Preconditioning layers affect osteoblastic cell adhesion to orthopedic implant surfaces

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Keywords: Biofilms, Laser spallation, Adhesion, MG 63, Blood plasma, Surface treatment

Bacteria can proliferate orthopedic implants, resulting in infection rates as high as 5%. A consistent problem across implantology is the development of surfaces which successfully promote the adhesion and propagation of healthy fibroblast and osteoblast cells while deterring formation of bacterial biofilms. Selecting surface configurations which favor cell adhesion will lead to decreased infection rates. Progress in identifying appropriate surface configurations is hindered by the lack of quantitative adhesion techniques capable of comparing adhesion of cells and biofilms directly. Recent advancements in adhesion techniques have allowed for quantitatively measured adhesion strengths of both bacterial biofilms and cell monolayers using the laser spallation technique. The quantified stress-based adhesion values allow surface and environmental factors that modulate both bacterial and cell adhesion to implant surfaces to be evaluated. During implantation blood propagates wound sites completely coating implant surfaces. Understanding the impact of preconditioning layers that accumulate on the implant surface on cell adhesion is vital to predict implant behavior. Previous work has demonstrated that these preconditioning layers either negatively or neutrally impact bacterial adhesion to titanium implant surfaces. This study focuses on the impact that blood plasma and fibronectin coatings have on the adhesion of osteoblastic (MG 63) cells and fibroblasts to the same titanium surfaces. Adhesion results indicate that preconditioning layers and increased surface roughness positively impact cell adhesion. Incorporating the increased adhesion values for cell adhesion into the Adhesion Index demonstrates that increased surface roughness, coupled with natural wound healing preconditioning of surfaces, yields positive biocompatibility.

Infections from medical implants, specifically orthopedic implants, can have infection rates as high as 5% [1-3]. These infections stem from the proliferation of bacteria into bacterial biofilms and in extreme cases can lead to loss of implant [4]. One problem that continually eludes implant developers is creating surfaces which adversely impacts bacterial adhesion while promoting attachment of healthy mammalian cells. Recent advancements in adhesion techniques have allowed for quantitatively measuring the adhesion strength of both bacterial biofilms and cell monolayers using the laser spallation technique [5-7]. The direct comparison now possible allows for the effects of surface and environmental characteristics to be understood and adjusted in order to achieve more favorable adhesion results for cells. However, previous research in this area lacks *in vivo* that would appropriately represent implant and wound healing environments.

During wound healing the first thing to contact an implant surface is blood [8, 9]. Since conditioning layers play a significant role in the initial adhesion and proliferation of cells during the wound healing process, the inclusion of relevant plasma proteins is vital to accurately predict cellular growth response onto implantable devices. In this work, the laser spallation technique is employed to measure the adhesion differential between two different bacterial biofilms and osteoblast-like cells on implant mimicking surfaces with a conditioning layer of either blood plasma or fibronectin. **Fig. 1.A** illustrates the laser spallation process and detachment of biological film layer while maintaining conditioning layer.

Cell adhesion onto typical orthopedic implant surfaces is the main focus of this study. MG 63 (ATCC CRL-1427), and immature osteoblastic cell line, is cultured onto preconditioned titanium substrates. Titanium is selected as it is one of the most common orthopedic implant materials [10]. Similarly the substrate surface is roughened to simulate typical orthopedic implant surface roughness [10]. The titanium substrates are then preconditioned with either human blood plasma, diluted to 55% by volume, obtained from the University of Kentucky's Biospecimens Core, or 10 μg/ml of human fibronectin, dissolved in PBS. Both preconditioning substances are applied to separate substrates for 1 hour before being aspirated to allow cell culturing. **Fig. 1.B.** illustrates typical protein layer thicknesses obtained during the preconditioning process. Scanning Electron Microscopy (SEM) images were taken from cross sectioned coating and a consistent layer, less than 2 μm in thickness, was measured. Cells are then cultured on top of preconditioned titanium substrates, using supplemented Eagle's Minimum Essential Medium (EMEM, ATCC 30-2003) for 24 hours before spallation experiments occur. Calcein AM (Thermo Fisher Scientific L3224) is applied to cell monolayer to visibly observe attachment and spallation of cells.

The laser spallation technique is a thin film adhesion technique recently applied to biological materials [5, 6, 11]. In our set up, a single laser pulse from an Nd:YAG, wavelength of 1064 nm, impinges upon the backside of our substrate surface. The laser pulse is converted into a mechanical wave through plasma gasification. The compressive wave travels through the substrate-cell systems before reflecting off of the free surface and loading the cell monolayer-titanium interface with a tensile load. Each cell layer system can be loaded multiple times resulting in adhesion quantification of the cell titanium system. The tensile loading of the cells, at sufficient energy, will result in detachment, or spallation, of the cells. The laser energy can be varied in order to find the minimum energy needed to initiate detachment. **Fig. 1.C** depicts what typical cell regions look like when loaded with both insufficient stress, and sufficient stress to spall from the surface.

As is the standard with laser spallation experiments a Michelson type interferometer is used to measure free surface displacement during loading of substrates. Un-treated titanium substrates are loaded with a typical range of fluence, energy per area, values to determine the free surface velocity. Calibration equations are applied to obtain the substrate stress profile. The substrate stress profile is then coupled with transmission and reflection wave coefficients in order to determine the interface stress of the cells and titanium surface. Identical procedure has been applied in Boyd *et al.* [6].

