

Ultrashort Pulse Laser Ablation as a Tool for Depth Profiling of *Staphylococcus epidermidis* Microbial Biofilms

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Introduction

- Biofilms are structured communities of microorganisms encapsulated within self-developed polymeric matrix & adhered to a solid surface.¹
- Biofilms can cause biofouling & hospital acquired infections. For example, they are observed in over 65% of bacterial infections,¹ with *Staphylococcus epidermidis* & other CoNS causative organisms in a majority of device related infections.²
- There is up to 70% difference in gene expression between biofilm & planktonic modes of microorganisms, by which biofilms develop increased resistance to environmental stresses including increased antibiotic resistance.¹
- Understanding the mechanisms of antibiotic resistance of biofilms would be aided by the ability to create 3D molecular maps of antibiotics, cathabolites, peptides & proteins therein.
- We are developing methods for laser desorption postionization mass spectrometric imaging for analysis of intact biofilms.^{6,9}
- Depth profiling of biofilms requires ability to remove the top layer of material while leaving the underlying biofilm unaltered.
- Sputtering with keV cluster ion beams suffers from very low material removal rates while cryomicrotoming requires difficult sample handling.^{3,4}
- We evaluate here the use of ultrashort (< 100 fs) pulse laser ablation for material removal for depth profiling of biofilms. This approach is motivated by studies showing ns laser pulses will damage tissue⁵. However, if laser pulse length is shorter than thermal diffusion time (>10ps), then thermally induced damage to underlying material is minimized due to "thermal confinement".⁵

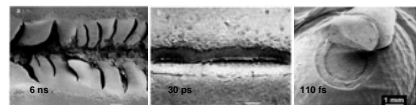


Fig. 1. Eye surgery: a) Cut in cornea by 6 ns pulse laser, b) by 30 ps, c) Lenticule dissected out of the corneal stroma by 110 fs pulse. Images borrowed from Ref. 5.

Goals

- Measure laser ablation threshold and define the best conditions for laser ablation.
- Demonstrate the ability to remove the top layer of material from the *S. Epidermidis* colony biofilms by ultrashort pulse laser ablation. Estimate penetration depth.
- Argue that analyte introduced into biofilm is not going to be damaged during ablation.

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Experimental Methods

Colony Biofilm Growth & Doping with Antibiotics:

- Colony biofilms were prepared from stock solutions of *Staphylococcus epidermidis* (ATCC 35984),⁶ grown on ITO coated glass slides for three days in a drip flow reactor according to published method⁷.
- 20 μ L of 50 mM solutions of tetracycline hydrochloride (TC) & sulfadiazine – sodium salt (SD) in water were spiked over surfaces of dried biofilms & allowed to dry.

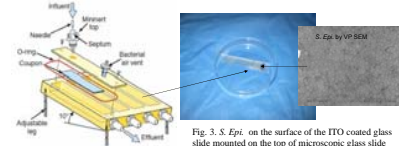


Fig. 2. Drip flow reactor. Image borrowed from Ref. 7.

Fig. 3. *S. Ep.* on the surface of the ITO coated glass slide mounted on the top of microscopic glass slide

Ultrashort Pulse Laser Ablation:

- 45 fs, 800 nm Ti-sapphire pulsed laser focused to a spot diameter of 200 μ m was used for laser ablation of biofilm samples. He-Ne laser was used as a guide. Experiments were monitored by surgical microscope equipped with CCD camera.

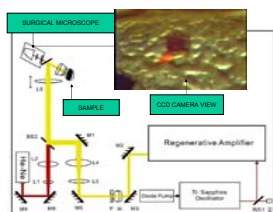


Fig. 4. Laser set up. Image borrowed from Ref. 8.

Scanning Electron & Two Photon-Laser Scanning Microscopy:

- Scanning electron microscopic (SEM) images of laser treated biofilm samples were obtained by S-3000N Hitachi scanning electron microscope with a tungsten electron source operating at 10 – 25 keV in variable pressure mode (0-270 Pa) and also in high vacuum mode after spin coating with Pt/Pd.
- Two photon-laser scanning microscopic (2P-LSM) images were obtained by custom built 2 photon confocal microscope after staining biofilm samples with 300 nM solution of 4',6-diamidino-2-phenylindole (DAPI) in PBS. 35 fs, 800 nm Ti-sapphire was used for excitation and fluorescence signal was collected through a short pass filter with cutoff of 650 nm.

Laser Desorption Postionization Mass Spectrometry:^{6,9}

- Mass spectral data was collected on home-built laser desorption post ionization (LDPI) instrument described below:
- 349-nm Nd:YLF desorption (3 – 60 mJ) laser (Spectra-Physics).
- 157-nm molecular fluorine (1 MW/cm²) postionization laser (Lambda Physik).
- Pulsed ion optics, Einzel lens, steering plates
- Home-built reflectron TOF analyzer, two-stage ion mirror, MCP detector
- Data acquisition: GaGe card (Dynamics Signals, LLC)
- Vacuum compatible translation stage (Micros, Germany)
- Nikon D300 camera with microscope lens, (Nikon ED, AF micro Nikkor 200mm, 1:4D)
- 100 Hz repetition rate, 100 shot averaging.

