

PI: Gordon, Vernita	Title: Assessing the roles of biofilm structure and mechanics in pathogenic, persistent infections	
Received: 11/07/2016	FOA: PAR16-242	Council: 05/2017
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IPF: 578403	Organization: UNIVERSITY OF TEXAS, AUSTIN	
Former Number:	Department: Physics	
IRG/SRG: BMBI	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1:  Year 2:  Year 3:  Year 4:	Animals: Y Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: Y Early Stage Investigator: N
<i>Senior/Key Personnel:</i>	<i>Organization:</i>	<i>Role Category:</i>
Vernita Gordon	The University of Texas at Austin	PD/PI
Kendra Rumbaugh	Texas Tech University Health Science Center	Other (Specify)-Collaborator
Christopher Gordon	Texas Tech University	Other (Specify)-Collaborator
Jason Shear PhD	The University of Texas at Austin	Other (Specify)-Collaborator

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APPLICATION FOR FEDERAL ASSISTANCE

SF 424 (R&R)

3. DATE RECEIVED BY STATE		State Application Identifier
1. TYPE OF SUBMISSION*		4.a. Federal Identifier AI121500
☐ Pre-application ☒ Application ☐ Changed/Corrected Application		b. Agency Routing Number
2. DATE SUBMITTED 2016-11-07	Application Identifier 201603679-001	c. Previous Grants.gov Tracking Number
5. APPLICANT INFORMATION		
Legal Name*: The University of Texas at Austin		Organizational DUNS*: [REDACTED]
Department:		
Division:		
Street1*: [REDACTED]		
Street2*: [REDACTED]		
City*: [REDACTED]		
County*: [REDACTED]		
State*: [REDACTED]		
Province:		
Country*: [REDACTED]		
ZIP / Postal Code*: [REDACTED]		
Person to be contacted on matters involving this application		
Prefix:	First Name*: Sofia	Middle Name: I. Last Name*: Zanabria Suffix:
Position/Title:	Grants & Contract Specialist	
Street1*:	[REDACTED]	
Street2:	[REDACTED]	
City*:	[REDACTED]	
County:	[REDACTED]	
State*:	[REDACTED]	
Province:	[REDACTED]	
Country*:	[REDACTED]	
ZIP / Postal Code*:	[REDACTED]	
Phone Number*:	Fax Number: [REDACTED]	Email: [REDACTED]
6. EMPLOYER IDENTIFICATION NUMBER (EIN) or (TIN)* [REDACTED]		
7. TYPE OF APPLICANT* H: Public/State Controlled Institution of Higher Education		
Other (Specify): ☒ Small Business Organization Type ☐ Women Owned ☐ Socially and Economically Disadvantaged		
8. TYPE OF APPLICATION*		If Revision, mark appropriate box(es).
☐ New ☒ Resubmission		☐ A. Increase Award ☐ B. Decrease Award ☐ C. Increase Duration
☐ Renewal ☐ Continuation ☐ Revision		☐ D. Decrease Duration ☐ E. Other (specify) :
Is this application being submitted to other agencies?* ☐ Yes ☒ No What other Agencies?		
9. NAME OF FEDERAL AGENCY* National Institutes of Health		10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER TITLE:
11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT* Assessing the roles of biofilm structure and mechanics in pathogenic, persistent infections		
12. PROPOSED PROJECT Start Date* Ending Date* 05/01/2017 04/30/2021		13. CONGRESSIONAL DISTRICTS OF APPLICANT TX-025

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name*: Vernita Middle Name: Last Name*: Gordon Suffix:

Position/Title: Assistant Professor

Organization Name*: The University of Texas at Austin

Department: [REDACTED]

Division: [REDACTED]

Street1*: [REDACTED]

Street2: [REDACTED]

City*: [REDACTED]

County: [REDACTED]

State*: [REDACTED]

Province: [REDACTED]

Country*: [REDACTED]

ZIP / Postal Code*: [REDACTED]

Phone Number*: [REDACTED] Fax Number: [REDACTED] Email*: [REDACTED]

15. ESTIMATED PROJECT FUNDING

- a. Total Federal Funds Requested* [REDACTED]
- b. Total Non-Federal Funds* [REDACTED]
- c. Total Federal & Non-Federal Funds* [REDACTED]
- d. Estimated Program Income* [REDACTED]

16. IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

- a. YES ☐ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:
- DATE: [REDACTED]
- b. NO ☒ PROGRAM IS NOT COVERED BY E.O. 12372; OR
- ☐ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

☒ I agree*

* The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION

File Name: [REDACTED]

19. AUTHORIZED REPRESENTATIVE

Prefix: First Name*: Courtney Middle Name: Last Name*: Frazier Swaney Suffix:

Position/Title*: Associate Director

Organization Name*: The University of Texas at Austin

Department: [REDACTED]

Division: [REDACTED]

Street1*: [REDACTED]

Street2: [REDACTED]

City*: [REDACTED]

County: [REDACTED]

State*: [REDACTED]

Province: [REDACTED]

Country*: [REDACTED]

ZIP / Postal Code*: [REDACTED]

Phone Number*: [REDACTED] Fax Number: [REDACTED] Email*: [REDACTED]

Signature of Authorized Representative*

Date Signed*

20. PRE-APPLICATION File Name: [REDACTED]**21. COVER LETTER ATTACHMENT** File Name: Cover_Letter1027054231.pdf

Project/Performance Site Location(s)**Project/Performance Site Primary Location**

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: The University of Texas at Austin
Duns Number: [REDACTED]
Street1*: [REDACTED]
Street2: [REDACTED]
City*: [REDACTED]
County: [REDACTED]
State*: [REDACTED]
Province:
Country*: [REDACTED]
Zip / Postal Code*: [REDACTED]
Project/Performance Site Congressional District*: TX-025

File Name

Additional Location(s)

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ☐ Yes ☒ No	
1.a. If YES to Human Subjects Is the Project Exempt from Federal regulations? ☐ Yes ☐ No If YES, check appropriate exemption number: 1 _ 2 _ 3 _ 4 _ 5 _ 6 If NO, is the IRB review Pending? ☐ Yes ☐ No IRB Approval Date: Human Subject Assurance Number	
2. Are Vertebrate Animals Used?* ☒ Yes ☐ No	
2.a. If YES to Vertebrate Animals Is the IACUC review Pending? ☐ Yes ☒ No IACUC Approval Date: 05-30-2016 Animal Welfare Assurance Number XXXXXXXXXX	
3. Is proprietary/privileged information included in the application?* ☐ Yes ☒ No	
4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ☐ Yes ☒ No	
4.b. If yes, please explain: 4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ☐ Yes ☐ No 4.d. If yes, please explain:	
5. Is the research performance site designated, or eligible to be designated, as a historic place?* ☐ Yes ☒ No	
5.a. If yes, please explain:	
6. Does this project involve activities outside the United States or partnership with international collaborators?* ☐ Yes ☒ No	
6.a. If yes, identify countries: 6.b. Optional Explanation:	
7. Project Summary/Abstract*	Filename Project_Summary_and_Abstract1026881782.pdf
8. Project Narrative*	Project_Narrative1026881781.pdf
9. Bibliography & References Cited	2016_11_06_references1027054170.pdf
10. Facilities & Other Resources	Facilities_and_other_resources__combined1026881827.pdf
11. Equipment	EQUIPMENT__Gordon1026881826.pdf
12. Other Attachments	Christopher G letter_of_commitment_signed_11_4_161027054119.pdf Rumbaugh_LOI1027054120.pdf

What spatial structure and mechanics develops in biofilm infections, and how such spatial structure and mechanics impact the persistence and virulence of biofilm infections, is not known. The long-term goal is to find diagnostic and treatment approaches that address the structure and mechanics of multicellular, three-dimensional biofilm infections within the host. The objective of this proposal is to determine the mechanics and structure of biofilm infections of the opportunistic pathogen *Pseudomonas aeruginosa* in chronic wounds, and how these physical properties impact disease course. The central hypothesis is that spatial structure and mechanics are the major physical factors controlling virulence, antibiotic resistance, and immune evasion in biofilm infections. The rationale underlying this proposal is that completion will identify key physical targets for preventing, disrupting, or ameliorating biofilm infections for an important biofilm-forming pathogen. The proposed work will also develop a widely-applicable platform for assessing the state and impact of biofilm structure and mechanics for other infecting organisms. The central hypothesis will be tested by pursuing three specific aims: 1) Determine the spatial structure and mechanics of *in vivo* biofilm infections; 2) Determine how spatial arrangements differentiate into distinct microenvironments; 3) Determine the role of spatial structure and mechanics in biofilm-neutrophil interactions. We will pursue these aims using an innovative combination of analytical and manipulative techniques from both biological and physical sciences. These include both recently-developed techniques specific to biofilm studies, and more-established techniques that have been applied very little to the study of biofilm materials. The proposed research is significant, because it will determine which structural and mechanical characteristics should be therapeutic targets. It is also significant because it will develop a platform that can be extended to study other pathogens (or commensals) and synergies to open new avenues for biofilm therapies. This work will develop foundational resources that will be used by other researchers, for *P. aeruginosa* and other organisms. The proximate expected outcome of this work an understanding of which biofilm structural and mechanical characteristics contribute to clinical impact. The results will have an important positive impact immediately because they will establish better understanding of biofilm infection, virulence, and resistance to antibiotics and the immune system for an important pathogens, and long-term because they lay the groundwork to develop a suite of techniques for better treatment of biofilm infections.

The proposed research is relevant to public health because understanding what biofilm structures and mechanics exist and the degree to which they influence the medical outcomes of biofilm infections is expected to give rise to new types of treatments and diagnostics for chronic biofilm infections that specifically target structure and mechanics. Thus, this proposal is relevant to the part of NIH's mission that pertains to fostering fundamental creative discoveries and innovative research strategies as a basis for ultimately protecting health.

FACILITIES AND RESOURCES: The University of Texas at Austin (Gordon and Shear)

Description of University of Texas: The University of Texas at Austin, founded in 1883, is the flagship campus of the University of Texas system and is a world-class center for basic and translational research. The Center for World University Rankings released the inaugural list of the world's top 100 universities and ranked UT Austin number 30. The university employs about 24,000 faculty and staff, enrolls about 51,000 students and confers 12,000 degrees each year. Research funding for the 2009-2010 academic year topped \$644 million and the amount awarded has increased each year. About \$1.1 billion was awarded in sponsored research over the past two years and the university recovered \$40 million in the past two years in revenue from the licensing of university technology from about 800 patents that have been awarded. UT Austin runs one of world's fastest supercomputers and one of the most powerful lasers as examples of the vibrant research environment on campus. This environment has fostered the careers of UT Austin faculty, who have been distinguished nationally and internationally and includes winners of the Nobel Prize, Pulitzer Prize, National Medal of Science and the Wolf Prize.

Vernita D. Gordon

Institutional Commitment: Dr. Gordon has a tenure-track appointment as assistant professor at the University of Texas at Austin. Institutional startup funds came with this appointment and were used in taking the preliminary data shown in this proposal, as were supplementary funds from internal graduate and undergraduate research fellowships used to support the graduate and undergraduate students who have been working on this project (stipend and supplies). UT Austin has provided two years of summer salary support to allow time dedicated solely for research, lab space as described below, and shared facilities and equipment also as described below. The graduate students who took the preliminary data in this proposal have been supported on a combination of internal fellowship, teaching, and startup funds. Administrative support is provided through the University, the College of Natural Sciences, the Department of Physics, and the Center for Nonlinear Dynamics. Access to shared and core facilities is described below. Dedicated travel funds in the startup package were used to allow the PI to attend grant-writing workshops held by NIH and NSF. Research personnel in Professor Gordon's group currently are 1 postdoc (PhD in Microbiology), 6 graduate students (5 in the Physics program at UT Austin, 1 in the Microbiology program at UT Austin), and numerous undergraduates.

Laboratory: Dr. Gordon's group has approximately 1500 square feet of lab space in the Department of Physics in the Robert Lee Moore Hall (RLM) at the University of Texas at Austin, and approximately 2000 additional square feet across the street in the Neuro-Molecular Sciences (NMS) building. The total lab space includes one large (2 microscopes) and 1 small specially-designed microscopy rooms (filtered air, light-tight, and temperature control) in RLM and an additional microscopy room in NMS, wet lab facilities for sample preparation in both RLM and NMS, and a Milli-Q pure water production system. Small equipment in the labs includes a Milli-Q pure water production system, a -80 freezer shared with one other biological physics faculty (Florin), -20 and -5 freezers, refrigerators, incubators, centrifuge, spectrophotometer, and vortexer as well as other small equipment and supplies. Additional small equipment for biophysical and biological work is ready available through colleagues in the Center for Nonlinear Dynamics, the Institute for Cell and Molecular Biology, and the Department of Biomedical Engineering.

Computer: Dr. Gordon has a laptop with docking station, external monitor, and external keyboard and mouse in her office. In Dr. Gordon's lab are three instrument computers, one to control each microscope, and a Linux server purpose-built for rapid image processing and analysis. This server runs doubly-redundant RAID. The group has a second Linux server dedicated to automated data backup. We do a second automated backup to tape, for long-term, off-site storage through the Texas Advanced Computing Center. The group has an additional desktop computer currently used by Dr. Gordon's postdoc and multiple personal laptops. The Gordon group has its own licenses for IDL and for Kaleidograph. UT Austin provides free copies of the Microsoft Office suite to students, faculty, and staff. Through the Center for Nonlinear Dynamics (see below), the group has access to Matlab site licenses and Adobe products. Offices and labs are all equipped with multiple Ethernet ports as well as wireless Internet access. Computer support is provided by University, Department, and CNLD IT staff.

Clinical: N/A

Animal: N/A

Office: Dr. Gordon has a 200 square foot office. Graduate students each have their own desks in shared offices. Undergraduates share desk space as appropriate. In addition, the Center for Nonlinear Dynamics has a seminar room for regular meetings and seminars. All the office space, the RLM lab space (including both microscopy rooms) and the seminar room are on the same floor; this greatly facilitates group interaction. Having the NMS space directly across the street makes regular traffic back and forth straightforward, and helps facilitate contact with microbiological colleagues also located in the NMS building. Through the Center for Nonlinear Dynamics (CNLD – see below), the group has access to Matlab site licenses and to Adobe Photoshop and other Adobe products. The group has its own licenses for IDL and for Kaleidograph. UT Austin provides free copies of the Microsoft Office suite to students, faculty, and staff. Offices and labs are all equipped with multiple Ethernet ports as well as wireless Internet access. There are CNLD shared printers (color and B/W), scanner, fax, and copier. Computer support is provided by University, Department, and CNLD IT staff.

Other Resources:

Support: PI Vernita Gordon is a member of the Center for Nonlinear Dynamics, a strength of UT Austin's physics department. The Center for Nonlinear Dynamics (CNLD) provides administrative support (administrator and an assistant), computer network infrastructure, and a visiting scientist program with typically 25 visitors/year. The CNLD has a well-established infrastructure for the education of students and postdocs with lecture courses, a weekly Nonlinear Dynamics Seminar, and a weekly group meeting (<http://chaos.utexas.edu/talks>). Every student has to present his/her project and results at regular intervals (at least once a year); students are strongly encouraged to practice their talks in front of a few critical peers before speaking before the full group. Group meetings are attended by all Center students, postdocs, and faculty (Swinney, Marder, Raizen, Swift, McCormick, Florin, Gordon). The weekly seminars also draw faculty and students from Microbiology, Physics, Mathematics, and Engineering. The biological physics groups in the CNLD (Gordon, Florin) also have a weekly journal club at which students and postdocs present recent relevant journal articles and distribute recent papers of interest.

Secretarial and administrative services are also provided by the Physics department.

The Center for Systems and Synthetic Biology (CSSB) has a regular (approximately biweekly) lunch for faculty interested in complex biological systems, such as the effects of population structure examined in this proposal. Gordon is a participant and speaker in these lunches, at which faculty members present current research in their groups. Over thirty other faculty participate in these lunches.

The PI is a member of the Institute of Cellular and Molecular Biology (ICMB) and has access to the Institute core facilities, described below. The ICMB has a regular seminar series with external speakers. The PI is a member of the Graduate Studies Committees for both Cellular and Molecular Biology and Microbiology and Molecular Genetics, and as such can supervise PhD students from both programs. This provides the group with infusions of fresh perspectives and skills. Currently, two members of the Gordon group are graduate students in the Microbiology program.

Jason B. Shear

Laboratory: The Shear laboratory occupies several rooms in the Welch Chemistry Building, and consists of ~2500 sq. ft. of wet lab, optical lab, and cell culturing facilities.

Computer: The Shear group uses numerous recent incarnations of Macintosh and PC computers for collecting and analyzing data and writing manuscripts. Image analysis is performed using a commercial package (Metamorph) and ImageJ shareware.

Clinical: N/A

Animal: N/A

Office: Dr. Shear maintains an office near his laboratories. In addition to the PI's office, office space exists for ~10 students including commons areas for meeting and discussing experimental plans and results.

Advanced Imaging: The Shear lab has access to various high-end imaging systems through users facilities in the Institute for Cellular and Molecular Biology and the Biomedical Engineering Department. These include an Asylum Research AFM with MFP3D software for both contact-mode imaging and force measurements, a FEI Tecnai transmission electron microscope, and several confocal fluorescence microscopes.

Other: Within Shear's laboratory or building is an ultrapure water system, ice-machines, a spectrophotometer, a fluorimeter, shaking incubators, a -80°F freezer, and several wide-field fluorescence microscopes. User and staff facilities in Welch Chemistry building or nearby provide access to scanning confocal laser microscopy, deconvolution microscopy, a time-resolved fluorimeter, mass spectrometry, machining, glass blowing, electronics design/repair, and high-field NMR. University facilities also exist for peptide and nucleic acid synthesis/analysis, scanning electron microscopy, and transmission electron microscopy. Dr. Shear is provided with administrative assistance and has access to fax and photocopier services.

Core Facilities at UT Austin (available to both Gordon and Shear)

Core Facilities: The University of Texas at Austin operates core facilities dedicated to providing the latest equipment and knowledge necessary to assist researchers on campus. From routine, though

essential, support services to advanced technical and consulting services, these cores facilitate and enhance the important research conducted at the university on a daily basis. The available core facilities are listed as follows.

- **College of Liberal Arts/College of Natural Sciences Facilities.**

- **Imaging Research Center.**
- **Institute for Cellular and Molecular Biology (ICMB) Core Research Facilities.** The ICMB core facilities support cellular and molecular biology research at The University of Texas at Austin. The facilities offer a full range of services in nucleic acid and protein sequencing, x-ray crystallography, and transgenic-knockout mice. The core facilities are open on an equal basis to all faculty with a philosophy to keep the core facilities as comprehensive and accessible as possible to increase faculty, staff, and student research productivity. The individual facilities are listed below.
 - DNA sequencing & Genomics Facility.
 - The Protein and Metabolite Analysis facility
 - UT Microarray Facility. Administered through the Center for Systems and Synthetic Biology (see above).
 - **Microscopy Core Facility.** This core facility is the most likely to be used for this project as it contains widefield (Zeiss Axiovert 200 M) and confocal (Leica SP2 AOBs) fluorescent microscopes as well as software for data processing and analysis, including Imaris (used to prepare the topographic views shown in this proposal) and Metamorph.
 - Macromolecular Crystallography Facility.
- **Department of Chemistry and Biochemistry.** The Department of Chemistry & Biochemistry features a variety of in-house services and facilities that support state-of-the-art teaching activities and high-level scientific research. Laboratory and classrooms are housed in over 500,000 sq. ft. of modern space and the department has numerous innovative resources to propel ground-breaking research. Individual facilities are listed below.
 - Center for Electrochemistry.
 - Mass Spectrometry.
 - Nuclear Magnetic Resonance.
 - Welch Computer Lab.
 - X-Ray Facility.
 - Fisher Scientific Research Storeroom.
 - Glassblowing Shop.
 - Instructional Media Laboratory.
 - Instrument Design and Repair.
 - Machine Shop.

