Fluorescent Probes

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Label-Free, Real-Time Glycosyltransferase Assay Based on a Fluorescent Artificial Chemosensor**

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Glycosyltransferases are involved in the biosynthesis of oligosaccharides, which are vital for all living systems. This family of enzymes plays a multifunctional role in cell physiology and has been associated with embryonic maturation, cell–cell recognition, cell development, and sperm–egg binding.[1] Selective inhibition of glycosyltransferase is thus of interest as it may lead to the development of new therapeutic agents.[2] However, the lack of a rapid and simple method for continuously monitoring glycosyltransferase activity prevents the efficient discovery of potent drug candidates.

Herein, we describe a real-time assay for glycosyltransferase on the basis of supramolecular chemistry. To date, supramolecular chemistry has stimulated the design and synthesis of a number of artificial chemosensors.[3] However, their applications in dynamic processes such as enzymatic reactions are still very limited.[4] Our newly proposed supramolecular method, which uses a fluorescent chemosensor for a multisubstrate enzymatic reaction, has many desirable features when compared with the conventional glycosyltransferase assay methods.[5–8] These advantages include rapid, real-time detection, high sensitivity based on the fluorometric response, suitability for high-throughput assays, and minimal required instrumentation, as well as general applicability to a wide range of glycosyltransferases without any special modification of the substrates.

In biological glycosyl transfer processes, a glycosylated nucleotide, such as uridine 5′-diphosphate (UDP)-glycoside, is used to form a new glycoside bond with a glycosyl acceptor. The glycosylated nucleotide is a universal glycosyl donor, whereas the acceptor may vary among structurally diverse (oligo)saccharides, glycolipids, and glycoproteins. As the glycosylated nucleotide is converted into the corresponding nucleotide during the reaction, it is conceivable that the monitoring of nucleotide formation is equivalent to measuring the progress of the glycosyl transfer reaction. Thus, sensing the generated nucleotide with an artificial chemosensor may provide a potentially general and unique glyco-

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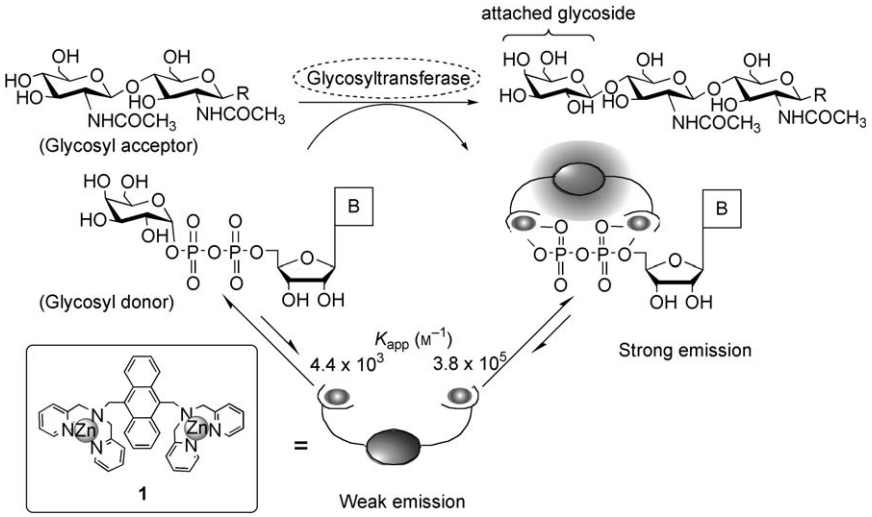
syltransferase assay, regardless of the structural complexity of the saccharide substrate.

