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

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Project Goals: Our team is focused on developing a comprehensive understanding for the actions of cellulose degrading enzymes. Our team's approaches to uncover mechanistic insights into cellulose degradation involve 1) Expression and purification of cellulases from bacterial and fungal sources 2) One-to-one conjugation of expressed enzymes to custom-designed fluorescent quantum dots, which provide constant, strong fluorescence for the imaging of cellulases on the single enzyme level over long timescales needed to observe their predicted slow, processive action 3) Real-time multi-scale 3D imaging of cellulases in complex with cellulose using a novel multi-resolution microscopy, allowing images of mobile cellulases in three dimensions with very high localization precision. These experiments will allow the generation of models for the mechanisms of different cellulases and determination of their synergistic effects, paving the way for the engineering of more efficient cellulases for biomass conversion.

As the negative environmental impacts of fossil fuel use become increasingly apparent, it is imperative that other renewal fuel sources with low carbon footprints are developed. Plant biomass, particularly lignocellulose, is an abundant source of bioenergy that could potentially be harnessed without any net carbon emissions and utilized without competing with existing food crops. However, current methods for breaking down lignocellulose into biofuel precursor sugars are costly and inefficient. Fungal and bacterial species, on the other hand, have evolved effective means to breakdown lignocellulose using a plethora of cell wall-degrading enzymes. Through a better understanding of these enzymes it may be possible to develop schemes to utilize, and even engineer, microorganisms and their enzymes to efficiently convert lignocellulose into a viable fuel source. Study of the enzymes that breakdown lignocellulose, including cellulases, has been hampered by the factors such as the heterogeneous nature of their substrate and the fact that they carry out interfacial catalysis on a solid substrate. Single-molecule experiments offer a means to characterize these cellulases by observing the movements of signal cellulase enzymes on natural substrates. For example, these experiments will shed light on processivity of these enzymes and give insight into how these enzymes can breakdown these large recalcitrant strands of cellulose. These constitute the first steps in developing these enigmatic enzymes into workhorses for biofuel production.

Team members at Princeton University and Penn State have been developing technologies to allow the study of cellulase enzymes on the single-molecule level. Currently, two fungal cellulases and one bacterial cellulase, all engineered with orthogonal tags to allow conjugation to luminescent quantum dots, have been expressed and purified. These three cellulases cover the three main modes of action for known cellulases, being reducing end exocellulase activity (*T.reesei* Cel7a), non-reducing end exocellulase activity (*T. reesei* Cel6a) and endocellulase activity (*T. fusca* Cel6a). As proper

glycosylation of fungal cellulases has been found to be important for activity and incorrect amounts of glycolyation has been seen when fungal enzymes as expressed in high expression strains, the tagged fungal cellulases in our study have been expressed from their native species *T. reesei*, with proper levels of glycosylation confirmed by mobility assays. To label the cellulases for imaging, we have carried out and optimized synthesis of giant quantum dots (gQDs), previously established to mitigate switching between fluorescent and dark states, known as 'blinking' behavior, seen with conventional quantum dots due to defects on the quantum dots surface (1). The synthesis of gQDs has been carried out through successive additions of inorganic layers to a CdSe core to mitigate these surface effects. Analysis of the spectral properties of these gQDs has revealed that they do not switch between fluorescent and dark states and display constant fluorescence for minutes on the single-molecule level using laser powers needed for single-molecule experiments. The conjugation of one gQD to one cellulase molecule is essential for real-time imaging of cellulases on their cellulose substrate. This one-to- one conjugation is not a trivial matter, as cellulases must be bound to the spherical surface of a quantum dot with the potential of multiple enzymes binding to each quantum dot. We have designed an electrophoretic separation procedure using very low gel concentrations and high voltage to allow the separation of gQD-cellulase conjugates of different stoichiometries. This procedure has been modified to allow the one-to-one conjugation of the different cellulases to their gQD labels. To follow the activity of single cellulase-gQD conjugates in real-time in the context of the substrates, we have developed an entirely new imaging modality. It capitalizes on the real-time capabilities previously developed in the Yang lab (2,3). This multi-resolution imaging system can concurrently image objects with large differences in size and has recently been used to observe the interaction of peptide-coated nanoparticles with living cells as a proof of concept (4). This multiple resolution capacity allows us to follow the movement of gQD- labeled cellulases in real-time, while simultaneously imaging the fluorescently labeled cellulose substrate that it is interacting with. This allows correlation of cellulase-cellulose interaction with cellulase movement and gives direct evidence of cellulase processivity. Single molecule trajectories of many individual cellulase molecules are being collected to gain a better understanding of the mechanisms of these enigmatic enzymes.

References:

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