- **College of Pharmacy.** The College of Pharmacy offers access to core facilities to the researchers on campus. These facilities are listed below.
 - **Protein and Metabolite Analysis Facility.**
 - **Drug Dynamics Institute: InstrUcTex Core.**
 - **Texas Institute for Drug and Diagnostics Development.**

- Automation and High Throughput Screening Facility.
- Genomic Sequencing and Analysis Facility.
- Macromolecular Crystallography Facility.
- Materials Physics Laboratory.

FACILITIES AND RESOURCES: Texas Tech University Health Sciences Center (Rumbaugh)

Laboratory:

Equipment available in the Dr. Rumbaugh's laboratories include laminar flow hoods for tissue culture, CO₂ incubators, bacterial incubators, high-speed refrigerated centrifuges with rotors, refrigerated microfuges, gel electrophoresis equipment and power supplies, orbital-shaking water baths, spectrophotometers and a luminometer, a monochromator-based multi-mode microplate reader for fluorescence and luminescence, analytical balances; autoclaves and drying ovens, -20°C and -80°C freezers, fridges, liquid nitrogen storage tank and thermocyclers for PCR.

Additional Facilities available at TTUHSC:

Dr. Rumbaugh has joint appointments in the Depts of Cell Biology and Biochemistry and Microbiology and Immunology. Additional equipment available in the Department of Microbiology and Immunology include an ABI7000 real time PCR machine, a Typhoon phosphor-imager and and gel imaging equipment. Specific to this project, the SilhouetteStar laser scanning wound imaging, 3D measurement and documentation system, will be used to document wound area and volume. The SilhouetteStar is a shared resource in the Department of Surgery. A Nikon TiE confocal microscope, which is equipped with an option known as N-STORM (Nikon Stochastic Optical Resolution Microscopy) is available in the Imaging Core and will be used for the described imaging studies.

FACILITIES AND RESOURCES: Texas Tech University (Christopher)

Institutional Commitment to ESI: Assistant Professor Christopher has strong support from Texas Tech University to conduct research as part of his tenure track appointment. His startup package as an assistant professor at Texas Tech University in the Mechanical Engineering Department included a major renovation of the room ME 133 bd, turning it into 400 ft² of brand new laboratory space and giving him funds to purchase a rheometer, inverted microscope and high speed camera all of which are necessary to complete the proposed work. Furthermore, Christopher currently has 3 PhD students supported by startup funds, teaching assistantships, and through additional support provided by the Graduate School Dean's office. As part of his assigned duties, Christopher focuses 65% of his time towards research related activities per the university's guidance. Christopher has access to the TTU Materials Characterization Center (described below) which offers a number of tools for visualizing soft matter. Finally, through the Texas Tech Teaching, Learning and Professional Development Center, the PI has access to training in grant writing, budgeting, ethics, and other faculty development programs.

Christopher Laboratory: Microfluidics and Microrheology: Christopher's primary lab space is the newly renovated ME 133 bd. It is 400 ft² of brand new laboratory space. The renovation included new water lines, sufficient power requirements, house air and nitrogen lines, and new safety showers and eyewashes. The laboratory has been outfitted with a 64 ft² class 10,000 modular clean room with UV filtering windows. The clean room is also outfitted with a fume hood for chemical work. Safe and sufficient storage, including a fire safe cabinet and a fridge, are available in the laboratory. Balances, ovens, UV exposure lamp, spinner, plasma cleaner, sonicator, stir plates and glassware for chemical work and solution prep have also been purchased and are ready for use.

Computer: The lab space is outfitted with 2 desktops and a lap top for data analysis, and a ReadyNAS Pro 6 level 5 Raid server for file backup and access over the internet. The College of Engineering IT-Division led by Nick Rinker who provides technical support and repairs to all computers in the laboratory facilities. Christopher, through the college of engineering, has access to licenses for Matlab, Microsoft Office, and Autocad software.

Office: Christopher has separate office space for himself and his graduate students to conduct non-experimental work and space for writing, reading and other activities. These offices are located in the same building as the laboratory space. Christopher has access to the administrative staff of the department of Mechanical engineering to assist in various administrative tasks.

Other Resources:

Intellectual Resources: Christopher is a member of 2 interdisciplinary faculty groups that provide a robust and helpful community for collaboration and high level academic discussions. The Complex Fluids Group is a weekly gathering of professors and students to present ongoing research, practice conference talks, and educational talks. This group provides a means of vetting early work, discussing possible problems and interpreting results. Of particular note in this group is Dr. Raj Khare of Chemical Engineering who has a background in simulating the rheology of confined biological soft matter and whose insight will provide great help to the proposed research. The Soft Matter group is a weekly meeting of professors working in soft matter to present their work, look for possible collaborations and identify possible large group funding sources.

TTU Materials Characterization Center: The center is designed to support and complete a range of materials studies from synthesis to characterization and includes the following equipment: atomic force microscope, spinning disc confocal microscope, differential scanning calorimeter, scanning electron microscope, fourier transform infrared spectrometer, microspore analyzer, and x-ray diffractometer. The center is staffed by a technician who provides training and support.

Major equipment available for this project

Our laboratories are fully equipped to perform the experiments described here, either using equipment physically located in our laboratories or through the use of shared equipment, either in institutional or departmental core facilities. Breakdown by participant follows:

GORDON (UNIVERSITY OF TEXAS AT AUSTIN)

The primary equipment to be used in this proposal are existing microscopes and a Linux server for data analysis. The server is described in the Facilities statement.

An inverted confocal epifluorescent laser-scanning microscope (Fluoview, Olympus) will be used on this project. The confocal microscope has three photomultiplier tubes for imaging as well as multiple laser lines, for which the 488 and 543 lines will likely be the most used on this project. This allows imaging and spectral resolution of GFP, YFP, and propidium iodide (PI) dead-stain fluorescence, with additional laser lines and filters available should the need arise.

A second inverted Olympus microscope is equipped with laser tweezers as well as epifluorescent, brightfield, and phase contrast imaging, and was used to acquire preliminary data shown in this proposal. The group has fluorescent filter cubes for GFP, YFP, and PI imaging, and can acquire additional filter cubes as needed.

Imaging on both microscopes is software-controlled, allowing long-term, automated data acquisition. The confocal microscope uses photomultiplier tubes and for the other microscope we have both a QImaging Blue camera and an Orca Flash 4.0 camera. The latter is a scientific CMOS camera with over 70% quantum efficiency at 600 nm, low noise (1.3 electrons at 100 frames/sec), 4 megapixels (with 6.5x6.5 μm pixels), and capable of 100 frames/sec at full resolution.

Each microscope has a temperature- and humidity-controlled enclosure. The enclosures (Precision Plastics) maintain temperature adjustable from room ($\sim 20^\circ\text{C}$) up to greater than 50°C , and stable to within 0.1°C over days. This allows long-term incubation on the microscope at physiological temperatures. There is also a temperature-controlled stage insert and objective heating/cooling collar that can be switched between the two microscopes to allow more-localized control of sample temperature. As well as two low-magnification 10x air objectives and a medium-magnification 40x air objective, there are two 100x oil objectives, a 60x oil objective, and a 60x air objective. All are from Olympus and fully sharable between all microscopes. Our primary intention is to use the 100x and 60x oil objectives in this study, along with the 60x air objective. This will allow high-resolution imaging of single-cell behavior and early structural development, and will allow measurements of the localization (and through this, the structural dependence) of antibiotic resistance and virulence gene expression. As needed, lower-magnification objectives can be used to image a larger area of biofilm structure.

Other equipment used in this proposal will be the microbiological storage, culture, and characterization equipment in the PI and collaborators' laboratories. These include -80 freezer, shaking and stationary incubators, spectrophotometers, 96 well plate reader, electroporation apparatus, electrophoresis apparatus, PCR thermocyclers, and other items detailed in the Facilities and Other Resources statement.

RUMBAUGH (TEXAS TECH UNIVERSITY HEALTH SCIENCES CENTER)

Dr. Rumbaugh's laboratories are fully equipped to perform the experiments described here, either using equipment physically located in their laboratories or through the use of shared equipment,

either in institutional or departmental core facilities. Dr. Rumbaugh has joint appointments in the Depts of Cell Biology and Biochemistry and Microbiology and Immunology. Equipment available in the PI's laboratories include laminar flow hoods for tissue culture, CO₂ incubators, bacterial incubators, high-speed refrigerated centrifuges with rotors, refrigerated microfuges, gel electrophoresis equipment and power supplies, orbital-shaking water baths, spectrophotometers and a luminometer, a monochromator-based multi-mode microplate reader for fluorescence and luminescence, analytical balances; autoclaves and drying ovens, -20°C and -80°C freezers, fridges, liquid nitrogen storage tank and thermocyclers for PCR. For fluorescent microscopy and bright field microscopy a Nikon 80i epifluorescence microscope with a color digital camera equipped with the Elements software analysis package is available in the Dr. Rumbaugh's lab. Additional equipment available in the Department of Microbiology and Immunology include an ABI7000 real time PCR machine, a Typhoon phosphor-imager and gel imaging equipment. Specific to this project, the SilhouetteStar laser scanning wound imaging, 3D measurement and documentation system, will be used to document wound area and volume. The SilhouetteStar is a shared resource in the Department of Surgery. A Nikon TiE confocal microscope, which is equipped with an option known as N-STORM (Nikon Stochastic Optical Resolution Microscopy) is available in the Imaging Core and will be used for the described imaging studies. Dr. Rumbaugh's laboratory also prepares their own tissue and slides for histopathology, using a Bright OTF5000 cryostat to produce thin, frozen tissue sections.

CHRISTOPHER (TEXAS TECH UNIVERSITY)

AFM: Dr. Christopher has access to an Asylum Research MFP-3D Infinity AFM in the TTU Materials Characterization Research Center. This system has a 35nm resolution in the Z axis, and 150pm resolution in the XY plane. Its software allows easy setup so it can operate in tapping, force-displacement, and AM-FM modes for all the proposed measurements. Furthermore, the self-calibration function allows precise and repeatable measurements. It is equipped to handle liquid environments as well. It can hold large samples (up to 80 mm in diameter). It will provide an ideal tool for the proposed measurements of the biofilm samples.

Confocal: Dr. Christopher has access to a 3i Marianas Spinning disk Confocal microscope in the TTU Materials Characterization Research Center. This tool allows sampling of large fields in a single image, unlike laser-scanning confocal microscopes. This results in fast image capture (1000 fps) and enables tracking of fluorescent particles, while maintaining high quantum efficiency. This allows its use in the microrheology application in the proposed work.

SHEAR (UNIVERSITY OF TEXAS AT AUSTIN)

Major equipment to be used for cell preparation and fluorescence microscopy currently resides within the Shear laboratory. This includes a Zeiss Axiovert fluorescence/DIC microscope outfitted with a scientific-grade fast low-noise readout CCD camera and cell-culturing facilities, including CO₂-controlled incubators, sterile hood space, water baths, table-top centrifuges, and standard solution preparation facilities (balances, analytical balances, high-purity water dispenser, chemical hoodspace). Various micro-3D printing instruments exist in the Shear lab, comprising femtosecond titanium:sapphire laser systems, associated optics (including one- and two-axis raster scanning mirrors and digital micromirror device dynamic masks), and inverted microscope systems with high-resolution vertical axis scanning capabilities.

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator				
Prefix:	First Name*: Vernita	Middle Name	Last Name*: Gordon	Suffix:
Position/Title*:	Assistant Professor			
Organization Name*:	The University of Texas at Austin			
Department:	[REDACTED]			
Division:	[REDACTED]			
Street1*:	[REDACTED]			
Street2:	[REDACTED]			
City*:	[REDACTED]			
County:	[REDACTED]			
State*:	[REDACTED]			
Province:	[REDACTED]			
Country*:	[REDACTED]			
Zip / Postal Code*:	[REDACTED]			
Phone Number*:	[REDACTED]	Fax Number:	[REDACTED]	
E-Mail*:	[REDACTED]			
Credential, e.g., agency login:	[REDACTED]			
Project Role*: PD/PI	Other Project Role Category:			
Degree Type:	Degree Year:			
Attach Biographical Sketch*:	File Name:	Vernita_Gordon_Biosketch1027054133.pdf		
Attach Current & Pending Support:	File Name:			

PROFILE - Senior/Key Person				
Prefix:	First Name*: Kendra	Middle Name	Last Name*: Rumbaugh	Suffix:
Position/Title*:	Assistant Professor			
Organization Name*:	Texas Tech University Health Science Center			
Department:	[REDACTED]			
Division:	[REDACTED]			
Street1*:	[REDACTED]			
Street2:	[REDACTED]			
City*:	[REDACTED]			
County:	[REDACTED]			
State*:	[REDACTED]			
Province:	[REDACTED]			
Country*:	[REDACTED]			
Zip / Postal Code*:	[REDACTED]			
Phone Number*:	[REDACTED]	Fax Number:	[REDACTED]	
E-Mail*:	[REDACTED]			
Credential, e.g., agency login:				
Project Role*: Other (Specify)			Other Project Role Category: Collaborator	
Degree Type:			Degree Year:	
Attach Biographical Sketch*:	File Name:	Kendra_Rumbaugh_Biosketch1027054136.pdf		
Attach Current & Pending Support: File Name:				

PROFILE - Senior/Key Person				
Prefix:	First Name*: Christopher	Middle Name F	Last Name*: Gordon	Suffix:
Position/Title*:	Assistant Professor			
Organization Name*:	Texas Tech University			
Department:	[REDACTED]			
Division:	[REDACTED]			
Street1*:	[REDACTED]			
Street2:	[REDACTED]			
City*:	[REDACTED]			
County:	[REDACTED]			
State*:	[REDACTED]			
Province:	[REDACTED]			
Country*:	[REDACTED]			
Zip / Postal Code*:	[REDACTED]			
Phone Number*:	[REDACTED]	Fax Number:	[REDACTED]	
E-Mail*:	[REDACTED]			
Credential, e.g., agency login:				
Project Role*: Other (Specify)			Other Project Role Category: Collaborator	
Degree Type:			Degree Year:	
Attach Biographical Sketch*:	File Name:	Gordon_Christopher_Biosketch1027054299.pdf		
Attach Current & Pending Support: File Name:				

PROFILE - Senior/Key Person			
Prefix: Dr.	First Name*: Jason	Middle Name	Last Name*: Shear
Suffix: PhD			
Position/Title*:	Associate Professor, PhD		
Organization Name*:	The University of Texas at Austin		
Department:	[REDACTED]		
Division:	[REDACTED]		
Street1*:	[REDACTED]		
Street2:	[REDACTED]		
City*:	[REDACTED]		
County:	[REDACTED]		
State*:	[REDACTED]		
Province:	[REDACTED]		
Country*:	[REDACTED]		
Zip / Postal Code*:	[REDACTED]		
Phone Number*:	[REDACTED]	Fax Number:	
E-Mail*:	[REDACTED]		
Credential, e.g., agency login:	[REDACTED]		
Project Role*: Other (Specify)	Other Project Role Category: Collaborator		
Degree Type:	Degree Year:		
Attach Biographical Sketch*:	File Name:	Shear_Biosketch_11_16_1027054297.pdf	
Attach Current & Pending Support: File Name:			

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Gordon, Vernita Diane

eRA COMMONS USER NAME (credential, e.g., agency login): [REDACTED]

POSITION TITLE: Assistant Professor of Physics

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.*)

INSTITUTION AND LOCATION	DEGREE (if applicable)	Completion Date MM/YYYY	FIELD OF STUDY
Vanderbilt University, Nashville, TN	B.S.	05/97	Physics and Math
Harvard University, Cambridge, MA	A.M.	06/01	Physics
Harvard University, Cambridge, MA	Ph.D.	11/03	Physics (experimental soft condensed matter)
University of Edinburgh, Edinburgh, Scotland	Postdoc	05/06	Physics of biomembranes
University of Illinois, Urbana-Champaign, IL	Postdoc	07/10	Biological physics of bacteria and biomembranes

A. Personal Statement

We aim to determine what physical structures and mechanics develop in chronic wounds infected by *Pseudomonas aeruginosa* biofilms, and how spatial structure and mechanics contribute to virulence, antibiotic resistance, and resistance to phagocytic clearance by the immune system. For this, we will take a multidisciplinary approach that combines innovative techniques from physical science and from microbiology. This work is a good fit to my group's expertise, since research currently based in my lab focuses on the mechanical and structural properties of single-cell and multicellular biological systems. My primary expertise lies in optical microscopy and quantitative image analysis of systems characterized by 1-100 μm lengthscales. My primary field of training is soft-matter physics and much of my microscopy work has focused on characterizing mechanics and sub-micron microstructure. More recently, I have acquired expertise in bacteria, specifically *P. aeruginosa*. After I began my faculty position I established another expertise in the spatial characterization and manipulation of dynamically developing systems of many bacteria. My lab can perform experiments combining mechanical, structural, and biological manipulation and measurement, a vanishingly rare ability. Thus, I am uniquely positioned to oversee the research described in this proposal. I have the experience and leadership to lead this project and to direct joint efforts with surgical, microbiological, engineering, and chemical collaborators as this project requires. This is substantiated by my track record of successful publication with medical and biological researchers. This includes a manuscript under review at mBio with Kendra Rumbaugh, who is a collaborator on this proposal.

I have New Investigator status; my Early Stage Investigator status ended in March 2015. I began my faculty position at UT Austin in 2010 and my tenure clock was stopped for the 2011-2012 and 2013-2014 academic years, so I have completed four years on the tenure clock.

Publications most-closely related to the present proposal:

1. [REDACTED]
[REDACTED]
[REDACTED]

2. *Role of multicellular aggregates in biofilm formation.* Kragh, K., J. Hutchison, G. Melaugh, C. Rodesney, A. Roberts, Y. Irie, P. Jensen, S. Diggle, R. Allen*, **V. Gordon***, and T. Bjarnsholt*. mBio, 2016. **7**: p. e00237-16 * = joint corresponding authors
3. *Tobramycin and bicarbonate synergise to kill planktonic Pseudomonas aeruginosa, but antagonise to promote biofilm survival.* Kaushik, K., J. Stolhandske, O. Shindell, H. Smyth, and **V. Gordon**. npj Biofilms and Microbiomes, 2016. **2**: p. 16006
4. *Single-Cell Control of Initial Spatial Structure in Biofilm Development Using Laser Trapping.* Hutchison, J., C. Rodesney, K. Kaushik, H. Le, D. Hurwitz, Y. Irie, and **V. Gordon**. Langmuir, 2014. **30**: p. 4522–4530

B. Positions and Honors.

Positions and Employment

2010- Assistant Professor, Department of Physics, University of Texas at Austin

Other Experience and Professional Memberships

2007-present American Physical Society (APS)
 2016-2020 Secretary/Treasurer for the Division of Biological Physics of the APS
 2009-2010; 2012-present American Society for Microbiology
 Symposium convener for the 2016 Microbe meeting.
 2014- Society of Rheology
 2015- Biophysical Society
 2013- NSF grant proposal reviewer
 2014 Instructor, Hands-On Research in Complex Systems School, International Center for Theoretical Physics , Trieste, Italy.

Honors

1995-1997 William and Nancy McMinn Honor Scholarship in Physics, Vanderbilt University
 2008-2010 Postdoctoral Fellow, Cystic Fibrosis Foundation
 2012 Contributed abstract selected for talk at the American Society for Microbiology annual meeting
 2012 Invited poster presentation, Human Frontiers Science Project annual meeting
 2013 Hyer Research Award, Texas Section of the American Physical Society
 For the best research done by a 2-person team of a faculty member and an undergraduate.