The key issue in this assay is the selectivity of a fluorescent probe that can distinguish a nucleotide from a glycosylated nucleotide in the multi-substrate enzymatic reaction medium (see Scheme 1). We found that the binuclear zinc complex-based fluorescent probe **1** can strongly bind to phosphate monoesters but not to phosphate diesters.[9] Under aqueous neutral conditions, **1** displayed a blue fluorescence at 415 nm (see Supporting Information), the intensity of which increased by about 200% upon the addition of a micromolar rage of UDP,[10] a representative nucleotide. The increase in fluorescence upon coordination toward the pyrophosphate monoesters is a consequence of the suppression of photoinduced electron-transfer (PET) quenching that results from the phosphate-assisted coordination of the second Zn^{II} atom, as clarified by us previously.[9] The apparent binding constant (K_{app}) of **1** to UDP was estimated from the saturation curve of the titration experiment to be $3.8\times10^5\,M^{-1}$. The binding of **1** to UDP-Gal, on the other hand, is weaker by about two orders of magnitude ($4.4\times10^3\,M^{-1}$) relative to UDP. In other words, its fluorescence response was observed from 1 μM for UDP and from 200 μM or higher for UDP-Gal (Figure 1a). The difference in the sensitivity of **1** gave a concentration window suitable for detecting UDP quantitatively in the range of 1 to 10 μM, without interference from UDP-Gal. A ^{31}P NMR study of the complex of **1** and UDP displayed a considerable downfield shift of both phosphorus atoms of UDP (see Supporting Information), which suggests that the involvement of both phosphate moieties of UDP in the binding of **1** may afford the strong binding affinity.[11]

The above-confirmed prerequisite encouraged us to perform the chemosensor-based glycosyltransferase assay. We initially employed β-1,4-galactosyltransferase (β-1,4-GalT, EC 2.4.1.22) as a representative enzyme, UDP-Gal as a glycosyl donor, and chitobiose as a glycosyl acceptor. The reaction was initiated by addition of chitobiose to a mixture of UDP-Gal, β-1,4-GalT, and **1**. After addition of chitobiose, the fluorescence intensity at 415 nm gradually increased (by nearly 100%; see Figure 1b). This spectral change is identical to the change induced by UDP (see Supporting Information), which indicates that this fluorescence effect arises from the production of UDP during the enzymatic galactosyl transfer reaction.[12] In contrast, no change in fluorescence was observed without chitobiose or β-1,4-GalT (Figure 1b, inset). The rate of fluorescence change was accelerated and saturated with increasing chitobiose concentration. This dependence obeys the Lineweaver–Burk plot (see Supporting Information), and yields a Michaelis constant K_m (a reciprocal of substrate affinity) of 0.15 mM for chitobiose.[13]



Scheme 1. The chemosensor-based glycosyltransferase assay. B = base content of the nucleotide.

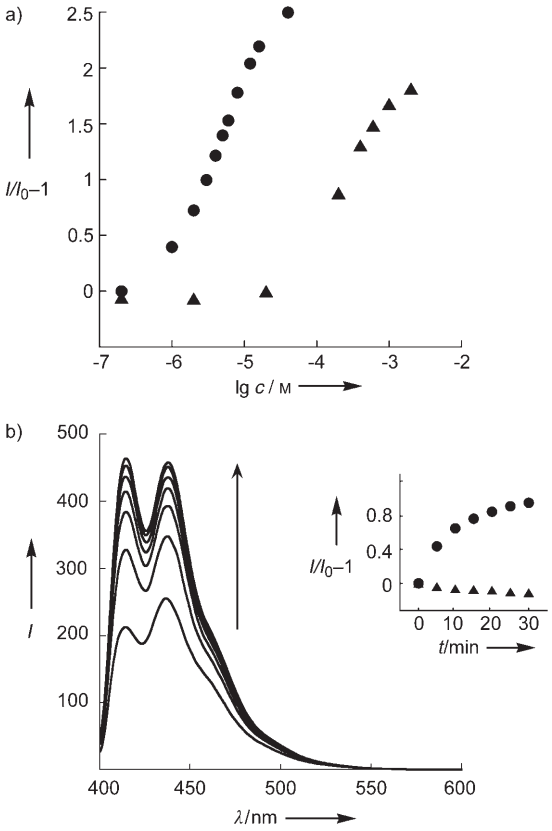


Figure 1. a) Fluorescence titration of **1** with UDP (●) and UDP-Gal (▲) presented as the emission ratio (I/I_0-1) at 415 nm. b) Real-time fluorescence detection of UDP by the β-1,4-GalT-catalyzed transfer of Gal from UDP-Gal to chitobiose by **1**. Inset: emission ratio (I/I_0-1) at 415 nm versus time during the GalT reaction in the presence (●) and absence (▲) of chitobiose. Assay conditions: **1** (4 μM), UDP-Gal (20 μM), chitobiose (100 μM), β-1,4-GalT (8 mU) in HEPES (50 mM), NaCl (50 mM), $MnCl_2$ (0.2 mM), pH 7.2, 25 °C, λ_{ex} = 380 nm. HEPES = 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

As this method does not require any modification of the enzyme substrates, the assay for β-1,4-GalT with various substrates, and the assay for another glycosyltransferase, α-1,3-galactosyltransferase (α-1,3-GalT, EC 2.4.1.90), were conveniently conducted according to the same procedure. The determined K_m values are summarized in Table 1. It is clear

Table 1: Substrate specificities of β-1,4-GalT and α-1,3-GalT determined by a chemosensor-based assay with **1**.

