Cohesive Living Protective Surfaces (CLIPS)
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Engineering of bacterial films through surface-display of adhesive proteins, and through expression of temperature- and pressure-sensitive cytoplasmic proteins, provides a powerful new approach to the creation of living materials that are capable of self-repair and response to environmental stimuli.

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Task Objectives

This project was designed to address ten tasks.

Task 1. Demonstrate growth of cohesive, thin bacterial films on the surface of perforated core scaffolds.

Task 2. Determine conditions that enable the viability of the bacterial film for at least one month.

Task 3. Establish the cohesive, interconnected structure of the surface layer.

Task 4. Develop thermal and pressure-sensitive bioswitches.

Task 5. Establish technologies for measuring the physical and mechanical properties of protective surface layers and their interaction with support materials.

Task 6. Demonstrate self-repair of the protective layer in response to tearing and determine the rate of material regeneration at the site of damage.

Task 7. Implement temperature sensing in engineered living materials.

Task 8. Demonstrate self-repair of the hybrid living material in response to mechanical shock and erosion.

Task 9. Demonstrate responsiveness to environmental stimuli at the tissue level.

Task 10. Demonstrate that living protective surfaces remain responsive to temperature and pressure following self-repair of mechanical or erosive damage.

Substantial progress was made toward the objectives proposed in Tasks 1-8 and Task 10. During the COVID-19 laboratory shutdown that extended from March 2020 unt l June 2020, and in consultation with the DARPA program management team, we redirected funds from Task 9 into Tasks 8 and 10. We did not achieve the objectives of Task 9.

Technical Problems

The methods developed in this project yield cohesive, viscoelastic bacterial films that are capable of self-repair and response to environmental stimuli. New methods of analysis were developed to determine the mechanical properties of such films. In many ways, the project went well beyond the objectives originally proposed. Perhaps the major technical problem encountered was the difficulty of achieving controlled regrowth of damaged films. Films injured by tearing or erosion did regrow, and mechanical properties were at least partially

recovered. But regrowth was found to be irregular, such that the initial film structure was not cleanly restored.

General Methodology

New experimental methods for preparation and characterization of engineered bacterial films, and for rendering such films responsive to temperature and pressure, were developed in this project.

Preparation of Engineered Bacterial Films. We evaluated alternative methods for creating thin bacterial layers on perforated scaffolds, for controlling intercellular interactions within the films, for autonomous synthesis and deposition of extracellular matrices, and for mineralization subsequent to film formation.

Film formation by filtration – Most of the work described in this report was performed on films that were formed initially by spreading bacterial cultures on polycarbonate membranes of 0.2 micron pore size. Membranes were placed in inexpensive vacuum filtration devices and subjected to modest vacuum to remove the culture supernatant. Membranes were then transferred to agar plates to allow film growth. This method proved to be reliable and reproducible in yielding films of 50 to 100 micron thickness and controlled lateral dimensions after a few days of growth.

Film formation by blade-coating – We also explored the use of a doctor blade to create the initial bacterial layer. Cultures were thickened by addition of a small amount of xanthan to allow improved control of the coating process. This method was used to create films 100 cm^2 in area, and may be preferred to the filtration method for preparation of large-area films.

Control of intercellular interactions – One of the key premises of our project was that the cohesiveness of engineered bacterial films would be increased by introducing strong intercellular interactions. Our solution to this challenge was to display adhesive proteins on the bacterial cell surface. Two systems proved to be of special interest: i). surface display of the SpyTag/SpyCatcher pair, which allows crosslinking of cells through covalent isopeptide bonds, and ii). surface display of cysteine-functionalized elastin linkers, which enables covalent crosslinking through disulfide bonds. Both methods were successful. We devoted primary effort to disulfide crosslinking because it provided the most attractive combination of simplicity and enhancement of mechanical properties.

Synthesis and deposition of extracellular matrix macromolecules – A second premise of our project was the hypothesis that cohesive films could be prepared by engineering bacterial cells to express and release extracellular matrix proteins. To test this idea, we designed artificial proteins that self-associate through β-sheet and α-helical domains. This approach also succeeded, but was set aside when we discovered the effectiveness of surface display of disulfide-crosslinkable elastin linkers.

