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Labeling and Purification of Cellulose-Binding Proteins for High Resolution Fluorescence Applications

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The study of enzymatic reactions through fluorescence spectroscopy requires the use of bright, functional fluorescent molecules. In the case of proteins, labeling with fluorescent dyes has been carried out through covalent reactions with specific amino acids. However, these reactions are probabilistic and can yield mixtures of unlabeled and labeled enzymes with catalytic activities that can be modified by the addition of fluorophores. To have meaningful interpretations of results from the study of labeled enzymes, it is then necessary to reduce the variability in physical, chemical, and biological characteristics of the labeled products. In this paper, a solid phase labeling protocol is described as an advantageous alternative to free solution labeling of cellulose-binding proteins and is applied to tag cellulases with three different fluorophores. The products from the labeling reactions were purified to remove the unreacted dye and separate labeled and unlabeled enzymes. Characterization of the catalytic and spectroscopic properties of the isolated labeled species confirmed that highly homogeneous populations of labeled cellulases can be achieved. The protocol for the separation of labeled products is applicable to any mixture of labeled proteins, making this an attractive methodology for the production of labeled proteins suitable for single molecule fluorescence spectroscopy.

Single molecule fluorescence spectroscopy (SMFS) techniques provide measurements of molecular events and reveal behaviors otherwise obscured by ensemble averages. SMFS has been used to study molecular associations,^{1,2} displacement,^{3,4} conformational

changes,^{5,6} and catalytic activity.^{5,7} The application of SMFS to enzyme studies requires tagging the proteins with bright, fluorescent moieties, while retaining wild-type activity. Organic dyes are small molecules that can confer fluorescence capabilities with minimal effect on enzymatic activity.^{8,9} Additionally, because the size of organic dyes lies in the 500–1500 Da range, they offer the advantage of minimally increasing the enzyme's mass even at high degrees of labeling (DoL). Limitations of performing measurements on molecules labeled with a single fluorophore are intensity fluctuations and bleaching,^{10,11} which calls for enzymes labeled with multiple fluorophores without loss of catalytic activity.

Labeling with organic dyes yields mixtures of labeled enzymes based on the quantity and accessibility of reactive sites.¹² This polydispersity of labeled molecules, along with unlabeled enzymes, can introduce significant variability, both in spectral and functional properties. Thus, to carry out SMFS and obtain results that accurately describe biological activities, it is important to purify the labeled mixtures by separating the different populations of labeled enzymes and characterize those populations to ensure wild-type catalytic activity. Few studies have fully characterized labeling through a variety of labeling molar ratios (LMRs),⁸ concentrations,¹³ and labeling methods,^{14–16} explored labeled product distribution, or evaluated the SM performance of labeled enzymes^{12,16–18} and their functionality.^{8,19} This manuscript presents methods to label *Thermobifida fusca* cellulases Cel5A, Cel6B, and Cel9A (as models of cellulose-binding proteins), with three

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different dyes, separate the labeled products, and test their catalytic and optical properties.

Cellulases are enzymes that catalyze the hydrolysis of cellulose by cleaving glucosidic bonds within cellulose chains (endocellulases) or from chains' ends (exocellulases) producing soluble oligosaccharides.²⁰ They are classified as either random or processive depending on whether they catalyze single or multiple bond cleavages in succession. Cellulases are key in the production of soluble sugars from lignocellulosic material that can be used for biofuel and bioproducts production and have become the focus of many fluorescence studies that explore their catalytic activity, processivity, and interaction with cellulosic materials.^{21–27} Yet, these studies have lacked the resolution to elucidate processivity and catalysis during cellulose depolymerization at the nanoscale, which could be provided by SMFs.

The goals of this paper were (1) to develop and optimize a solid phase (SP) labeling method, where cellulases are immobilized on cellulose, as an advantageous alternative to free solution (FS) labeling, where the proteins freely diffuse in an aqueous medium; (2) to separate the labeled mixtures obtained into their components; (3) to characterize the activities and optical properties of the separate labeled products.

EXPERIMENTAL SECTION

Cellulase Production and Labeling. *T. fusca* cellulases Cel5A, Cel6B, and Cel9A were expressed and purified by size exclusion as previously described.^{21,28} Cellulases were labeled with amine-reactive AF350, AF488, or AF647 (Invitrogen, Carlsbad, CA) in FS or on SP. In both strategies, 3000 pmols of enzyme were labeled at 10 μM concentration with LMRs of 10:1, 30:1, or 100:1. All reactions were conducted in triplicate at 4 °C, for 24 h, in the dark, and were stopped by unreacted dye removal.

