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Synthetic Assembly of Bifluorescence-Labeled Glycopolymers as Substrates for Assaying α-Amylase by Resonance Energy Transfer

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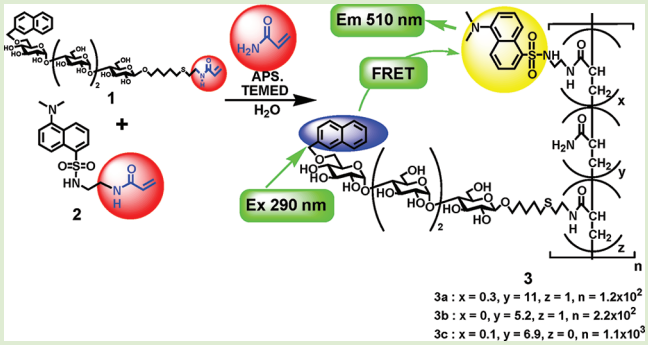
Supporting Information

ABSTRACT: To meet the need for a convenient substrate for sensitive and continuous assay for α-amylase, we developed a fluorescence resonance energy transfer (FRET)-based polymer substrate. Radical copolymerization of FRET-component monomers in different ratios of fluorogenic donor and acceptor was utilized to prepare such polymers. A glycomonomer as a fluorogenic donor was derived from naphthylmethylated maltotetraose, and a dansyl derivative monomer was used as an acceptor. Their mixture and acryl amide were copolymerized in a typical radical polymerization to yield a bifluorescence-labeled polymer in good yield. All of the polymers showed effective FRET and were used for the continuous assay of human salivary α-amylase. The time course of α-amylase reactions led to the apparent kinetic parameters of K_m = 4 μM and V_{max} = 0.29 nmol/min. The results strongly suggested that FRET-sensitive polymers are conveniently accessible and applicable for the sensitive determination of biochemical events.



Fluorescence resonance energy transfer (FRET) was introduced as a spectroscopic ruler by Strayer and Haugland,[1,2] and FRET techniques have frequently been used in chemical, biochemical, biomedical, and other fields to evaluate biological events.[3−5] An appropriate pair of fluorescence probes in which spectral overlap of the donor emission spectrum with the absorption of the acceptor gives rise to effective FRET.[6] In addition to the combination of an appropriate pair of the probes, the efficiency of intramolecular resonance energy transfer is strongly dependent on the distance between the donor fluorophore and acceptor fluorophore, and the most suitable distance is estimated to be around 10−60 Å for a pair of naphthyl-dansyl fluorophores. In light of the glycoside clustering effect,[7−9] a polymeric substrate containing multiple units of both donor and acceptor in the same molecule is expected to offer new possibilities of increased sensitivity because of multiple simultaneous occurrences of FRET.

FRET techniques have been applied to continuous monitoring of proteases,[10−12] nucleases,[13,14] and glycosidases[15−18] to yield kinetic parameters. Since a complicated synthetic sequence is normally needed to obtain a substrate based on FRET, simple and convenient access of the substrate is desirable. We chose to use general radical polymerization, which gives polymers with a reasonably controllable number of monomer units in the large molecule[19] for the preparation of a FRET-sensitive polymer.

α-Amylase (EC.3.2.1.1) was selected as the enzyme since it is used not only for biochemical applications but also for medicinal applications.[20−25] α-Amylases are *endo*-type enzymes that selectively cleave α1→4 glucosidic linkages in an amylose and maltooligosaccharides[26] and are extensively found in various species, including animals, plants, fungi, yeasts, and bacteria.[27] Although synthetic studies on bifluorescence-labeled substrates for a continuous assay of human salivary α-amylase have been carried out by means of multistep chemical synthesis, no polymer-based substrate has been reported.[28−31] In this paper, we describe the preparation of FRET-sensitive polymers by means of one-step assembly of fluorogenic polymerizable donors and the corresponding pair acceptors using simple radical polymerization.

