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TECHNICAL NOTE

A novel FRET approach for in situ investigation of cellulase–cellulose interaction

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Abstract A novel real-time in situ detection method for the investigation of cellulase–cellulose interactions based on fluorescence resonance energy transfer (FRET) has been developed. FRET has been widely used in biological and biophysical fields for studies related to proteins, nucleic acids, and small biological molecules. Here, we report the efficient labeling of carboxymethyl cellulose (CMC) with donor dye 5-(aminomethyl)fluorescein and its use as a donor in a FRET assay together with an Alexa Fluor 594 (AF594, acceptor)–cellulase conjugate as acceptor. This methodology was successfully employed to investigate the temperature dependency of cellulase binding to cellulose at a molecular level by monitoring the fluorescence emission change of donor (or acceptor) in a homogeneous liquid environment. It also provides a sound base for ongoing cellulase–cellulose study using cellulosic fiber.

Keywords FRET · Cellulase · Cellulose · Carboxymethyl cellulose (CMC)

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Introduction

Cellulose is the major component of the cell wall of most bioresources, consisting of β-1,4-D-glucose units, that can be enzymatically hydrolyzed by cellulases to glucose which is the key intermediate for the biological generation of the second-generation biofuels [1, 2]. Cellulases are produced by a variety of fungi and bacteria, and are classified into three categories based on their hydrolytic functionalities [3]: exo-cellulases (cellobiohydrolases) that release disaccharide cellobioses at the reducing or non-reducing ends of cellulose; endo-cellulases (endoglucanases) that degrade the cellulose randomly at internal positions; and β-glucosidases (cellobiases) that cleave cellobiose to glucose. Considerable efforts have been made to investigate and understand the fundamental interactions between cellulase and cellulose to improve the enzymatic depolymerization of cellulose. Water-soluble celluloses, such as carboxymethyl cellulose (CMC) [4], provide a facile model for studies on enzyme functionality, though the mode of action on water-soluble oligosaccharides or cellulose derivatives may be different from those on water-non-soluble polymeric substrates. A host of analytical techniques have been adopted and utilized to investigate the catalytic activity, affinity, or binding properties of a variety of cellulases or their cellulose binding/catalytic domains (CBD/CD), such as UV–Vis spectroscopy [4], liquid scintillation [5], nuclear magnetic resonance (NMR) [6], fluorescence microscopy [7] and spectroscopy [8], scanning electron microscopy (SEM) [9], quartz crystal microbalance (QCM) [10], and mass spectrometry (MS) [11]. Although these methods have provided useful information on cellulase–cellulose interactions, new analytical methods are desired for real-time analysis of cellulase–cellulose

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interactions. For instance, NMR [6], fluorescence microscopy [7], and QCM [10] have recently been adapted to study in situ the binding preference of cellulases to cellulose oligomers, the adsorption of cellulases to the cellulose fiber [7], or the enzymatic hydrolysis kinetics of immobilized cellulose film [10].

Fluorescence resonance energy transfer (FRET) has been frequently employed in the biological and biophysical fields to study the interactions between biomolecules, such as association between enzyme and its substrate [12]. Here, we describe a proof of concept for a novel application of FRET for the investigation of cellulase–cellulose interaction based on the fluorescence resonance energy transfer from donor–CMC conjugate to acceptor–cellulase conjugate (Fig. 1), allowing for rapid real-time in situ investigation of cellulase–cellulose interaction at the molecular level. CMC, which is traditionally used to determine the cellulase activity in homogeneous solution, was chosen as a model substrate compound, labeled with a donor fluorophore, and cellulase was labeled with an acceptor fluorophore. The labeling of cellulase with the FRET acceptor was accomplished by following well-established literature methods. Whereas we developed an improved technique for labeling CMC in an aprotic solvent. Upon mixing, the labeled cellulases could interact with labeled CMC. It was anticipated that the FRET signal would be detected provided that the FRET acceptor, bound to cellulase, is at a distance satisfying the FRET requisite as summarized in Fig. 1. The feasibility of this proposed novel FRET methodology has been verified by successfully distinguishing the temperature dependency of cellulases binding to CMC at 4.0 and 20.0 °C.