Free Solution Labeling. Enzymes and dye were mixed in a low adhesion tube; the volume was completed to 270 μL with 35 mM boric acid, 50 mM NaCl buffer pH 8.3 (labeling buffer), and 30 μL of 1 M sodium bicarbonate was added. Removal of unreacted dye from labeling mixtures was performed as previously described through native polyacrylamide gel electrophoresis (n-PAGE), and protein was recovered via electroelution.²⁶

Solid Phase Labeling. An 8:1 (w/w) mixture of CF11-BMCC resuspended at 32 mg/mL was prepared as the SP binding matrix (CF11 cellulose powder from Whatman, Piscataway, NJ, and bacterial microcrystalline cellulose (BMCC) from Monsanto Cellulon, San Diego, CA). The ratio of the components was chosen

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Table 1. Fluorophore and Enzyme Properties^a

molecule	λ_{EX} (nm)	CF	ε	MW (Da)	charge (at pH 7.0)	lysine residues (accessible/total)
AF350	346	0.19	19000	410	0	
AF488	495	0.11	71000	643	-1	
AF647	650	0.03	239000	~1300	-3	
Cel5A			97100	46300	-16	3/10
Cel6B			115150	59600	-37	4/11
Cel9A			210670	90400	-52	10/20

^a Data for fluorophores from Alexa Fluor Amine-Reactive Probes Catalog, Molecular Probes. The number of accessible lysine residues was calculated as a minimum of 30% surface exposure.

based on binding capacity (Figure S-1 in the Supporting Information). SP labeling was done in Spin-X columns (0.45 μm nylon membrane, Costar, Lowell, MA). For the optimization of SP labeling, two protocols were tested: SP_A was carried out with constant agitation under the same volume conditions as FS, while SP_B used reduced volume and no agitation (details for the two protocols are available in the Supporting Information). The binding matrix was loaded onto the columns, and excess buffer was removed by centrifugation. Then, enzymes were added in 300 μL of labeling buffer and allowed to bind for 1 h at 4 °C with end-over-end agitation, after which the buffer was removed by centrifugation. The fluorophore was added, and the volume was completed to the specified volume by addition of labeling buffer and 1 M sodium bicarbonate. After labeling, unreacted dye was removed by six consecutive centrifugations (5000g, 2 min), followed by additions of 500 μL of labeling buffer. Between washes, samples were agitated using a magnetic stir bar (500 rpm, 5 min), after which the sidewalls of the tube were scrubbed using an external magnet. The labeled enzymes were recovered by three elutions with chilled ethylene glycol (EG). Two washes were done with 400 μL of EG, incubation in ice for 10 min, and centrifugation (6000g, 5 min). The third was done with 200 μL of EG and centrifugation (10 000g, 10 min). The flow-throughs were immediately diluted 3× in labeling buffer and kept in ice.

Concentration and Characterization. Removal of EG, buffer exchange with 20 mM MES pH 6.0 (MES buffer), and concentration of labeled products was done in Vivaspin4 columns (10 kDa MWCO, Sartorius, Bohemia, NY). Protein concentrations (C_p) and D/P were calculated by absorbance according to

$$C_p = \frac{[A_{280} - (A_{\text{EX}} \times CF)]}{\varepsilon_p} \quad (1)$$

$$\text{D/P} = \frac{A_{\text{EX}}}{\varepsilon_F \times C_p} \quad (2)$$

where A_{280} is the absorbance at 280 nm, A_{EX} is the absorbance at the fluorophore's excitation wavelength, CF is the fluorophore correction factor, and ε is the extinction coefficient (Table 1). The term D/P was used to denote the presence of labeled and unlabeled proteins in the labeled mixture and differentiate it from the DoL for pure labeled species.¹⁸

Separation of Labeled Mixtures. Separation of labeled mixtures was done by fast protein liquid chromatography (FPLC) at 21 °C with MES buffer as the mobile phase, using an ÄKTA

Explorer 10S FPLC system and a Resource Q column (1 mL, GE Healthcare, Piscataway, NJ). The column was equilibrated to the initial NaCl concentration (80 mM NaCl for Cel5A, 250 mM for Cel9A, and 250 mM for Cel6B), and 1 mL of labeling mixture was injected. Proteins were eluted by applying a 250 mM linear salt gradient (3 mL/10 mM NaCl) at a 1.5 mL/min flow rate. Absorbance was recorded at 280 nm for proteins and at 346, 495, or 650 nm for samples labeled with AF350, AF488, and AF647, respectively. Eluted proteins were collected in 1 mL fractions and partitioned according to the peaks observed in the chromatograms. Fractions were consolidated and concentrated, and buffer was exchanged with 50 mM sodium acetate pH 5.5 using Vivaspin 4 columns (Sartorius, Bohemia, NY). C_p and DoL were measured for the separate labeled products.