Four different preconditions were tested in this set of experiments, all cultured with MG 63, including: smooth titanium coated in 55% blood plasma, smooth titanium coated in 10 μg/ml of human fibronectin, rough titanium coated in 55% blood plasma, and rough titanium coated in 10 μg/ml of human fibronectin. Almost 50 separate substrates were culture, with each substrate loaded more than 10 times with increasing fluence values. The failure statistics for these experiments are reported in **Fig. 1.D**. Early results indicate a significant difference in adhesion between substrates coated with human plasma and those coated with fibronectin. More stress is required to initiate detachment of cells on surface pretreated with human plasma than those with fibronectin.

Additionally, there appears to be no correlation with the surface roughness used in this study and adhesion on human plasma coated substrates. While a difference in adhesion can be observed when examining both fibronectins coated smooth and rough titanium. In previous studies the increase in macroscopic adhesion resulted in a large increase in adhesion strength for MG 63 onto titanium surfaces. The change of the impact of surface roughness on adhesion is believed to be caused by the surface topography change that occurs when preconditioning layers are applied to the surface. Similarly, when compared to the adhesion statistics of uncoated samples it is observed that adhesion decreases when the surface proteins are attached. Further work seeks to examine the relationship that these preconditions play in the competition of both bacteria and cell adhesion.

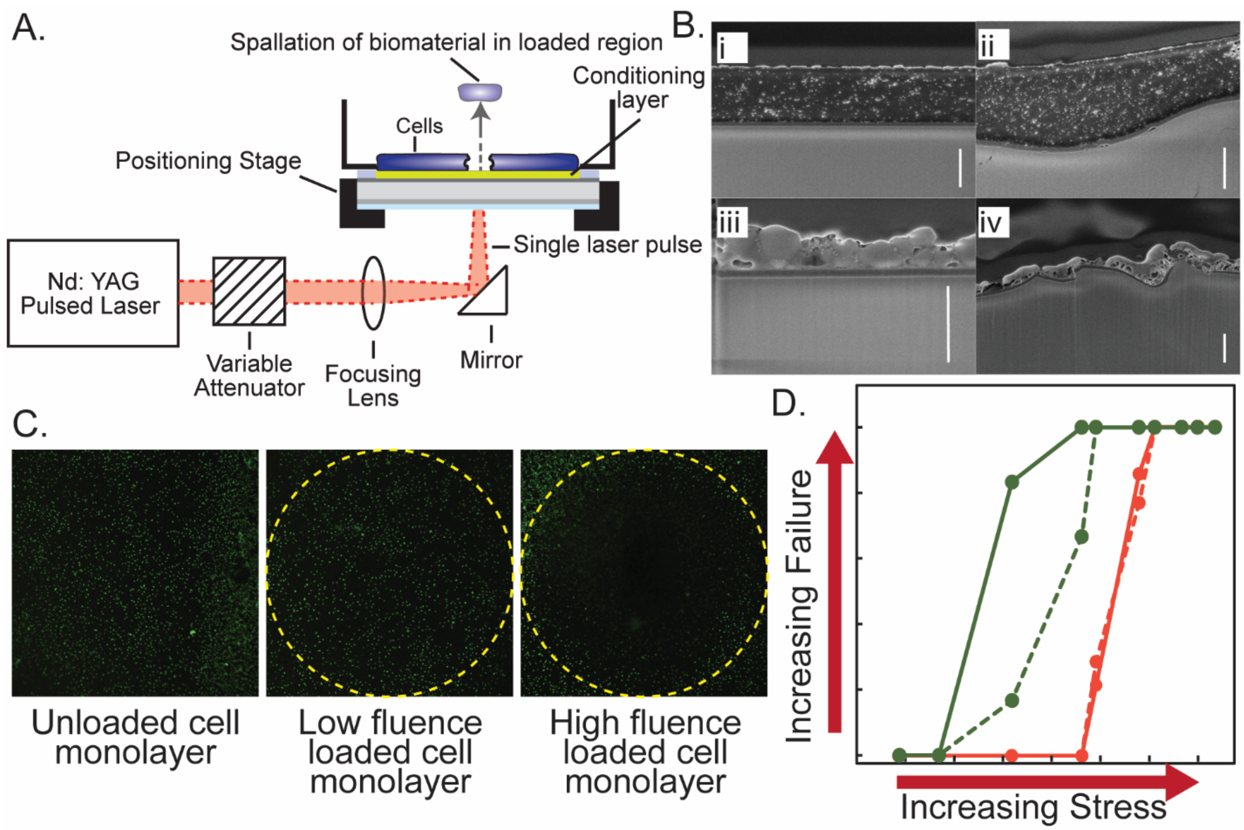

Fig. 1 A. The laser spallation set up used during delamination experiments. The Nd: YAG laser used reflected 90° to allow for horizontal orientation of the substrate assembly used. **B.** (i, ii) Depicts SEM images of cross sections for plasma coated titanium, (iii, iv) are SEM images of cross sections for fibronectin coated titanium surfaces. Scale bars represent 1 µm vertically. **C.** Illustrate typical failure for MG 63. Yellow circle depicts the 2 mm diameter loaded region. **D.** Graph of increasing failure of films as a result of increasing interface stress. Green line depicts fibronectin coated samples while red indicates plasma coated samples. Solid lines represent smooth titanium surfaces and dashed lines represent roughened surfaces.

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