Invited Talks (since joining UT Austin faculty in 2010)

2011 National Society of Black and Hispanic Physicists annual meeting, Austin, TX
 Physics Department Seminar, Trinity College, San Antonio, TX
 2012 Workshop on Biomembranes, Kavli Institute of Theoretical Physics – China, Beijing, China
 Center for Theoretical Biology Seminar, Beijing University, Beijing, China
 2013 Physics and Biology Departments joint seminar, Austin College, Sherman, TX
 Holliday Lecture (named Physics colloquium), Vanderbilt University, Nashville, TN
 2014 Biophysics Seminar, Center for Theoretical Biological Physics, Rice University, Houston, TX
 Condensed Matter Physics Seminar, University of Edinburgh, Edinburgh, United Kingdom
 Chemistry Seminar, University of Leeds, Leeds, United Kingdom
 Physics Seminar, Texas Christian University, Fort Worth, TX
 2015 Mechanical Engineering Seminar, Dept. of Mechanical Engineering, Texas Tech, Lubbock, TX
 Biomedical Engineering Seminar, Dept. of Biomedical Engineering, UT Brownsville, Brownsville, TX
 Soft Matter Seminar, Soft Matter Institute, Dept. of Physics, Georgetown University, Washington DC
 Center for Biofilm Engineering (CBE) Seminar, CBE, Montana State University, Bozeman, MT
 Biophysics Colloquium, Cornell University, Ithaca, NY

Physics Colloquium, Emory University, Atlanta, GA
 Biophysics Seminar, Massachusetts Institute of Technology, Cambridge, MA
 New England Complex Fluids Meeting, Harvard University, Cambridge, MA

- 2016 March Meeting of the American Physical Society, Baltimore, MD
 Colloquium, Dept. of Physics, University of Massachusetts – Amherst, Amherst, MA
 Colloquium, Dept. of Physics, Trinity University, San Antonio, TX
 Center for Bioengineering Seminar, University of California – Santa Barbara, Santa Barbara, CA
 American Society of Microbiology Microbe 2016 annual meeting, Boston, MA
 Texas Soft Matter meeting, Dallas, TX
 American Chemical Society National Meeting, Philadelphia, PA
 Seminar, Chemical Engineering, University of Pennsylvania, Philadelphia, PA
 Biosoft Seminar, Dept. of Physics, Georgia Institute of Technology, Atlanta, GA
 Colloquium, Chemical Engineering, Stanford University, Stanford, CA
 Biophysics Seminar, Dept. of Physics, University of California – Irvine, Irvine, CA
 American Chemical Society Southwest Regional Meeting, Galveston, TX
 Biophysics Seminar, Dept. of Physics, Princeton University, Princeton, NJ
 Soft Matter Seminar, Dept. of Chemistry, Columbia University, New York, NY
- 2017 Systems Biology Seminar, Bioinformatics Program, Boston University, Boston, MA

C. Contribution to Science

1. A major theme in my lab is understanding how spatial structure develops in multicellular bacterial systems such as biofilms, and how spatial structure impacts the biological responses of the bacteria, such as growth. Examples of bacterial processes that could lead to structure include aggregative tendencies, chemotaxis of motile bacteria, and growth instabilities.
 1. *Singly flagellated Pseudomonas aeruginosa chemotaxes efficiently by unbiased motor regulation.* Cai Q, Li Z, Ouyang Q, Luo C, **Gordon VD**. 2016. mBio 7(2):e00013-16. doi:10.1128/mBio.00013-16.
This paper received a one-star recommendation from Faculty of 1000.
 2. *Role of multicellular aggregates in biofilm formation.* Kragh KN, Hutchison JB, Melaugh G, Rodesney C, Roberts AEL, Irie Y, Jensen PO, Diggle SP, Allen RJ*, **Gordon V***, Bjarnsholt T*. * = **joint corresponding authorship**. 2016. mBio 7(2):e00237-16. doi:10.1128/mBio.00237-16
This paper received a two-star recommendation from Faculty of 1000.
 3. *Shaping the Growth Behaviour of Biofilms Initiated from Bacterial Aggregates.* Melaugh G, Hutchison J, Kragh KN, Irie Y, Roberts A, Bjarnsholt T, **V. D. Gordon**, RJ Allen. (2016) PLoS ONE 11(3): e0149683. doi:10.1371/journal.pone.0149683
 4. *Single-cell control of initial spatial structure in biofilm development using laser trapping.* J. B. Hutchison, C. A. Rodesney, K. S. Kaushik, H. H. Le, D. A. Hurwitz, Y. Irie, **V. D. Gordon**. 2014 Langmuir 30:4522-4530
This work was featured in
“Laser trapping to show the effects of bacterial arrangement on biofilm infection” 2014 SPIE Newsroom, DOI:10.1117/2.1201412.005727
and in “‘Laser Trapping’ technique could lead to better management of bacterial infections in CF patients” 2015 Cystic Fibrosis News Today
2. In bacterial biofilms, structure and mechanics are intimately connected, because the same extracellular polymers (EPS) that make up the biofilm matrix also control the biofilm’s viscoelastic mechanics. A single strain of biofilm-forming bacteria can produce more than one type of EPS molecule – for example *P. aeruginosa* makes Psl, Pel, and alginate. Another major theme in my lab is understanding how different types of EPS materials impact the mechanics of biofilms and of single bacteria.

1. [REDACTED]

2. *Asymmetry and inequity in the inheritance of a bacterial adhesive*. Benjamin J Cooley, Sheri Dellos-Nolan, Numa Dhamani, Ross Todd, William Waller, Daniel Wozniak **and Vernita D Gordon**. 2016. doi: [10.1088/1367-2630/18/4/045019](https://doi.org/10.1088/1367-2630/18/4/045019)
3. *The extracellular polysaccharide Pel makes the attachment of P. aeruginosa to surfaces symmetric and short-ranged*. B.J. Cooley, T.W. Thatcher, S.M. Hashmi, G. L'Her, H.H. Le, D.A. Hurwitz, D. Provenzano, A. Touhami, **V.D. Gordon**. 2013 Soft Matter 9:3871-3876

3. To find new approaches to antibiotic treatment that combine standard antimicrobials with spatial properties of a multicellular bacterial system, we use the opportunistic human pathogen *Pseudomonas aeruginosa* and the aminoglycoside antibiotic tobramycin. I have studied this as a postdoc and a PI.

1. *Tobramycin and bicarbonate synergise to kill planktonic Pseudomonas aeruginosa, but antagonise to promote biofilm growth*. Karishma S Kaushik, Jake Stolhandske, Orrin Shindell, Hugh D Smyth and **Vernita D. Gordon**. npj Biofilms and Microbiomes (2016) 2, 16006; doi:10.1038/npjbiofilms.2016.6
2. *A low-cost, hands-on module to characterize antimicrobial compounds using an interdisciplinary, biophysical approach*. K.S. Kaushik, A. Kessel, N. Ratnayeke, **V.D. Gordon**. 2015 PLoS Biology, DOI:10.1371/journal.pbio.1002044
3. *The spatial profiles and metabolic capabilities of microbial populations impact the growth of antibiotic-resistant mutants*. K.S. Kaushik, N. Ratnayeke, P. Katira, **V.D. Gordon**. 2015 Journal of the Royal Society Interface 12:20150018
4. *The PEL polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas aeruginosa*. Colvin, K. M., **Gordon, V. D.**, Murakami, K., Wozniak, D. J., Wong, G. C. L., Parsek, M. R. 2011 PLOS Pathogens 7:e1001264

4. The site of adhesion of a cell to another cell, an extracellular matrix, or an antimicrobial peptide is frequently associated with the formation of heterogeneities and topological changes in the membrane. I have studied this as a postdoc and a PI.

1. *Specific adhesion of membranes simultaneously supports dual heterogeneities in lipids and proteins*. O. Shindell, N. Mica, M. Ritzner and **V. D. Gordon**. 2015 Physical Chemistry Chemical Physics 17:15598-15607 DOI: 10.1039/c4cp05877a
2. *Mechanism of a prototypical synthetic membrane-active antimicrobial: Efficient hole-punching by targeting lipids with negative spontaneous curvature*. L. Yang, **V. D. Gordon**, D. R. Trinkle, M. A. Davis, C. DeVries, A. Som, J. E. Cronan, Jr., G. N. Tew, G. C. L. Wong. 2008 Proceedings of the National Academy of Sciences of the USA 105:20595-20600
3. *Adhesion promotes phase separation in mixed-lipid membranes*. **V. D. Gordon**, M. Deserno, S. U. Egelhaaf, W. C. K. Poon. 2008 Europhysics Letters 84:48003
4. *Synthetic antimicrobial oligomers induce composition-dependent topological transition in membranes*. L. Yang, **V. D. Gordon**, A. Mishra, A. Som, K. Purdy, M. A. Davis, G. N. Tew, and G. C. L. Wong. 2007 Journal of the American Chemical Society 129:12141-12147

Complete List of Published Work in MyBibliography:

<http://www.ncbi.nlm.nih.gov/sites/myncbi/1vqypcpindo5i/bibliography/47369830/public/?sort=date&direction=ascending>

D. Research Support

Ongoing

[REDACTED]

[REDACTED]

[REDACTED]

Completed

National Science Foundation (PI: Touhami; Gordon is co-PI)

9/1/13-8/31/15

MRI: Acquisition of an Integrated Fluorescence and Atomic Force Microscope for Biophysical and Biomedical Research and Education

This funding has been used to acquire and install a microscope with integrated epifluorescence imaging and atomic force microscopy.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: **Kendra P. Rumbaugh**

eRA COMMONS USER NAME (credential, e.g., agency login): XXXXXXXXXX

POSITION TITLE: Associate Professor

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.*)

INSTITUTION AND LOCATION	DEGREE (if applicable)	Completion Date MM/YYYY	FIELD OF STUDY
University of Texas at El Paso	B.S.	1993-1996	Microbiology
Texas Tech University Health Sciences Center (TTUHSC)	Ph.D.	1996-2001	Medical Microbiology
University of California at San Francisco (UCSF)	Post-doc	2001-2002	Microbial Pathogenesis

A. Personal Statement

The research in my laboratory is focused on bacterial pathogenesis in acute and chronic wound and soft tissue infections. We have over 16 years' experience investigating bacterial pathogenesis utilizing rodent wound models, and our group has made seminal contributions towards our understanding of the roles quorum sensing and biofilms play *in vivo*. My laboratory also has expertise in investigating how polymicrobial interactions and bacterial biofilms affect pathogenesis and modulate innate immune responses in the local wound environment. We have performed numerous efficacy studies of antimicrobial and antibiofilm compounds for academic collaborators and private companies, the data for which have been used to justify Phase II clinical trials. I have sat on wound infection-related study sections for a number of organizations including the American Diabetes Association, the Department of Defense, NIH and VA. Thus, I am extremely familiar with the most current advances in the field. As a principal investigator I have successfully administered several wound infection-related basic science, translational, and clinical projects. I have also participated in many long-term, productive collaborations. Most notable, for the past 6 years my group has worked closely with Dr. Marvin Whiteley's laboratory to advance our understanding of how polymicrobial interactions affect microbial metabolism and pathogenesis *in vivo* by combining their cutting-edge genomic approaches with our animal models. Thus, we have all the necessary reagents, animal models, and productive collaborative relationships already in place to make us extremely well-suited to complete this new project in collaboration with Dr. Gordon.

B. Positions and Honors**Positions and Employment**

2001-2002	Postdoctoral Research Fellow, UCSF, Dept. of Anesthesia and Preoperative Care, San Francisco, CA (mentor, Jeanine Wiener-Kronish)
2002-2003	Postdoctoral Research Fellow, TTUHSC, Dept. of Surgery, Lubbock, TX
2003-2004	Instructor, Department of Surgery, TTUHSC, Lubbock, TX
2004-2005	Research Assistant Professor, Department of Surgery, TTUHSC, Lubbock, TX
2006-2012	Assistant Professor, Department of Surgery
2012-present	Associate Professor Department of Surgery, with joint appointments in Department of Cell Biology and Biochemistry and Microbiology and Immunology, TTUHSC, Lubbock, TX

Other relevant experience and professional associations

2004	Ad hoc member NIH 'Host Interactions with Bacterial Pathogens' (HIBP) Study Section
2008	Ad hoc member Department of Defense, War Supplement
2008-present	Association for Women in Science (West-Texas chapter co-founder and previous President)
2009	Ad hoc member Defense Medical Research and Development Program (DMRDP)
2009-2014	American Diabetes Association Research Grant Review Committee
2011-2013	American Society for Microbiology (Texas Branch President-elect)
2012	Ad hoc member NIAID Investigator Initiated Program Projects (P01)
2012	Reviewer US Army Medical Research and Materiel Command
2012-present	Editorial board, FEMS Microbiology Letters
2013	Reviewer US Army Combat Casualty Care Research Program (CCCRP)
2013-present	American Society for Microbiology (Texas Branch President)
2013-present	Ad hoc member NIAID "Anti-Infective Therapeutics" Special Emphasis Panel ZRG1 IDM-10
2013-present	Panel member, Department of Veterans Affairs Veterans Health Administration Office of Research and Development (Infectious Disease B (INFB))
2014	Ad hoc member NIAID AREA (R15) ZRG1 IDM S (81)
2015, 2016	Ad hoc member Congressionally Directed Medical Research Programs (CDMRP) and Military Infectious Diseases Research Program (MIDRP)
2016	Ad hoc member NIH ZRG1 Musculoskeletal, Oral and Skin Sciences (MOSS) T02 Special Emphasis Panel
2016	Ad hoc reviewer Wellcome Trust/ DBT India Alliance
2016	Panel Chair-Defense Medical Research and Development Program (DMRDP), Joint Program Committee (6)- Burns, Wounds and Fractures

Honors and Awards

2004	Review of published work in ASM magazine "Microbe", Current Topics Section
2006	Review of published work in ASM magazine "Microbe", Current Topics Section
2006	TTUHSC President's Young Investigator Award Recipient
2007, 2006, 2004	Cover Photos: J Bacteriol April 04, Appl Environ Microbiol July 06, Infect Immun Aug 07
2009	Outstanding Faculty Mentor, Texas Tech Univ. & Howard Hughes Medical Institute
2009	Paper included in the 'Editor's Choice' section of <i>Science</i> (Vol 323)- March ed.
2009	Review of published work in ASM magazine "Microbe", Journal Highlights
2009	Work discussed in News Feature "Tinker, Bacteria, Eukaryote, Spy" <i>Nature</i> (Vol 459)
2014	Outstanding Faculty Teaching Award (from TTUHSC Student Government)
2014	Susan G. Talkmitt Science Motivator Award (from TTU)

C. Contributions to Science

1. My early work as a graduate student and postdoctoral fellow focused on understanding the pathogenesis of *Pseudomonas aeruginosa* during infection. In particular I was interested in how *P. aeruginosa* used quorum sensing (a relatively new term at the time) to control its virulence. As a graduate student, I was the first to show that knocking out quorum sensing genes significantly impaired *P. aeruginosa* virulence in a murine burn wound model, results that were subsequently confirmed by several other groups in several other models of infection. When I started my own laboratory group my interest in *P. aeruginosa* quorum sensing evolved into understanding how the small chemical signals (autoinducers) made by bacteria affected host cells in a process called 'interkingdom signaling'. Our seminal contributions in this area (selected papers chronologically listed below) provided mechanistic information about how quorum signals induced apoptosis and inflammation in host cells. Several of these studies were highlighted in *Microbe*, *Science* and *Nature*.

- a. Williams, S.C. Patterson, E. K., Carty, N.L., Griswold, J. A., Hamood, A. N. and **Rumbaugh, K.P.** 2004. *Pseudomonas aeruginosa* Autoinducer Enters and Functions in Mammalian Cells. J. Bacteriol. 186(8): 2281-7. PMID:15060029 *Cover photo*
 - b. Beale, E., Li, G., Tan, M.W., **Rumbaugh, K.P.** 2006. *Caenorhabditis elegans* Senses Bacterial Autoinducers. Appl Environ Microbiol. 72(7):5135-7. PMID:16820523 *Cover photo (recommended by F1000)*
 - c. Shiner, E.K., Terentyev, D., Bryan, A., Sennoune, S., Martinez-Zaguilan, R., Li, G., Gyorke, S., Williams, S.C. and **Rumbaugh, K.P.** 2006. *Pseudomonas aeruginosa* Autoinducer Modulates Host Cell Responses through Calcium Signaling. Cellular Microbiol. 8(10):1601-10. PMID:16984415
 - d. Jahoor A., Patel R., Bryan, A., Do C., Krier J., Watters C., Wahli W., Li, G., Williams S.C., **Rumbaugh K.P.** 2008. Peroxisome Proliferator Activated Receptors Mediate Host Cell Pro-inflammatory Responses to *P. aeruginosa* Autoinducer. J Bacteriol. Jul;190(13):4408-15. Epub 2008 Jan 4. PMID:18178738
2. My laboratory has also had a long time interest in role of bacterial biofilms in wound infections, healing dynamics and antimicrobial tolerance. We have developed a murine chronic wound model to study how biofilms impair healing and other host tissue processes and how the host environment modulates biofilm formation. One major area of focus has been to determine how the diabetic environment influences biofilm processes.
- a. **Rumbaugh, K. P.** and Carty, N. L. 2010. In vivo models of biofilm infection. In: Biofilm Infections. Bjarnsholt, Jensen, Østrup, Høiby (Eds.). Springer, New York, NY. ISBN:978-1-4419-6083-2
 - b. Watters C., DeLeon K., Trivedi U., Griswold J.A., Lyte M., Hampel K.J., Wargo M.J., **Rumbaugh K.P.** 2013. *Pseudomonas aeruginosa* biofilms perturb wound resolution and antibiotic tolerance in diabetic mice. Med Med Microbiol Immunol. 2013 Apr;202(2):131-41. (*recommended by F1000*)
 - c. Watters C, Everett JA, Haley C, Clinton A, **Rumbaugh KP**. Insulin Treatment Modulates the Host Immune System To Enhance *Pseudomonas aeruginosa* Wound Biofilms. Infect Immun. 2014 Jan;82(1):92-100.
 - d. Trivedi U, Parameswaran S, Armstrong A, Burgueno-Vega D, Griswold J, Dissanaik S, **Rumbaugh KP**. Prevalence of Multiple Antibiotic Resistant Infections in Diabetic versus Nondiabetic Wounds. J Pathog. 2014;2014:173053. PMID: 25054067
3. Utilizing our animal models of infection we have collaborated on several translational projects to understand the clinical consequences of biofilms in wounds. Several of these projects involved working with small companies on Phase I Pre-clinical trials.
- a. DeLeon, K., Watters, C., Baldin, F., Hamood, A., Griswold, J., Sreedharan, S., and **Rumbaugh, K.P.** 2009. Efficacy of gallium maltolate in treating *Pseudomonas aeruginosa* infection in a thermally-injured mouse model. Antimicrob Agents Chemother. Apr; 53(4):1331-1337. PMID:19188381
 - b. Wolcott, R.D., **Rumbaugh, K. P.**, James, G., Schultz, G., Phillips, P., Yang, Q., Watters, C., Stewart, P. S. and Dowd, S. E. 2010. Biofilm maturity studies indicate sharp debridement opens a time-dependent therapeutic window. J Wound Care 19(8) 320-328. PMID:20852503 (*recommended by F1000*)
 - c. Gawande PV, Clinton AP, LoVetri K, Yakandawala N, **Rumbaugh KP**, Madhyastha S. Antibiofilm Efficacy of DispersinB® Wound Spray Used in Combination with a Silver Wound Dressing. Microbiology Insights 2014;7, 9-13. PMID: 24826078
 - d. Rai A, Pinto S, Velho TR, Ferreira AF, Moita C, Trivedi U, Evangelista M, Comune M, **Rumbaugh KP**, Simões PN, Moita L, Ferreira L. One-step synthesis of high-density peptide-conjugated gold nanoparticles with antimicrobial efficacy in a systemic infection model. Biomaterials. 2016; 85:99-110.
4. Chronic wound infections tend to be polymicrobial, so much of my more current work has focused on studying the interspecies interactions occurring in wound infections. Many of these studies have focused on the two most prominent bacterial species found in chronic wounds, *P. aeruginosa* and *S. aureus*. Independently, and in collaboration with Marvin Whiteley's group we have uncovered new and important mechanisms of interaction between these two pathogens that influence infection.

