Wireless Nanoplasmonic Dressings for Long-term Spatiotemporal Imaging of Pseudomonas Aeruginosa Biofilms in Wound Models

Aditya Garg[1], Meitong Nie[1], Elieser Mejia[1], Stacie Deaver[2], Erin Gloag[2], Wei Zhou[1]

1. Department of Electrical and Computer Engineering, Virginia Tech
2. Department of Biomedical Sciences and Pathobiology, Virginia Tech

Email: aditya14@vt.edu

OBJECTIVE

To enable long-term spatiotemporal imaging of Pseudomonas aeruginosa biofilms in wound models.

BACKGROUND

Pseudomonas aeruginosa (PA) biofilms in wounds

- Bacterial biofilms in wounds contribute to infection and delay wound healing.
- *Pseudomonas aeruginosa* (PA), a gram-negative bacteria, is among the most common bacterial species observed in wounds and is known for its ability to form biofilms.
- The presence of PA biofilms in wounds is associated with negative wound outcomes. Wounds containing PA biofilms are often larger in size and experience prolonged duration compared to wounds that do not contain PA biofilms.

Conventional Detection methods and their limitations

1. **Clinical signs and symptoms:** Wounds colonized with PA may exhibit a greenish color or emit a sweet odor due to the production of pyocyanin, a blue–green pigment that causes oxidative stress in the host. However, high bacterial loads are required for detection, delaying treatment.
2. **Ex-situ microbiological analysis:** PA infection confirmation through culture-dependent methods takes 24–48 hours, further delaying treatment.
3. **In-situ receptor-based sensing:** While various receptor-based biosensors exist, they suffer from poor long-term performance in protein-rich wound environments due to bioreceptor instability, sensor surface biofouling, and irreversible analyte-receptor interactions.
4. **In-situ receptor-free sensing:** Receptor-free approaches can enable reliable long-term performance. Fluorescence imaging detects pyoverdine, a biomolecule with a unique cyan fluorescent signature produced by the Pseudomonas family, but lacks comprehensive information for therapy guidance, such as bacterial strain and biofilm state information. Vibrational spectroscopy (e.g., Raman spectroscopy) provides molecular fingerprint information for a more comprehensive analysis but lacks the sensitivity for non-invasive in-situ operation.

Surface-enhanced Raman spectroscopy (SERS)

- SERS is an extension of Raman spectroscopy, where the Raman signal of molecules near plasmonic nanostructures is significantly enhanced, enabling the possibility of non-invasive in-situ analysis (Figure 1A).
- Nanoplasmonic devices can be strategically designed to detect bacterial components according to their properties.
- Spatiotemporal SERS signals from bacterial components (e.g., metabolites, cell proteins, extracellular polymeric matrix (EPS) polysaccharides, nucleic acids (NA)) can be used to identify bacterial strains, the presence of biofilms, and biofilm growth stages (Figure 1B).

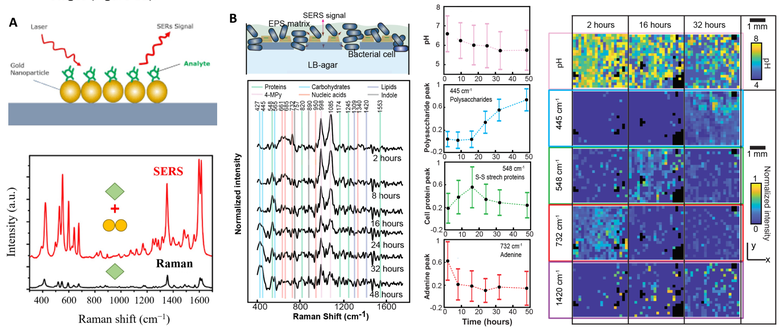

Figure 1. A) Illustration of SERS mechanisms. B) Illustration of spatiotemporal SERS imaging of bacterial biofilm growth. Figure adapted from ref[1]

Limitations of state-of-the-art nanoplasmonic devices impeding wound-interfaced SERS imaging

1. **Unreliable wound-interfaced spatiotemporal analysis:** Unbound devices, such as plasmonic nanoparticles, face issues with reproducible spatiotemporal analysis due to uncontrolled diffusion, aggregation, and distribution of nanoparticles (Figure 2A). Conversely, surface-bound devices can trigger an inflammatory response at the wound bed due to their rigid and non-porous structures (Figure 2A). Thus, novel nanoplasmonic devices need to be developed for wound-interfaced spatiotemporal analysis.
2. **Biofouling of the transducer surface:** Long-term sensing in protein-rich wound environments is challenging because the plasmonic transducers can get fouled by the proteins, preventing other molecules from accessing the transducer surface (Figure 2B). Therefore, transducer regeneration methods need to be developed.