Hydrolysis Assay. The hydrolytic activities of labeled cellulases were quantified on BMCC by measuring the total oligosaccharides produced and comparing them to those of unlabeled enzymes. Hydrolysis reactions were carried out in 600 μ L of sodium acetate buffer with 1.5 mg of BMCC and 5 pmol of enzyme. Hydrolysis reactions were performed in triplicate at 50 °C for 24 h under constant agitation. Filtration through the Spin-X columns (5000g, 5 min) stopped the reaction by removing the cellulose substrate. The flow-through was incubated for 1 h on ice in a column with fresh BMCC to capture remaining enzymes and then recovered by centrifugation (5000g, 5 min). Oligosaccharides were measured by refractive index using a Shimadzu HPLC (Shimadzu, Columbia, MD). Hydrolysis samples (injection volume: 50 μ L) were eluted at 0.6 mL/min through an HPX-87P Bio-Rad Aminex column (BioRad, Hercules, CA), heated to 84 °C, using 18.2 MΩ water as the mobile phase. The chromatograms for the products were baseline corrected, and the peaks were fitted to Gaussian functions using Origin8Pro (OriginLab, Northhampton, MA). Standards containing cellobiose, cellotriose, cellooligosaccharides, and glucose were used to calculate the oligosaccharide concentrations from peak areas. The total oligosaccharides produced were converted into either cellobiose (Cel5A and Cel6B) or cellotetraose equivalents (Cel9A), and hydrolytic activities were expressed as nmol of cellobiose or cellotetraose equivalents/nmol enzyme/h.

Characterization of Photon Output and Imaging. SM fluorescence from AF647 labeled Cel9A cellulases was recorded using an inverted microscope (IX-71 Olympus, Center Valley, PA) equipped for laser-induced fluorescence. A 647 nm laser beam (ArKr, Melles-Griot, Carlsbad, CA) incident on a dual-band dichroic mirror (488/647, Chroma Technology, Rockingham, VT) was focused on a microfluidic channel through a 60×/1.2 NA water-immersion objective (UPlanSAPO, Olympus, Center Valley, PA). Fluorescence was filtered through an emission filter (680/40M, Chroma Technology, Rockingham, VT) and collected using a 100 μ m diameter core multimode optical fiber (OZ Optics, Ottawa, ON, Canada). Photons emitted by single AF647-labeled cellulases were detected by avalanche photodiodes (APDs, PerkinElmer, Waltham, MA). A high-speed correlator (correlator.com) was used to record the APD photon count. Microfluidic channels were fabricated as previously described^{29,30} to constrict the

excitation volume to submicrometer dimensions and achieve SM confinement at 1nM concentration.

The microfluidic channels were initially filled with running buffer (4× PBS containing 0.5% Triton X-100 v/v, both from Sigma Aldrich, St. Louis, MO). Electrokinetic drive was established by applying 50 V across the fluidic channel. The combined autofluorescence of the channel and buffer was determined by illumination with 500 μ W laser power. The channels were then cleared and filled with 200 pM labeled enzymes in running buffer, and fluorescence from the SMs was recorded. Fluorescence photon counting/burst histogram analysis was performed using a custom Matlab routine.³⁰ The threshold for a SM event was established at 5σ above background. The photon output was collected for a total of 10 000 molecules. A histogram of SM data was plotted using 25 photon bin, and the resulting distributions were fitted to Gaussian functions to determine average photon output. Images of Cel9A cellulases (100 fM) bound to fluorescently labeled cellulose were acquired using a FV1000 confocal microscope and a 100×/1.45 NA oil immersion objective (Olympus, Center Valley, PA).

Statistical Analysis. All statistical analyses were performed with raw data using Origin8Pro (OriginLab, Northhampton, MA). The comparison between D/P ratios for all labeling treatments and cellulases was performed via a two-way ANOVA and Bonferroni significance test using confidence intervals (CI) set to 99%. D/P ratios for each cellulase were compared by pairing the data grouped by LMR with either the fluorophore or labeling method (while maintaining the other factor constant). Statistical analysis of catalytic activity was performed via a two-tail Student *t* test for the mean of unpaired samples. The activity of each assayed labeled fraction was directly compared with the unlabeled enzyme and tested at 95 and 99% CI.

RESULTS AND DISCUSSION

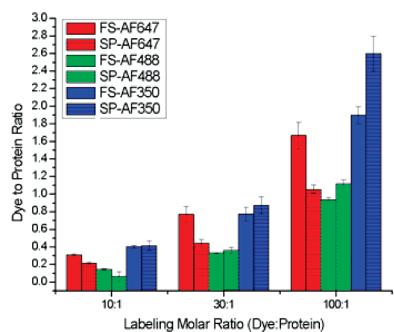
Cellulase Labeling. Removal of unreacted dye after labeling is important to stop the labeling reaction and avoid excess dye that contributes to fluorescence background in quantitative applications. The two labeling protocols applied different strategies for unbound fluorophore removal: in FS, n-PAGE followed by electroelution of the labeling mixtures was used to effectively separate the free dye from the proteins (Figure S-2 in the Supporting Information); in SP, free dye was eluted through centrifugation and washing (Figure S-3 in the Supporting Information). Both strategies successfully removed excess dye from labeled samples.