- a. Dalton, T., Dowd, S. E., Wolcott, R.D., Sun, Y., Watters, C., Griswold, J.A. and **Rumbaugh, K.P.** 2011. An *in vivo* polymicrobial biofilm wound infection model to study interspecies interactions. *PLoS One*. 2011;6(11):e27317. Epub 2011 Nov 4. PMID:22076151
 - b. Korgaonkar, U. Trivedi, **K.P. Rumbaugh**, M. Whiteley. 2013. Community surveillance enhances *P. aeruginosa* virulence during polymicrobial infection. *Proc Natl Acad Sci U S A*. 2013 Jan 15;110(3):1059-64. PMID:23277552
 - c. DeLeon S, Clinton A, Fowler H, Everett J, Horswill AR, Rumbaugh KP. Synergistic Interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an In Vitro Wound Model. *Infect Immun*. 2014 Aug 25. pii: IAI.02198-14. PMID:25156721
 - d. Gabriliska R and **Rumbaugh KP**. Biofilm Models of Polymicrobial Infection. *Future Microbiol*. 2015. Dec;10:1997-2015. PMID:26592098
5. For the past 6 years my group has worked closely with Dr. Marvin Whiteley's laboratory to advance our understanding of how polymicrobial interactions affect microbial metabolism and pathogenesis *in vivo* by combining their cutting-edge genomic approaches with our animal models. One major focus of this work has been on the interspecies interactions among oral pathogens. For these studies we developed a murine abscess model to study polymicrobial infection and optimized techniques such as RNAseq and Tnseq to uncover new mechanisms of bacterial synergy that promote infection.
- a. Ramsey, M.C., **Rumbaugh, K.P.** and Whiteley, M. Metabolic cross-feeding enhances virulence in a model polymicrobial infection. *PLoS Pathogens* 2011 Mar;7(3):e1002012. Epub 2011 Mar 31. PMID:21483753 (*recommended by F1000*)
 - b. Jorth P, Trivedi U, **Rumbaugh K**, Whiteley M. Probing bacterial metabolism during infection using high-resolution transcriptomics. *J Bacteriol*. 2013 Nov;195(22):4991-8. [Epub ahead of print]. PMID:23974023. *Chosen for special Commentary (23974023)*
 - c. Stacy A, Everett J, Jorth P, Trivedi U, **Rumbaugh KP**, Whiteley M. Bacterial fight-and-flight responses enhance virulence in a polymicrobial infection. *Proc Natl Acad Sci U S A*. 2014 May 13. pii: 201400586. [Epub ahead of print]. PMID: 24825893
 - d. Turner KH, Everett J, Trivedi U, **Rumbaugh KP**, Whiteley M. Requirements for *Pseudomonas aeruginosa* Acute Burn and Chronic Surgical Wound Infection. *PLoS Genet*. 2014 Jul 24;10(7):e1004518. PMID: 25057820

Complete List of Published Work in MyBibliography:

<http://www.ncbi.nlm.nih.gov/sites/myncbi/kendra.rumbaugh.1/bibliography/47366413/public/?sort=date&direction=ascending>

D. Research Support Ongoing

NIH/R01 (Rumbaugh, Sub-contract PI; Whiteley, PI) 2016-2020
 [REDACTED] (total subcontract)

"Mechanisms of *Pseudomonas aeruginosa* virulence in polymicrobial wound infections"

The overall goal of this research plan is to determine how interactions between *Pseudomonas aeruginosa* and other microbes that commonly co-infect wounds impact wound severity. To accomplish this goal, *in vivo* murine wound models and high-throughput genomics techniques will be employed to identify and characterize microbial genes required for enhanced pathogenesis during co-infection.

NIH/NIAID R15AI105763 (Rumbaugh, PI) 2014-2017

"*Pseudomonas aeruginosa* and *Staphylococcus aureus* Interspecies Interactions in Wounds"

The goals of this study are to 1) characterize *P. aeruginosa* and *S. aureus* interspecies interactions in an *in vitro* wound environment; 2) determine how components of the wound environment inhibit *P. aeruginosa*'s ability to kill *S. aureus* and thus promote stable coinfections; and 3) investigate what consequences *P. aeruginosa*/*S. aureus* coinfection has on wound resolution *in vivo*.

Army Research Office grant 62507-LS (Rumbaugh, Sub-contract PI; Whiteley, PI) 2013-2017

“Polymicrobial virulence mechanisms in chronic wounds”

The overall objective of this project is to study peptidoglycan-mediated interactions between *P. aeruginosa* and *S. aureus* or *Acinetobacter baumannii* in a murine chronic wound model, primarily using RNAseq technology.

[REDACTED]

Completed

NIH/NIAID R21AI107570 (Rumbaugh, Sub-contract PI; Whiteley, PI)

2014-2016

“Genetic determinants of bacterial fitness in polymicrobial wound infections”

The overall goal of this research plan is to examine, from a genomic standpoint, how interactions between opportunistic pathogens impact in vivo persistence in acute and chronic wound infections. To accomplish this goal, high-throughput genomics techniques will be employed to identify microbial genes that are required for and expressed during polymicrobial synergy.

[REDACTED]

NIH/NIDCR R01DE020100 (Rumbaugh, Sub-contract PI; Whiteley, PI)

2011-2015

“Metabolite sensing in an oral polymicrobial community”

The major goals of this project are to define the interspecies relationships between the oral pathogens *A. actinomycetemcomitans* and *S. gordonii*.

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

BIOGRAPHICAL SKETCH

NAME Christopher, Gordon F.	POSITION TITLE Assistant Professor of Mechanical Engineering		
eRA COMMONS USER NAME (credential, e.g., agency login) 			
EDUCATION/TRAINING (<i>Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.</i>)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	MM/YY	FIELD OF STUDY
Columbia University (New York, NY)	BS	5/02	Mechanical Engineering
Columbia University (New York, NY)	BA	5/03	Film
Carnegie Mellon University (Pittsburgh, PA)	MS	5/04	Mechanical Engineering
Carnegie Mellon University (Pittsburgh, PA)	MS	5/08	Chemical Engineering
Carnegie Mellon University (Pittsburgh, PA)	PhD	5/08	Mechanical Engineering
National Institute of Standards and Technology	Post Doc	1/10	Rheology and Complex Fluids

A. Personal Statement

The goal of the proposed research is to characterize the physio-chemical properties of wound biofilms both *in-vivo* and *ex-vivo*. I have a strong background in mechanical and chemical engineering, with a research focus on the rheology of complex fluids and interfaces. During my Ph.D. in mechanical engineering from Carnegie Mellon University under the guidance of Prof. Shelley Anna, I worked extensively in the characterization of complex fluids and their use in microfluidics using both shear and extensional rheology. During my post doctoral work as an NRC fellow at NIST, I continued to develop skills in the modeling and characterizing of complex fluids, development of novel microrheology techniques, and in the use and fabrication of MEMS devices. My lab at TTU focuses on the development of novel rheology techniques to examine the effects of confinement on soft matter/complex fluids and methods to study interfacial rheology. We have focused on a wide range of problems including bulk protein solution rheology, interfacial rheology of proteins solutions, and the interfacial rheology of biofilms at air/water interfaces. We have developed skills in handling and characterizing biomaterials in our lab, and are well versed in a wide range of material characterization techniques including bulk, interfacial, and micro rheology. Based on my work and record, I have received several NSF grants in the areas of rheology and biomaterials.

1. Zhang, Z. and **G.F. Christopher**, "Effect of particulate contaminants on the development of biofilms at air/water interfaces." *Langmuir*, 2016. 32 (11) p. 2724-2730.

B. Positions and Honors

Positions and Employment

2009-2010 National Institute of Standards and Technology, NRC Postdoctoral Fellow
 2010- Texas Tech University Department of Mechanical Engineering, Assistant Professor

Other Experience and Professional Memberships

2004- Member, Society of Rheology
 2008- Reviewer for Scholarly Journal: Journal of Applied Polymer Science, Journal of Rheology, Microfluids and Nanofluids
 2010- Member, American Institute of Chemical Engineers
 2011 Panel Reviewer, NSF Particulate and Multiphase Flow

Honors

2002	Magna Cum Laude, Columbia University, New York, NY
2005	DOWD ICES Fellow, Carnegie Mellon University, Pittsburgh,
2009	NIST Exploratory Research Grant, Gaithersburg, MD
2012	Sandia National Laboratories MEMS Design Competition, Texas Tech University, Lubbock, TX

C. Contributions to Science

Role of particle behavior on interfacial rheology of particle laden interfaces

Using advanced rheology and microscopy techniques developed in my lab, I have studied how changing the properties of an interface effect the individual behavior, collective microstructure, and interfacial rheology of particle laden interfaces. The work is supported by 2 separate NSF grants, and has been widely cited in publications.

1. Supakar, T., Moradiafrapoli, M. **Christopher, G. F.**, and J. Marston. "Spreading, encapsulation and transition to arrested shapes during drop impact onto hydrophobic powders." *Journal of Colloid and Interface Science*, 2016. 468 p.10-20
2. Barman, S. and **G.F. Christopher**, "Role of Capillarity and Microstructure on Interfacial Viscoelasticity of Particle Laden Interfaces." *Journal of Rheology*, 2015. 60 (1) p. 35-45.
3. Snoeyink, C., Barman, S., and **G.F. Christopher**, "Contact Angle Distribution at Fluid Interfaces." *Langmuir*, 2014. 31(3), p891-897.
4. Barman, S. and **G Christopher**, "Simultaneous Interfacial Rheology and Visualization of Densely Packed Aggregated Particle Laden Interfaces." *Langmuir*, 2014. 30 (32), p. 9752-8760.

Interfacial Behavior of Biological Materials

We have done a number of studies focusing on how interfacial properties are affected by biomaterials. This work has primarily focused on 2 systems:1) how do globular proteins on and interface change interfacial viscoelasticity, and 2) how can a liquid interface be modified to disrupt the growth of pellicles. We have several publications in this area, and are supported by a NSF grant.

2. Zhang, Z. and **G.F. Christopher**, "Effect of particulate contaminants on the development of biofilms at air/water interfaces." *Langmuir*, 2016. 32 (11) p. 2724-2730.
3. Zhang, Z and **G.F. Christopher**, "The nonlinear viscoelasticity of hyaluronic acid and its role in joint lubrication." *Soft Matter*, 2014. 11(13), p.2596-2603.
4. Zhang, Z., Barman, S., and **G.F. Christopher**, "The Role of Protein Content on The Steady and Oscillatory Shear Rheology of Model Synovial Fluids." *Soft Matter*, 2014. 10 (32), p. 5965-5973.
5. Zhang, Z., Barman, S., and **G.F. Christopher**, "Effect of Interfacial Viscoelasticity on the Bulk Linear Viscoelastic Moduli of Globular Protein Solutions." *Physical Review E*, 2014 89(5): p. 052306-052310

Elastic instabilities in microfluidics

Using microfluidic channels, we have examined how viscoelastic Mach number is a useful tool in characterizing purely elastic instabilities around microfluidic cylinders. In this work we have discovered a new class of flow instabilities around cylinders that had been previously un-reported. This work was supported by the American Chemical Society, and resulted in several well received publications

1. Shi, X. and **G.F. Christopher**, "Growth of Elastic Instabilities around Linear Arrays." *Physics of Fluids*, Submitted.
2. Shi, X. and **G.F. Christopher**, "Mechanisms of Onset for Moderate Mach Number Instabilities of Viscoelastic Flows around Confined Cylinders." *Rheologica Acta*, 2015. 54 (9-10), p.805-815.
3. Kenney, S., K. Poper, G. Chapagain, and **G.F. Christopher**, "Large Deborah number flows around confined microfluidic cylinders." *Rheologica Acta*, 2013. 52(5): p. 485-497.

Droplet behavior in microfluidics

During my PhD, I published several influential papers on the behavior of droplets in microfluidic channels detailing their generation, deformation, and coalescence.

Lin, Y., Barman, S., **Christopher, G.F.**, S.L. Biswal, "In situ Microstructure Visualization and Interfacial Shear Rheology of Asphaltenes at Air/Oil-Water Interfaces." *Journal of Rheology*, Submitted.

1. **Christopher, G F** and Anna, S L "Passive breakup of viscoelastic droplets and filament self-thinning at a microfluidic T-junction." *Journal of Rheology*. 2009. 53 (3): p 663-683
2. **Christopher, G F**, Bergstein, J, *et al.* "Coalescence and splitting of confined droplets at microfluidic junctions." *Lab on a Chip* 2009. 8 (9): p1102-1109
3. **Christopher, G F**, Noharuddin N, *et al.* "Experimental observations of the squeezing-to dripping transition in T-shaped microfluidic junctions." *Physical Review E*, 2008.78 (3) *
4. **Christopher, G F** and Anna, S L "Microfluidic methods for generating continuous droplet streams." *Journal of Physics D: Applied Physics*, 2007.40 (19):p R319-R336

URL to complete list of published work: <https://scholar.google.com/citations?user=sPO48d4AAAAJ&hl=en>

D. Research Support

Current Research Support

Using Active Materials at Liquid Interfaces to Regulate Bacterial Biofilm Mechanical Properties

National Science Foundation

Role: PI

Dates 9/1/2016-8/31/2019

Description: The goal of this project is to understand how surface active materials can be used to disrupt the growth of a biofilm at an air/water interface.

Characterization of the Dynamic Behavior of Particle Contact Angle at an Oil/Water Interface

National Science Foundation

Role:co-PI

Dates 9/1/2016-8/31/2019

Description: The goal of this project is to characterize the dynamics of particles at an oil/water interface by measuring changes in three phase contact line and contact angle.

Testing Special Additive Manufacturing (AM) Materials Project

DOE/Consolidated Nuclear Security, LLC-PANTEX

Role: co-PI

Dates 3/10/2016-12/31/2017

Description: The goal of this project is to develop a 3D printer capable of printing high binder loading pastes for weapons manufacture.

Role of Composition on Mesostructure-Flow Interaction and Rheology of Particle Laden Interfaces

National Science Foundation

Role: PI

Dates 9/1/2014-8/31/2017

Description: The goal of this project is to characterize how changes to particle properties affect the microstructure and rheology of particle laden interfaces.



BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.

NAME Jason Shear	POSITION TITLE Professor Dept. of Chemistry & Biochemistry and the Institute for Cellular & Molecular Biology		
eRA COMMONS USER NAME 			
EDUCATION/TRAINING (<i>Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.</i>)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
University of Texas at Austin	B.S.	1985 – 89	Chemistry
Stanford University	Ph.D.	1989 – 94	Chemistry/Neuroscience
Cornell University	Post-doc	1994 – 96	Biophysics

A. Personal Statement

Prof. Shear is an expert in materials bioengineering, optics, imaging, bio-analytical measurements, and use of cell-culture model systems, a knowledge base ideal for the micro-3D printing studies on bacterial micro-encapsulation the Shear group will undertake in this collaborative project. The Shear group has been involved in a broad range of collaborations with microbiologists, organic chemists, physicists, and engineers, and has published extensively in areas related to the proposed work. Some publications relevant to the Shear group's proposed contributions include:

1. "3D Printing of Microscopic Bacterial Communities," J. L. Connell, E. T. Ritschdorff, M. Whiteley, and J. B. Shear, *Proc. Natl. Acad. Sci. USA* (2013) **110**, 18380–18385.
2. "Oxygen Limitation within a Bacterial Aggregate," A. K. Wessel, T. A. Arshad, M. Fitzpatrick, J. L. Connell, R. T. Bonneau, J. B. Shear, M. Whiteley. *mBio*, (2014) **5**, e00992-14 [1–9].
3. "Probing Prokaryotic Social Behavior with Bacterial Lobster Traps," J. Connell, A. Wessel, M. R. Parsek, E. Ellington, M. Whiteley, and J. B. Shear, *mBio* (2010) **1**, e00202-10.
4. "3D Printing of Photoresponsive Biomaterials for Control of Bacterial Microenvironments," J. L. Connell, E. T. Ritschdorff, J. B. Shear, *Anal. Chem.* published on web, Oct 2016 (DOI: 10.1021/acs.analchem.6b03440).

B. Positions and Honors

Positions: (1) Assistant Professor of Chemistry, University of Texas at Austin (9/96 – 8/02); (2) Associate Professor of Chemistry, University of Texas at Austin (9/02 – 9/08); (3) Professor of Chemistry, University of Texas at Austin (9/08 – present); (4) K. Bala Texas Instruments Visiting Professor in Bioengineering, Rice University (8/10 – 5/11).

Honors: National Merit Scholarship, 1985–1989; Phi Beta Kappa, 1987; Fannie and John Hertz Foundation Research Fellowship Grant, 1990; Howard Hughes Predoctoral Fellowship, 1990–1994; NSF Postdoctoral Fellowship, 1994–1996; ONR Young Investigator Award, 1997; Beckman Foundation Young Investigator Award, 1997; Searle Scholars Award, 1998, Alfred P. Sloan Research Fellowship, 1999; Top 100 Young Innovator citation, MIT Technology Review, 1999; Eli Lilly Grantee, 2000; University of Texas ICMB Fellow, 1999–present; Beckman Scholars Advisory Panel (2003–04); Noted for a "Chemical Development of the Year" by *C&E News* (2003); Texas Academy of Sciences protégé (2004); Speaker, Paul Flory Meeting, Stanford University (2004); American Chemical Society Arthur F. Findeis Award in Analytical Chemistry (2005); Analytical Chemistry News & Features Advisory Panel (2007–present); Beckman Foundation Scientific Advisory Council member (2009–present); K. Bala Texas Instruments Visiting Professorship in Bioengineering, Rice University (8/10–5/11); Beckman Foundation Scientific Advisory Council (2009–present); Beckman Young Investigator Program Executive Committee (2012–present).

C. Contributions to Science

1. The Shear group developed methods for performing solution-phase chemical separations on time frames more than 1000-fold shorter than previously accomplished, offering insights into reaction pathways of transient reaction products that are more easily characterized from their electrophoretic mobilities than from measurable spectroscopic properties. This method, based on photochemical preparation of reaction intermediates, enabled compounds to be electrophoretically probed using extremely large electric fields over distances as small as several micrometers on timescales as small as several microseconds. Relevant publications include:

- "Electrophoretic Characterization of Transient Photochemical Reaction Products," M. J. Gordon, E. Okerberg, M. L. Gostkowski, J. B. Shear, *J. Am. Chem. Soc.* **2001**, 123, 10780–10781.
- "Microsecond Electrophoresis," M. L. Plenert and J. B. Shear, *Proc. Natl. Acad. Sci. USA* **2003**, 100, 3853–3857.
- "Microsecond Analysis of Transient Molecules using Bi-Directional Capillary Electrophoresis," E. Ritschdorff, M. L. Plenert, and J. B. Shear, *Anal. Chem.* **2009**, 81, 8790 – 8796.

2. The Shear group has developed micro-3D-printing technologies for organizing cellular environments, a technology that allows cellular populations to be characterized under well-defined conditions and on scales in which ensemble behaviors begin to emerge. Of particular impact has been our use of these methods to probe bacterial group behaviors that underlie enhanced virulence, including quorum sensing and population-dependent antibiotic resistance. Relevant publications include:

- "Multiphoton fabrication of chemically responsive protein hydrogels for microactuation." B. Kaehr and J. B. Shear, *Proc. Natl. Acad. Sci. USA* **2008** 105, 8850 – 8854.
- "Probing Prokaryotic Social Behavior with Bacterial Lobster Traps," J. Connell, A. Wessel, M. R. Parsek, E. Ellington, M. Whiteley, and J. B. Shear, *mBio* **2010**, 1, e00202-10.
- "3D Printing of Microscopic Bacterial Communities," J. L. Connell, E. T. Ritschdorff, M. Whiteley, and J. B. Shear, *Proc. Natl. Acad. Sci. USA* **2013**, 110, 18380–18385.
- "3D-Printed Microfluidic Microdissector for High-Throughput Studies of Cellular Aging," E. C. Spivey, B. Xhemalce, J. B. Shear, I. J. Finkelstein, *Anal. Chem.* **2014**, 86, 7406 – 7412.