A schematic diagram of our synthetic target **3** is shown in Figure 1. Control of the distance between the fluorogenic donor unit *z* and the acceptor unit *x* in the bifluorescence-labeled polymer **3** would be achieved by varying the monomer ratio of the naphthylated carbohydrate monomer **1**, the dansylated monomer **2**, and acrylamide (AA), and FRET can be observed when the distances between donor fluorophores and acceptor fluorophores in the polymer are appropriate. A naphthyl moiety and a dansyl moiety were selected as the donor−acceptor combination on the basis of results of a previous study.[32] In this work, the naphthyl moiety was introduced into a carbohydrate monomer **1**,[33] and the dansyl moiety was used as a known fluorogenic monomer **2**.[34]

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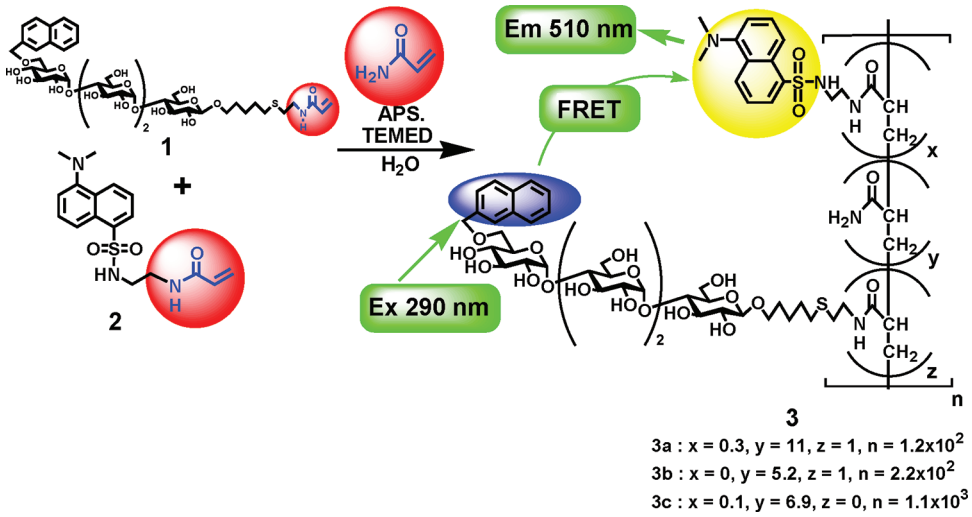

Figure 1. Schematic diagram of FRET-sensitive polymers derived from polymerizable fluorescence donor **1**, polymerizable fluorescence acceptor **2**, and acrylamide by radical polymerization. The naphthyl group in the polymer is excited at 290 nm, giving emission around at 340 nm, which in turn excites the dansyl group in the polymer to furnished fluorescence emission at 510 nm. Unit ratios in the polymer are indicated as *n*, *x*, *y*, and *z*.

Table 1. Results of Polymerizations Using Different Ratios of 1, 2, and Acrylamide (AA)

monomer ratio			total yield[a]	polymer composition[b]				sugar content	$\bar{M}_w$[c]	
1	**2**	AA	%	*x*	*y*	*z*	*n*	wt %	kDa	$\bar{M}_w/\bar{M}_n$
1	0.1	5	87	0.27	7.0	1	53	65	120	1.5
1	0.1	10	85	0.33	10.7	1	1.2×10^2	55	300	1.4
1	0.1	20	95	0.32	13.3	1	1.3×10^2	40	375	1.1
1	0	5	90	0	5.2	1	2.2×10^2	68	291	1.2
0	0.1	5	83	0.1	6.9	0	1.1×10^3		420	1.2

[a]Total yields were calculated on the basis of quantities of monomers used. [b]Polymer compositions of *x*:*y*:*z*:*n* were estimated on the basis of the results of ^{1}H NMR. [c]The weight-average molecular weights ($\bar{M}_w$) were estimated by size-exclusion chromatography in dimethyl sulfoxide−water (1:1, v/v) solution using a Shodex GF-510HQ column. Calibration curves were obtained using pullulan standards (5.9, 11.8, 22.8, 47.3, 112, 212, 404, and 788 kDa; Shodex P-82).