The common measure of labeling efficiency is the ratio of dye to protein molecules present in the sample (referred to as dye to protein ratio (D/P) for labeled mixtures or DoL for labeled products after FPLC separation). Preliminary experiments labeling Cel9A with AF647 showed that, for FS, the D/P varied linearly with LMRs between 10 and 200 (Figure S-4 in the Supporting Information). Thus, only LMRs of 10, 30, and 100 were compared in the labeling experiments. Figure 1 shows that, as the LMR increases, the D/P for all enzyme-fluorophore-labeling method combinations significantly increases (CI > 99%) due to a greater reaction rate between fluorophores and reactive sites. Additional

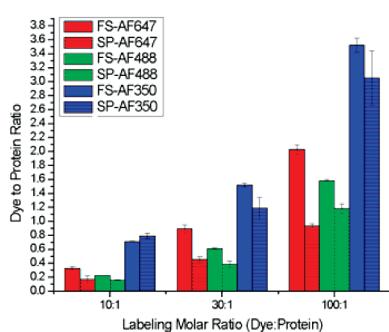
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Cel5A



Cel6B



Cel9A

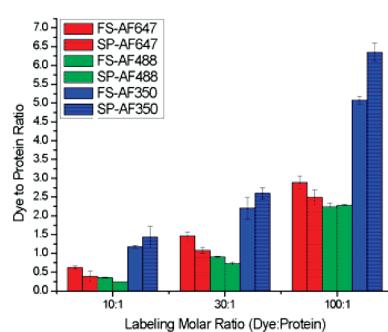


Figure 1. Comparison of labeling efficiency for the different fluorophore–enzyme combinations measured through D/P. SP_A and FS labeling protocols were compared for labeling with three fluorophores (AF350, AF488, and AF647) at three LMRs (10:1, 30:1, 100:1).

labeling experiments comparing SP_A and SP_B protocols showed that D/P was also improved if the reaction volume was reduced, increasing the fluorophore concentration (Figure S-5 in the Supporting Information). Thus, SP_B enabled labeling reactions with minimal volume and was used to increase the D/P without increasing the fluorophore loading.

Under identical labeling conditions, each fluorophore had different labeling efficiency (Figure 1), with AF350 exhibiting the highest and AF488 exhibiting the lowest efficiency. This was attributed to the structural characteristics of each fluorophore. Because AF350 is the smallest fluorophore, it has greater accessibility to lysine residues and yields higher D/P. Charge and hydrophobicity can also affect the interactions with proteins and alter the labeling efficiency. It was also observed that, for any given fluorophore, the D/P for each cellulase is different (Figure 1) and correlates with the number of accessible lysine residues (Table 1). Thus, both the amino acid sequence and protein conformation are as important as the size, charge, and hydrophobicity of the fluorophore and can affect the labeling efficiency.

Comparison of the labeling methods in FS and SP showed that their D/P was significantly different for all fluorophore–enzyme combinations (Figure 1, CI > 99), except Cel5A-AF488, Cel6B-AF350, and Cel9A-AF488, where no difference was observed. Overall, labeling of any enzyme with AF647 was more efficient in FS, labeling of Cel6B with AF488 was more efficient in FS, and labeling of Cel5A and Cel9A with AF350 was more efficient with SP. Other fluorophore–enzyme combination labeling efficiencies were similar for FS or SP. The general trend was that, under identical conditions, SP yielded lower D/Ps than FS. Cellulose binding changes the accessibility to lysine residues either via conformational changes or by steric hindrance. The fact that AF647 consistently labeled more efficiently with FS suggests that, as the enzymes bind to cellulose, access to lysine residues becomes restricted, preventing labeling by large fluorophores.

Separation of Labeled Mixtures. FPLC chromatograms (Figure 2 and Figures S-6 and S-7 in the Supporting Information) show the separation of mixtures obtained from labeling cellulases in FS or SP with AF647, AF350, and AF488. The first eluted peak represents the unlabeled protein, which only shows absorbance at 280 nm. All other peaks showing absorbance at both 280 nm and the fluorophore absorbance wavelength represent labeled products. Consistent with an increase in D/P, as LMR increased, the amount of unlabeled protein decreased and in some cases disappeared, while the total labeled products increased. A surpris-

ing find was that labeled mixtures with D/Ps above two still contained unlabeled proteins, meaning that characterization of mixtures based solely on D/P does not guarantee that all molecules possess at least one label. The separated fractions were partitioned according to the peaks observed in the chromatograms and characterized for their DoL.