Experimental

Chemical reagents and apparatus

CMC (MW=90,000, DS=0.7), *Trichoderma reesei* cellulase, dimethyl sulfoxide (DMSO), H-type exchange resin, and tributylamine were obtained from Sigma-Aldrich. Ethyl-3(3-dimethylaminopropyl)carbodiimide (EDAC), *N*-hydroxysulfosuccinimide (sulfo-NHS), 5-(aminomethyl)fluorescein, and

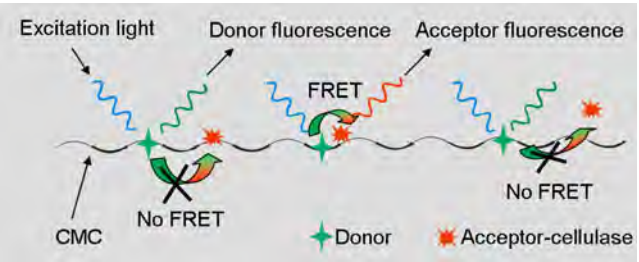

Fig. 1 Demonstration of FRET phenomena when acceptor-labeled cellulases bind to donor-labeled CMC

Alexa Fluor 594 succinimidyl ester (AF594) were acquired from Molecular Probes (Invitrogen, Carlsbad, CA). PD-10 desalting columns were purchased from GE Healthcare. Dialysis tubing was from Spectrum Laboratories (molecular weight cutoff (MWCO) 3,500–5,000 D).

Instrumentation

Fluorescence measurements were performed by using a steady-state fluorescence spectrometer (PC1, ISS, Champaign, IL). The excitation wavelength was 450 nm and emission spectra were collected between 500 and 640 nm using a 1-nm step size and an integration time of 0.5 s.

UV absorption measurements were made using a UV–Vis spectrometer (UV-2450, Shimadazu). The absorption spectra were acquired from 200 to 800 nm, and scan rate and step were 1 s/point and 2 nm respectively.

CMC labeling, purification, and characterization

CMC was labeled with donor fluorophore 5-(aminomethyl) fluorescein in DMSO. To dissolve CMC in DMSO, the carboxylic groups of CMC were first converted to the tributylammonium salt as shown in Fig. 2. Briefly, an aqueous 2% (w/w) sodium salt of CMC solution was passed through an H-type exchange column and tributylamine was then added until the pH of the solution reached 7 to form compound III, the CMC tributylammonium salt. The carboxylic groups of CMC (1.00 ml 10.0 mg/ml in DMSO) were activated with 8.0 mg 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDAC) and 8.0 mg*N*-hydroxysulfosuccinimide (sulfo-NHS), before adding 0.10 ml of 10.0 mg/ml 5-(aminomethyl)fluorescein DMSO solution (Fig. 2). The mixture was stirred overnight, then diluted with water (20.00 ml) and dialyzed against water. A PD-10 desalting column was used to further purify the fluorescein–CMC conjugate. The obtained fluorescein–CMC conjugate was freeze-dried. A fixed amount of the dry conjugate (10.0 mg) was dissolved in a 0.10 M pH 7.4 phosphate buffer solution and characterized by using UV–Vis spectroscopy. The concentration of the fluorescein fluorophore in the solution was calculated based on the absorption calibration curve of the free fluorescein in buffer. The amount of fluorescein conjugated to CMC was determined and the degree of labeling (DOL, molar number of fluorophore molecules per mole of CMC) of fluorescein–CMC conjugate was calculated.