Mineralization of bacterial films – The methods described above allowed us to prepare cohesive bacterial films with moduli of tens of kilopascals. In order to form stiffer films, we engineered cells to express alkaline phosphatase, which catalyzes the formation of insoluble calcium phosphate minerals from calcium glycerophosphate, a soluble substrate. This strategy allowed us to increase the stiffness of our films by three orders of magnitude, yielding moduli in the megapascal range. We have not yet achieved the toughness that we've sought in these films; they are hard but brittle. We are continuing to evaluate strategies for increasing toughness.

Characterization of Engineered Bacterial Films. This project developed an innovative bulge-testing method for determination of the mechanical properties of engineered bacterial films. Films prepared as described above were removed from their membrane supports and clamped across an orifice in a fluidic device that allowed for imposition of a controlled pressure difference across the film. Optical coherence tomography (OCT) was used to record the deformation of the film as the pressure increased. In some experiments, tracer particles in the film allowed determination of local changes in dimensions and provided insight into heterogeneity in film properties. Time-dependent film properties could also be determined.

Design of Films that Respond to Temperature and Pressure. Bacterial cells were outfitted with genetic circuits that turn gene expression "on" or "off" in response to changes in temperature. Use of these circuits to control expression of β-galactosidase yielded films that produce dark pigment selectively at low temperatures. The rationale for this design was the expectation that increased light absorption at low temperature would cause cold films to warm in autonomous fashion. Pressure-sensitive films were prepared by expression of gas vesicles in the constituent bacterial cells. Because intact gas vesicles scatter light, the films are turbid. Application of ultrasound pressure waves causes collapse of the vesicles and clarification of the film. Taken together, these two approaches allowed us to prepare both temperature-sensitive and pressure-sensitive films.

Technical Results

Preparation of Cohesive Bacterial Films. Although several methods of preparation of cohesive bacterial films were developed, primary attention was devoted to the filtration method described in the previous section. The method is illustrated in **Figure 1**, and allows simple preparation of films that are tens of microns in thickness and centimeters in diameter. We used this method successfully for films of many different compositions.

Figure 2 shows the consequences of displaying cysteine residues on the bacterial cell surface. Both films shown in **Figure 2** were prepared from *Escherichia coli* cells that were engineered to display elastin-like proteins, roughly 125 amino acids long, on the cell surface. In **Figure 2a**, the displayed proteins lack crosslinking functionality, and the film is soft and minimally cohesive. In **Figure 2b**, the elastin linker is outfitted with a single cysteine residue near the *N*-terminus of the chain. Because the linker is anchored at the *C*-terminus, the cysteine residue is expected to be unencumbered with respect to crosslinking to adjacent cells. In contrast to the behavior

shown in **Figure 2a**, the film in **Figure 2b** is elastic and highly cohesive. We attribute the change in behavior to the formation of intercellular disulfide bonds. Elastic, highly cohesive films were obtained irrespective of the length of the elastin linker, as long as cysteine residues were included for disulfide crosslinking. We explored linkers that ranged in length from roughly 75 amino acids to roughly 300. By using the bulge-test method described in the next section, we discovered that film modulus is insensitive to linker length in this class of films.

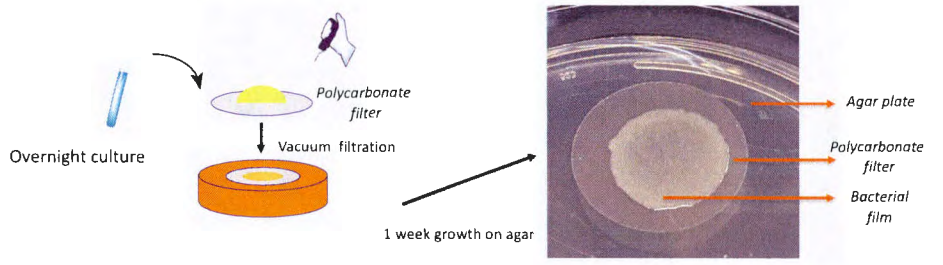


Figure 1. Engineered bacterial films were prepared by depositing overnight cultures of *E. coli* cells on porous polycarbonate membranes. After vacuum filtration to remove culture supernatant, membranes were moved to agar plates to allow film growth. The Figure shows a typical film after 7 days of growth on agar. The membrane is 2.5 cm in diameter.

