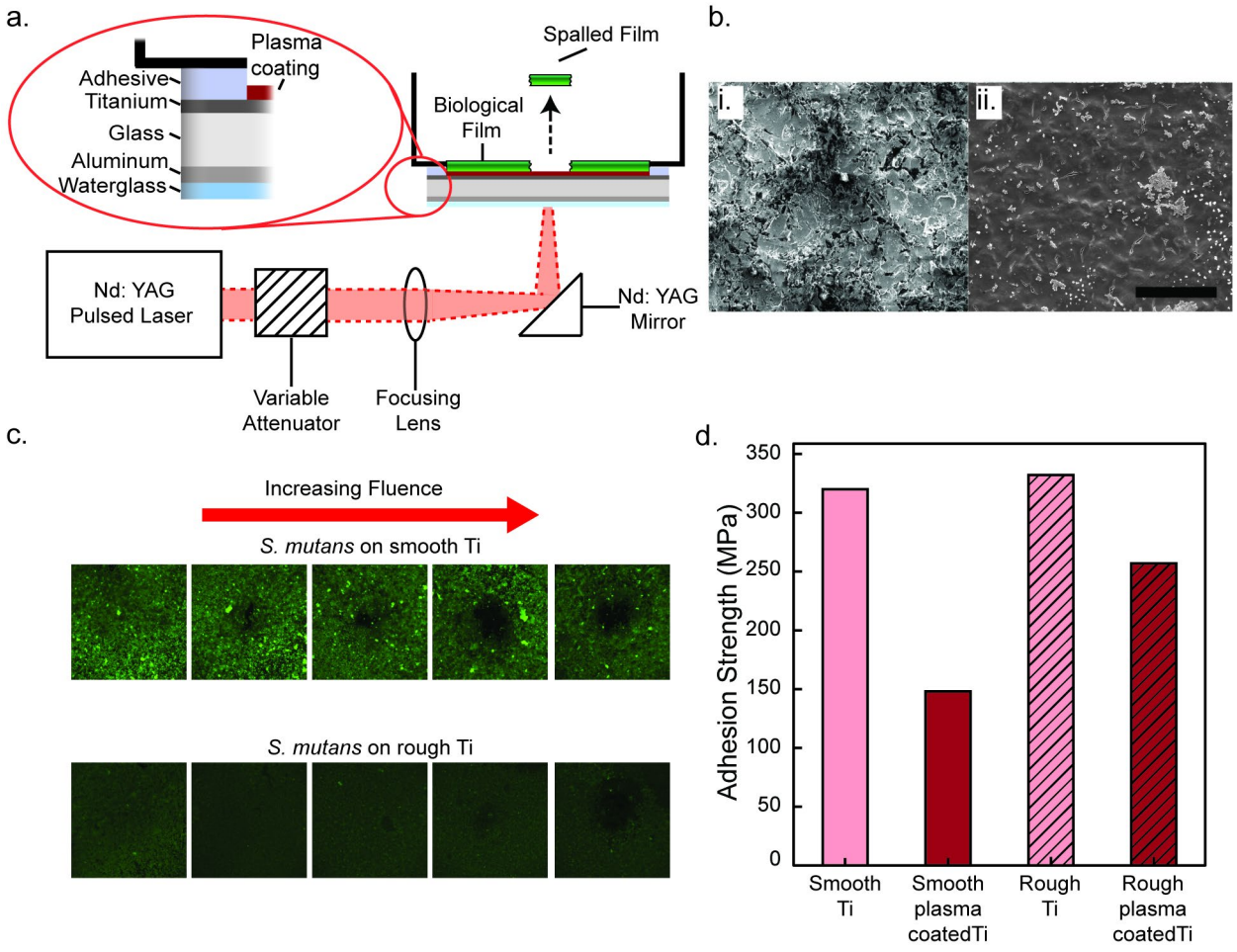

Fig. 1 (**a**) The laser spallation set up used during delamination experiments. The Nd: YAG laser used reflected 90° to allow for horizontal orientation of the substrate assembly used. (**b**) (i.) Depicts an SEM image obtained of roughened titanium substrate used to mimic dental implant thread roughness, (ii.) is an SEM image of the plasma protein coating on top of the titanium surface, scale bar is set to 100 μm. (**c**) Illustrate typical failure for both *S. mutans* plasma coated smooth and rough titanium surfaces due to increasing fluence values. (**d**) Graph of interface strength obtained after calibration experiments and Weibull analysis, for smooth and rough titanium and plasma coated smooth and rough titanium for *S. mutans*.