Cellulase labeling, purification, and characterization

The labeling of *Trichoderma reesei* (*T. reesei*) cellulase with acceptor AF594 was accomplished by following published methods [13]. In brief, dye AF594 (1.00 mg) was added into

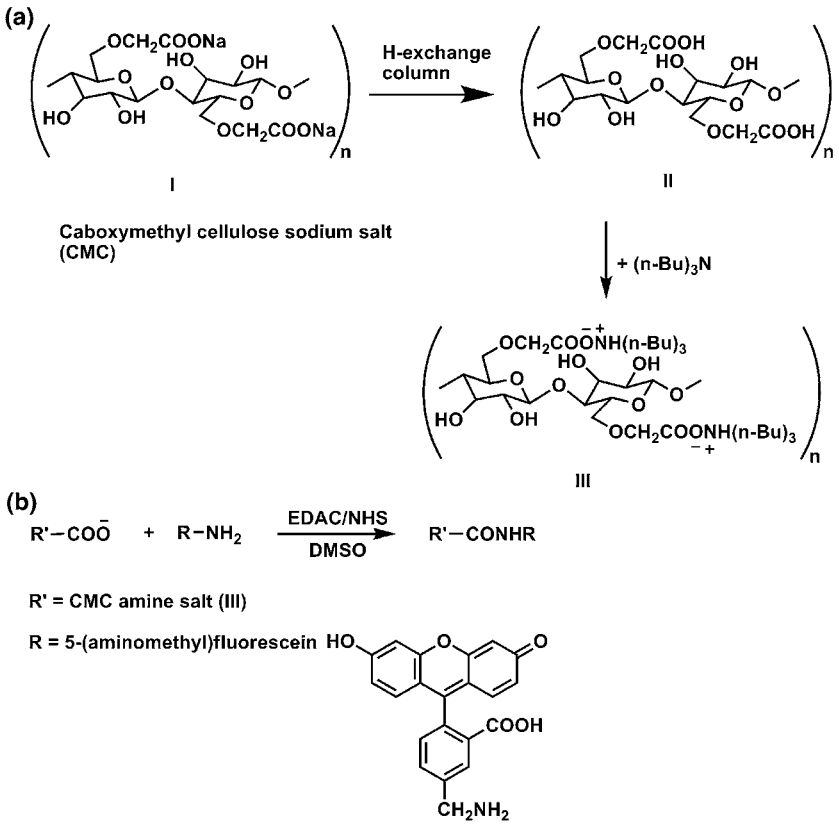

Fig. 2 Schematic of deriving carboxymethyl cellulose sodium (CMC) to DMSO-dissolvable CMC amine salt and of labeling of CMC amine salt

1.00 ml of 10.0 mg/ml cellulase aqueous solution. After stirring for 2 h in the dark at room temperature, the reaction solution was dialyzed to remove free dye. A PD-10 desalting column was then used to further purify the AF594–cellulase conjugate. The DOL of labeled cellulase was determined by UV–Vis spectroscopic measurements [13].

Determination of labeled cellulase activity

The hydrolytic activity of labeled and starting cellulase with CMC and labeled CMC was measured by monitoring the release of reducing sugars after enzymatic hydrolysis [4]. DNS (dinitrosalicylic acid) reagent was used to determine the concentration of reducing sugars in the hydrolysis solutions [14]. Enzymatic deconstruction experiments were performed at 50.0 °C for 1 h by mixing 1.00 ml 0.2% (w/w) CMC and 0.50 ml 0.10 mg/ml cellulase, which were dissolved in 0.05 M pH 5.0 sodium acetate buffer. Then, 1.50 ml DNS reagent was added into the solution for the color development following hydrolysis. The absorbance was measured at 540 nm using a UV–Vis spectrophotometer. A standard curve was prepared in the range of 0.4–2.0 mM glucose.