Characterization of Engineered Bacterial Films. As shown in **Figure 2**, *E. coli* films bearing surface-displayed cysteine residues could be peeled from their membrane scaffolds and transferred to testing devices for analysis of mechanical properties. Bulge-testing of these films proved to be especially instructive. To implement the bulge test, circular sections of films, 3 mm in diameter, were cut from larger film samples and clamped across a 1.5 mm orifice in a fluidic device, as shown in **Figure 3**. The pressure difference across the film was varied and film deformation was monitored by OCT imaging. Plotting stress vs strain provided a measure of film modulus.

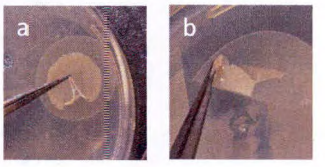
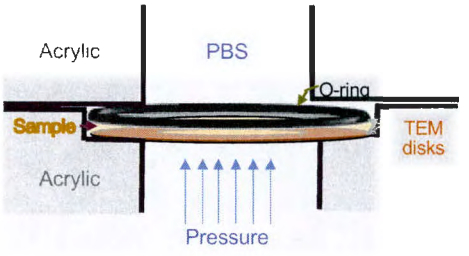
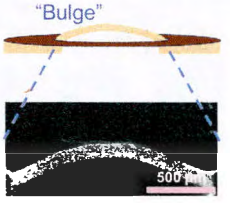


Figure 2. *E. coli* films deposited on porous membranes and grown for 7 days. (a) Cells equipped with elastin linker. (b) Cells bearing elastin linker functionalized with cysteine residue to enable disulfide crosslinking.





The effect of introducing cysteine residues into the surface-displayed elastin linker is illustrated in striking fashion by comparison of the extent to which films with and without cysteine functionality differ in their response to applied stress (**Figure 4**). At comparable levels of stress, the measured strain in films prepared from cysteine-bearing cells

Figure 3. Bulge-testing method developed in the course of this work. (Top) A circular section of film is clamped across a 1.5-mm orifice and subjected to a pressure difference controlled by the heights of fluid columns in channels leading to the upper and lower surfaces of the sample. OCT imaging (bottom) is used to record film deformation.

(designated CE6-AT to signify the cysteine (C) residue, the 150-residue elastin linker (E6), and the autotransporter (AT) system used for surface display) is approximately one-fourth that observed in films made from cells without cysteine functionality (designated E6-AT). E6-AT

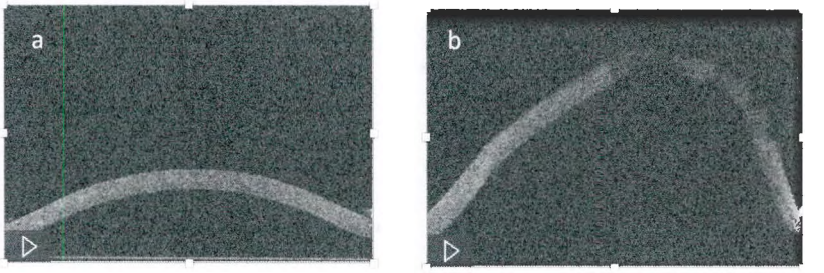


Figure 4. OCT images of *E. coli* films undergoing bulge testing. (a) CE6-AT film (b) E6-AT film. Applied stresses are roughly equal in panels (a) and (b). The E6-AT film is significantly softer than the CE6-AT film, and fails at high stress.

films characteristically failed as stress was increased (**Figure 4b**). Failure was not observed for CE6-AT films (**Figure 4a**), although there is no reason to doubt that failure could be induced at higher stresses than we've applied to date.

Figure 5 shows quantitative stress – strain curves for CE6-AT and E6-AT films. The failure event shown in **Figure 4b** corresponds to the yielding behavior indicated by the flattening of the curves for E6-AT films in **Figure 5**. The slopes of the curves at low strain provide values of the Young's moduli of 57.6 +/- 13.3 kPa for CE6-AT films and 14.3 +/- 4.1 kPa for E6-AT samples. Introduction of cysteine residues (and putative disulfide crosslinking of cells) increases the modulus four-fold and defers film failure to higher stresses than those applied in these experiments.

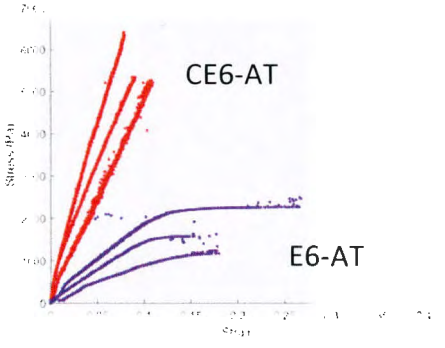


Figure 5. Stress – strain curves determined by bulge-testing of CE6-AT and E6-AT films.

