Microcontact Printing of Thiols: Changing the Way Cell Attachment is Investigated

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Introduction

Biofilms are complex three-dimensional structures formed by microorganisms. Biofilms protect microorganisms (for example, pathogenic bacteria) against antimicrobial compounds, therefore posing a threat to human, animal, and plant health. Despite numerous biofilm formation studies, details of factors influencing bacterial cell-to-cell and cell-surface interaction mechanisms are often unclear. We have been observing the unique association of disciplines and new techniques dedicated to examining how living and non-living entities adhere or detach to/from particular substrates. There are a number of mechanical effects, physical forces, and chemical changes that are critical in these interactions [1]. The importance of this cell attachment/adhesion phenomenon is grounded in the fact that bacterial pathogens will alter their physiological-biochemical and molecular status, once anchorage has been established, by activating pathogenicity factors. Specific connections to host surfaces at a molecular level seem to be a critical first step. Bacterial cells attach to surfaces, above all, because it is a survival mechanism. Pathogenic bacteria will attach to surfaces to more effectively colonize the host, thus providing them a competitive advantage over unattached cells. That is why attraction of human pathogens to medical instruments, tubing, and connectors is currently being investigated by several research groups. Not surprisingly, xylem-limited plant pathogenic bacteria are considered successful if they are able to establish themselves on the xylem walls in numbers that help them achieve massive growth [2].

Cell attachment to non-host surfaces is also a survival strategy, allowing the pathogen to wait for an appropriate opportunity to be transferred to a host organism. For instance, fresh produce can carry food-borne pathogens without causing any disease to the plant. In this case, the bacterial cells are attached to produce surfaces until bacterial cells meet an ideal environment (host) to thrive. Once attachment is established, it is almost impossible to eliminate these contaminants by regular washing, exposing us to danger even when we follow proper food processing recommendations [1].

For the present work, we tested the xylem-limited colonizing plant pathogen *Xylella fastidiosa* [3]. These bacterial cells are able to attach themselves to the internal walls of xylem vessels and form colossal amounts of biofilm, eventually clogging these vessels and killing the host by restricting access to water and nutrients. The disorder is known as Pierce's disease in grapevines and mainly affects California wine industry, though it is present in most of the southern areas of the US. The objectives of the present work are: (1) to demonstrate a new and less time-consuming approach for studying biofilm formation and cell attachment and (2) to evaluate factors influencing cell attachment during initial stages and subsequent steps of bacterial colonization.

Microcontact Printing of Thiols

Standardized testing method needed. To quickly assess attachment of *X. fastidiosa* cells to a surface, we used a technique called microcontact printing (http://education.mrsec.wisc.edu/294.htm). In simple wording, the technique allows the fabrication of modified surfaces on top of an inert substrate. The advantage of this technique is the creation of a surface to test surface-related phenomena, such as physical attraction, attachment, adhesion (stronger than attachment), and repulsion. Additionally, this constitutes an optimum scenario to verify the influence of chemicals that may enhance or hinder these developments. There is a clear demand from numerous food and medical industries to understand attachment to equipment surfaces. The lack of standardization and known sensitivity of methods has been pointed out [4]. The use of standard methods capable of measuring the bulk properties of cell aggreates rather than the behavior of isolated cells could provide a unique insight into understanding and controlling biofilm formation. For example, quorum sensing, a well-known phenomenon that characterizes a system of stimuli and responses correlated to population density could greatly benefit from a measurement technique that can provide insights into triggering factors. Moreover, we envision that such a technique would speed up cell surface studies because of its ability to scale up experimental conditions.

Why microcontact printing of thiols?. Microcontact printing of thiols (MCT) is a technique that attaches a monolayer of thiol-functionalized molecules to a silver or gold surface. The methodology was developed by George Lisensky, based on the Tollens's Test and the well-known self-assembly of thiol monolayers (SAM) on gold surfaces [5, also http://education.mrsec.wisc.edu/294.htm]. In other words, the surface properties of a substrate can be altered depending on the properties of the monolayer molecules. The printing can be accomplished using any pattern as long as there is a strong affinity of the printed molecules for the substrate surface.