FRET experiments

FRET experiments were performed in 0.10 M phosphate buffer (pH 7.4) at 4.0 and 20.0 °C respectively, using fluorescein–CMC with a relatively high DOL ca. 8 and AF594-labeled cellulase. The concentration of fluorescein–CMC conjugate was kept constant (2.0 μM) in these experiments. The concentration of AF594–cellulase conjugate was varied (i.e., 6.0, 12.0, and 24.0 μM respectively) to observe the corresponding FRET signal changes after 1 or 4 h of mixing with fluorescein–CMC conjugate in the buffer solution. Control experiments were performed by using free 5-(aminomethyl)fluorescein and AF594 fluorophores.

Results and discussion

Cellulase activity on CMC and labeled-CMC

The labeling of cellulase with AF594 yielded a DOL of 6 as determined by UV–Vis method [13]. The labeling of CMC with 5-(aminomethyl)fluorescein yielded a DOL of 8 as determined by the method mentioned in the previous section. Thus, there is about one fluorescein substitution per 50 glucose units for the obtained fluorescein–CMC conjugate.

The enzyme hydrolysis results are summarized in Table 1 which reveals a slight decrease in activity for *T. reesei* cellulase for both CMC and labeled CMC after labeling with AF594. The lowest activity (i.e., 9% reduction in activity) was obtained with labeled cellulase hydrolyzing

Table 1 Hydrolysis activity of AF594-labeled and non-labeled cellulases for fluorescein-labeled and non-labeled CMC

	Fluorescein-labeled CMC, mM	CMC, mM
AF594-labeled cellulase	1.210±0.074	1.235±0.043
T. reesei cellulase	1.322±0.089	1.332±0.050

Values in the table are the concentrations of sugar reducing end in hydrolysis solutions

labeled CMC which was attributed to the steric interactions caused by the dye labels.

FRET experiments

Experimental FRET results using labeled cellulose and labeled cellulase at different concentrations are shown in Fig. 3. Figure 3a and b summarize the experimental results performed at 4.0 °C after 1 h of mixing: Fig. 3a provides the fluorescence emission of fluorescein–CMC, and of mixtures of fluorescein–CMC and AF594–cellulase; Fig. 3b provides the calculated absolute signals generated from FRET phenomena in the region 500–600 nm for the mixtures of labeled cellulose and labeled cellulase. These data illustrate that the donor fluorophore fluorescein generated an emission in the region of 500–580 nm; acceptor fluorophore AF594 generated an emission in the region of 600–640 nm. The calculated absolute FRET signal in the region of 500–580 nm presented in Fig. 3b–d is equal to the emission spectrum of pure fluorescein–CMC solution subtracted from that of mixture solution collected under the same experiment temperature, and the mixture solution contained the same concentration of fluorescein–CMC as the pure fluorescein–CMC solution.

The results shown in Fig. 3a indicate that the donor fluorescence emission in the region 500–580 nm decreased and the acceptor fluorescence emission in the region 590–640 nm increased with the addition of AF594–cellulase conjugate into the fluorescein–CMC solution. The calculated absolute FRET signals demonstrate this tendency more clearly (Fig. 3b). With the addition of AF594–cellulase into the mixture solution, the absolute FRET signal increased, suggesting increased binding of AF594-labeled cellulase to the fluorescein-labeled CMC chain.

The same samples freshly prepared were also used for FRET experiments performed at 20.0 °C. The calculated absolute FRET signals that resulted from mixing fluorescein–CMC and AF594–cellulase conjugates for 1 and 4 h are shown in Fig. 3c and d, respectively. From this data it is apparent that the FRET phenomena occurred after mixing fluorescein–CMC and AF594–cellulase conjugates. When compared to the FRET generated after 4 h mixing at 20.0 °C, the FRET signal generated after 1 h mixing at 20.0 °C was stronger. We believe this can be attributed to the enzymatic hydrolysis of CMC yielding lower-binding CMC fragments for the labeled cellulase. The substitution of carboxymethyl groups on the CMC backbone is not uniform [15] and this undoubtedly influenced the binding of cellulases to the CMC polymer chain especially at the shorter reaction time. However, compared to the FRET phenomena generated at 4.0 °C after mixing for 1 h, the FRET signals generated at 20.0 °C were about 37% weaker for sample S3 (mixture of 24.0 μM AF594–cellulase and 2.0 μM fluorescein–cellulose) based on the emission of fluorescein at 520 nm. This result may be interpreted as that the lower temperature favors the cellulase binding to substrate CMC.