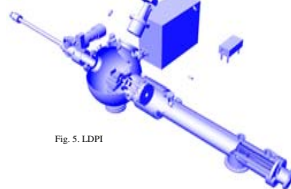


Fig. 5. LDPI

Results

Laser Ablation Thresholds Determined by Microscopy:

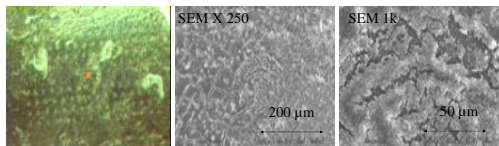


Fig. 6. Ablation craters on the surface of thin biofilms by CCD camera

Fig. 7. and 8. Ablation crater on the surface of thin biofilm obtained by applying threshold fluence $\Phi_T = 0.48 \text{ J/cm}^2$

- Ablation thresholds were determined by observing changes by CCD camera. They vary from sample to sample & within single inhomogeneous sample.

~ 25 μ m (thin) biofilms grown by the drip flow method for ~ 24 hr showed ablation threshold to be:
 $\Phi_T = 0.42 \pm 0.15 \text{ J/cm}^2$.

~ 100 μ m (thick) biofilms grown by the drip flow method for ~ 72 hr showed ablation threshold to be:
 $\Phi_T = 0.79 \pm 0.24 \text{ J/cm}^2$.

Depth Penetration of Ablated Biofilms by Microscopy:

- Single shot by fs laser pulse creates an elliptical crater ~ 20 μ m long and ~ 10 μ m wide in the thick biofilm.

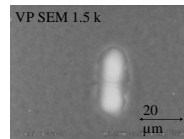


Fig. 9. Single shot done by 0.64 J/cm² on surface of thick biofilm

- It appears only laser beam waist removes material from surface of thick (~ 100 μ m) biofilm under these ablation conditions.
- Ablation of 0.5 x 0.5 mm area was performed by applying laser fluence slightly above ablation threshold (1.04 J/cm²) and rastering laser beam across the surface with 10 μ m spacing between shots.

Depth penetration was estimated to be ~ 10 μ m.

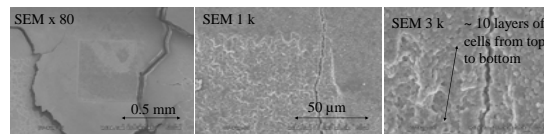


Fig. 10. Ablated area of 0.5 x 0.5 mm on the surface of the biofilm

Fig. 11. Corner of the ablated area at higher magnification (x 1000)

Fig. 12. Corner of the ablated region at higher magnification (x 3000)

- Integrity of individual bacterial cells at the bottom of the ablated region is disrupted compared to intact surface of the biofilm.

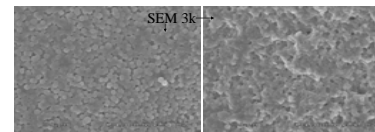


Fig. 13. Intact surface of *S. epidermidis* bacterial biofilm

Fig. 14. Bottom of laser ablated region. Applied laser fluence is 1.04 J/cm²

- Channels created in a biofilm by applying laser in the CW mode (100 Hz, 0.48 J/cm²) were scanned by 2P-LSM showing that laser created squared rather than curved craters in the biofilm.

Thickness of the biofilm was measured to be ~ 100 μ m.

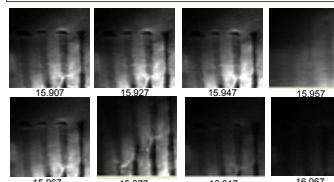
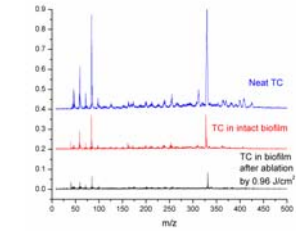
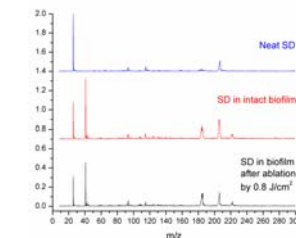


Fig. 15. 2P-LSM images of laser created channel in a biofilm taken at different depths. Units are nm.

Results (cont.)

LDPI-MS of Antibiotics within Native & Ablated Biofilms:

- Biofilms spiked with solutions of antibiotics: tetracycline (TC) and sulfadiazine (SD) were laser ablated & analyzed by LDPI.
- Spectra were taken from the surface of intact biofilm & from the bottom of laser ablated region & compared to spectra of neat antibiotics.
- Results indicate that fs laser ablation does not degrade analyte introduced into biofilm.



Conclusions

- Biofilm ablation by 45 fs, 800 nm Ti-sapphire pulse laser show:
- Removal of ~10 μ m of biofilm – similar to LDPI penetration depth.
- Antibiotic analytes in biofilm not degraded by ultrashort pulses.
- Microbes at bottom of ablation crater are lysed by laser ablation.

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