The separation between unlabeled and labeled enzymes increased with increasing fluorophore charge (Figure 2, Figures S-6 and S-7 in the Supporting Information, and Table 1). This was also true for the separation of the labeled products, where more negatively charged fluorophores had labeled peaks that were better resolved than those from less charged fluorophores. Peaks that were well resolved represent pure labeled species, as their measured DoL was always close to an integer. On the other hand, convoluted peaks can represent a mixture of two or more labeled species. Products with the same DoL, but different elution times, can also represent different combinations of labeled sites. Overall, molecules with higher DoL eluted later than those with lower DoL, and protein distribution shifted from lower to higher DoL products as LMR increased (Figure 2 and Figures S-6 and S-7 in the Supporting Information). The results suggest that anionic exchange FPLC can separate enzymes labeled with few fluorophores better than those that contain multiple tags. Yet, products with high DoL represent combinations of few labeled products and are still more homogeneous than labeled mixtures.

The chromatograms obtained from enzymes labeled in FS versus SP showed significantly different distributions. Although some of the labeled peaks coincide for both labeling methods, the profiles are different in number of peaks, their size, and distribution (Figure 2). Because, under identical conditions, the D/P was higher for FS than for SP, differences in the amplitude of the labeled species were expected between methods. However, differences in the number and separation between species were also observed. Chromatograms for SP usually had fewer and better resolved peaks than those for FS. Thus, the comparison between the separations obtained from mixtures labeled with FS and SP showed that cellulose binding to cellulose alters the labeling by modifying the accessibility of some primary amines.

Catalytic Activity of Labeled Cellulases. Fluorescence labeling of enzymes can affect their binding and catalytic activity through steric hindrance and accessibility to functional sites, changes in hydrophobicity and charge, and even a disruption of the overall 3D structure of the enzyme. The effect of the labeling method and DoL on oligosaccharide production for cellulases

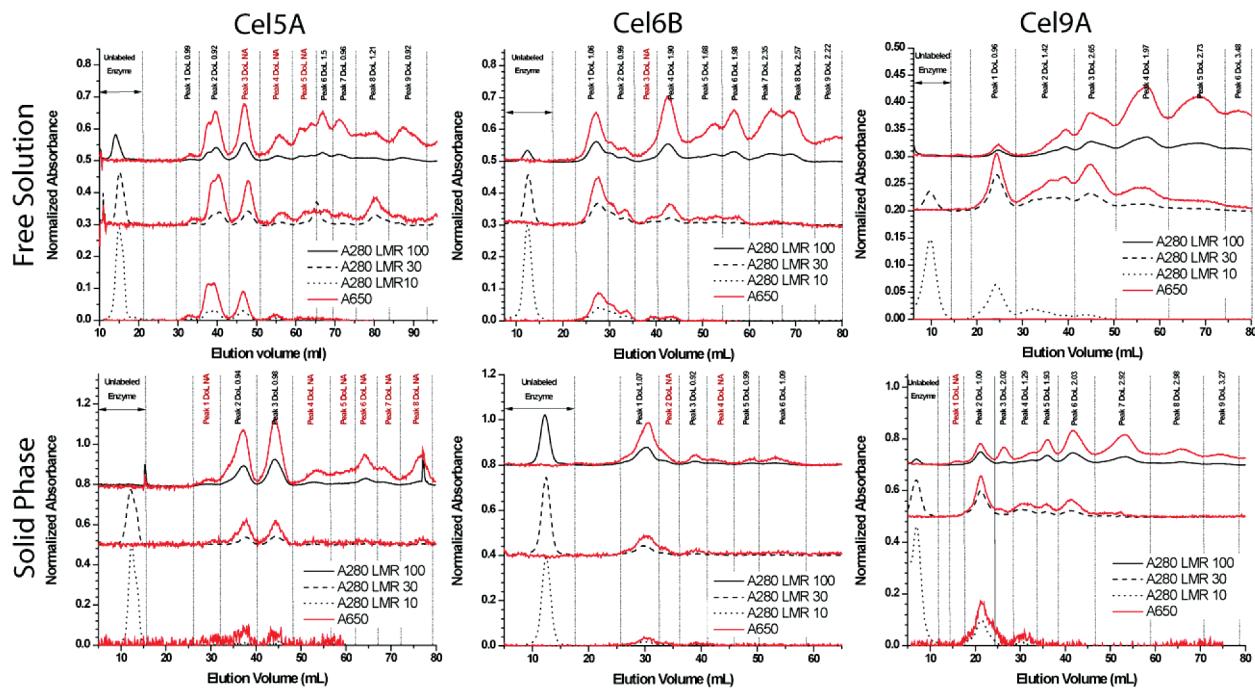


Figure 2. FPLC chromatograms of the separation of samples labeled with AF647 either by FS or by SP_A. Chromatograms have been normalized to the total area under the A₂₈₀ curve. Unlabeled protein elutes earlier than labeled products. Identified peaks representing populations of labeled products were consolidated and characterized for their DoL. Labels in red represent products where not enough enzyme was recovered to measure DoL.