Control experiments were performed by using the same concentrations of free donor and acceptor fluorophores (i.e., fluorescein and AF594) in a 0.10 M phosphate buffer solution in the absence and presence of comparable concentrations of non-labeled CMC and cellulase. No FRET signals were observed in the control experiments in the absence of non-labeled CMC and cellulase neither at 4.0 nor 20.0 °C. When control experiments were performed in the presence of non-labeled cellulase and CMC, a decrease of the signal at the position of fluorescence emission of fluorescein (520 nm) was observed upon mixing the free fluorophores, i.e., fluorescein and AF594, and there were no significant differences between control experiments performed at 4.0 or 20.0 °C. No FRET signal should be observed in control experiments as the donor and acceptor fluorophores will not be close enough to each other in solution. To figure out the reasons that caused the FRET from control experiments, one set of comparison experiments was performed by adding fluorescein and AF594 into the mixture solution of cellulase and CMC, or by adding these two dyes into PBS buffer, followed by dialysis of these two samples in PBS buffer for 48 h. The fluorescence measurements were done on these two dialyzed samples, and the results show that the sample with the presence of CMC and cellulase has ca. 30% more fluorescein dye remaining after 48 h dialysis compared to the sample without the presence of CMC and cellulase. Therefore, the background signal could be due to the absorption of the two fluorophores to the unlabeled cellulase or CMC. The FRET result from the control experiments with the presence of non-labeled cellulase and CMC was compared with that arising from labeled cellulase and labeled CMC interaction based on the

