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08-ERD-071 Final Report: New Molecular Probes and Catalysts for Bioenergy Research

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BACKGROUND AND PROJECT RATIONALE

A major thrust in bioenergy research is to develop innovative methods for deconstructing plant cell wall polymers, such as cellulose and lignin, into simple monomers that can be biologically converted to ethanol and other fuels. Current techniques for monitoring a broad array of cell wall materials and specific degradation products are expensive and time consuming. To monitor various polymers and assay their breakdown products, molecular probes for detecting specific carbohydrates and lignins are urgently needed. These new probes would extend the limited biochemical techniques available, and enable real-time imaging of ultrastructural changes in plant cells. Furthermore, degradation of plant biomass could be greatly accelerated by the development of catalysts that can hydrolyze key cell wall polysaccharides and lignin. The objective of this project was *to develop cheap and efficient DNA reagents (aptamers) used to detect and quantify polysaccharides, lignin, and relevant products of their breakdown*. A practical goal of the research was to develop electrochemical aptamer biosensors, which could be integrated into microfluidic devices and used for high-throughput screening of enzymes or biological systems that degrade biomass.

Several important model plant cell wall polymers and compounds were targeted for specific binding and purification of aptamers, which were then tested by microscopic imaging, circular dichroism, surface plasmon resonance, fluorescence anisotropy, and electrochemical biosensors. Using this approach, it was anticipated that we could provide a basis for more efficient and economically viable biofuels, and the technologies established could be used to design molecular tools that recognize targets sought in medicine or chemical and biological defense projects.

Apart from the well-known genetic functions of DNA and RNA, nucleic acids are capable of binding a variety of target molecules with high affinity and specificity, imparted by complementarity to molecular shape [1]. Short, single stranded DNA molecules known as aptamers can be generated through combinatorial methods, isolated and enriched through reiterative cycles of target binding. Then, using standard DNA sequencing and synthesis techniques, individual aptamers can be characterized and scaled up. Aptamers have been selected that bind to small molecules as well as proteins, and recognize these targets in real biological systems including viral particles and mammalian cells [1]. Prior characterization of aptamers that bind cellulose (β1→4 linked glucose polymer)[2] and cellobiose (β1→4 linked glucose disaccharide)[3] provided technical precedents and a practical starting point for this project.

The research and development of molecular probes to be used in an important problem in bioenergy research at LLNL is relevant to the bioenergy mission in the DoE Office of Science, and complementary with ongoing projects funded by the DoE Genome Sciences Program both at LLNL and at the Joint BioEnergy Institute (JBEI).

EXPERIMENTAL CHALLENGE

Relatively small, single stranded DNA known as aptamers can be easily synthesized and, once selected, used as highly specific probes of molecular structure. However, even though this exciting technology has been developed over the last 15 years, the most challenging aspect is the selection stage of the process of aptamer isolation. In general terms, a combinatorial approach is used to synthesize a library of randomized DNA sequences that are tested for binding affinity to one or more target molecules. In our project, the targets were chosen for relevance to biofuel processing: either plant cell wall materials such as cellulose, or the small molecule products of polysaccharide degradation.

Aptamer binding must be separated into non-specific and specific fractions, where the latter contains many fewer DNA sequences and is at very low concentration. These sequences are amplified using polymerase chain reaction (PCR) techniques and then taken through the binding regime again. After

several rounds, a final fraction is selected that contains only a few DNA sequences that can be prepared for analysis. It is well known that these last steps of selection incur the most risk, but the investment of time and energy in many cases has paid off with patents and scientific discoveries. One further risk in our project was the attempt to find aptamers that bind specifically to *insoluble polymers*, namely cellulose and its associated polysaccharides. Based on the lack of reports in the current literature, the field of polysaccharide- and disaccharide- specific aptamers is very open. This could also mean that attempts to acquire these have failed. In addition, there may be a great deal of sensitivity to small changes in experimental conditions, as we found that even though previously published aptamer sequences could be obtained, the results of our binding studies did not generally match those reported by other investigators.

RESULTS

Cellulose and cellobiose targets. Several cellulose-specific aptamer sequences were found in recent literature, and for our experiments five of these were synthesized to incorporate a covalently bound fluorescent compound. After mixing with crystalline cellulose, these were examined using fluorescence microscopy and found in most cases to have no appreciable binding affinity (**Figure 1**). However, one aptamer appeared promising due to the high level of fluorescence intensity in the cellulose particles. This aptamer, Cel183[3], was found to disassociate from the bound complex in the presence of the disaccharide cellobiose (a major product of the enzymatic hydrolysis of cellulose), indicating competition for binding site(s) on cellulose by cellobiose.

A. Imaging cellulose particles soaked with Cel183

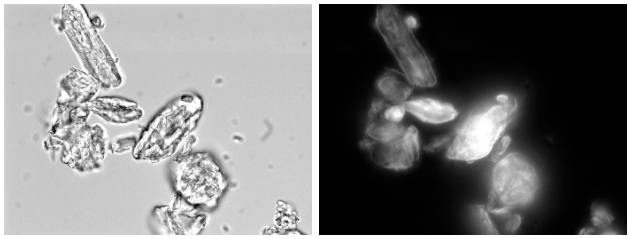

Figure 1. Analysis of Cel183 aptamer binding to cellulose. Microscopic imaging of cellulose crystals, average size 20μm, after soaking with the aptamer Cel183. Cellulose alone is not fluorescent, and other aptamers did not cause cellulose to fluoresce intensely (not shown). Left, brightfield; right, fluorescence.