Cel5A, Cel6B, and Cel9A was evaluated through a hydrolysis assay. Results show that Cel9A produced cellobetraose, cellotriose, cellobiose, and glucose, Cel5A produced cellobiose and cellotriose, and Cel6B produced almost exclusively cellobiose (Figure 3a). When binding to the cellulose strand, Cel6B and Cel9A depolymerize cellulose by processively cleaving multiple oligosaccharide units (of cellobiose and cellobetraose, respectively) before detaching from the strand.^{31–33} On the other hand, Cel5A cleaves a single cellobiose unit every time it binds to the cellulose strand.³¹ Thus, Cel5A and Cel6B matched the expected oligosaccharide production, while Cel9A showed products other than cellobetraose, suggesting the enzymatic conversion of cellobetraose in solution.

The results from cellulose depolymerization are presented in Figure 3b–d. Cel9A yielded the most labeled products, and only a few were assayed to cover the spectrum of DoLs obtained. On the other hand, all the AF647-labeled Cel5A and Cel6B products were assayed. None of the labeled products recovered for Cel5A presented significant changes in activity (Figure 3b, CI 95%), indicating that adding a single AF647 fluorophore to Cel5A does not alter its catalytic activity. Labeled Cel6B (Figure 3c) showed differences only for products labeled in FS (DoLs of 1.00, 1.90, and 1.98), where activity was increased by the addition of one fluorophore but decreased to wild-type levels upon further labeling. The increase in activity for AF647-labeled Cel6B was an unexpected result that cannot be fully explained with current data. Cel9A showed significant decrease in activity for products labeled in FS with DoLs of 4.09 and 4.50 and in SP with a DoL of 6.21

(Figure 3d). The decrease in activity observed for labeled enzymes from FS with DoL > 4 and from SP for DoL > 6 supports the hypothesis that SP labeling protects the enzymatic activity.

Single Molecule Characterization of Photon Output. The effect of DoL on photon output was evaluated for AF647-labeled Cel9A. A sample plot of binned data for the photon output measured from molecules with DoL 2.03 is presented in Figure 4a. Figure 4b presents the binned data for all the labeled products tested. In addition to labeled products obtained after FPLC separation, a labeled mixture was run as a comparison (dashed/gray line). Products with DoL of 1 labeled with FS or SP have overlapping distributions, showing that their emission properties are similar. As DoL is increased (Figure 4b), the distributions become wider as a result of the combinations of excitation states for the fluorophores incorporated in the molecule. While molecules that have a single fluorophore can only be in either a dark or a bright state, molecules with higher DoL can have all possible combinations of dark and bright states for each fluorophore attached. The trend for the photon output (Figure 4b) was that, as DoL increased (up to a DoL of 5), the distribution shifted to the right, i.e., more photons were emitted per molecule. When the average photon output is plotted against DoL (Figure 4c), it is observed that there is a monotonic increase as a function of DoL, until the DoL surpasses a value of 4. Molecules with DoL > 4 do not show a linear increase in the average photon emission. The extreme case is the DoL of 6.21, where a significant reduction in photon output is observed. In theory, each added fluorophore should increase the photon output by an equal amount. In practice, the addition of multiple fluorophores can increase the photon output only if they are incorporated at distances greater than their Förster radius. Thus, the decrease in the photon output for heavily labeled molecules can be attributed to quenching. These results