3. The Shear group has pioneered high-sensitivity multiphoton-based sensing technologies for microanalyses, developing various strategies for characterizing picoliter-sized biological samples using capillary electrophoretic analysis. Using these methods, the Shear group demonstrated strategies for analyzing volumes commensurate with subcellular volumes for spectrally diverse native chromophores present in attomole to zeptomole quantities. Relevant publications include:

- "Characterizing Spectrally Diverse Biological Chromophores Using Capillary Electrophoresis with Multiphoton-Excited Fluorescence," M. L. Gostkowski, J. B. McDoniel, J. Wei, T. E. Curey, J. B. Shear, *J. Am. Chem. Soc.* **1998**, 120, 18–22.
- "Sub-Attomole Fluorescence Determination of Catecholamines in Capillary Electrophoresis Effluent Streams," M. L. Gostkowski, J. B. Shear, *J. Am. Chem. Soc.* **1998**, 120, 12966–12967.
- "Measurements of Serotonin and Related Indoles Using Capillary Electrophoresis with Multiphoton-Induced Hyperluminescence," M. Gostkowski, J. Wei, J. B. Shear, *Anal. Biochem.* **1998**, 260, 244-250.
- "Determination of Fluorogen-Labeled Neurotransmitters at the Zeptomole Level Using Two-Photon Excited Fluorescence with Capillary Electrophoresis," J. Wei, M. L. Gostkowski, M. J. Gordon, J. B. Shear, *Anal. Chem.* **1998**, 70, 3470–3475.

4. The Shear group was involved in foundational work developing broad-based sensor array devices for analysis of various solution-phase sample types, ranging from measurements of bodily fluids such as saliva to determination of small-molecule components in consumables. In collaboration with a number of science and engineering labs at the University of Texas, Prof. Shear spearheaded a proposal during his pre-tenure period to the Beckman Foundation to support sensor-array development. Relevant publications include:

- "Solution-Based Analysis of Multiple Analytes by a Sensor Array: Toward the Development of an Electronic Tongue," J. J. Lavigne, S. Savoy, M. B. Clevenger, J. E. Richie, J. B. McDoniel, S.-J. Yoo, E. V. Anslyn, J. T. McDevitt, J. B. Shear, D. Neikirk, *J. Am. Chem. Soc.* **1998**, 120, 6429–6430.
- "Development of Multianalyte Sensor Arrays Composed of Chemically Derivatized Polymeric Microspheres Localized in Micromachined Cavities," A. Goodey, J. J. Lavigne, S. M. Savoy, M. D. Rodriguez, T. Curey, A. Tsao, G. Simmons, J. Wright, S. -J. Yoo, Y. Sohn, E. V. Anslyn, J. B. Shear, D. P. Neikirk, J. T. McDevitt, *J. Am. Chem. Soc.* **2001**, 123, 2559–2570.
- "Characterization of Multicomponent Monosaccharide Solutions Using an Enzyme-Based Sensor Array," T. E. Curey, A. Goodey, A. Tsao, J. J. Lavigne, J. T. McDevitt, E. V. Anslyn, D. P. Neikirk, J. B. Shear, *Anal. Biochem.* **2001**, 293, 178–184.
- "Enzyme-Based Sensor Arrays for Rapid Characterization of Complex Disaccharide Solutions," T. E. Curey, M. A. Salazar, P. Oliveira, D. J. Javier, P. J. Dennis, P. Rao, J. B. Shear, *Anal. Biochem* **2002**, 303, 42–48.

D. Research Support

Ongoing

[REDACTED]

Army Research Office
Role: Co-PI

6/13 – 5/17

"Polymicrobial virulence mechanisms in chronic wounds"

Goal: Determine mechanisms of polymicrobial interactions relevant to infections in chronic wounds

Completed in last 3 years

National Institutes of Health (1R21AI097929-01)

2/12 – 1/15

Role: PI

"Mechanisms of antibiotic resistance in confined microcolonies"

Goal: Use of multiphoton protein fabrication to create defined, microscopic environments for understanding population-dependent antibiotic resistance in *P. aeruginosa* and other bacteria.

[REDACTED]

Senior/Key Personnel: Vernita Gordon, Ph.D. PI. (effort = 2 summer months). Dr. Gordon will be directly responsible for image analysis of data from Aim 1 and for *in vitro* structuring of biofilms and measuring the effects of biofilm structure on microenvironment and interactions with neutrophils. She will also coordinate research and exchange of data, personnel, and training between all participants and Austin and Texas Tech as the project PI. She will direct experimental design, data analysis, and preparation of manuscripts

Other Personnel: Years 1-3, two full-time Ph.D. students will be involved. One student will do image analysis to determine *in vivo* biofilm structure (Aim 1) and *in vitro* measurements of the effects of biofilm structure on microenvironment, antibiotic resistance, and virulence (Aim 2). The second student will measure the effects of mechanics and virulence on resistance to neutrophil phagocytosis (Aim 3). Effort will be full-time for both students.

In addition, in Year 2, one student will be supervised by Prof. Jason Shear to make microfabricated enclosures for bacteria (Activity 2-2). Student effort will be half time. Dr. Shear will contribute 0.02 summer months in scientific leadership for the half time GRA.

In Year 4, one full-time Ph.D. student will do *in vitro* measurements of the effects of biofilm structure on microenvironment, antibiotic resistance, and virulence (Aim 2).

Tuition and fees: Tuition and fees for graduate students are included, according to effort described above.

Travel: [REDACTED] per year is requested for the PI and one student to attend one meeting per year to present data from this project, and for the students to travel to Texas Tech to meet face-to-face with collaborators.

Other Direct Costs: [REDACTED] per year is requested for Materials and Supplies in year 1, 3, and 4. We are requesting [REDACTED] in year 2. This includes chemicals, media, glassware, and other supplies for culturing, and [REDACTED]/yr for confocal microscope maintenance contract. [REDACTED] per year is requested for Publication Costs, which are standard for leading journals in our fields.

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

Expiration Date: 10/31/2018

1. Human Subjects Section

Clinical Trial? ☐ Yes ☐ No*Agency-Defined Phase III Clinical Trial? ☐ Yes ☐ No

2. Vertebrate Animals Section

Are vertebrate animals euthanized? ☒ Yes ☐ No

If "Yes" to euthanasia

Is the method consistent with American Veterinary Medical Association (AVMA) guidelines?

☒ Yes ☐ No

If "No" to AVMA guidelines, describe method and provide scientific justification

.....

3. *Program Income Section

*Is program income anticipated during the periods for which the grant support is requested?

☐ Yes ☒ No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount (\$)	*Source(s)
----------------	--------------------------	------------

PHS 398 Cover Page Supplement

4. Human Embryonic Stem Cells Section

*Does the proposed project involve human embryonic stem cells? ☐ Yes ☒ No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s) (Example: 0004):

5. Inventions and Patents Section (RENEWAL)

*Inventions and Patents: ☐ Yes ☒ No

If the answer is "Yes" then please answer the following:

*Previously Reported: ☐ Yes ☐ No

6. Change of Investigator / Change of Institution Section

☐ Change of Project Director / Principal Investigator
Name of former Project Director / Principal Investigator

Prefix:

*First Name:

Middle Name:

*Last Name:

Suffix:

☐ Change of Grantee Institution

*Name of former institution:

PHS 398 Research Plan

OMB Number: 0925-0001

Expiration Date: 10/31/2018

Introduction	
1. Introduction to Application (Resubmission and Revision)	2016_11_06_Introduction1027054233.pdf
Research Plan Section	
2. Specific Aims	2016_11_06_Specific_Aims1027054147.pdf
3. Research Strategy*	2016_11_06_Research_strategy1027054151.pdf
4. Progress Report Publication List	
Human Subjects Section	
5. Protection of Human Subjects	
6. Data Safety Monitoring Plan	
7. Inclusion of Women and Minorities	
8. Inclusion of Children	
Other Research Plan Section	
9. Vertebrate Animals	2016_11_04_Vertebrate_Animals1027054127.pdf
10. Select Agent Research	
11. Multiple PD/PI Leadership Plan	
12. Consortium/Contractual Arrangements	combined_letters_of_commitment1027054129.pdf
13. Letters of Support	Gordon_letter_from_Dan_Wozniak1027054146.pdf
14. Resource Sharing Plan(s)	2016_11_02_Resource_Sharing_Plan1026881603.pdf
15. Authentication of Key Biological and/or Chemical Resources	2016_11_02_Authentication_of_Resources1026881604.pdf
Appendix	
16. Appendix	

Introduction: This application is a re-submission of 1-R01-AI121500-01, which was reviewed in June 2015 by the Biomaterials and Biointerfaces Study Section. It got an impact score of 38 and was scored in the 30th percentile. From the Summary Statement: “The strengths of the application are the unstudied area with innovative convincing ideas; the experience of the investigators with expertise in such measurements using many tools, and the study of structures that could identify new targets—‘there is much to be gained.’”

We thank all reviewers for their efforts on this. In response to their critiques, this resubmission is substantially changed from the original submission, as follows:

Significance

Criticism: Original proposal too broad in scope. *Response:* We have sharpened the focus by making this resubmission only about *P. aeruginosa* in chronic wounds, rather than polymicrobial infections in multiple anatomical sites of chronic infection.

Criticism: Difficult to translate results between *in vivo* and *in vitro*. *Response:* We have added information on how we will measure disease outcomes (Specific Aim 1) and how we will compare those with our *in vitro* results (Specific Aim 2). We have sketched how we will respond if *in vivo* and *in vitro* results do not correspond (page 10). Our new Activity 3-3 gives an *in vivo* validation of the most novel *in vitro* results.

Criticism: Some aspects of the work were already done by the team. *Response:* We have revised the scope of our proposal, so that the work proposed is entirely new, both to us and to the state-of-the-art.

Investigator

Criticism: PI Vernita Gordon is very junior and does not have enough related publications. *Response:* In 2015 and 2016, the PI published eight original research articles in leading microbiology and interdisciplinary journals. She led multidisciplinary teams, located in four countries on three continents, to complete work that has been published in mBio, npj Biofilms and Microbiomes, Langmuir, and PLoS ONE. Her leadership in physics and microbiology is reflected in her professional service and experience. Please see Biosketch. The collaborators add deep expertise in specific areas needed for this proposal.

Innovation

Criticism: No explanation of how this study will lead to new targeted drug or treatment development. *Response:* We have included new Preliminary Studies that indicate the molecular mechanisms underlying the different mechanical contributions of different matrix components (Aim 1). This points toward a therapy using competitive binding or degradation of specific matrix elements. Development of such a therapy would be guided by the results of Aim 3. We have changed the antibiotic-resistance work so that it now focuses on tobramycin (tob), PEG-y-lated tob, and bicarbonate (Aim 2). We expect to learn how to interfere with biofilm structure and matrix functionality such that tob and bicarbonate synergize in treatment of biofilms.

Criticism: Some of the rheological measurements and “leukocyte invasion” are standard practice. *Response:* This is incorrect. We have highlighted the novel nature of our rheology studies. We have also highlighted that how viscoelastic mechanics impacts susceptibility to phagocytosis is entirely unknown.

Approach *Criticism:* Using three different mouse models, at two different geographical sites, could cause site-specific factors to affect outcome. *Response:* We now use only one mouse model at only one site.

Criticism: Statistical treatment and identification of potential relationships were not discussed; there was a lack of quantifiable hypotheses. *Response:* We have expanded our discussion of statistical treatment (Research Strategy & Vertebrate Animals). We indicate the expected functional form of relationships where this is supported by physical understanding. Elsewhere, we describe the properties of the expected function.

Criticism: Not enough preliminary data to ensure success. *Response:* All our preliminary studies are now published in peer-reviewed journals and have been referenced in the resubmission.

Criticism: Not clear that biofilm microrheology would work. *Response:* We have explained our rheology plan in much greater detail. Microrheology is secondary to AFM work and is not required for success.

Criticism: PNA-FISH is not needed. *Response:* We agree. We have changed to fluorescent bacteria.

Criticism: Experimental plans unclear. *Response:* We have clarified and increased detail in all Aims.

Criticism: Requested number of mice not well-supported. *Response:* Expanded Vertebrate Animals.

Environment

Criticism: Three performance sites, one foreign, would make collaboration difficult.

Response: Now, only sites and colleagues in Texas are involved.

Biohazards

Criticism: Any biological safety facility would be used. *Response:* This is incorrect. All animal work will be done in a certified animal facility and laboratory. All *in vitro* work will be done in BSL-2 laboratories. *P. aeruginosa* is a BSL-2 organism, therefore this is an appropriate level of biological safety.

Foreign Organizations *Criticism:* Foreign participant unjustified. *Response:* We have removed him.

Specific Aims:

This proposal's objective is to determine the impact of the spatial structure and mechanics of *Pseudomonas aeruginosa* biofilm infections, in chronic wounds, on virulence, antibiotic resistance, and immune evasion.

Most chronic bacterial infections are caused by biofilms, aggregated bacteria that are embedded in a matrix of polymer and protein. Unlike well-mixed, liquid cultures, biofilm infections have well-defined spatial structure. This spatial structure is given by the sizes of bacterial aggregates, the relative positions of aggregates, and matrix heterogeneity. Aggregates also have viscoelastic mechanical properties that are conferred by the matrix. Basic principles of material transport indicate that the spatial structure of biofilm infections must impact intercellular signaling, virulence, and antibiotic resistance; comparison of biofilm mechanics with known phagocytic forces indicate that resistance to deformation and breakup likely help biofilms resist immunological clearance. However, there is little to no in-depth, quantitative knowledge regarding the impact of spatial structure and mechanics on disease course. Completion of the work we propose here will open new possibilities for therapeutic strategies that specifically target biofilm structure and/or mechanics.

Our long-term goal is to find new strategies for remediating biofilm infections by addressing physical properties. Here, our central hypothesis is that spatial structure and mechanics are the major *physical* factors controlling the development of pathogenicity, antibiotic resistance, and immune evasion in biofilm infections. This hypothesis is based on a synthesis of our own and others' published work. The rationale is that completion will identify key physical targets for preventing, disrupting, or ameliorating biofilm infections for an important biofilm-forming opportunistic human pathogen. The work we propose here will also develop experimental techniques and understanding of an important model system that together will constitute a widely-applicable platform for assessing the impact of biofilm structure and mechanics for other infecting organisms.

We will test our central hypothesis and attain our objective *via* the following specific aims:

1: Determine the spatial structure and mechanics of biofilm infections in wounds. For this, we will use sophisticated imaging to determine, in three dimensions, the size, number, locations, and heterogeneous matrix content of bacterial aggregates in a mouse model of chronic wound infection. We will simultaneously measure the density and distribution of neutrophils around the biofilm aggregates. At present, no good technique for measuring the mechanics of biofilm infections exists. We will develop such a technique using AFM microindentation and abrasion of *ex vivo* biofilms. Working hypothesis: The structure and mechanics of *in vivo* biofilm infections in chronic wounds will follow development trajectories arising from the matrix-producing capabilities of the bacteria and pressure from the host immune defense.

2: Determine how spatial arrangements impact bacterial growth, biofilm microenvironments, antibiotic resistance, and virulence. For this, we will use manipulative techniques that we recently developed to re-create biofilm structures found in *in vivo* and *in vitro* environments and measure the biological changes induced by specific structures. Working hypothesis: Virulence and antibiotic resistance will depend on key structural characteristics, such as the sizes of aggregates and the distances between aggregates, through the development of microenvironments that differentiate as a result of the material transport and consumption of growth substrate, bacterial products, and antibiotics.

3. Determine the role of spatial structure and mechanics in biofilm-leucocyte interactions. For this, we will add freshly-isolated human neutrophils to biofilms at different stages of formation and with different structures and mechanics and monitor the attack by neutrophils and the bacterial response. Working hypothesis: Biofilm tolerance and evasion of neutrophils and their action will depend both on the neutrophils' ability and speed in breaking off and engulfing pieces of biofilm, and on the ability of biofilms to kill immune cells.

The expected outcome of this work is a comprehensive understanding of what structures and mechanics develop in biofilm infection of chronic wounds, and the degree to which these structures and mechanics give rise to pathogenicity, antibiotic resistance, and evasion of the immune system. The results will have an important positive impact because they lay the groundwork to develop a new class of targeted treatments.

Research Strategy

A) Significance:

Chronic infections caused by biofilms annually affect 17 million Americans, cause at least 550,000 American deaths, and cost the US healthcare system billions of dollars [2-8]. Chronic wounds in particular can cost, per patient, tens of thousands of dollars per year, and are prevented from healing because they are infected by bacterial biofilms dominated by *Pseudomonas aeruginosa* [10]. Biofilm infection in chronic wounds afflicts both diabetic and non-diabetic patients and can lead to amputation [14].

Biofilms resist antibiotics and evade the host immune defense [10, 15-18]. In biofilms, a heterogeneous matrix of differentiated extracellular polymers (EPS) and proteins holds bacteria in place [19-22], thus controlling intercellular associations and differentiation of microenvironments [19, 23-34]. Matrix polymers and proteins also confer intercellular cohesion on biofilm bacteria, thereby determining the mechanical resistance of the biofilm to physical breakup. The impact of spatial structure and matrix mechanics on biofilm properties such as virulence, antibiotic resistance, and immune evasion are largely unknown. Indeed, we know little about what specific structures and mechanics develop in biofilm infections, and extant techniques to probe these properties are largely lacking. *These are significant and critical unaddressed problems.*

We will bring to bear a unique combination of techniques, a number of which we have recently developed, for measuring and controlling the *in vivo* and *in vitro* structural and the *in vitro* mechanical properties of biofilms [9, 11, 35]. Using our distinctive combination of microbiological and physical expertise, we will also develop new approaches to measuring the mechanical properties of biofilms *in vivo* and how they impact resistance to immunological clearance. We will determine: what mechanical and structural properties develop for biofilm infections in chronic wounds *in vivo*; how the spatial structures and associated material transport in biofilm infections impact antibiotic resistance and host-damaging virulence; how virulence and biofilm mechanics impact resistance to immunological clearance. In short, we will contribute knowledge of what biofilm structures and mechanics develop in *P. aeruginosa* biofilm infections in chronic wounds, and the degree to which these structures and mechanics impact the virulence, antibiotic resistance, and persistence of the biofilm infection. *This contribution will be significant because it will identify cases where structure and/or mechanics should be or inform specific therapeutic targets.* We expect that treatments addressing physical properties could also be combined with traditional therapeutics for enhanced clinical outcome. In addition to treatments addressing virulence, antibiotic resistance, and immune evasion, our work is also likely to lead to improved approaches for debridement and negative pressure wound therapy.

In the short term, our work will advance understanding of an important pathogen that forms biofilm infections in wounds and many other anatomical sites [36-51]. Moreover, the work proposed here will develop a platform of complementary techniques and knowledge that will be extensible to future studies of other infection sites and other organisms, including multi-species infections and engineered microbial consortia [52-54]. Thus, this platform will be a foundational resource for the emerging field of physical microbiology & medicine. We expect that our immediate results, as well as the subsequent work building on the platform we develop, will lead to improved quality of life and medical outcomes, and reduced healthcare costs.