Mineralization of Bacterial Films. Surface-display of adhesive proteins yields *E. coli* films with moduli of tens of kilopascals. We were interested in the possibility that stiffer films could be obtained by mineralization – in particular by deposition of calcium phosphate, an essential constituent of hard biological tissues. To that end, we engineered *E. coli* cells to express alkaline phosphatase (PhoA), which catalyzes the conversion of soluble calcium glycerophosphate into insoluble calcium phosphate minerals. Treatment of films of PhoA-equipped cells with calcium glycerophosphate resulted in mineral deposition within a few minutes and a mineral content of 30-40% by mass after 24 h (as determined by thermogravimetric analysis). The calcium-to-phosphorus ratio in mineralized films (as determined by energy-dispersive x-ray spectroscopy) ranged from approximately 1.30 to 1.67, depending on location within the film. The latter value is characteristic of stoichiometric

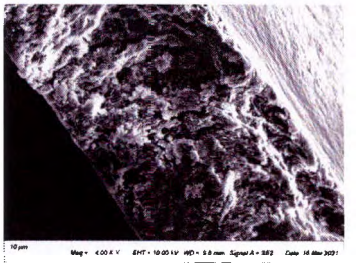


Figure 6. Cross-sectional scanning electron micrograph of mineralized *E. coli* film. The top surface of the film is at right, and appears to be covered by a thin crust of calcium phosphate. Scale bar: 10 microns.

hydroxyapatite. As expected, mineralization increased the stiffness of the films by several orders of magnitude, as determined by nanoindentation. Typical values of the modulus were approximately 2 MPa, 400-fold higher than those measured for unmineralized films. **Figure 6** shows a representative scanning electron micrograph of a cross-section of a mineralized film.

Design of Films that Respond to Temperature and Pressure. We sought to engineer bacterial films that would express different recombinant proteins at high and low temperatures. The long-term objective of this work is the preparation of films that are capable of thermal self-regulation, absorbing light when cold and reflecting light when warm.

Figure 7 shows a genetic circuit that allows differential protein expression at 30°C and 38°C. The key element of the circuit is a temperature-sensitive repressor (*tlpA36*) that is active at temperatures below 36°C. At 30°C, TlpA36 is active and represses expression of the green fluorescent protein (GFP) and the repressor cI_{wt}. Under these conditions, the LacZα peptide is expressed and complements the enzymatically inactive LacZω fragment expressed from the genome of the host (*E. coli* strain DH10B). LacZ catalyzes the deposition of black pigment through the hydrolysis of 3,4-cyclohexenoesculetin and subsequent reaction of the hydrolysis product with ferric ammonium citrate. At 38°C, Tlp36 is inactivated, GFP and cI_{wt} are expressed, and cI_{wt} represses expression of the LacZa peptide.

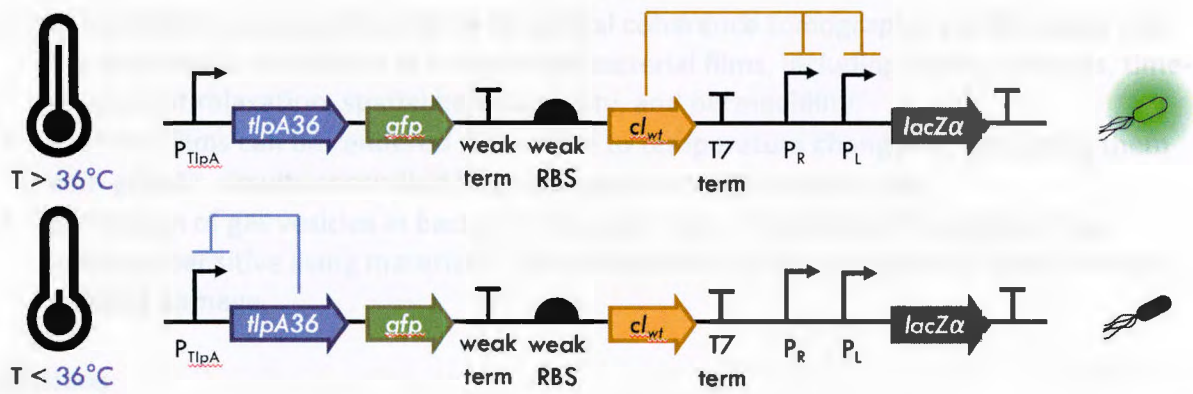


Figure 7. Genetic circuit that controls deposition of black pigment at low temperature and expression of GFP at high temperature.