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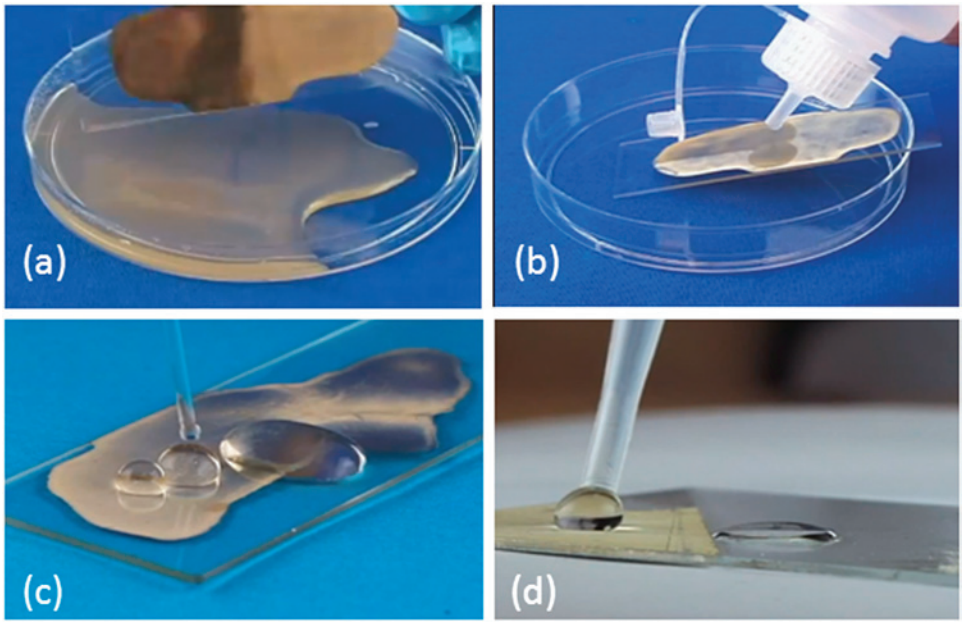

Figure 1: Octadecanethiol monolayer on silver. (a) Diamminesilver ($Ag(NH_3)_2$) is reduced to Ag metal, evidenced by the mirror-like slide bottom, (b) this silver layer is then coated with octadecanethiol, which makes the surface hydrophobic, and (c) water hydrophobicity is demonstrated on a newly formed silver coat. Images (a), (b), and (c) show these results on different prepared surfaces. (d) Distinct behavior of water droplets on side-by-side surfaces: thiol-coated on the left, glass on the right. Images are from a movie posted online by the MRSEC group *(https://www.youtube.com/watch?v=SvgEd71nHUg.).* Notice that the process is the same for regular slides or coverslips.

materials science and nanotechnology research. The group is supported by the UW Interdisciplinary Education Group (UW MRSEC IEG), funded by NSF (National Science Foundation). The missions of the group are dedicated to making complex knowledge available to the public and, at the same time, to stimulate the potential for the resolution of modern and old problems, all under an interdisciplinary umbrella. The UW MRSEC Education Group (IEG) creates and distributes a broad range of educational products that are widely used for K–12 and college-level instruction on topics that build on the center's materials science discoveries and expertise.

Bacterial plant pathogen. Auburn University's Department of Entomology and Plant Pathology is the home of a plant pathology lab researching the ionomics of the plant pathogen *Xylella fastidiosa* and the role of trace elements and nutrients in the infection and progression of disease. The lab is especially interested in infection processes, phases in host colonization, biofilm formation, and molecular characterization of bacterial plant pathogens. There is a particular aspect of the interaction of *X. fastidiosa* that poses a major concern for these investigators. The question is related to the fact that cell attachment and biofilm formation have been shown to be affected by the xylem chemistry. The group is investigating the influence of calcium, calcium chelators, and ions [6, 7]. It is this work where artificially prepared surfaces could have a major impact.

Materials and Methods

Thiol surfaces. For this investigation, we printed an octadecanethiol pattern on gold to assess a pathogen's preferential binding to this highly hydrophobic surface. Glass slides were washed with water, ethanol, and acetone. The dry glass slides were coated with a thin layer of gold after sputtering for 1 minute. Polydimethylsiloxane (PDMS) stamps were prepared by pouring Sylgard 184 silicone elastomer onto a microfluidic channel mold and curing at 100°C for 1 hour. After curing, the pattern was cut away from excess silicone. A few drops of octadecanethiol in ethanol (~1 mg/mL) were placed on the pattern and allowed to dry. The thiol-coated PDMS was then placed on the gold-coated slide, and light pressure was applied to ensure thiol transfer between the PDMS and substrate. The PDMS stamp was carefully removed from the substrate, leaving behind a layer of hydrophobic octadecane molecules.