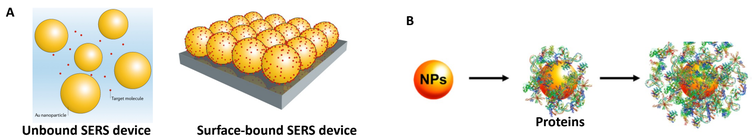

Figure 2. A) Illustration of unbound and surface-bound nanoplasmonic devices. Figure adapted from ref[2] B) Illustration of the biofouling of plasmonic transducers by proteins.

NANOPLASMONIC DRESSINGS

Fabrication of the nanoplasmonic dressings

- The underlying nanoplasmonic device was fabricated using CMOS-compatible processes including photolithography, nanoimprint lithography, physical vapor deposition, dry etching, and wet etching.
- The nanoplasmonic device was integrated with a transparent wound dressing (Nexcare Tegaderm, 3M), to create the nanoplasmonic dressing (Figure 3A).

Structural properties of the nanoplasmonic dressings

- Ultra-flexible and porous structure for an inflammation-free interface with the wound bed (Figure 3B).
- Uniformly distributed plasmonic transducers over large areas for reliable spatiotemporal analysis (Figure 3C).
- Mechanically adhered transducers for long-term stability.

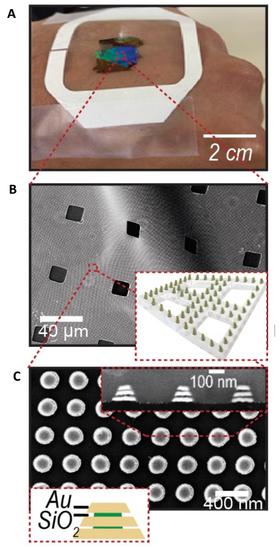

Figure 3. A) Images of the nanoplasmonic dressings at various magnifications.

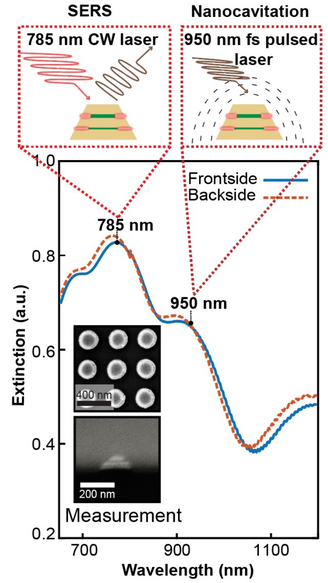

Figure 4. Extinction spectra of the nanoplasmonic dressing, illustrating resonant modes at 785 nm and 950 nm.

Plasmonic Properties of the nanoplasmonic dressings

- Multiresonant plasmonic response due to the multilayered metal-insulator-metal structure of the plasmonic transducers: Enables wavelength multiplexed operation (Figure 4).
- The resonant wavelength at ~785 nm was used for SERS measurements under 785nm continuous wave (CW) laser excitation (Figure 4).
- The other resonant wavelength at ~950nm was used to regenerate the sensor surface via nanocavitation under 950nm fs pulsed laser excitation (Figure 4).
- Working principle of nanocavitation: Plasmonic nanostructures generate local photothermal heating upon excitation leading to vapor nanobubble formation. These nanobubbles can expand and collapse following the laser pulse train, generating photoacoustic pressure waves for cleaning the transducer surface.

WOUND MODEL

- Agar block biofilm assay (ABBA) was used for the *in vitro* wound model, whereby *PA* was inoculated into wound-like media with molten agar and allowed to solidify.
- The oxygen gradient through the agar gel permits larger aggregates to develop closer to the agar-air interface, and smaller aggregates to develop deeper into the agar gel (Figures 5A-B).
- The model recapitulates both nutritional environment conditions and biofilm phenotypes observed *in vivo* during wound infections.
- Three different PA strains were used
  - PAO1: Wild type.
  - PAO1ptsP: Mutant strain associated with higher pyocyanin production.
  - PAO1wspF: Mutant strain associated with elevated cyclic di-GMP production. Cyclic di-GMP is an intracellular signaling molecule associated with improved biofilm formation.

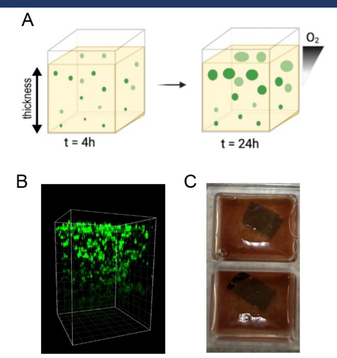

Figure 5. A) Schematic illustration, B) fluorescence image and C) camera image of the ABBA wound model.

TRANSDUCER REGENERATION VIA NANOCAVITATION

Experimental Setup

- Nanoplasmonic dressings were incubated in blood serum for 24 hours.
- Pyocyanin was added at different concentrations.
- Nanocavitation was performed to regenerate the transducer surface.
- SERS measurements were performed before and after nanocavitation.

