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Ratiometric sensing of β -galactosidase based on excited-state intramolecular proton transfer (ESIPT) and solid-state luminescence enhancement†

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Glycosidases play important roles in modulating the structural and functional integrity of glycoproteins and glycolipids, and thus are promising biomarkers for disease diagnosis. While current approaches for glycosidase detection mainly rely on an enhancement of the UV-vis absorbance or fluorescence emission of glycosyl indicators, here we develop a ratiometric fluorescent probe for the sensitive and selective detection of glycosidase activity based on the combined mechanisms of excited-state intramolecular proton transfer (ESIPT) and solid-state luminescence enhancement (SSLE). The probe behaves like a typical SSLE when glycosylated, and exhibits a ~140 nm red-shift in fluorescence owing to activation of ESIPT after deglycosylation. Such a large Stokes shift may facilitate the unbiased analysis of glycosidase activities when used in diagnostic and drug-screening assays.

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Glycosylation and deglycosylation reactions of biomacromolecules including proteins, peptides and lipids are implicated in a myriad of biological and pathological processes.^{1–3} Deglycosylation is the removal of a glycosyl residue from a glycoconjugate as mediated by glycosidases, which are conserved in almost all eukaryotes. In human cells, they mainly distribute in the endoplasmic reticulum (ER), Golgi apparatus and

lysosomes.^{4,5} Glycosidases localized in the ER and Golgi apparatus are responsible for tailoring the *N*-glycans on proteins after translation, and lysosomal glycosidases are known to hydrolyze the glycosyl residues on glycoconjugates endocytosed by cells.

However, the abnormal expression of glycosidases is associated with human diseases. For example, during cell senescence, β -galactosidase (β -Gal) and α -fucosidase are overly expressed,^{6,7} and the abnormally high expression of β -Gal is closely related to the tumorigenesis and metastasis of ovarian cancer.⁸ In addition, a recent proteomics study suggests that the expression level of cytoplasmic β -glucocerebrosidase in liver cancer tissues is significantly lower than that in para-carcinoma tissues.⁹ As a consequence, the effective detection of glycosidase activities is important for glycobiological studies and disease diagnosis.

The current approaches for analysis of glycosidase activity mainly rely on colorimetric assays, which use glycosylated indicators such as 4-nitrophenol as the colorimetric substrate. However, assays that are dependent on color changes are easily compromised by the intrinsic color of the sample itself and are generally of low sensitivity. To overcome these issues, activatable fluorescent probes that exhibit a “turn-on” fluorescence upon enzymatic hydrolysis have been developed.^{10–17} Based on a variety of fluorescent dyes, molecular probes capable of sensing glycosidases in cells and *in vivo* with emission wavelengths that range from the visible to the near-infrared region, have been synthesized in recent years.^{18–27}

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Here, we report the construction of a ratiometric fluorescent probe that exhibits a large Stokes shift of the fluorescence emission wavelength upon hydrolysis by glycosidases based on the combination of excited-state intramolecular proton transfer (ESIPT) and solid-state luminescence enhancement (SSLE).^{28–30} The probe is a typical SSLE system when glycosylated, and after deglycosylation, the ESIPT process is activated, thereby achieving a ~ 140 nm red-shift in fluorescence emission.

To construct the ratiometric glycosidase probe, we first synthesized a fluorescent reporter with dual photophysical mechanisms. In the *ortho* position relative to the phenol group of tetraphenylethylene (TPE), benzothiazole (BT) was introduced. The resulting conjugate (**TPE-BT**) formed by the cycloaddition of 2-hydroxy-5-(1,2,2-triphenylethenyl)-benzaldehyde and 2-aminothiophenol exhibits ESIPT due to the intramolecular hydrogen bonding interaction between the nitrogen atom and the phenolic proton.^{31,32} Then, galactose was introduced to the phenolic site to inhibit ESIPT by removing the hydrogen-bond donor. A benzyl group was used to connect the **TPE-BT** and galactose (producing the **Gal-TPE-BT** probe) in order to enhance the sensitivity for glycosidases.³³ After deglycosylation, the benzyl moiety undergoes a “self-immolation” process resulting cleavage (Fig. 1), thereby recovering the ESIPT nature of **TPE-BT**. The synthetic details for the probe are shown in Scheme S1.†

With the probe in hand, we evaluated its photophysical properties in the absence and presence of β -Gal. β -Gal isolated from *Escherichia coli* was used as a model enzyme for the analysis. We first determined that **Gal-TPE-BT** exhibited typical SSLE properties. The fluorescence of the probe (excited at 360 nm) was negligible in dimethyl sulfoxide (DMSO) as the good solvent, whereas a gradual increase in the H₂O fraction of the solvent system led to a gradual fluorescence enhancement at $\lambda_{\max} = 470$ nm, which is characteristic of TPE (Fig. 2a and b). The fluorescence of the probe dropped slightly in pure water, which is common for TPE-based fluorogens.³⁴ This

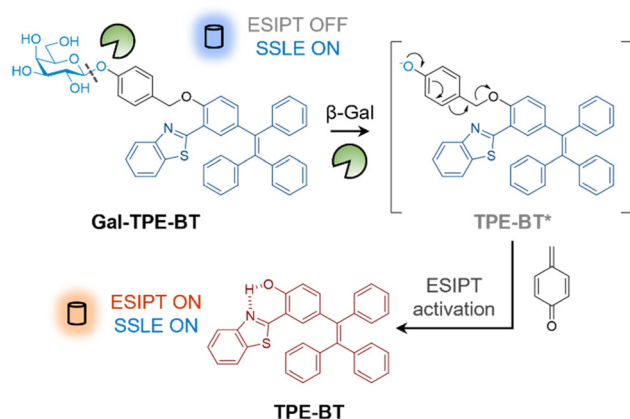


Fig. 1 Schematic illustration of the ratiometric detection of β -galactosidase based on excited-state intramolecular proton transfer (ESIPT) and solid-state luminescence enhancement (SSLE).