The method promotes localized hydrophobicity by bonding the thiol group to gold and/or silver surfaces, leaving hydrophobic tail exposed (Figure 1). In order to evaluate bacterial biofilm formation, we compared *X. fastidiosa* cultures after growth on a rich growth medium (PD2) by inoculating PD2 medium (control), PD2 medium supplemented with calcium ($CaCl_2$), and PD2 medium plus EGTA (a calcium chelator that binds to calcium).

Self-assembled monolayers. The self-assembled monolayers of thiolates on metals is becoming an exceptional form of nanotechnology, as anticipated by a group from University of Illinois – Champaign, Urbana [8]. Self-assembled monolayers (SAMs) are convenient, flexible, and easy to replicate. Assembled monolayers of thiol on top of silver, using a US penny coin pattern (Figure 2), as well as a designed pattern coated with gold (Figure 3), were analyzed in preparation for quantitative biofilm studies. The penny experiment was a proof of concept experiment; additional data were generated with the experiment employing the micro fluidity chamber pattern.

Bacterial growth. Microscope cover slips (1 cm^2) previously sputter-coated with gold were printed with octadecanethiol. These coated and printed cover slips were sterilized twice with ethanol inside the laminar flow hood. Subsequently, slides were placed inside Falcon tubes containing PD2 [6] growth media and incubated for 5 days at 28°C. Slides were removed, dried inside the laminar flow hood, and fixed with osmium tetroxide. Treatments were: (1) control PD2 medium + *Xylella fastidiosa* (inoculated media used as positive control); (2) PD2 + 2mM $CaCl_2$ + *Xylella fastidiosa* (complex media PD2 supplemented with calcium); and (3) PD2 + 1.5 mM EGTA + *Xylella fastidiosa* (complex media PD2 supplemented with the calcium chelator EGTA). The inoculation and growth were carried out at Auburn University laboratories.

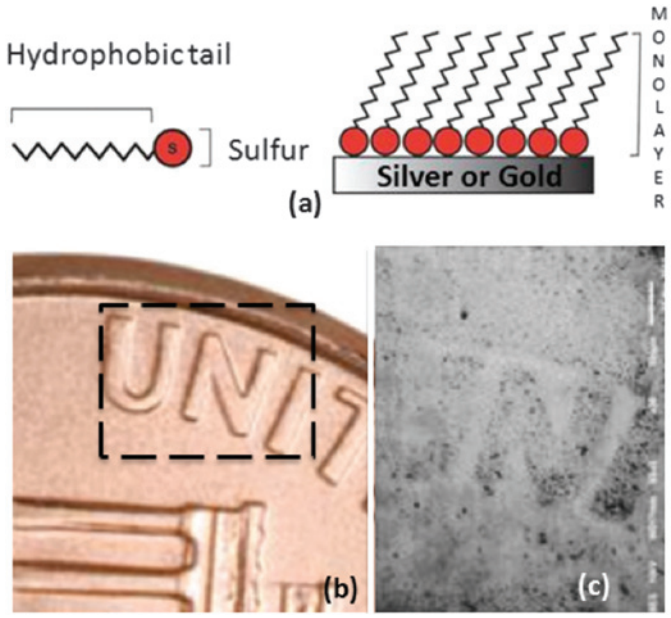

Figure 2: Microcontact thiol printing (MCT). (a) Hydrophobicity results from forming a monolayer of octadecanethiol molecules, with thiol moieties from the octadecanethiol bound to gold or silver and the tail of the molecules extending from the surface. (b) and (c) together indicate differential biofilm growth for the letters of US penny print when compared to the background. The first three letters of "United" are shown in the SEM image (c) obtained using an off-center backscattered electron detector.