Results

- The pyocyanin peaks at 415 and 1350 cm^{-1} exhibited increased intensities and consistent quantitative trends after nanocavitation, indicating improved and reliable access of pyocyanin to the transducer surface (Figure 6A-B)
- The peaks at 1000 and 822 cm^{-1} from blood serum proteins significantly reduced after nanocavitation at different pyocyanin concentrations, indicating the removal of adsorbed proteins from the transducer surface (Figure 6A,C).

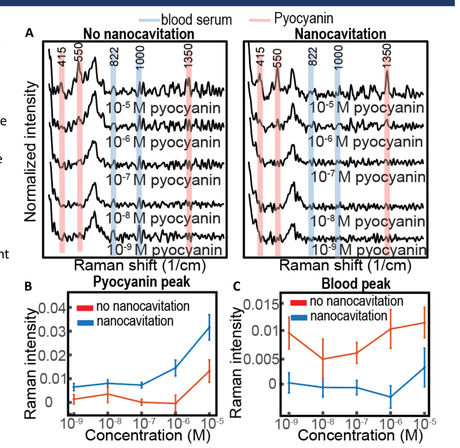

Figure 6. A) SERS spectra acquired before and after nanocavitation. The corresponding peak intensities from B) pyocyanin (1350 cm^{-1}) and C) serum protein(1000 cm^{-1}).

LONG-TERM IMAGING OF PA BIOFILMS IN WOUND MODELS

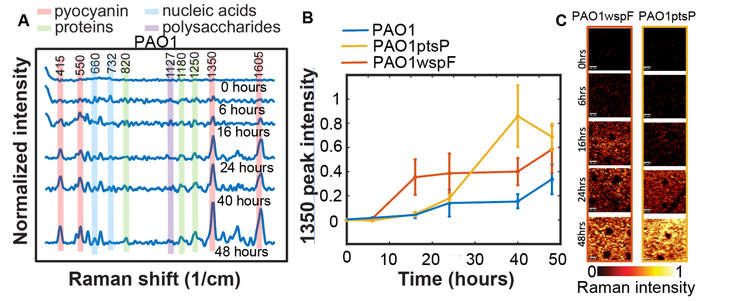

Figure 7. A) SERS spectra of PA01 between 0-48 hours. B) Temporal evolution of the 1350 cm^{-1} pyocyanin peak in the 3 PA strains. C) Spatial maps of the 1350 cm^{-1} pyocyanin peak in PAO1wspF and PAO1ptsP.

Experimental setup

- Nanoplasmonic dressings were placed on the wound models and SERS imaging was performed at 0,6,16,24,40 and 48 hours. Nanocavitation was performed before each measurement to regenerate the plasmonic transducers.

Results

- Temporally evolving SERS peaks were observed, attributed to components such as pyocyanin, proteins, nucleic acids, and polysaccharides (Figure 7A).
- The pyocyanin peaks can be visually observed within 6 hours, indicating potential for early detection (Figure 7A).
- The three strains showed unique trends temporal trends for pyocyanin production (Figure 7B).
- The PAO1wspF strain showed a rapid increase in pyocyanin production within 16 hours, likely due to the role of cyclic di GMP in biofilm formation, as reported in previous studies (Figure 7B). A positive correlation between pyocyanin production and biofilm formation has also been reported in previous studies.
- The PAO1ptsP strain showed the highest pyocyanin production at later time points, consistent with expectations for the pyocyanin overproducing mutant (Figure 7B).
- Pyocyanin SERS maps indicated the strongest signals near the porous openings of the device, attributed to the highest availability of oxygen in those regions (Figure 7C).

CONCLUSIONS

- Nanoplasmonic SERS dressings were developed for the inflammation-free and long-term imaging of PA biofilms in wound models.
- A transducer regeneration method utilizing plasmon-induced nanocavitation was developed, enabling long-term sensing in protein-rich wound environments.
- Long-term imaging of PA in wound models was successfully achieved, with strong signals from pyocyanin within 6 hours and distinctive strain-specific temporal trends, highlighting the potential for rapid strain-strain-level detection.

REFERENCES

1. Garg, A.; Mejia, E.; Nam, W.; Vikesland, P.; Zhou, W., Biomimetic Transparent Nanoplasmonic Meshes by Reverse-Nanoimprinting for Bio-Interfaced Spatiotemporal Multimodal SERS Bioanalysis. *Small* 2022, 2204517.
2. Wang, X.; Huang, S.-C.; Hu, S.; Yan, S.; Ren, B., Fundamental understanding and applications of plasmon-enhanced Raman spectroscopy. *Nature Reviews Physics* 2020, *2* (5), 253-271.