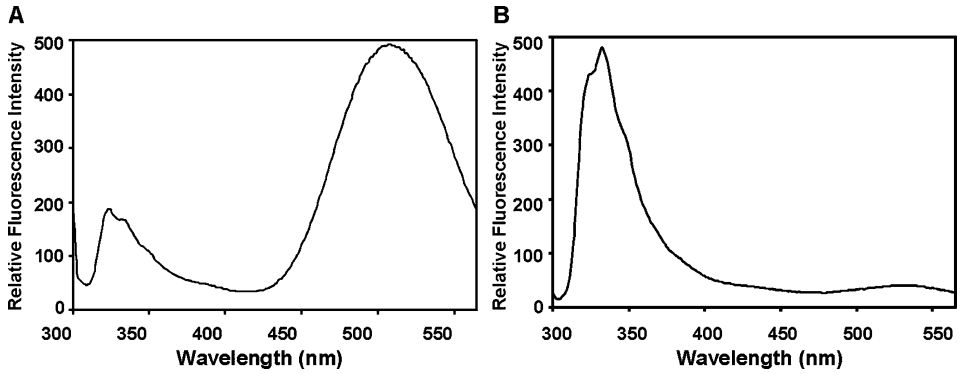

Figure 2. (A) Fluorescence spectra of FRET-sensitive polymer **3a** (*x*:*y*:*z* = 0.3:11:1) in 10 mM HEPES buffer (pH 7.0) at 37 °C. The 2-naphythyl moiety as a donor fluorophore in the polymer was excited at 290 nm, and the emission of the dansyl moiety as an acceptor fluorophore was observed at around 510 nm. (B) Fluorescence spectra of a polymer mixture of fluorogenic polymers **3b** and **3c** in 10 mM HEPES buffer (pH 7.0) at 37 °C. The 2-naphthyl moiety in **3b** was excited at 290 nm as for A, but emission from the dansyl moiety as the acceptor fluorophore in **3c** was not observed.

Radical polymerizations of the monomers including AA were performed in aqueous media at room temperature in the presence of ammonium persulfate (APS) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED),[35] and the polymerization proceeded smoothly to give a highly viscous mixture. After dialysis against water followed by lyophilization, white powdery polymers **3** were obtained. The results of polymerizations are summarized in Table 1. All fluorogenic polymers are soluble in water and have appropriate molecular weight on the basis of size-exclusion chromatography (SEC) analyses. Polymer composition and sugar content were estimated by the results of ^{1}H NMR spectra.[33]

The fluorescence measurement of the bifluorescence-labeled polymer **3a** was preliminarily performed in 10 mM HEPES buffer (pH 7.0) at 37 °C, and FRET was observed when an excitation wavelength of 290 nm was used. Figure 2A