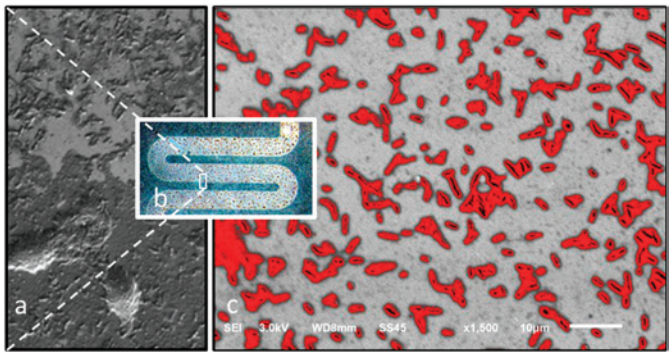

Figure 3: MCT was used to print a custom-designed microfluidic channel pattern on a gold substrate as experimentation evolved because gold coating is easier to obtain. (a) Secondary electron image showing bacterial growth of *X. fastidiosa* cells at the interface between thiol-coated (bottom) and gold only coated (top). SEM image width = 60 µm. (b) Light optical microscope image of the custom-designed pattern used. (c) Threshold image generated by the software *Image J* (NIH). In the process, bacterial cells were colorized (red) to distinguish them from the background. The % area of bacterial growth occupied was calculated and plotted in Figure 4. The area in this case corresponds to a non-thiol printed (gold only) surface (similar to top area on (a)).

SEM analysis. The treatments were later analyzed by scanning electron microscopy (SEM) at the JEOL USA, Inc. facilities in Peabody, MA. Samples were imaged in the JSM-6610 SEM. A low accelerating voltage (between 1 and 3 keV) was used to avoid charging because the treatments to be examined did not receive a conductive surface coating. Most of the images were obtained in a way to highlight differences between regions outside and inside the thiol-printed areas. Biofilm formation and/or cell colonization were assessed by processing the data with the National Institutes of Health *ImageJ* software (http://imagej.nih.gov/ij/). Images were converted into 8-bit grayscale format and submitted to a thresholding routine in order to separate definite cell forms from the image background (Figure 3).

Results

Biofilm studies. Processed data revealed that *X. fastidiosa* biofilm had more bacterial cells in thiol-printed areas compared to non-printed areas, an indication that hydrophobicity is an important factor for *X. fastidiosa* biofilm formation (Figure 4). The colonized area % corresponded to the fraction of the total area with bacterial cell forms. The experiment was repeated with similar results.

Effect of added calcium. We also observed that supplementation of the divalent calcium (Ca^{2+}) resulted in even more biofilm and more cells within the biofilm architecture. Thus, in media supplemented with calcium, the biofilm exhibited more cells per area, and, comparatively, less debris accumulation (Figures 4 and 5).

Effect of calcium chelator. Media with calcium chelator EGTA exhibited a reduced number of cells within the biofilm; however, the presence of EGTA was not enough to prevent some biofilm formation (Figure 4). The influence of calcium on *X. fastidiosa* biofilm formation was confirmed and is a result of calcium bridging; the phenomenon was demonstrated in nutritional media aggregation and biofilm studies [9].

Discussion

The present work evaluates for the first time the importance of calcium in a *X. fastidiosa* biofilm using an artificially modified surface. *X. fastidiosa* biofilm seems to be affected by: (a) the xylem vessel's internal surface chemistry, (b) the chemical composition of the xylem fluid (Figure 4), (c) the presence of morphological structures, and (d) other unknown factors (not evaluated in this work). In a recent study at Auburn University, *in vitro* studies [6] demonstrated that *X. fastidiosa* mutants responded positively to the presence of calcium in the medium and induced more biofilm formation; the authors concluded that strong cell-to-cell attachment and biofilm formation are directly related to calcium concentrations. In another recent investigation, it was verified that a number of ions accumulate in developed biofilms of *X. fastidiosa*, including a two-fold increase in calcium [10]. Our results strongly support a multi-layered stepwise progression toward biofilm formation: (1) free cells, (2) cell aggregation, and (3) adhesion to surfaces mediated by free calcium. Moving forward, calcium accumulation seems to play a key role in vessel plugging because calcium ions can form insoluble minerals (for example, calcium phosphates and calcium oxalates) resulting in biofilm hardening.

This intersection of surface science and biological techniques provides a unique platform for examining biofilm formation from a surface chemistry perspective. Functionalization of the substrate surface chemistry allows us to probe the cell-surface interactions that affect biofilm formation. Biofilm development appears to begin with the random attachment of cells to the surface, followed by

