Development of Quantum Dot Probes for Studies of Synergy Between Components of the Wood-Degrading Fungal Enzymes

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The overarching goal of this research program is to understand the cellular and molecular mechanisms that underlie the fungus-enzyme-lignocellulose gestalt that will enable the efficient and economical production of liquid fuels from plant biomass. The current project aims to develop quantum dot-based tagging and imaging technologies to simultaneously monitor several lignocellulosic enzymes in real-time and in the natural fungal/lignocellulose environment. The specific goals are: 1) Synthesis of commercially unavailable multiplexed luminescent quantum dots optimized for the study of lignocellulosic degradation. 2) Development of bio-orthogonal tags and protocols that can be generalized to other wood-degrading organisms for labeling secreted enzymes under realistic enzyme-biomass conditions. 3) Advancement of single-molecule imaging technology and the development of assays for studying individual lignocellulosic enzymes.

To achieve these objectives a team with highly complementary expertise was assembled: Tien of the Pennsylvania State University (co-PI) is the co-discoverer of the lignin-degrading enzyme lignin peroxidase, and is a world expert in fungal lignin biodegradation. He has extensive experience and knowledge of the enzymology, genetics, and biogenesis of lignocellulosic enzymes, as well as the culturing and genetic manipulation of white-rot fungi. Nixon of The Pennsylvania State University (co-PI) is the co-discoverer of the ubiquitous two-component signaling system, a central signal transduction pathway in bacterial, fungal and plant cells involved in response to environmental stimuli. He has experience using genetic, molecular, biochemical and structural approaches to link two-component signal transduction to the gene regulation machinery. Yang of Princeton University (PI) is an expert in the development of single-molecule assays and optical probes, and in using them to solve biological and biochemical problems. One of the instruments developed in his group allows real-time tracking of individual nanoscale optical probes in three dimensions with microsecond (μs) time resolution and nanometer localization resolution and will be used as the primary tool for this work. This team is uniquely able to discover how fungal enzymes synergistically degrade lignocelluloses, and the tools developed to accomplish this will be broadly applicable to study other systems in their natural microenvironments.

Goal 1 is to synthesize multiplexed luminescent quantum dots specifically optimized for the study of lignocellulosic degradation which are otherwise unavailable. The design stage for synthesis of compact and water-soluble non-blinking quantum dots is finished. Doped quantum dots that have multiple emitters in a single dot were developed as an improvement to conventional quantum dots which need a thick shell to suppress blinking behavior. The blinking behavior is overcome in doped quantum dots because all of the emitters (dopants) are unlikely to stay in the dark state at the same time when the quantum dots have a high doping concentration. With the bio-orthogonal tagging technologies being developed, the compact and

water-soluble non-blinking quantum dots can be used as markers to follow the translational motions of enzymes.

Goal 2 is to develop bio-orthogonal tags and protocols that can be generalized to other wood-degrading organisms for simultaneously labeling multiple secreted enzymes under realistic enzyme-biomass conditions. The designs for engineering *Trichoderma reesei* to express bio-orthogonally tagged variants of Cel7A, Cel6A and Cel7B are finished. Our strategy improves upon the work reported in the existing literature by providing forward and reverse selections for introducing any recombinant form of the cellulase genes of interest in a way that leaves absolutely no footprint other than the desired change. In accomplishing changes this way, our manipulations will be cleanly restricted to the desired changes in a targeted cellulase. This maximizes our ability in the future to examine the behavior of secreted enzymes in a wood matrix that is being normally degraded by the fungus. The strategy also streamlines the process of testing many recombinant genes by providing for quick construction of desired genes and introducing them back into *T. reesei* via a single homologous recombination event. Strain construction is underway, with preliminary results anticipated by the time of the meeting.

Goal 3 is to advance single-molecule imaging technology and develop assays for studying individual lignocellulosic enzymes. To conquer the multiple scales covered by the action of lignocellulosic enzymes, the previously reported real-time single-particle spectroscopy (RT-3D-SPTS) method has been combined with a scanning two-photon microscope to create a high-resolution, multiscale spectral imaging system. The RT-3D-SPTS is implemented in the near infrared (NIR, 650 nm - 800 nm) region of the electromagnetic spectrum with the intent to avoid intrinsic autofluorescence of biological samples, which is typically confined to shorter wavelengths. The RT-3D-SPTS system has spatial localization resolution down to 10 nm in the lateral dimensions and 20 nm in the axial dimensions, with temporal resolution of down to 10 microseconds. The system is also fitted with a z-locking mechanism that counteracts objective drift to ensure fidelity of position measurement during tracking. The scanning-two photon microscope covers the larger length scales (up to 200 microns) allowing concurrent sampling of the substrate environment while the quantum dot tagged enzyme is investigated spectroscopically by the RT-3D-SPTS system. Crosstalk between the two modules is minimized by spectral separation, reaching further into the NIR (<800 nm) for two-photon excitation and collecting images in the visible (400 nm – 600 nm). This allows for imaging of conventional dyes, fluorescent proteins or autofluorescence. The system is further outfitted for biological samples with an onboard temperature control chamber. This multiscale imaging system has already been used to track freely diffusing nanoparticles in the presence of fluorescently labeled single cells.

This material is based upon work supported by the Department of Energy under Award Number DE-SC0006838.