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References

1. Velnar, T., T. Bailey, and V. Smrkolj, *The wound healing process: an overview of the cellular and molecular mechanisms.* Journal of International Medical Research, 2009. **37**(5): p. 1528-1542.
2. Park, J.B., *Biomaterials science and engineering*. 2012: Springer Science & Business Media.
3. Barbosa, J.O., et al., *Streptococcus mutans Can Modulate Biofilm Formation and Attenuate the Virulence of Candida albicans.* PLoS One, 2016. **11**(3): p. e0150457.
4. Kreth, J., J. Merritt, and F. Qi, *Bacterial and Host Interactions of Oral Streptococci.* DNA and Cell Biology, 2009. **28**(8): p. 397-403.
5. Chang, Y.-Y., et al., *Biological characteristics of the MG-63 human osteosarcoma cells on composite tantalum carbide/amorphous carbon films.* PloS one, 2014. **9**(4): p. e95590-e95590.
6. Shapira, L. and A. Halabi, *Behavior of two osteoblast-like cell lines cultured on machined or rough titanium surfaces.* Clin Oral Implants Res, 2009. **20**(1): p. 50-5.
7. Oshida, Y., *Bioscience and bioengineering of titanium material* Elsevier, 2013. **2nd Edition**
8. Gaviria, L., et al., *Current trends in dental implants.* Journal of the Korean Association of Oral and Maxillofacial Surgeons, 2014. **40**(2): p. 50-60.
9. Boyd, J.D., et al., *Biofilm and cell adhesion strength on dental implant surfaces via the laser spallation technique.* Dent Mater, 2021. **37**(1): p. 48-59.
10. Duarte, P.M., et al., *Bacterial adhesion on smooth and rough titanium surfaces after treatment with different instruments.* J Periodontol, 2009. **80**(11): p. 1824-32.
11. Bayoudh, S., et al., *Assessing bacterial adhesion using DLVO and XDLVO theories and the jet impingement technique.* Colloids Surf B: Biointerfaces, 2009. **73**(1): p. 1-9.
12. Stoodley, P., et al., *Biofilm material properties as related to shear-induced deformation and detachment phenomena.* J Ind Microbiol Biotechnol, 2002. **29**(6): p. 361-367.
13. Grady, M.E., et al., *Molecular tailoring of interfacial failure.* Langmuir, 2014. **30**(37): p. 11096-102.
14. Wang, J.L., R.L. Weaver, and N.R. Sottos, *A parametric study of laser induced thin film spallation.* Experimental Mechanics, 2002. **42**(1): p. 74-83.
15. Boyd, J.D., N. Korotkova, and M.E. Grady, *Adhesion of biofilms on titanium measured by laser-induced spallation.* Experimental Mechanics, 2019. **59**(9): p. 1275-1284.
16. Hu, L., et al., *Cell adhesion measurement by laser-induced stress waves.* Journal of Applied Physics, 2006. **100**(8): p. 084701.
17. Hagerman, E., et al., *Evaluation of laser spallation as a technique for measurement of cell adhesion strength.* J Biomed Mater Res A, 2007. **82**(4): p. 852-60.
18. Kearns, K.L., J.D. Boyd, and M.E. Grady, *Biofilm rupture by laser-induced stress waves increases with loading amplitude, independent of location.* ACS Applied Bio Materials, 2019.
19. Kandula, S.S.V., et al., *Adhesion strength measurement of polymer dielectric interfaces using laser spallation technique.* Thin Solid Films, 2008. **516**(21): p. 7627-7635.

20. Boyd, J.D. and M.E. Grady, *The effect of surface roughness on laser-induced stress wave propagation.* Applied Physics Letters, 2020. **117**(12): p. 121601.
21. Mei, L., et al., *Influence of surface roughness on streptococcal adhesion forces to composite resins.* Dent Mater, 2011. **27**(8): p. 770-8.
22. van der Mei, H.C., J. de Vries, and H.J. Busscher, *Weibull analyses of bacterial interaction forces measured using AFM.* Colloids Surf B: Biointerfaces, 2010. **78**(2): p. 372-5.