Substrates (acceptors)	K_m [mM]	
	β-1,4-GalT	α-1,4-GalT
Chitobiose (GlcNAcβ1-4GlcNAc)	0.15	>10
GlcNAc	1.3	>500
Glc	987	>2500
LacNAc (Galβ1-4GlcNAc)	>5	0.20
Lac (Galβ1-4Glc)	>10	0.66
Gal	>1000	130

that the substrate specificity of the two enzymes is quite different, that is, β-1,4-GalT prefers a member of the glucose family such as chitobiose (highest affinity: $K_m = 0.15$ mM) or GlcNAc (moderate affinity: $K_m = 1.3$ mM), whereas α-1,3-GalT shows a preference for the galactose-terminated saccharides such as LacNAc (lowest affinity: $K_m = 0.20$ mM), Lac, and Gal. It is also apparent that both GalTs have higher affinities toward disaccharides than monosaccharides, which is in good agreement with literature comments on the crystal structure of GalT enzymes.[14] In addition, assays of α-2,3-sialyltransferase (EC 2.4.99.5) were also preliminarily performed in the same manner with cytidine 5′-monophosphate-N-acetylneuraminic acid (CMP-NeuAc) as a glycosyl donor and LacNAc as an acceptor (see Supporting Information). These results imply that the present assay is applicable to almost all kinds of glycosyl donors, regardless of their complicated structures.

As a benefit of the simplicity in evaluating the GalT activity, we subsequently conducted high-throughput inhibitor screening by this supramolecular assay.[15] Twelve individual inhibitor candidates (1 mM; see the Supporting Information) were mixed with a solution containing the fluorescent probe **1**, β-1,4-GalT, GlcNAc, and UDP-Gal and the fluorescence intensities at 415 nm were measured by the plate reader technique (Figure 2a). Compared to some positive intensity changes, the presence of compound **d** (uridine) induced a negligible increase in the fluorescence intensity. The increase of fluorescence intensity was suppressed gradually in proportion to the uridine concentration (see Supporting Information). This suppression behavior, with a typical saturation manner, was plotted against the uridine concentration (Figure 2b), and yielded an IC_{50} value (50% inhibitory concentration) of 0.8 mM, which is consistent with literature values.[16] Among the compounds tested, uridine was the most potent for the β-1,4-GalT inhibitor, whereas other uracil derivatives (compounds **a**–**c**, **e**–**g**) showed no capacity to inhibit the enzyme. The aminosugar derivatives (compounds **h**–**l**), which are all known as inhibitors for glycosidase, also did not affect the glycosyl transfer activity of β-1,4-GalT. This experiment demonstrates that our method is convenient