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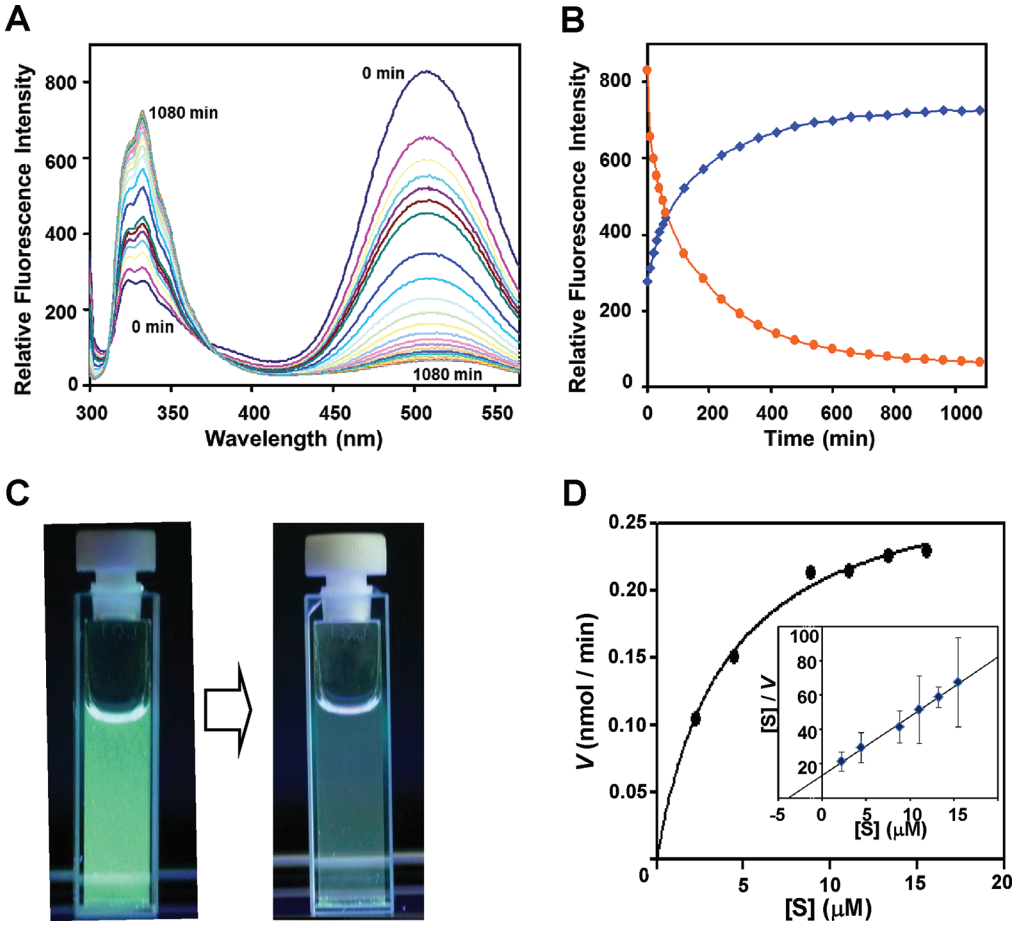

Figure 3. (A) Time course (0, 10, 20, 30, 40, 50, 60, 120, 180, 240, 300, 360, 420, 480, 540, 600, 660, 720, 780, 840, 900, 960, 1020, and 1080 min) of the relative fluorescence emission of FRET-sensitive polymer **3a** during hydrolysis with human salivary α-amylase in 10 mM HEPES buffer (pH 7.0) at 37 °C. The concentration of 8.86 μM **3a** (based on the sugar unit, z) was used for the assay. (B) Time course of the relative fluorescence intensity from both the dansyl moiety (red ●) at 510 nm and the naphthyl moiety (blue ◆) at 333 nm in FRET-sensitive polymer **3a** during hydrolysis with α-amylase. (C) Visible change in the fluorescence emission of FRET-sensitive polymer **3a** before the enzymatic reaction (left) and after the reaction (right) in a cuvette excited at 254 nm using a typical handy-type UV lamp. (D) Initial rates of hydrolysis by α-amylase as a function of sugar concentration based on sugar unit in the polymer. The inset shows the Hanes–Woolf plot of hydrolysis of FRET-sensitive polymer **3a** by α-amylase (means ± SE, n = 5).

simultaneously shows a smaller emission of naphthyl moieties at around 330 nm, which was not used for FRET and a larger emission of dansyl moieties at around 510 nm, which was resonance energy transferred from the naphthyl group. When the excitation of a mixture of polymer **3b** (having only a naphthyl moiety) and polymer **3c** (having only a dansyl moiety) was performed, no FRET was observed (Figure 2B). The results strongly suggested that intermolecular FRET was not observed when a similar concentration of each polymer was used as the polymer mixture. In addition to the polymer mixture, a solution of a mixture of both monomers **1** and **2** with appropriate concentrations was also excited, but no FRET was observed. Therefore, intramolecular FRET was exclusively observed when the bifluorescence-labeled polymer was used as the substrate.