B) Innovation:

The common approaches to developing new treatments for biofilms are either to find genes important for forming biofilms or to directly kill the bacteria in biofilms by novel drugs. These approaches have met with only limited success [55]. Our goal is to determine the structural and mechanical characteristics of biofilms *in vivo* and *ex vivo* and to identify the impact of these physical properties on disease course. For this, we will implement an innovative combination of techniques from both biological and physical sciences. Our approach allows elucidation of the mechanistic relationship between the physical characteristics of biofilm infections and the course of biofilm disease that are not accessible by conventional methods.

C) Approach:

Specific Aim 1: Determine the spatial structure and mechanics of biofilm infections in chronic wounds

Introduction: The objective of this aim is to determine the spatial structures and mechanics that develop in *P. aeruginosa* biofilm infections in chronic wounds. Here “spatial structure” means the size and number density of

bacterial aggregates and the matrix heterogeneity within aggregates, and “mechanics” means viscoelastic responses to applied forces. The working hypothesis for this aim is that the structure and mechanics of *in vivo* biofilm infections in chronic wounds will follow development trajectories arising from the matrix-producing capabilities of the bacteria and pressure from the host immune defense. Our approach to testing this working hypothesis will be to use advanced imaging to assess biofilm structure and to use atomic force microscopy (AFM) and microrheology to measure mechanics. Both structure and mechanics will be measured *in situ*. The rationale for this aim is that *P. aeruginosa* is an important biofilm-forming human pathogen, but the types of structure and mechanics that arise in *P. aeruginosa* biofilm infections are largely unknown. These gaps in knowledge preclude identification of physical properties of biofilms as therapeutic targets.

Justification and Feasibility:

Review of Relevant Literature:

In vitro, biofilms can be millimeters or more in extent, but not *in vivo* [56-59]. Recent advances in staining and microscopy of tissue have allowed visualization of bacterial biofilms in chronic wounds, cystic fibrosis (CF) lungs, and other anatomical sites [1, 12, 60]. In general, *in vivo* biofilms are collections of aggregates ~10-100 µm in diameter, containing $\sim 10^2 - 10^6$ bacteria per aggregate. In chronic wounds and other sites, different species are typically spatially segregated by tens of microns or more [12, 60, 61], making it reasonable to study single-species *P. aeruginosa* infections as a first step to understanding how physical factors impact biofilm infections.

Much work has been done to understand *P. aeruginosa* biofilm infections in the lungs of CF patients [62, 63]. Much less work has been done to understand biofilm infections in wounds, although wounds affect many more people: there are ~30,000 CF patients in the U.S., and ~6 million people annually are burdened with chronic wounds [10, 64]. Therefore, we will use *CF lung infections as a test case which can guide investigation of wound biofilms*. Biofilms in the CF lung can last for decades, under pressure from antibiotics and the immune system. Wound biofilm infections are not typically as long in duration as CF infections, but they are subject to similar pressures *in vivo*. Therefore, we expect that *characteristics that are adaptive in CF infections will be beneficial to biofilms in chronic wounds as well*. Chronic wound models are also expedient for our study because the proximity of biofilms to the surface should greatly facilitate the work in *Specific Aim 1*.

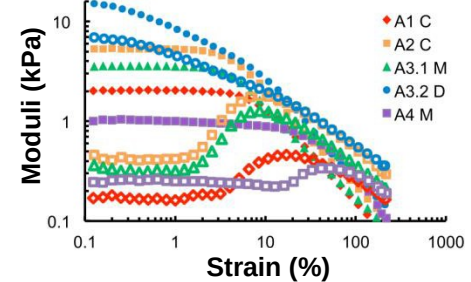
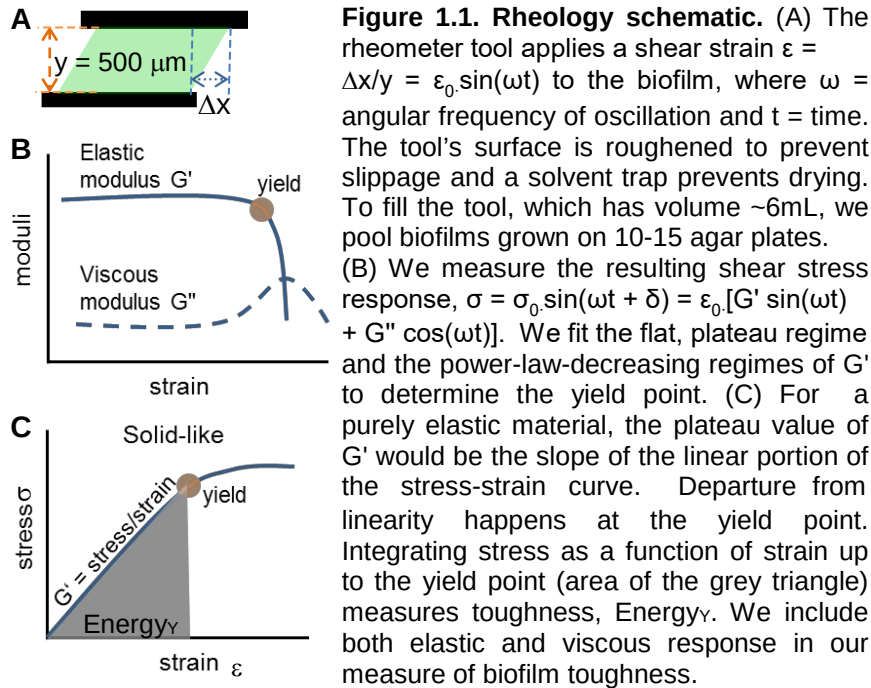
The most important EPS materials for *P. aeruginosa* biofilms are polysaccharides (Psl, Pel, and alginate) and extracellular DNA (eDNA) [65-70]. In CF infections, production of alginate increases over time [71, 72] and worsens outcomes for patients. Alginate chemically protects biofilms by chelating reactive oxygen species from inflammatory cells [73, 74] and preventing activation of the complement immune system [75]. CF infections also evolve to increase the production of Psl, and likely Pel, with worse outcomes for patients [76-81]. Psl protects biofilms against antibiotics, likely by chemical binding [82], and blocks the binding of opsonins that promote phagocytosis [83]. We have shown that Pel also provides antibiotic protection [84]; cationic Pel binds to eDNA, a polyanion which binds to cationic antibiotics, such as the aminoglycoside tobramycin [85].

Thus, it is well-appreciated that *biofilm matrix polymers can provide chemical protection against clearance*. The role of *mechanical protection has not been characterized*, and the distinct contributions of matrix materials to biofilm mechanics is largely unknown. *In vitro*, increased alginate softens biofilms [86, 87] and Psl decreases but Pel increases creep compliance, the tendency to slowly deform under constant stress [88]. The state of the art has relied on bulk rheology, microrheology, or AFM measurements of *in vitro* biofilms [89-100]. The mechanics of biofilms grown *in vivo* has not been measured, even though growth conditions are likely to change material properties.

Summary of preliminary data and findings:

The mechanics of biofilm infections evolve within patients. We recently determined how evolutionary changes in EPS production by biofilms in CF lungs are linked to changes in biofilm mechanics [11]. We use *P. aeruginosa* strains that were isolated from the sputum of four CF patients [77]. These isolates were taken at well-resolved timepoints over ~200-3000 days of infection, and have been characterized to determine the changes arising from *in vivo* evolution [76, 77]. We use a parallel-plate rheometer tool to apply an oscillatory

shear strain to biofilms (**Figure 1.1**) [101, 102]. We measure both elastic (solid-like) modulus G' and viscous (fluid-like) modulus G'' . All moduli show only weak frequency dependence from 0.1 to 10^3 Hz. Plateau G' is typically $\sim 10\times$ greater than plateau G'' , thus *biofilms are dominantly solid-like* (**Figure 1.2**). Material failure begins at a yield strain ε_Y and a yield stress σ_Y . The toughness is the energy cost to cause yielding, Energy_Y .



We group descendent isolates by whether production of alginate, Psl, or both has increased from that of the ancestor. We find increased Psl production alone does not change Energy_Y , but increased alginate alone reduces Energy_Y by $\sim 2/3$. However, increasing Psl along with alginate entirely rescues the loss of Energy_Y caused by increased alginate alone. Increased alginate alone decreases plateau G' by 90% and decreases σ_Y by over 60%. However, if both alginate and Psl increase, G' decreases by only 40% and σ_Y is maintained at the ancestral value. Thus, increased Psl can counteract softening, weakening and loss of toughness caused by increased alginate. To investigate the mechanisms by which distinct types of EPS give rise to distinct mechanical properties, we use isogenic strains in the PAO1 lab-strain background. We find that biofilms with high Psl ($\Delta wspF \Delta pel$) have 3x the Energy_Y , 1.8x the plateau G' , and 2.5x the σ_Y of wild-type (WT) biofilms. However, the mechanical benefits of increased Psl arise only if the protein CdrA, which binds mannose on Pel, is co-produced [11, 103, 104]. Microscopy shows that the volume fraction of bacteria in biofilms is $\ll 50\%$. Therefore, attachment of bacteria to the matrix should not impact the biofilm's bulk mechanics. Thus, we conclude that CdrA crosslinking of Psl undergirds Psl's mechanical benefits to biofilms. This is the first insight into a molecular mechanism causing the distinct mechanical impacts of EPS types.

We find that high Pel ($\Delta wspF \Delta psl$) increases Energy_Y and σ_Y by increasing the yield strain ε_Y but not G' . We use atomic force microscopy (AFM) to separate pairs of bacteria. These are the first measurements of interbacterial cohesion. High Psl increases the energy cost to separate two bacteria by 4-6x, but high Pel decreases the work to separate two bacteria by 20x [11]. Thus, unlike Psl, Pel's toughening effects are an emergent property of the biofilm state. Pel binds to eDNA [85], which is a significant component of the biofilm matrix [105-107], but is not present for the pairs of bacteria in our AFM studies. Therefore, we infer that Pel-eDNA binding likely toughens biofilms and increases yield strain. A possible analogy lies with double-network gels, in which mobile junctions and hidden length can allow large deformation without yielding [108-115].

By identifying the specific matrix components and interactions needed to generate specific mechanical properties, we have identified candidate approaches for disrupting specific properties via competitive binding and/or enzymatic degradation of matrix material. The results of Specific Aim 3 will establish which mechanical characteristics would be the most important to disrupt for better immunological clearance.

Research Design:

Methodology: Bacterial strains: Two libraries of single-gene transposon mutants, in two different WT backgrounds (PAO1 and PA14), are available at nominal cost and allow us to elucidate the roles of specific gene products [116, 117]. We will focus on PAO1 because it has more possibilities for matrix variation.

Animal models for biofilm infection: We will use a mouse model for chronic wounds [118-120]. We have previously shown that *P. aeruginosa* in mouse wounds are in a biofilm state [121-123]. Mice will be shaved and administered full-thickness, surgical excision wounds. The wounds will then be covered with a semipermeable dressing to prevent contractile healing, and bacteria will be injected under the dressing (a detailed description of the model, power calculations and an animal usage table can be found in the Vertebrate Animals Section). Bacterial strains to be used are PAO1 WT, ΔmucA (high alginate), $\Delta\text{wspF } \Delta\text{pel}$ (high Psl), $\Delta\text{wspF } \Delta\text{psl}$ (high Pel) and ΔcdrA (no production of the Psl-crosslinking protein CdrA). These strains will constitutively express fluorescent proteins (red or green fluorescent protein (GFP) or mCherry) so that biofilms can be imaged using confocal laser-scanning microscopy (CLSM) (**Figure 1.3**). We will use DAPI to visualize inflammatory cells. We will sacrifice animals at 24 & 72 hours and 7 days after infection and harvest tissue from the infection site. Harvested samples will be fixed and sectioned.

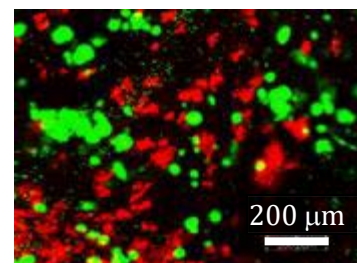


Figure 1.3. Aggregates of *P. aeruginosa* (green) and *S. aureus* (red), in a mouse wound. We visualized these using CLSM (unpublished).

As assays for disease outcomes, for all animals we will determine: systemic spread of bacteria, as a proxy for virulence; quorum sensing, *ex vivo* using fluorescent reporter strains; antibiotic efficacy, *ex vivo* using measurements of colony-forming units; and inflammation, wound closure and healing, as follows:

To measure systemic bacterial load, spleens will be excised, weighed, dispersed in sterile PBS, serially diluted, and plated on *Pseudomonas* isolation agar to quantitate CFU/g tissue. To measure antibiotic efficacy, we will use a similar procedure on tissue sections from infected wounds, which will first be suspended in 90 $\mu\text{mol/L}$ tobramycin or PBS for 5 hours, and then treated as above. We will image wounds daily using the SilhouetteStar laser-scanning wound imaging, 3D measurement and documentation system (ARANZ Medical) and percent wound closure will be calculated. Wound tissue histology *via* hematoxylin & eosin and Masson's trichrome staining will be used to assess wound healing as previously described [123-125]. The inflammatory response will be assessed using a Qiagen Cytokine Multi-Analyte ELISArray Kit and histological assessment of neutrophil infiltration. Also, since weight loss indicates a progressing infection, all mice will be weighed daily.

Activity 1-1: Determine the structures of *P. aeruginosa* biofilm infections as a function of infection time and matrix production capability. We will use confocal laser-scanning microscopy (CLSM) to measure the spatial structures of *P. aeruginosa* biofilm infections as they form *in vivo*. We will use quantitative image analysis, in Matlab, to turn micrographs into quantitative measurements. The PI has expertise in quantitative microscopy and image analysis [9, 35, 84, 126-144]. Thus we are well-positioned to develop new measurement metrics as needed during the progress of our work. Initially, we will determine the size distribution of bacterial aggregates and the three-dimensional positions and number density of bacterial aggregates with respect to each other. We will also measure the number and spatial distribution of neutrophils, as in [1, 145], and correlate neutrophil density with the size and position of aggregates. We expect the aggregate size distributions to show a peak value that increases with early infection time and biofilm growth, and then plateaus as the aggregates hit size limits that arise from limiting oxygen, which we expect to depend on the density of surrounding neutrophils [1]. Because this limits aggregate growth beyond a certain size, we also expect the width of the size distribution to decrease with time. We expect that if matrix production capabilities impact the size distribution, then matrix content with diminished mechanics (*Activity 1-3*) linked with better clearance by neutrophils (*Specific Aim 3*) should be associated with smaller aggregate sizes.

We expect the sizes of individual aggregates to impact the development of microenvironments *via* diffusion within aggregates (*Aim 2*). For an isotropic 3D environment, the density of diffusing material ϕ is a function of position $\vec{r}$ and time t , thus: $\frac{\partial \phi(\vec{r}, t)}{\partial t} = D \nabla^2 \phi(\vec{r}, t)$; ∇ is the gradient operator and D is the diffusion

coefficient, which has dimensions of length²/time. Therefore, for a structural metric characterizing diffusive transport between aggregates, we will use spatial moment analysis to find the mean and variance of the square of the inter-aggregate distance, which we expect to remain roughly constant with time. If not, we will use knockout mutants to probe the roles of motility, dispersal, and chemotaxis in developing biofilm structure.

In addition to possible interchanges and exchanges of motile bacteria with each other and their environment, we expect that aggregates may be able to interact through the exchange of diffusible signals and sequestration of diffusible antibiotic. To date, no good technique for measuring the transport properties of *in vivo* infection sites exists. We will work to develop a new technique to do so by co-inoculating fluorescent beads and quantum dots of different sizes and surface functionalities into animal models, along with bacteria, and measuring how the spatial distribution of these tracer particles changes with time. This will give us measures of the effective diffusion constants, plus additional transport through flow or animal movement, for materials of different sizes and surface functionalities within the wound bed. If our analysis of these results indicates that transport properties of the environment change significantly with time, we will add differently-labeled tracers to the wound at different timepoints after inoculation, and measure how their positions vary as a function of time. Since transport is fundamental to any intercellular process, especially so in biofilm infections containing multiple spatially-segregated, but interacting, pathogens, plus host cells, we expect that our technique will be foundational for future studies of biofilm infections and of the healthy microbiome.

Determining the internal structure of biofilms in terms of heterogeneous matrix content is a challenge, since most stains for biofilm matrix materials also stain host tissue. To overcome this difficulty we will use quantitative image analysis to distinguish differential binding – we expect that stains for biofilm matrix materials will have higher affinity for the biofilm (which we will measure as greater brightness) than the host tissue. We will validate this expectation by comparing biofilms that over-express specific EPS materials with WT biofilms. There, we expect over-expressing biofilms to be brighter in the relevant stain, with respect to the surrounding tissue, than the WT. We used a similar approach recently to distinguish specific and non-specific binding of a Psl stain [129]. To determine the spatial distribution of Pel, Psl, alginate, and eDNA, we will stain harvested biofilms using antibody and lectin for Psl, lectin for Pel, propidium iodide for eDNA, and cationic dyes for the polyanion alginate, as in [146]. We will get Psl stains from Prof. Dan Wozniak and Pel stain from Prof. Matt Parsek (see *letters*). The other stains are commercially available.

In comparing the matrix content and heterogeneity of biofilms grown *in vivo* with those of biofilms grown *in vitro* (Specific Aim 2), we will determine whether eDNA content is higher, overall or near the periphery, for biofilms grown *in vivo*. If so, this would be consistent with our and others' finding that *P. aeruginosa* biofilms can incorporate eDNA from neutrophils [121, 147]. Neutrophils can be lysed by bacterial virulence factors and can also release eDNA-rich extracellular traps as part of the innate immune response [148-152].

Activity 1-2. Determine biofilm mechanics as a function of matrix content and infection time. The primary means of mechanical characterization of the *in-vivo* wound biofilms will be through *ex-vivo* measurements done using an AFM. We will use *ex vivo* samples, specifically biopsies containing biofilms that will be harvested from mouse wound beds at the timepoints and for the bacterial strains described above under Methodology. These samples will be placed onto an agar plate and kept submerged within a growth broth. These samples will then be characterized using an Asylum Research MFP-3D SA AFM in a liquid environment. We can characterize samples over their entire 2D area; samples with lateral dimensions ~100 μm up to orders of magnitude larger can be probed. Keeping samples in a liquid environment will ensure that the tested material is as close to *in-vivo* state as possible. All tests will occur as quickly as possible after the biofilms are excised, always less than 12 hours.

First, we will use the AFM in tapping mode to determine both the topography of the biopsy sample and the location of the biofilm. In tapping mode, the AFM will provide a topographical height map and a qualitative stiffness map of the sample, which we will use to distinguish regions of biofilm from regions of tissue [153-155]. Then, we will do a Force-Volume mapping. The AFM tip will be rastered over the 2D sample area, and will measure force-displacement curves using a cantilever with a round, large silica probe. From these curves, biofilm elasticity can be obtained using an appropriate contact model. Typically a Hertz or Sneddon model is

used [100, 153, 154], assuming no adhesion between probe and sample; however, many other possible models include adhesion and can be used for biological samples [156]. Thus, we will measure the relative stiffness of the biofilm with respect to tissue. We will then run the AFM in a constant force mode while measuring the displacement. This will yield the relaxation time spectrum of the biofilm directly, from which we will estimate viscosity by assuming the biofilm acts as a Maxwell element (*i.e.*, a spring and dashpot in series).

The above measurements will provide relative stiffness, elasticity, and relaxation times, but will not truly probe system viscoelasticity. To get truer values of biofilm G' and G'' , we will use a more advanced technique, Amplitude and Frequency Modulation [153]. By forcing the cantilever to vibrate at a controlled frequency and amplitude before contact with the biofilm, the amplitude change and frequency change due to the biofilm can be related to viscoelastic moduli; this is similar to results obtained in a rheometer as described in Preliminary Data, but with *in situ* measurement and high spatial resolution. This mode runs automatically on our AFM. Rastering over the sample will provide a map of these moduli similar to the Force-volume stiffness map.