Using circular dichroism spectroscopy to measure changes in molecular shape, the initial spectrum of Cel183 was shifted significantly by mixing in 1mM cellobiose, demonstrating a conformational change in the aptamer (**Figure 2**). From this data, we concluded that Cel183 has a "relaxed" shape in solution, in which perhaps many nucleotide residues are unshielded, and that when specific contacts are made between the DNA bases and the disaccharide, the aptamer snaps into a specific shape that fits around the bound ligand. The spectrum of the aptamer:cellobiose complex distinguishes it from either component alone and from other combinations, such as Cel183 with the disaccharide lactose. This specific signal was not seen with other cellulose-specific aptamers mixed with cellobiose, for example, Cel16[3].

A relatively high concentration (1mM) of cellobiose was needed to achieve the putative conformational change in Cel183. To select new aptamers with higher affinity to cellulose and cellobiose, i.e. with dissociation kinetics (K_D) in the nanomolar range, we performed a competition experiment in which randomized DNA sequences were mixed with low concentrations of Cel183, and aptamers that bound to cellulose were eluted with cellobiose and amplified. However, no additional aptamers were isolated in several attempts of this experiment. Also, we used both fluorescence anisotropy and surface plasmon

resonance (SPR) to measure the kinetics of aptamer binding to cellobiose and cellulose, respectively, but these experiments also failed. The anisotropy instrument did not take simple measurements properly, and was too expensive to repair. For SPR, the chemistry for immobilizing either cellobiose or cellulose on the SPR chip proved too difficult for us to carry out. Without having a second measure besides CD to assess binding, the development of this aptamer was not pursued any further.

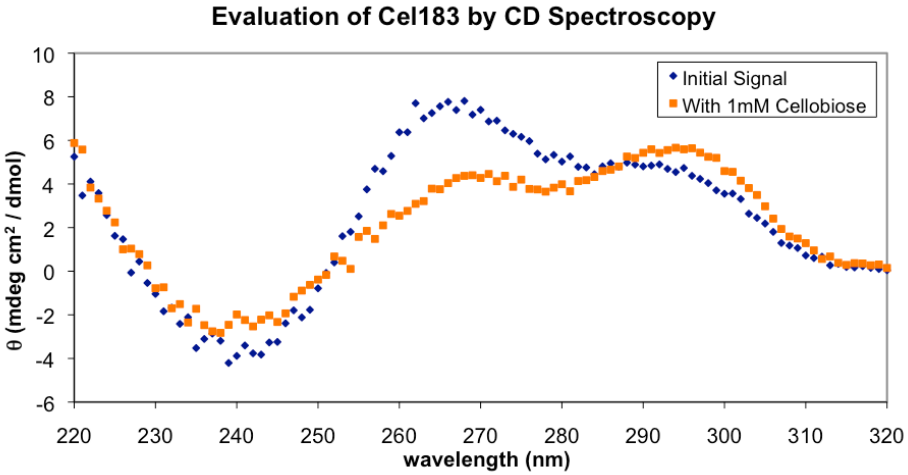

Figure 2. Analysis of Cel183 aptamer binding to cellobiose. Circular dichroism (CD) spectroscopy of Cel183 before and after addition of cellobiose at 1mM. The change in spectrum is quite apparent and indicative of a conformational change in the aptamer immediately upon binding.

Apart from cellulose (β1→4 glucose$_n$), which is completely insoluble, we performed aptamer selections using the following targets: β1→3 glucose$_n$, known as curdlan, or callose – a plant wound polymer that is gelatinous in aqueous suspension; β1→4 xylose$_n$ (xylan) – a hemicellulose that is partially soluble; and, α1→4 galacturonic acid – a pectin that is completely soluble. For these 3 polysaccharides, 30 aptamers each were isolated and sequenced. After the sequences were analyzed for predicted base-pairing and potential 2D folding patterns, for each target we synthesized several aptamers to include a fluorescent tag. Experiments targeting these polysaccharides over the duration of the project were mostly taken up with method development, which included: removal of experimental artifacts observed during PCR amplification (that appeared only in cycles 18 and over); application of different chemical techniques for immobilizing polysaccharides onto column resins; cloning and sequencing potential aptamer candidates; and, incubation of derivitized polystyrene beads and cultured plant cells with fluorescent aptamer candidates for localization of cell wall features.

SUMMARY

Short, single-stranded DNAs (aptamers) that bind with high affinity to plant disaccharide and polysaccharides were targeted to provide new assay tools for cell wall deconstruction during biofuel processing. A collection of aptamers that recognize each of several polymers and polymer byproducts would be extremely valuable in biofuel process engineering. To achieve this, we synthesized end-tagged, randomized nucleotide libraries, and attempted to select those that bound specifically to each of the carbohydrate targets. Although many aptamers were isolated, cloned and sequenced, and tested in biochemical and cell biology assays, we concluded that no consensus sequences were enriched, and that we had not selected any truly target-specific aptamers.

REFERENCES

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