Given the success of the preparation of a FRET-sensitive polymer, our attention was turned toward continuous monitoring of hydrolysis of the oligosaccharide part in the FRET-sensitive polymer by human salivary α-amylase. The fluorescence measurement of the time course of the enzymatic reaction was carried out in the presence of α-amylase in 10 mM HEPES buffer (pH 7.0) at 37 °C. The emission spectrum was taken immediately after addition of the enzyme, and this was regarded as the 0 min spectrum. Subsequently, spectra were taken at appropriate intervals, and the results of the continuous measurement are shown in Figure 3A. As expected, enzymatic digestion caused a decrease in dansyl emission at around 510 nm by diminishing the energy transfer from the naphthyl moieties. It also caused an increase in naphthyl emission at around 330 nm by eliminating the quenching by dansyl moieties. In addition to the spectral transformation, since these emission spectra clearly showed an isoemissive point at around 375 nm, an efficient reaction, such as enzymatic scission by α-amylase for the oligosaccharide part of the FRET-sensitive polymer, was supported. The changes in fluorescence intensities of the FRET-sensitive polymer by measurement of both naphthyl emission and dansyl emission was monitored at the corresponding naphthyl emission (333 nm) and dansyl emission (510 nm) from the value at 0 min until 1080 min, respectively (Figure 3B). The naphthyl emission at 333 nm gradually increased up to 600 min, after which the reaction tapered off until it had almost stopped at 1080 min. The decreases in dansyl emission at 510 nm as the reaction progressed were also measured. Figure 3C shows fluorescence

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emission of the starting solution of the FRET-sensitive polymer **3a** in a cuvette and the resulting solution of the polymer after enzymatic digestion excited both at 254 nm by using a UV-handy lamp. The results suggested that enzymatic reaction can occur with a FRET-sensitive polymer when an appropriate concentration of the polymer is used. Kinetic analysis of the enzymatic reaction for the FRET-sensitive polymer was also performed, and the results are shown in Figure 3D. The initial velocities were analyzed by a nonlinear regression fitting, and the inset in Figure 3D shows the Hanes–Woolf (HB) plot. From both plots according to the enzymatic reactions, kinetic parameters were estimated to be K_m = 4.0 μM (nonlinear regression) or 3.8 μM (HB plot) and V_{max} = 0.29 nmol/min (both analyses). Since K_m using the FRET-sensitive polymer was smaller than those determined by using other types of substrates [K_m = 2.2 mM (maltotriaoside derivative)[36] and 0.887 mM (maltotetraoside derivative)[37]], we estimated that the affinity for α-amylase was enhanced by means of the polymeric substrate.

In summary, we have shown that bifluorescence-labeled polymers can be conveniently prepared from corresponding fluorogenic monomers and that FRET of the polymer can be clearly observed. FRET can be effectively applied to assaying α-amylase activity using the synthetic polymer-type substrates prepared in this study. One fluorogenic acceptor in the FRET-sensitive polymer can receive resonance energy from several neighboring fluorogenic donors, and enhanced excitation of the acceptor followed by emission is therefore observed. This concept includes simplicity, rapidity, and precision of assaying an enzymatic reaction using the novel FRET-sensitive polymer. An appropriate combination of fluorogenic polymerizable donors and the corresponding acceptors provides a wide variety of applications based on FRET from the viewpoint of biochemical, medicinal, and other fields. Further applications of this concept are now under investigation, and the results will be reported elsewhere.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures: radical polymerization, SEC method, and ^{1}H NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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