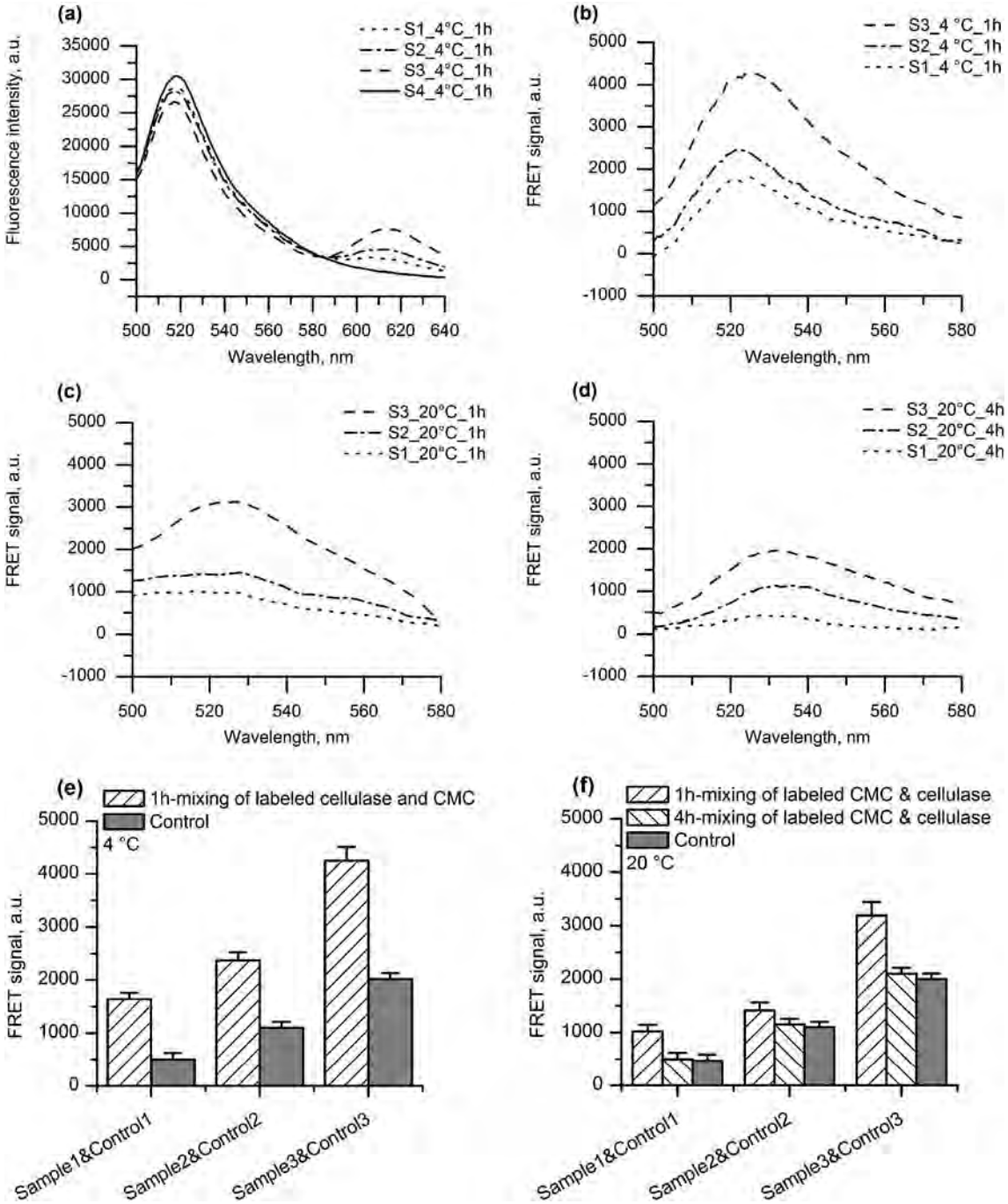

Fig. 3 **a** Fluorescence signals change with the addition of AF594–cellulase into fluorescein–CMC phosphate buffer solution (0.1 M, pH 7.4). Experiments performed at 4.0 °C. **b** Calculated FRET signals for different mixture solutions after mixing for 1 h. Experiments performed at 4.0 °C. **c** Calculated FRET signals for different mixture solutions after mixing for 1 h. Experiments performed at 20.0 °C. **d** Calculated FRET signals for different mixture solutions after mixing for 4 h. Experiments performed at 20.0 °C. **e** Comparison of FRET signals generated by mixtures of labeled CMC and labeled cellulase, and by controls. Experiments performed at 4.0 °C. **f** Comparison of FRET signals generated by mixing labeled CMC and labeled cellulase for 1 and 4 h, and by controls. Experiments performed at 20.0 °C. Sample 1 (S1): 6.0 μM AF594–cellulase + 2.0 μM fluorescein–CMC; sample 2 (S2): 12.0 μM AF594–cellulase + 2.0 μM fluorescein–CMC; sample 3 (S3): 24.0 μM AF594–cellulase + 2.0 μM fluorescein–CMC; sample 4 (S4): 2.0 μM fluorescein–CMC (*a.u.* arbitrary unit)

emission of fluorescein at 520 nm, and these results are displayed in Fig. 3e and f. The FRET signal differences between mixtures of conjugates after 4 h mixing and corresponding controls at 20.0 °C were not significant which was attributed to the enzymatic hydrolysis of CMC.

Conclusion

This study has reported for the first time that the cellulase–cellulose interaction can be investigated at a molecular level by using the FRET method. This study represents a proof of concept for the rapid real-time in situ investigation of cellulase–cellulose interaction using donor-labeled CMC and acceptor-labeled cellulase. This FRET method is promising for a rapid real-time in situ study on the kinetics of cellulase–cellulose interaction, cellulose binding domain–cellulose interaction, cellulase screening, or competition of cellulases binding to cellulose in homogeneous liquid phase, and shows a perspective to apply this technique for the investigation of cellulose–cellulase interaction using cellulose fiber.

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