AFM work requires removing the samples from the animal. Mechanical characterization *in vivo* is more difficult, but it would allow us to verify that the *ex vivo* biofilm mechanics correspond to the *in vivo* biofilm mechanics. Therefore, we will attempt microrheology *in vivo*. In two-particle passive microrheology, the cross-correlated thermal motion of pairs of particles can be related to viscoelastic moduli, over a range of frequencies ω [157, 158]. The displacement $u(\omega)$ of a single particle is given by the Stokes-Einstein relationship $u(\omega) =$

$\frac{f(\omega)}{6\pi aG(\omega)}$, where $f(\omega)$ is a Brownian force and $G(\omega) = G' + iG''$ is the complex shear modulus. Here, if colloidal probes from *Activity 1-1* incorporate into biofilms, we will take high-speed, 3D movies of colloids moving in the biofilms *in vivo*. A high-speed confocal fluorescence microscope is available in the Materials Characterization Facility at TTU. If tracer particles from *Activity 1-1* incorporate into biofilms, we will also use magnetic beads for active microrheology. Inexpensive, high-precision magnetic tweezers can apply stresses up to ~25 kPa, comparable to the yield stresses we measure for biofilms grown *in vitro* [159]. Thus, we expect to measure local yielding as well as viscoelasticity. Microrheology *in vivo* has a number of potential difficulties, and so will be a secondary analysis technique intended to supplement the primary AFM analysis [160, 161].

Expected Outcomes: The overall outcome of this aim will be comprehensive knowledge of the structural and mechanical characteristics of *P. aeruginosa* biofilm infections that develop in chronic wounds. This knowledge will be used to guide our work in Specific Aims 2 and 3.

Potential Pitfalls and Alternative Strategies: We expect the results from *in vivo* models to reveal common themes of structure and mechanics controlled by infection time and matrix production capability. If we measure changes in structure or mechanics that do not result from changes in EPS or protein content, we will investigate whether this could arise from varying pH or salt concentration between sites. We will also investigate other possible causes such as cross-linking by pili or flagellae, or degradation by host enzymes or reactive oxygen species from host neutrophils [103, 162].

Specific Aim 2: Determine how spatial arrangements differentiate into distinct microenvironments

Introduction: The objective of this aim is to determine how distinct spatial structures result in the development of microenvironments that impact biofilm disease. The working hypothesis for this aim is that virulence and antibiotic resistance will depend on key structural characteristics, such as the sizes of aggregates and the distances between aggregates, through the development of microenvironments that differentiate as a result of the material transport and consumption of growth substrate, bacterial products, and antibiotics (**Figure 2.1**). Our approach will be to recreate the salient structural features of biofilm infections *in vitro* and then measure the oxygen gradients, antibiotic resistance, virulence, and quorum sensing in the resulting microenvironments. The rationale for this aim is that knowing the effect of structure on clinical outcomes will indicate when it is appropriate to target a particular structure for treatment, and could help open the way for treatments based on interfering with the physico-chemical microenvironments of biofilms.

Justification and feasibility:

Review of the relevant literature:

In vitro, the size and proximity of bacterial aggregates impacts virulence and antibiotic resistance. Small, dense populations of ~150-1000 cells in a pL volume can activate quorum sensing and develop phenotypic antibiotic resistance [163-169]. Quorum sensing controls gene expression according to the density of like neighbors [170-174]. Bacteria produce autoinducers and sense their concentration. When autoinducer concentration is high, quorum sensing activates [175] and, for *P. aeruginosa*, increases the production of virulence factors [176-178]. Even aggregates that are separated by tens of microns or more can interact through chemical signals [179, 180]. Oxygen depletion, which arises in aggregate interiors as bacteria near the surface consume oxygen, can also impact quorum sensing and virulence [181-187]. Oxygen depletion also slows growth, which can lead to antibiotic resistance because the mechanism of activity of many antibiotics makes them more effective against rapidly-growing bacteria: e.g., aminoglycosides bind to the ribosome and disrupt protein synthesis.

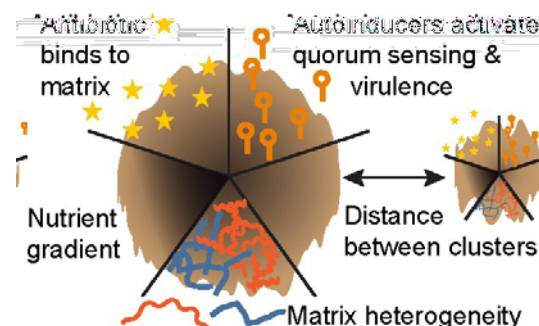


Figure 2.1. Structural characteristics of biofilm infection, such as aggregate size, inter-aggregate spacing, and heterogeneous distribution of matrix materials, should give rise to differentiated microenvironments that enhance antibiotic resistance and virulence.

The composition and structure of biofilm matrices impacts antibiotic resistance. Psl protects biofilms against antibiotics, likely by chemical binding to antibiotics [82]. We have shown that Pel also provides antibiotic protection [84]. This likely arises because cationic Pel binds polyanionic eDNA along the biofilm periphery, where it is thought to electrostatically bind to cationic antibiotics, such as the aminoglycoside tobramycin [85]. Binding antibiotics at the biofilm periphery would hinder high concentrations of antibiotic from penetrating the biofilm and thus reduce the effective concentration of antibiotic to which bacteria are exposed, as long as the surrounding EPS is not saturated with antibiotic.

Preventing antibiotics from binding to the biofilm matrix can increase antibiotic efficacy. Tobramycin (tob) is an aminoglycoside antibiotic that is the front-line drug for many *P. aeruginosa* infections [188]. Prof. Hugh Smyth (Pharmacy, UT Austin), has recently found that tob that has been chemically conjugated with polyethylene glycol (tob-PEG) is 3x more effective against mature *P. aeruginosa* biofilms (treated after 24 hours of growth, the concentration corresponding to 80% inhibition of activity (MIC_{80}) is 27.8 $\mu\text{mol/L}$) than is conventional tob ($MIC_{80} = 89.8 \mu\text{mol/L}$) [189]. This likely results because PEG functionalization reduces the binding of tob to biofilm matrix polymers and not from an intrinsic increase in drug activity: tob-PEG is 10x less effective against planktonic *P. aeruginosa* ($MIC_{80} = 14 \mu\text{mol/L}$) than conventional tob ($MIC_{80} = 1.4 \mu\text{mol/L}$).

Summary of preliminary data & findings:

Biofilm-specific antagonisms between antimicrobials. We and others have shown that high pH synergizes with aminoglycosides in killing exponentially-growing planktonic bacteria [13, 190-192]. Thus, combining an aminoglycoside with an adjuvant base has promise for re-empowering an established class of antibiotic. This could combat rising antibiotic resistance and the dwindling supply of new antibiotics and reduce toxic side effects by lowering required dosage [193-195]. However, we find high pH antagonizes with tob in biofilm treatment (**Figure 2.2**) [13]. Tob+base acts additively against stationary-phase planktonic bacteria, so antagonism *per se* does not result from low growth. Biofilms with high Psl show the greatest antagonism (**Figure 2.2D**), so we anticipate that antagonism most likely arises because tob binds to the biofilm matrix.

Controlling biofilm structure. We have developed a novel way to structure flow-cell biofilms using an optical trap to build arbitrary configurations of bacteria with single-cell resolution, including co-patterning of distinct strains [9]. Under the microscope, we can measure how subsequent biofilm development depends on structure (**Figure 2.3**) [9]. The microscope stage is enclosed in a light-tight incubator to allow biofilm growth at physiological temperature with continual monitoring. This approach imposes no barriers to inter-aggregate transport or accessibility of aggregates to immune cells, and it results in structures comparable in size to those measured *in vivo* (compare **Figure 2.3** and **Figure 3.1**). Other methods of structuring biofilms either enclose bacteria or structure biofilms on lengthscales much greater than *in vivo* [30, 196-201].

Research Design:

Methodology: We will use optical trapping to create biofilms with spatial structure that mimics what we find in *Activity 1.1* in terms of aggregate size, inter-aggregate proximity, and intra-aggregate heterogeneity. We will use bacterial strains with different profiles of matrix production; strains are optically-distinguishable *via* production of different fluorescent proteins. To confirm and measure local polymer content, we will use staining as in *Activity 1-1*.

Optical trapping is uniquely advantageous as a way to structure biofilms because it allows construction of biofilms with user-controlled structure. However, in wound infections and many other scenarios, bacterial aggregates are suspended, not attached to a surface [15, 202, 203]. Furthermore, we expect material transport in wound infections to be diffusive (c.f. *Activity 1-2*), not convective as for inter- and extra-aggregate transport in a flow cell. Therefore, to better re-create environmental properties of *in vivo* biofilms, we will also use biofilms grown as suspended aggregates in three-dimensional soft gels of agarose or agar. Others have found that *P. aeruginosa* and *Staphylococcus aureus* aggregates ($\sim \leq 100 \mu\text{m}$ in radius) display biofilm traits such as antibiotic resistance, differentiation, and growth limitation due to nutrient and oxygen depletion [204, 205].

To best represent wound conditions, we will also use an *in vitro* model based on chopped-meat media, with heparinized bovine plasma and horse red blood cells [206]. This contains physiological concentrations of blood components. We and others have used this model to grow polymicrobial biofilms that accurately reflect the microbial populations and morphologies of human and mouse wound infections [124, 206-208].

By varying the number density of bacteria initially incorporated into the gel, we will vary the inter-aggregate spacing; the size of aggregates varies with distance from the interface where nutrient and oxygen are supplied [204]. Thus, we will reproduce the aggregate sizes and inter-aggregate spacings that we measure *in vivo*. Gels also allow high-throughput measurements in a way that optical trapping does not.

Others have shown that the EPS Psl can act as a signal to increase levels of the second messenger cyclic-di-GMP, which changes the expression patterns for many genes [209]. Therefore, to confirm that the effects of EPS result from transport and not from signaling, we need to restrict full physical and chemical access to bacterial microcolonies for a subset of experiments. For this, we will use micro-3D (μ3D) printing that we developed to encapsulate bacteria and grow microcolonies within picoliter containers of defined size and arbitrary shape [196, 197, 210]. Walls are formed from cross-linked proteins and provide a physical

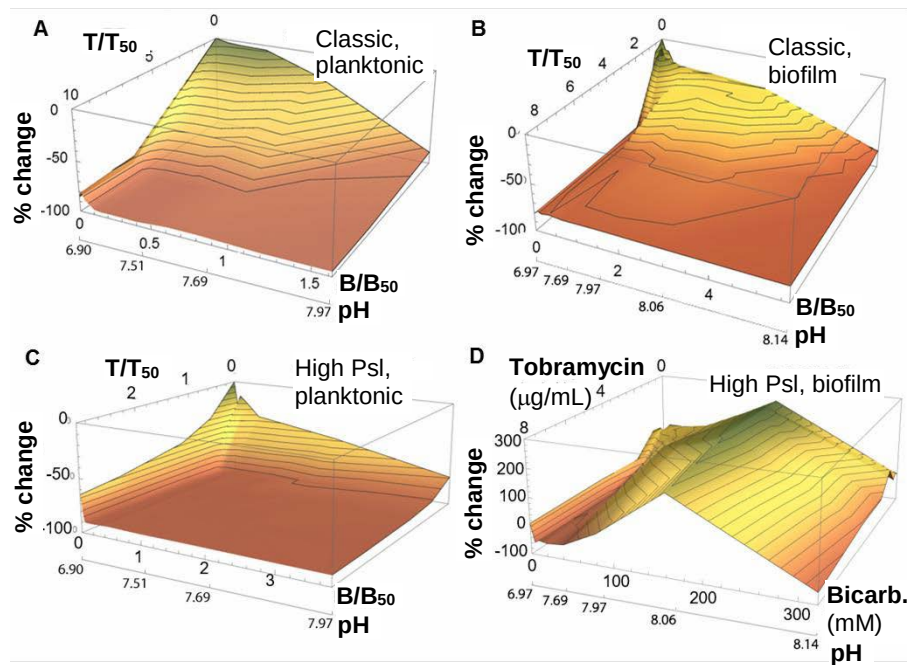
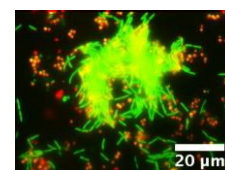


Figure 2.2 We use response surfaces to measure the effect of tob and bicarbonate (bicarb) combinations on (A,C) planktonic bacteria and (B,D) biofilms of (A,B) a CF clinical isolate with “classic” phenotype and (C,D) a CF isolate with high Psl. (A-C) Tob and bicarb concentrations are plotted as the fraction of the concentration corresponding to 50% inhibition of growth (MIC_{50}). (D) MIC_{50} is not well-defined for this system, so actual concentrations are used. (A,C) The planktonic response surfaces are concave-upward, reflecting synergy between tob and bicarb. (B,D) The biofilm response surfaces are concave-downward, reflecting antagonism between tob and bicarb. (C,D) The high-Psl isolate shows greater antagonism in the biofilm state than does the classic isolate, even though the high-Psl isolate shows greater synergy in the planktonic state. (D) Portions of the biofilm response surface extend above 0% change, indicating that treatment has enhanced biofilm activity. All panels from [13].



■ *P. aeruginosa*
● *S. aureus*

Figure 2.3: Biofilm structures created by optical trapping. We placed 10 *P. aeruginosa* in an isotropic field of *S. aureus*. Six hours later, the initial structure has propagated into the developing biofilm [9].

boundary for segregating cells while supporting diffusion of different molecular types and sizes depending on the matrix composition and crosslinking density (**Figure 2.4**).

We will begin by recreating published *in vivo* biofilm structures [61, 211], and then build on the results from *Activity 1-1*. We will use wide-field epifluorescence microscopy and confocal laser-scanning microscopy (CLSM) for measurements. We will measure antibiotic resistance using live-dead staining, and quorum sensing and virulence using reporter strains of bacteria that express green fluorescent protein (GFP) in proportion to the activity of the *rsaL* and *rhl* genes. We will record growth physiology using a plasmid for GFP [212] that will only be expressed in metabolically-active cells, and using quantitative FISH [213]. We will measure oxygen concentration and pH using fiber optic and electrochemical microsensors [12, 214-217].

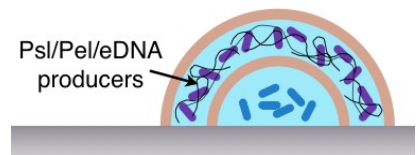


Figure 2.4: Bacterial microenclosure.

We will verify the *in vivo* relevance of the *in vitro* microbiology studied below by comparing the results found below with the disease outcomes found with different biofilm structures in Specific Aim 1.

Activity 2-1: Determine the effect of biofilm spatial structure on growth physiology and microenvironment. We will determine the degree to which the distinct structures found in biofilm infections alter bacterial growth and microenvironment, both of which are linked to antibiotic resistance. We expect to find that bacteria in small aggregates, near biofilm exteriors, and near the inlet or interface where growth substrate is supplied, will grow more quickly than bacteria in biofilm interiors due to oxygen and nutrient limitation. Our published work and the work of others indicates that, for a given exterior concentration of growth substrate, the periphery of aggregates will contain a layer of actively-growing bacteria that is constant in thickness regardless of aggregate size, and an interior of quiescent or very slow-growing bacteria that fills the rest of the aggregate volume [35, 218, 219].

Activity 2-2 Determine the degree to which structure impacts the antibiotic tolerance of biofilm infections. We expect this to depend on biofilm structure both directly, *via* the matrix content and heterogeneity, and indirectly, *via* growth physiology and microenvironment. We believe that PEG-tob should be able to overcome tob's tendency to bind to the matrix and thereby make tob+bicarbonate combinations synergistic against biofilms. Moreover, we expect that the spatial structure of heterogeneous matrix content will maximize resistance to tobramycin, and antagonism between tob+bicarbonate, when tob-binding matrix polymers such as Psl and eDNA are located near the biofilm periphery, because this will minimize transport of tob into the biofilm interior.

In addition to optical trapping to control matrix heterogeneity, we will also use μ 3D-printed core-shell communities to confirm that EPS confers antibiotic protection by binding to antibiotic and thereby hindering transport, and not *via* any mechanism requiring direct contact with cells. EPS non-producers will be encapsulated in cores surrounded by shells, of varying thickness, containing EPS producers or EPS extracts (**Figure 2.4**). We will tune the wall thickness, composition, and cross-linking density to make barriers permeable to antibiotics but impermeable to high molecular-weight EPS. We also have the option to eliminate the outer shell, so that producers in bulk solution cover a space extending essentially to infinity.

Measurement endpoints will be killing of bacteria if this happens, and the achievement of steady-state antibiotic distributions if not. We will measure the distribution of antibiotic using fluorescently-tagged drugs and using hydrophilic fluorophores without drug conjugation.

Activity 2-3: Determine how structure impacts the upregulation of virulence. We expect virulence in the quorum-sensing regulon to depend on the number density and size of aggregates. We will use GFP reporter strains to measure the upregulation of *P. aeruginosa* virulence factors and of quorum sensing. As controls, we will use *P. aeruginosa* mutants that are incapable of responding to quorum sensing signals. We expect that the structure of *P. aeruginosa* biofilms that maximize virulence upregulation caused by quorum sensing will be set by a competition between high cell number and density (which will tend to increase the local concentration of autoinducers) and low metabolism in oxygen- and nutrient-limited large bacterial aggregates.

Expected Outcomes: The outcomes of this aim will be an understanding of how key characteristics of biofilm structure impact the development of microenvironments that lead to two important properties of biofilms – antibiotic tolerance and virulence. This will open up the possibility of manipulating biofilm structure and microenvironments to make them more amenable to antibiotic treatment, or to treatment with less-toxic combinations of antibiotics, and/or to make biofilms less virulent.

Potential Pitfalls and Alternative Strategies: Our working hypothesis is that virulence and antibiotic resistance will depend on key structural characteristics. This hypothesis is based on basic principles of mass transport and is unlikely to prove invalid *in vitro*. However, it is possible that our *in vitro* measurements may not correspond with *in vivo* outcomes. This could result from differences in the transport in *in vitro* and *in vivo* environments, or pH that alters chemical stability. Published measurements of biofilm structure *ex vivo* are compatible with aggregates that are patterned laterally, in a 2-D plane, as we did in [9]. If we find in Activity 1-1 that we need to structure biofilms in the z-direction as well, we will use μ 3D-printed microenclosures [163, 180, 210] or solidify gel on top of bacteria placed by optical trapping [28, 220, 221], and then arrange more bacteria on top. We can also use low-cost bioprinting available from SE3D, who will consult with us on developing their current technology to be able to structure biofilms (see *letter* from CEO Dr. Mayasari Lim).

Specific Aim 3: Determine the role of mechanics and virulence in biofilm-neutrophil interactions

Introduction: The objective is to determine how mechanics and virulence interplay to impact biofilm evasion of the host defense. The working hypothesis the bacterial tolerance and evasion of neutrophils will depend both on the neutrophils' ability and speed in breaking off and engulfing pieces of biofilm, and on the ability of biofilms to kill immune cells. Our approach to testing this working hypothesis will be to add freshly-isolated human neutrophils to gels with tunable mechanics that recreate biofilm mechanics and to structured biofilms and measure the neutrophils attack on the bacteria and the bacterial counterattack. The rationale is that chronic biofilm infections are not removed by neutrophils, but little is known about why. In CF most lung damage comes from reactive oxygen species (ROS) released as a result of frustrated phagocytosis [222, 223]. Reducing ROS release by enhancing phagocytosis would slow the cumulative destruction of lung tissue. We have found that in wounds biofilms are surrounded by high numbers of neutrophils, but many of them are dead and, counter-intuitively, enhanced neutrophils response enhances biofilm formation (perhaps because eDNA from neutrophils is incorporated into biofilms) [121]. Enhancing phagocytosis should cause neutrophils to repress, rather than enhance, biofilm development. Moreover, mechanics-targeting strategies that enhance the susceptibility of biofilms to phagocytic clearance would not be hindered by the rise in antibiotic resistance.