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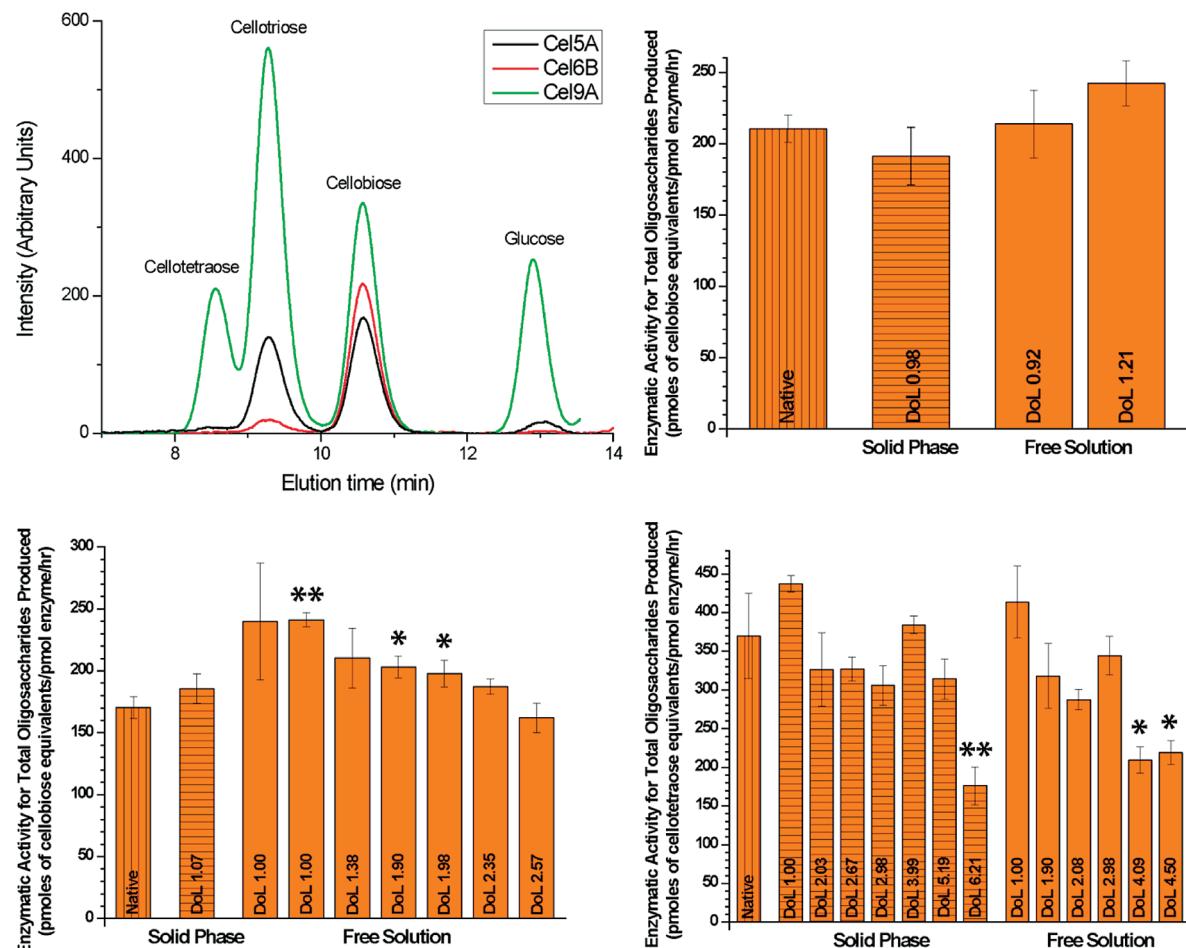


Figure 3. Evaluation of catalytic activities of labeled cellulases. (a) HPLC chromatogram with sample profile of oligosaccharides produced. (b)–(d) Comparison of catalytic activities of labeled and native Cel5A, Cel6B, and Cel9A. Statistical significance ($n \geq 3$) is marked as * for 95% and ** for 99%.