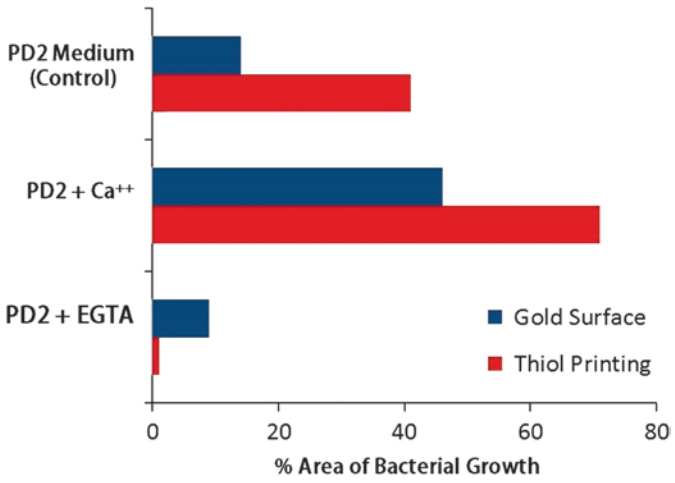

Figure 4: The % area of bacterial growth occupied by *Xylella fastidiosa* measured from SEM images of isolated cells and cells within biofilm. Area measurements are straightforward and can quickly represent how weak or vigorous was the colonization on each of the modified surfaces, that is, naked gold (blue bar) or thiol-printed areas (red bar). The PD2 medium without additives was the control. Bacterial growth was greater when $CaCl_2$ was added to the medium. Growth was negatively affected by the addition of EGTA, a calcium chelator.

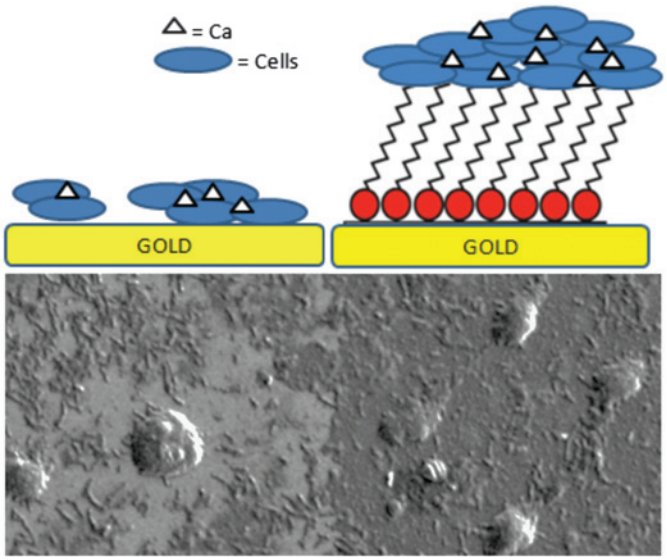

Figure 5: Idealized diagram (above) to explain the robust growth on the thiol-print side (right side) compared with the pure gold-coated surface (left). Drawings are for illustration purposes only and are not to scale. The hydrophobic thiol layer is likely to hold a greater number of bacterial cells building a solid base because there is less repulsion between bacterial cells. This allows further development of colonies of cells, evolving quickly to a complex biolfim (city of cells). The image is from the treatment with $CaCl_2$ (see Figure 4) that enhanced colonization. Image width = 60 µm.

subsequent cell-to-cell interactions. Later stages of biofilm development exhibit deposition of extracellular material around the attached cells [9].

However, the advantage of performing biofilm surface analysis also has a limitation. We think that the appropriate time to assess, count, and compare attachment of cells seems to be prior to biofilm formation. The reasoning is simple: while trying to count only cells during the thresholding of the image, one may end up selecting cells plus debris or not accounting for cells located just below the layer exposed. Published data [10] dealing with biofilm growth indicate that PD2 medium amended with calcium caused a 2-fold growth increase; whereas, we are reporting here only a 1.73-fold growth increase using the same medium and treatments, which could be an indication that we may be missing some information by having a two-dimension SEM image that does not factor in the possibility of layers. This difference might be statistically meaningless considering that all treatments in this proposed method carry the same error.

Conclusion

MCT provided a tunable platform for assessing cell-surface attachment. We have: (a) confirmed that *X. fastidiosa* cells attach preferentially to surfaces with higher degrees of hydrophobicity; (b) shown that calcium ($CaCl_2$) significantly enhances the tendency of these cells to attach and aggregate; and (c) shown that EGTA reduces attachment and consequently compromises the rest of the colonization process. These results are encouraging and validate previous results. Importantly, the combination of the MCT method and SEM required less time for data acquisition than conventional methods. With appropriate standardization this method may develop as a routine screening step in studying cell surface chemistry and intercellular attachment during the colonization process of many pathogens. In future work we foresee an increase in data generation capabilities allowing: (a) the establishment of a common basis for attachment involving plant and human pathogens and (b) greater understanding of the surface interaction between cells and their environment.

Acknowledgements

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