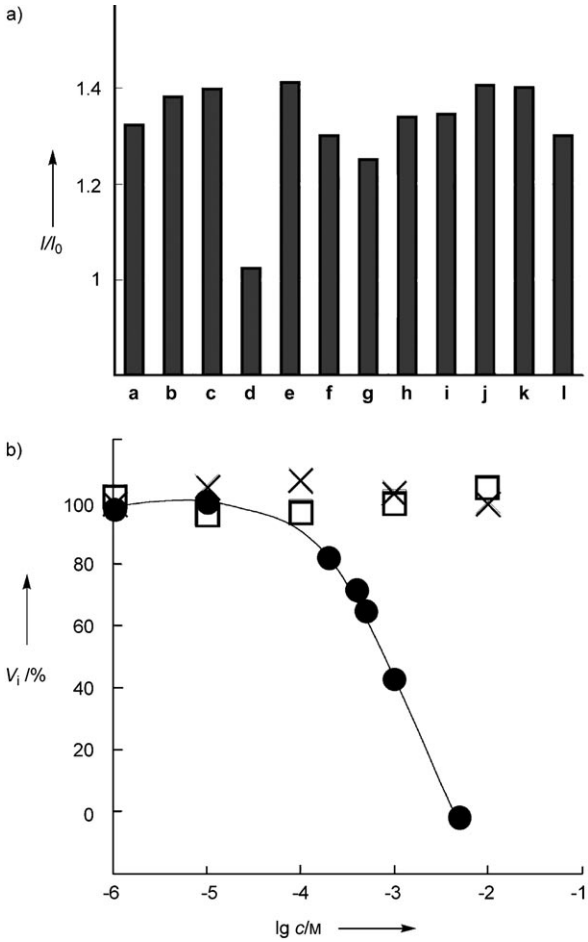


Figure 2. a) Screening of the β-1,4-GalT inhibitor with a fluorescence microplate reader. I/I_0 refers to the emission ratio at 415 nm. Assay conditions: **1** (4 μM), UDP-Gal (20 μM), GlcNAc (1 mM), β-1,4-GalT (8 mU) in HEPES (50 mM), NaCl (50 mM), $MnCl_2$ (0.2 mM), pH 7.2, 25 °C, $\lambda_{ex} = 380$ nm. b) Concentration-dependent inhibition profiles of uridine (**d**, ●) and its derivatives **c** (×) and **e** (□). V_i [%] refers to the relative initial velocity.

and useful for the high-throughput screening of potent glycosyltransferase inhibitors, which is still considered to be a significant challenge in the research field of glycobiology and medicinal chemistry.

In summary, we have developed a method for the label-free, real-time monitoring of glycosyltransferase activity based on an artificial chemosensor. Normally, a radiolabeling method with a radioactive saccharide is the most frequently used assay for glycosyltransferase,[5] but this has several drawbacks such as the tedious procedure, high cost, and inevitable radiochemical waste. Although chemically modified substrates have recently provided an alternative for glycosyltransferase assay techniques, such as fluorescence resonance energy transfer (FRET), enzyme-linked immunosorbent assay, and enzyme-coupling methods,[6] enormous efforts are needed in labeling the substrates. Assays based on mass spectrometry are powerful with regard to speed and accuracy,[7] but are not widely used because of the requirement of a special sophisticated instrument. In contrast, our supramolecular method, which is the first to apply the

selective recognition ability of a chemosensor to the glycosyltransferase assay, is easy to use and sensitive on account of the fluorescence detection, with a reliable output even under multisubstrate enzymatic conditions. We believe that our successful example may facilitate the development of artificial chemosensors that are useful under more complex conditions, such as cell lysate or living systems.

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[10] Compound **1** preferably binds to polyphosphates (e.g., adenosine 5′-triphosphate (ATP; $K_{app} = 4.0 \times 10^5\,\text{M}^{-1}$), adenosine 5′-diphosphate (ADP; $K_{app} = 1.6 \times 10^5\,\text{M}^{-1}$)), whereas it shows weaker binding to monophosphate species (e.g., adenosine 5′-monophosphate (AMP; $K_{app} = 9.1 \times 10^3\,\text{M}^{-1}$)) and extremely weak binding to phosphate diesters (e.g., cyclic AMP (cAMP), CMP-NeuAc). See reference [9] for K_{app} values to the phosphate species and details of the fluorescence sensing mechanism of **1**. Phosphate and citrate buffers should be avoided to maintain the highest sensitivity of **1** to UDP.
[11] The stronger binding of **1** to the nucleoside polyphosphates rather than the monophosphate (20-fold) might be reasonably attributable to the multiple interactions between the two phosphate groups of UDP and the two Dpa-Zn^{II} sites through the strong metal–ligand coordination and/or the additional electrostatic interactions.
[12] MALDI-TOF mass spectrometry confirmed the production of Gal-chitobiose qualitatively, as a main product of the β-1,4-GalT-catalyzed galactosyl transfer reaction to chitobiose.
[13] The activity of glycosyltransferase is often sensitive to the specific metal cation, such as Mn^{II} or Mg^{II}. In the case of β-1,4-GalT, for example, an excess amount of Mn^{II} (0.2 mM) was used to keep the high activity of the enzyme, and 0.1 mM of Mg^{II} was added in the case of α-1,3-GalT for the same purpose. Under these conditions, it is not possible that the metal bound to the enzyme was extracted with Dpa sites of **1** at a concentration of only 0.004 mM. Based on the kinetic parameters obtained herein, it is apparent that the Zn^{II}-based fluorescent probe did not disturb the enzyme activity.
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