Justification and feasibility:

Review of the relevant literature:

Neutrophils are first responders to infection [224] that easily engulf planktonic bacteria [217, 225]. However, mature biofilms are protected against phagocytosis despite being surrounded by continually-recruited neutrophils that do not penetrate the biofilm [1, 12, 15, 16, 18, 145, 217, 226-232] (Figure 3.1). The mechanism(s) by which biofilms are protected are not fully known [18, 217, 233-236], although physical hindrance, slow bacterial growth, and virulence have been implicated. *In particular, the role of biofilm mechanics in evasion of the immune system is not known.*

Because $\sim 10\ \mu\text{m}$ neutrophils are an order of magnitude smaller than the $\sim 100\ \mu\text{m}$ biofilm aggregates (Figure 3.1), for neutrophils to phagocytose biofilm bacteria they must be able to break off a piece of the biofilm which is small enough for them to ingest [237]. *It has been estimated that neutrophils apply a stress of $\sim 1\ \text{kPa}$ during phagocytosis [238]. This stress is within the range of G' values we measure for biofilms (Figure 1.2) and suggests that the mechanics of a biofilm is likely to impact its resistance to phagocytosis.* Phagocytosing blood granulocytes and macrophages also exert stresses of $\sim 1\ \text{kPa}$ [239, 240]; phagocytosing *Dictyostelium* and *Entamoeba* can break off and ingest small pieces of a target [241, 242].

Biofilm mechanics are also likely to impact the timescale of phagocytosis. Changes in timescale matter

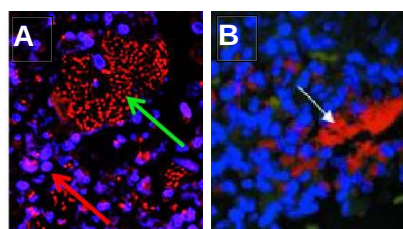


Figure 3.1. *P. aeruginosa* biofilm aggregates surrounded by neutrophils (A) in a CF lung [1] and (B) in a chronic wound [12]. Bacterial aggregates (red, green and white arrows) are surrounded by neutrophils (blue, red arrow).

because bacteria-produced virulence factors can kill neutrophils and other immune cells [243-245]. Delays that give more time for virulence factors to act should help protect the biofilm. Mature, large biofilms produce rhamnolipid that lyses neutrophils [244]. Quorum sensing upregulates production of pyocyanin, which induces neutrophil apoptosis and impairs neutrophil-mediated host defense [245]. Therefore, we expect that the ability of biofilms to kill neutrophils using virulence factors will also depend on the biofilm structure (see *Activity 2-3*).

Research Design:

Activity 3-1. Determine the role of mechanics in the biofilm's evasion of neutrophil phagocytosis. We will use gels to recreate dominant aspects of the biofilm mechanics we measure in our *preliminary studies for Specific Aim 1* and in *Activity 1-2* – namely the elastic modulus G' , the yield stress σ_Y , and the yield strain ϵ_Y . Agarose and polyacrylamide gels are well-established for studying the effects of substrate mechanics on eukaryotic cells. For agarose gels, G' can be tuned using both concentration and molecular weight, from below 1 kPa to over 1 MPa [246]. For polyacrylamide, both empirical and analytical relationships for G' as a function of acrylamide and bis-acrylamide content exist [247, 248]; G' can be tuned from 0.5 to 50 kPa. Thus, the range of gel moduli available compares well to the range of biofilm moduli we measure (*Figure 1-2*).

We will add freshly-isolated human neutrophils to gels, in liquid serum media. The isolation protocol comes from Prof. David Greenberg, U. T. Southwestern Medical Center (see *letter*), and is similar to published protocols [1, 18, 249]. We will incorporate live bacteria into gels to make them immunogenic. We will use two assays: (1) Incubate neutrophils in multi-well plates coated with gel containing fluorescent beads. After rinsing, we will measure neutrophil fluorescence using a plate reader or flow cytometer; the greater the increase in fluorescence, the more successful the neutrophils at phagocytosis. We will also use fluorescence to measure the change in gel volume in wells. This high-throughput approach is inspired by previous work on phagocytosis of biofilms [83] and will allow us to rapidly survey the impact of a wide range of elasticity and yielding values. (2) Record the interactions of neutrophils with gels (formed on glass coverslips coated with fibrinogen or poly-L-lysine as in [83]), at 37°C, using a microscope. Bacteria and embedded beads will be tracers for traction force microscopy measurements of the time-dependent stresses, strains, yielding, and engulfment resulting from neutrophils' phagocytic attempts [250, 251].

We expect to find ranges of G' and σ_Y such that, for long incubation times, susceptibility to phagocytosis decreases with increasing G' and σ_Y . Outside these ranges, we expect phagocytosis to be constantly high for low G' and σ_Y , and constantly zero or very low for high G' and σ_Y . Thus we will bracket a “window” of G' and σ_Y for which disrupting biofilm mechanics could increase susceptibility to phagocytosis. We also expect to find a range of ϵ_Y such that, for constant σ_Y , the timescale required to complete phagocytosis will increase with ϵ_Y . This will reveal a “window” of ϵ_Y where disrupting biofilm mechanics could increase the speed of phagocytic clearance. Should we find that the timescale for clearance is not well-described as an increasing function of ϵ_Y/σ_Y , we will investigate the role played by viscous contributions to Energy_Y . We will also determine if the stresses exerted by phagocytosing neutrophils vary with G' - substrate G' impacts neutrophil morphology and migration [252], stresses exerted by migrating fibroblasts and *Dictyostelium* [253-255], the macrophage foreign body response and adhesivity [256, 257], and phagocytosis of small solid particles [258].

Activity 3-2. Determine how upregulation of virulence impacts immune evasion. *In vitro*, we will create configurations of aggregates found *in vivo* (*Activity 1.1*) that have different levels of virulence (*Activity 2-3*). We will use microscopy and reporter strains for virulence genes to determine the biofilms' response to and killing of neutrophils. We will also confirm the link between structure, virulence, and resistance to neutrophils using bacterial strains that constitutively over-express rhamnolipids and/or pyocyanin and strains for which virulence is inducible by addition of arabinose to the growth medium. We expect that killing by virulence factors will likely be described by a Hill function, which describes a variable ligand concentration $[L]$ (here the concentration of a particular virulence factor) binding randomly to receptors with disassociation constant K_d :

$$H([L]) = \frac{[L]^m}{K_d^m + [L]^m}; m \text{ measures the self-cooperativity of the ligand and the slope of the linear regime of the sigmoidal curve } H([L]).$$
 This form of dose-response function has been seen for many antimicrobials, by us

and others [13, 259-261]. We will analyze the interaction of multiple types of virulence factors using dose-response surfaces, as we and others have done for interacting antibiotics (*Figure 2.2*) [13, 262-264]. Here we expect to find additive or synergistic interactions. We will also use dose-response surfaces to analyze the interaction of mechanics with virulence. For this, one “concentration” axis will measure the timescale required for phagocytic engulfment (*Activity 3-1*). We expect synergy between virulence and engulfment timescale.

Activity 3-3. Validate immune evasion results from the previous activities *in vivo*. To confirm the the *in vivo* impact of mechanics and virulence on neutrophil phagocytosis of biofilms, we will make “model” biofilms by creating gel beads with the most starkly distinct absolute susceptibility to phagocytosis and timescale for phagocytosis, as determined in *Activity 3-1*. We will embed bacteria into gel beads, using both WT and a highly-virulent strain so that we can probe the interplay between phagocytosis timescale and virulence. Bacteria-impregnated gels will be inoculated into mouse wounds and inflammation, wound closure and healing, and bacterial load will be measured at 4 and 7 days post-innoculation. We expect to find that low mechanical susceptibility to phagocytosis and high virulence synergize to worsen disease outcomes.

Expected Outcome: The expected outcome is knowledge of what windows of elasticity and yielding, virulence levels, and changes therein have potential for promoting better immunological clearance. This will open up the possibility of manipulating biofilm mechanics and/or structure and microenvironments (which we expect to impact the production of virulence factors) to counteract biofilms’ evasion of the immune system.

Potential Pitfalls and Alternative Strategies: The working hypothesis for this aim is that a biofilm’s evasion of the immune system depends on both the mechanics of the biofilm and its production of virulence factors. This hypothesis is based on the literature and our own results but it carries a measure of risk, since very little is known about how biofilms evade the host immune system, and because these types of measurements are new and new protocols will have to be developed. If the agarose and polyacrylamide gels resist phagocytosis despite provoking an immune response, we will first test whether they are too stiff, and/or have yield stress too high, for neutrophils to overcome. To extend the range of mechanics assayed to softer, weaker materials, we will use alginate and other hydrogels with moduli that are tunable even lower [265-269]. Should we find that, even in this case, mechanics do not contribute to immune evasion, we would use AFM and shear flow to measure the strength of adhesive forces between neutrophils and biofilms with different polysaccharide production patterns. We anticipate that stronger adhesion between neutrophils and the biofilm target will be linked to a greater probability of phagocytosis [270].

Timeline:	Year 1	Year 2	Year 3	Year 4
Aim 1	Activity 1 & 2	Activity 1 & 2	Activity 1 & 2	
Aim 2	Activity 1	Activities 1 & 2	Activities 2 & 3	Activity 3
Aim 3	Activity 1	Activity 1	Activity 2	Activity 3

Structure of the Collaboration: P.I. Vernita Gordon will lead the collaboration and coordinate efforts. She will directly supervise image analysis of biofilm structure and wound transport properties (Activities 1-1&2) and *in vitro* determination of the effects of structure on antibiotic resistance and virulence (Specific Aim 2) and the impact of mechanics on phagocytosis (Activities 3-1&2). Collaborator Kendra Rumbaugh will supervise animal work in Specific Aim 1 and Activity 3-3. Collaborator Gordon Christopher will supervise *ex vivo* measurements of biofilm mechanics in Activity 1-3. Collaborator Jason Shear will supervise μ 3D-printing experiments in Activity 2-2. Students and senior personnel will meet in person at least yearly. Skype meetings will be held at least monthly. Students will prepare written reports bimonthly, for distribution to all senior personnel. Gordon and Rumbaugh have a joint manuscript under review at mBio.

General Methods. Scientific Rigor. and Biological Variables Criteria:

Blinded studies: the bacterial strains used will be coded so that researchers at Texas Tech (Rumbaugh, Christopher) will not know which strains were used until experiments and analysis are completed.

It is possible that characteristics of the mouse host, such as strain, sex, and age, may impact our findings. However, the focus of the present study is on characterizing bacterial behaviors and properties and it is outside the scope of this study to examine all the host factors that might impact this.

VERTEBRATE ANIMALS

All experiments will be carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center (Protocol Number: 07044).

1) Description of the proposed use of animals: Adult, female, Swiss Webster mice will be used in the experiments proposed. Swiss Webster is a well-characterized outbred strain of white mice, which is susceptible to infection with ESKAPE wound pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species) and have been used extensively by our group. The non-lethal surgical excision mouse model will be used to study chronic infection as previously described.¹⁻⁴ Specifically, mice will be anesthetized by intraperitoneal injection of sodium pentobarbital. After a surgical plane of anesthesia is reached the backs will be shaved and mice administered a full-thickness, dorsal, 1.0 x 1.0 cm excisional skin wound to the level of panniculus muscle with surgical scissors. Wounds will then be infected with approximately 10⁵ colony forming units of bacteria and covered with a semipermeable polyurethane dressing (OPSITE dressing). Mice will then be given warmed lactated ringers as fluid replacement and allowed to recover under warming lights. The adhesive dressings prevent contractile healing and ensure that these wounds heal by deposition of granulation tissue, much like human wounds. We have shown that this infection can persist for weeks and is highly recalcitrant to antibiotic treatments.

2) Justify the use of animals: Although artificial skin models have been used for studying wound healing, there are currently no good *in vitro* models for evaluating the host response to wound infection. For the experiments described in this proposal, we will require a model that, as closely as possible, reflects the infection sequela seen in human wound patients. Dr. Rumbaugh has over 16 years' experience utilizing acute and chronic wound mouse models^{2, 5-14}. The numbers of animals to be used for the efficacy experiments are based on our experience of determining the minimum number of mice needed to generate statistically significant data. With our prior experience with this model, to power the study with a 85% confidence interval, a minimum of 9-12 animals will be needed for each experimental group (depending on the number of parameters being assessed). Smaller group sizes can often be used when larger effects are expected. However, until we know the expected variance in outcome, exact sample size estimates cannot be made. We have carefully tried to design the experiments so that data from as many parameters possible will be obtained from as few mice possible. These experiments are detailed in Table 2 of the Research Plan. In our experimental design we have conscientiously attempted to plan as many assays per mouse as possible.

3) Provide information on the veterinary care of the animals involved: Animals are housed in the centrally managed TTUHSC Animal Facility under the supervision of a staff vivarium director, a veterinarian (Dr. Scott Trasti), a staff of trained support personnel, which is overseen by the TTUHSC Animal Care Committee. TTUHSC is fully accredited by the AALAC. An Institutional Animal Care and Use Committee reviews all the proposals to ensure the ethical and humane treatment of animals. The vivarium follows the NIH Guide for the Care and Use of Laboratory Animals (revised 1985) prepared by the Institute of Laboratory Animal Resources, National Research Council, all applicable government regulations, and TTUHSC policies governing the care and use of laboratory animal. All physical plant construction, as well as plant maintenance, husbandry, and transportation, are in compliance with the Animal Welfare Act (PL89-544, PL91-579 and PL94-279).

4) Describe the procedures for ensuring that discomfort, distress, pain, and injury will be limited to that which is unavoidable in the conduct of scientifically sound research: To alleviate discomfort, distress and pain, after surgery and during wound care, analgesics will be given (1-2% lidocaine (max dose of 10 mg/kg) + 0.25-0.5% bupivacaine in equal volume) twice daily (AM and PM) pre-emptively. Analgesics will also be given if there are signs of sickness; inactivity, sunken eyes, hunched posture, matted fur, abdominal vacuolization when handled. A mouse showing signs of morbidity (recumbent posture, labored breathing, unresponsiveness) will be euthanized. In addition, moist food within reach and additional bedding materials will be provided. Euthanasia is carried out by intracardial injection of 'Fatal Plus', a method approved by the American Veterinary Medical Association Panel on Euthanasia.

5) Describe any method of euthanasia to be used and the reasons for its selection: Euthanasia is carried out by intracardial injection of 'Fatal Plus', a method approved by the AVMA Panel on Euthanasia.

Table 1. Breakdown and numbers of mice to be used

Experimental Group	Data to be obtained	# of mice/ group	# of repeats	# of PA strains tested	# of time points assessed	Total number of mice
<u>Specific Aim 1: Determine the spatial structure and mechanics of biofilm infections in chronic wounds</u>						
<u>Activity 1.1- Determine the structures of <i>P. aeruginosa</i> biofilm infections as a function of infection time and matrix production capability</u>						
Uninfected control	Bacterial load, wound closure/healing, spatial distribution	4	3	-	3	36
<i>P. aeruginosa</i> infection	Bacterial load, wound closure/healing	4	3	6	3	216
<u>Activity 1.2 Determine biofilm mechanics as a function of matrix content and infection time</u>						
Uninfected control	mechanics of biofilm material in wounds	4	3	-	3	36
<i>P. aeruginosa</i> infection	mechanics of biofilm material in wounds	4	3	5	3	180
<u>Specific Aim 3: Determine the role of mechanics and virulence in biofilm-neutrophil interactions</u>						
Uninfected, hydrogel alone	Bacterial load, wound closure/healing, biomarkers	4	3	-	2	24
<i>P. aeruginosa</i> in hydrogel	Bacterial load, wound closure/healing, biomarkers	4	3	2x4 gel variants	2	192
					Total	684

ETHICAL ASPECTS OF PROPOSED RESEARCH

We intend to use mice for experiments described in this study. We have conscientiously attempted to design our experiments to evaluate as many parameters per mouse as possible. Procedures to reduce discomfort, distress, pain, and injury have been put in place including the use of a preemptive analgesic. Mice used in these experiments will be treated humanely and in accordance with the protocol approved by the Animal Care and Use Committee at Texas Tech University Health Sciences Center (Lubbock, TX).

References for this section:

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Resource Sharing Plan

New bacterial strains generated or harvested will be permanently stored at -80C, in replicate. We will make them available to other researches upon request. After publication and upon request, we will also share experimental protocols, software developed, and data in the form of microscopy movies and analysis and rheology and analysis. Protocols, software, and data will be shared over email or by electronic transfer; strains will be shipped at the requester's expense, if pick-up in person is not practicable. Gene sequences will be deposited in the National Center for Biotechnology Short-Read Archive, the Pseudomonas Genome Database and the Sequence Read Archive. If new genetic sequences are measured, these will be stored as textfiles and deposited in publically-accessible repositories at the same time as any relevant publications. If transcriptome data is measured, it will be stored as textfiles.

Software will be stored as Matlab, IDL, or other code, as appropriate. All microscopy images and associated analysis are stored both in proprietary format and in open format (TIFF for images, TXT for analysis) on a doubly-redundent RAID system on a dedicated Linux server. Rheometer data and AFM force measurements will be stored in open-reading-format textfiles along with metadata, as well as in supported proprietary formats. ELISA, immunoblot, and plate-reader data will be stored as textfiles, with accompanying metadata, and redundantly recorded in accompanying hardcopy. Immunoblot data and the staining data acquired by microscopy will also be stored as tif files, with accompanying metadata, as for the micrographs above. Additional backups are done regularly to removable external hard drives. Archival data will be stored on stable long-term media, currently archive grade DVD-RW. We will upgrade this as necessary as technology progresses, ensuring that our data are always in a readable format. In addition, researchers will keep careful lab notebooks with full details of experiments, so that experiments can be reconstructed as fully as possible even after students have graduated and left the group.

We will promptly prepare and submit for publication, with authorship that accurately reflects the contributions of those involved, all significant findings. Post-publication, we will share data and metadata with other researchers promptly upon request at only the incremental costs thereby incurred. Sufficiently-small computer files will be shared using email or network transfer, incurring no incremental costs. If this is not practical, data will be made available to other researchers on portable media, such as removable hard drive, as supplied by the requester. Bacterial strains generated will be shipped in accordance with standard BSL-2 protocols. Sequences generated, if any, will be deposited in publically-accessible repositories.

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Authentication of Key Biological and/or Chemical Resources

Strains will be acquired from the *P. aeruginosa* transposon mutant libraries – we have in hand those strains that we intend to use in the proposed research. Strains will be authenticated using polymerase chain reaction (PCR) on the respective genes, for wild-type and mutant strains. Strains that have had a gene disrupted by transposon mutagenesis (Δ strains) will show an increase in the size due to the insertion of the transposon. We used this approach previously to authenticate strains used in our work on self-inhibition of bacteria in the presence of antibiotic [1].

Bacteria strains will be stored in a -80 freezer and will periodically be reisolated and checked for their appropriate phenotypic/antibiotic markers.

We will verify antibodies using Western blot and immunoprecipitation using EPS extracts from wild-type, over-expressor, and knockout strains. We used this approach previously to authenticate a Psl antibody and to show that it had a low degree of non-specific binding to non-Psl EPS material [2].

We will use outspread white mice for our animal model. Therefore, we will not perform any authentication per se on the mice, but we will closely review health monitoring reports from the vendor, as well as health reports from the sentinel animals in our laboratory animal facility.

We buy high-quality chemical reagents (including antibiotics) from sources such as Sigma-Aldrich and Invitrogen. Chemicals will be validated by checking pH, mass spectrometry, and confirming known biological activity (such as known minimal inhibitory concentrations of antibiotic for well-characterized strains of bacteria such as *E. coli*).

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