Figure 8 shows the behavior of bacterial films outfitted with this genetic circuit. Black pigment is deposited in the films at 30°C, but not at 38°C. In contrast, expression of GFP turns on at the higher temperature, off at low temperature. Additional work remains to be done to enable systems such as this one to change color repetitively upon temperature cycling.

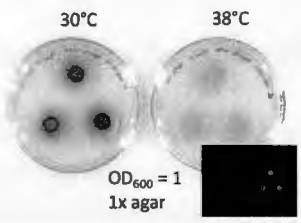


Figure 8. Temperature regulation of transcription by the genetic circuit shown in Figure 7. Black pigment is deposited at 30°C but not at 38°C. The inset shows expression of GFP at 38°C but not at 30°C.

We approached the design of temperature-sensitive films by using bacterial strains that express gas vesicles (GVs) that can be engineered to collapse at specified values of applied pressure.

Because the light-scattering properties of intact and collapsed vesicles differ, the opacity of bacterial films is reduced upon vesicle collapse. **Figure 9** shows an *E. coli* film supported on a porous polycarbonate membrane. In **Figure 9a**, a portion of the film has been exposed to ultrasound to effect vesicle collapse, as illustrated by the appearance of a region of reduced opacity. This process is reversible. **Figure 9b** shows the same film 9 hours later, after re-expression of GVs has increased the opacity of the film.

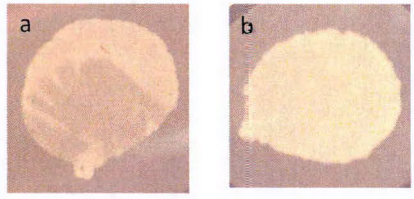


Figure 9. An *E. coli* film that expresses pressure-sensitive GVs. (a) Exposure to ultrasound causes collapse of GVs and reduction in opacity in the lower portion of the image. (b) 9 hours later, re-expression of intact GVs has increased the opacity of the exposed region.

Important Findings

The key findings of this project are the following:

- Surface display of adhesive proteins provides a versatile strategy for engineering of bacterial films with elastic moduli of tens of kilopascals.
- Mineralization of engineered bacterial films increases the modulus into the megapascal range.
- Bulge-testing, coupled to imaging by optical coherence tomography, yields insight into the mechanical properties of engineered bacterial films, including elastic modulus, time-dependent relaxation, spatial heterogeneity, and permeability.
- Bacterial films can be rendered responsive to temperature changes by equipping them with genetic circuits controlled by temperature-sensitive repressors.
- Expression of gas vesicles in bacterial films provides a mechanism for engineering pressure-sensitive living materials. Films engineered in this way recover after pressure-induced damage.

Conclusions

Engineering of bacterial films through surface-display of adhesive proteins, and through expression of temperature- and pressure-sensitive cytoplasmic proteins, provides a powerful new approach to the creation of living materials that are capable of self-repair and response to environmental stimuli.

Implications for Further Research

This project developed many of the elements needed to create multifunctional living materials. As described in earlier sections of this report, we have introduced effective methods for surface display of adhesive proteins, for determination of mechanical properties, for mineralization of bacterial films, and for designing materials that respond to changes in temperature or pressure. An exciting prospect for future work is the integration of these – and other – elements to yield materials that are capable of a broad spectrum of responses, e.g., combining self-repair with changes in optical, mechanical and transport properties in a single material. Further work on

controlled regrowth in response to damage, and on long-term behavior, e.g., in films that combine vegetative cells and spores, also offers substantial promise.

Publications and Presentations

The following publications are complete or in preparation. Many more are anticipated as we gather additional data on the systems developed in this project.