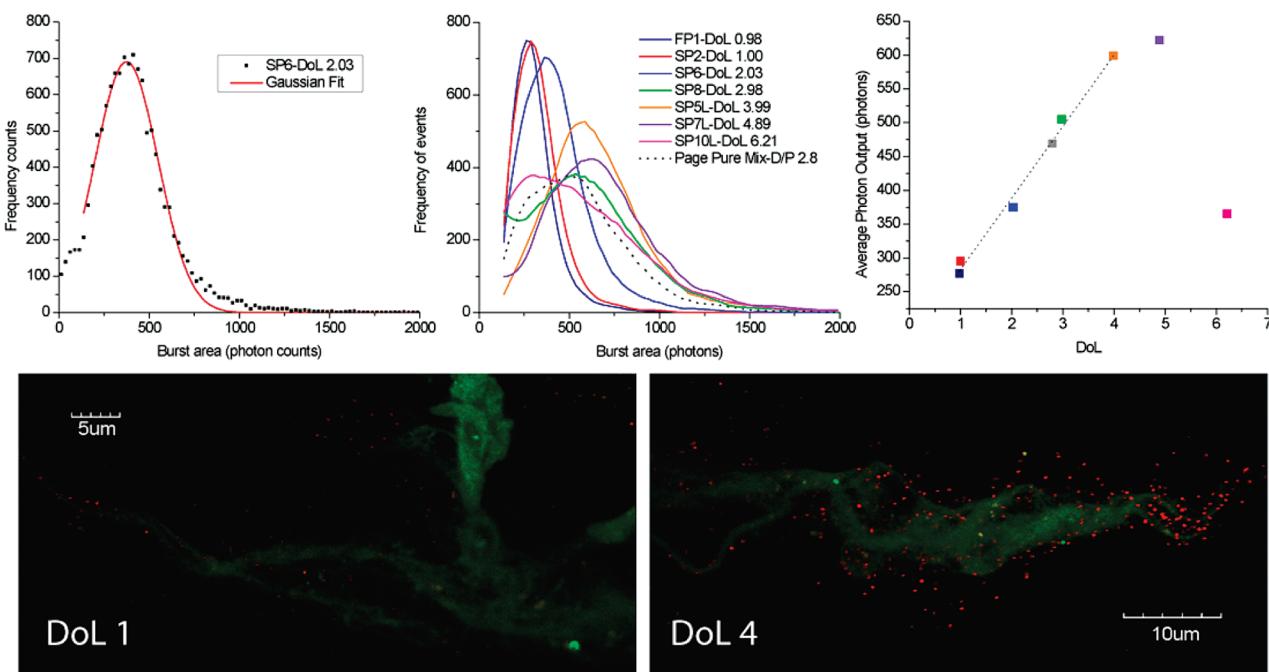


Figure 4. Photon output for single AF647 labeled Cel9A cellulases. (a) Raw distribution of binned data for photon output collected from ~10 000 enzymes with DoL 2.03. Gaussian fit presented in red. (b) Distribution of photon output for data collected from labeled products assayed. (c) Average photon output plotted vs DoL. (d) Confocal images of AF647 labeled Cel9A cellulases (red) bound to fluorescently labeled cellulose (green). Cellulases with a DoL of 1 and 4 were incubated at 100 fM and imaged under identical conditions.

show that overlabeling of Cel9A with AF647 does not necessarily translate into brighter molecules and that a DoL of 4 is optimal. Figure 4d exemplifies the advantage of the use of highly labeled molecules in SM imaging. The comparison shows Cel9A molecules with a DoL of 1 and 4 bound to fluorescently labeled cellulose. The higher average photon output for a DoL of 4 allows greater signal-to-noise ratio, which makes SM localization more accurate.

CONCLUSIONS

In this paper, methods have been described to label cellulose-binding proteins and separate the resulting mixtures into labeled products with well-defined optical and functional characteristics. A solid phase labeling method for cellulose-binding proteins was developed and optimized as an alternative to free solution labeling. Although the FS labeling protocol can be applied to any protein, the SP method presents several advantages: (1) protection of binding and catalytic residues during labeling, (2) reduced volume for labeling reactions, (3) ease of removal of unreacted dye through centrifugation and washes, and (4) high recovery of labeled proteins. Provided a suitable immobilization matrix, these advantages can be extended to other proteins. Comparison of the labeling methods, solely through D/P, indicated that an increase in LMR yields higher D/Ps; fluorophore size, charge, and hydrophobicity affect the labeling efficiency, with AF350 being the dye that labels cellulases most efficiently; and the labeling efficiency for cellulases depends on the number of surface-accessible lysines. Significant differences were observed between the labeling efficiency of FS and SP, with some dye-cellulase combinations exhibiting higher labeling efficiency with FS and others with SP.

Separation of the labeled mixtures through the FPLC method developed showed significant amounts of unlabeled enzymes present, along with a multiplicity of labeled products. Thus, characterization solely based on the D/P is not an accurate depiction of the labeled mixtures. Use of labeled mixtures in SM experiments could translate into the observation of an average behavior of the different labeled products. Therefore, separation of labeled mixtures into its components is necessary for the isolation of more homogeneous labeled molecules, with known functional characteristics. A comparison of the separated mixtures from FS against those from SP showed differences in the distribution of labeled products. This is a reflection of the changes in lysine accessibility induced by cellulase binding onto the cellulosic matrix. With the FPLC protocol developed, it was

possible to eliminate the presence of any unlabeled enzymes and separate labeled species with well-defined DoLs. The separation of labeled species was done based on the charge carried by the fluorophore, which allows this separation method to be applied to separate any mixture of labeled proteins.

The purification of more homogeneous labeled products allowed the thorough characterization of functional properties of AF647-labeled cellulases. Measurement of the catalytic activity of labeled cellulases on BMCC showed that labeling with few fluorophores did not change their catalytic activity, but overlabeling could reduce it. This could be due to labeling of lysine residues that participate in binding or catalysis or to an overall disruption of the enzyme structure. The threshold for overlabeling was lower for FS than for SP, which shows that functional residues are protected by cellulose binding. A special case was Cel6B, where activity increased by 30% for a DoL of 1 when labeled in FS. The root of the increase cannot be traced with current data but could be related to enhancement of hydrophobic interactions with cellulose. Finally, the characterization of the emission characteristics of labeled cellulases by SM experiments showed that overlabeling does not always result in an increased photon output. The labeled cellulases obtained from the labeling and separation methods can be used in the future for SMFS studies with confidence that their performance mirrors that of native enzymes.

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SUPPORTING INFORMATION AVAILABLE

Seven additional figures available as Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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