Publications

M. T. Kozlowski, B. R. Silverman, C. P. Johnstone and D. A. Tirrell, Genetically Programmable Microbial Assembly, *ACS Synth. Biol.* **10**, 1351 (2021)

G. J. Lu, L. Chou, D. Malounda, A. K. Patel, D. S. Welsbie, D. L. Chao, T. Ramalingam and M. G. Shapiro, Genetically Encodable Contrast Agents for Optical Coherence Tomography, *ACS Nano* **14**, 7823 (2020)

L. Xiong, M. Garrett, J. Kornfield and M. Shapiro, Engineering Thermally Self-Regulating Bacteria with Temperature-Dependent Light Absorption (in preparation)

L. Ginsberg, S. Sim, P. Chittur, J. Kornfield, D. A. Tirrell and G. Ravichandran, Multiscale Inverse Analysis to Determine Optimal Elastic Properties of *Bacillus subtilis* and N-(hydroxymethyl)acrylamide (in preparation)

K. Şahin, H. Liu, D. A. Tirrell and G. Ravichandran, Time Dependent Mechanical Behavior of Genetically Engineered Bacterial Biofilms (in preparation)

K. Şahin and G. Ravichandran, Investigating Coupled Poroviscoelastic Response of Engineered Bacterial Biofilms, Volume in honor of Prof. Igor Emri's 70th Anniversary (in preparation)

P. Chittur and J. Kornfield, Multiscale Properties of Freestanding Biofilms Using Optical Coherence Tomography (in preparation)

P. Chittur and J. Kornfield, Microstructural Basis of Strain, Softness and Failure of Engineered Elastin-Like Surface-Display Biofilms (in preparation)

Presentations

L. Xiong, Towards a Thermally Self-Regulating Living Material, *American Chemical Society National Meeting* (2021)

G. J. Lu, Biomolecular Engineering of Gas-filled Protein Nanostructures for Imaging Cellular Function in Deep Tissue, *Engineering Biology Research Consortium (EBRC) virtual seminar series* (2020)

G. J. L, Gas-filled Protein Nanostructures for Imaging and Controlling Cellular Function, *Seminar Series at Houston Community College (HCC)* (2020)

L. Ginsberg, P. Chittur, S. Sim, J. Kornfield, D. A. Tirrell and G. Ravichandran, *Bacillus subtilis* as Polymeric Crosslinker and Particle Reinforcement in NHMAA Hydrogel, *Society of Engineering Science Virtual Technical Meeting* (2020)

P. Chittur, H. Liu, D. A. Tirrell and J. Kornfield, Using Freely Suspended Biofilms to Study Interactions Among Bacterial Cells, *International Congress of Rheology* (2020)

P. Chittur, H. Liu, D. A. Tirrell and J. Kornfield, Mechanics of Engineered Biofilms, *American Institute of Chemical Engineers (AIChE) Annual Meeting* (2020)

P. Chittur, H. Liu, D. A. Tirrell and J. Kornfield, Biofilms Get Stressed Out: Engineered Living Materials Under (Mechanical) Stress, *Division of Chemistry and Chemical Engineering Seminar Day, Caltech* (2020)

G. J. Lu, Reporter Genes for Optical Coherence Tomography, *Synthetic Biology: Engineering, Evolution & Design (SEED)* (2019)

L. Ginsberg and G. Ravichandran, Extracting Mechanical Properties of Thin Biofilms Using Inverse Analysis, *Society for Experimental Mechanics (SEM) Annual Conference & Exposition on Experimental and Applied Mechanics* (2019)

K. Şahin, H. Liu, D. A. Tirrell and G. Ravichandran, Mechanical Response of Bacterial Biofilms as Living Engineering Materials, *Society of Engineering Science (SES) 56th Annual Technical Meeting* (2019)

K. Şahin, H. Liu, D. A. Tirrell and G. Ravichandran, Mechanical Properties of Bacterial Biofilms as Living Engineering Materials, *SEM 2019 Annual Conference & Exposition on Experimental and Applied Mechanics* (2019)

G. J. Lu, Genetically Encoded, Acoustically Erasable OCT Contrast Agents, *World Molecular Imaging Congress (WMIC)* (2018)

G. J. Lu, Biomolecular Engineering of Acousto-magnetic Protein Nanostructures for Non-invasive Imaging of Cellular Function, *Biomedical Engineering Society (BMES) Annual Meeting* (2018)

G. J. Lu, Biomolecular Engineering of Acousto-Magnetic Protein Nanostructures for Non-Invasive Imaging of Cellular Function, *AIChE Annual Meeting* (2018)

Follow-on Funding

We have not yet obtained follow-on funding for this work. We are interested in doing so.