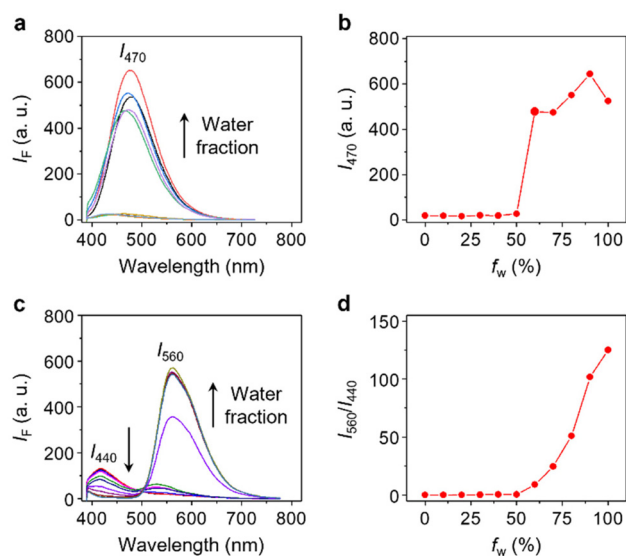


Fig. 2 Fluorescence emission spectra of (a) **Gal-TPE-BT** (10 μ M) and (c) **TPE-BT** (10 μ M) in DMSO with increasing water fractions. (b) Plotting the maximum fluorescence emission intensity of **Gal-TPE-BT** at 470 nm in a mixed solvent of H₂O/DMSO as a function of water fraction (f_w). (d) Plotting the ratios of the maximum fluorescence emission intensity of **TPE-BT** at 560 nm and 440 nm in a mixed solvent of H₂O/DMSO as a function of water fraction (f_w). The excitation wavelength used for **Gal-TPE-BT** and **TPE-BT** is 360 nm.

suggests the successful suppression of the ESIPT process in **Gal-TPE-BT** through the masking of the phenolic proton.

In the presence of β -Gal, the fluorescence emission spectra of **Gal-TPE-BT** in mixed H₂O/DMSO solvents changed substantially (Fig. 2c). We observed a new red-shifted emission band with λ_{\max} at 560 nm that was sharply enhanced as the water fraction increased, and the original emission band with λ_{\max} at 440 nm gradually decreased. The newly emerged emission band is assignable to the keto-state emission of **TPE-BT**,³¹ suggesting the recovery of the ESIPT mechanism of the probe.^{35,36} More interestingly, the gradually enhanced fluorescence at $\lambda_{\max} = 560$ nm with increasing water fraction suggests the maintenance of the SSLE mechanism in **TPE-BT**, which favours sensing applications in an aqueous phase.

To confirm the enzymatic hydrolysis, mass spectroscopic (MS) analysis generated a MS peak assignable to **TPE-BT** detected after treatment of **Gal-TPE-BT** with β -Gal (Fig. S1†), corroborating that the galactosyl substrate can be deglycosylated by the enzyme. We also determined that the ratiometric fluorescence changes (I_{560}/I_{440}) of the probe were dependent on the concentration of β -Gal (Fig. 3a), and a good linearity from 1–6 U mL⁻¹ was determined (Fig. 3b). The limit of detection of the probe for β -Gal was determined to be 0.03 U mL⁻¹ ($3\sigma/k$, where σ is the standard deviation of ten blank samples, and k is the linear slope of the ratiometric changes of the probe as a function of β -Gal concentration). In addition, a kinetic study indicated a 13.5-fold increase in the I_{560}/I_{440} ratio after the reaction between the probe and the enzyme for 300 min (Fig. S2a and b†), and the K_m and V_{\max} were deter-



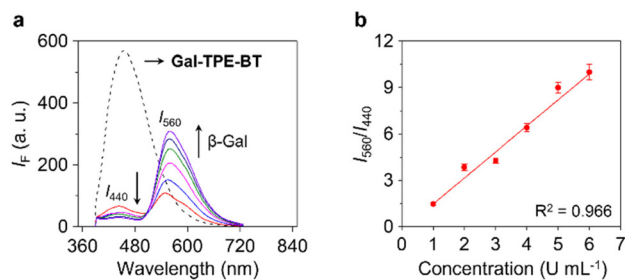


Fig. 3 (a) Fluorescence emission spectra of Gal-TPE-BT (10 μM) incubated with increasing concentrations of $\beta\text{-Gal}$ (0–6 U mL^{-1}) for 2 h. (b) Plotting the ratios of the maximum fluorescence emission intensity of Gal-TPE-BT (10 μM) after treatment with increasing concentrations of $\beta\text{-Gal}$ (1–6 U mL^{-1}) for 2 h at 560 nm and 440 nm as a function of $\beta\text{-Gal}$ concentration (error bars mean S. D., $n = 3$). All measurements were performed in a solvent mixture of phosphate buffered saline (PBS) (0.01 M, pH 7.4)/DMSO (7 : 3, v/v) with an excitation of 360 nm.



Fig. 4 (a) Fluorescence emission spectra of Gal-TPE-BT (10 μM) in the presence of $\beta\text{-Gal}$ (5 U mL^{-1}) and other biospecies including lysozyme (5 U mL^{-1}), ribonuclease A (RNase A) (5 U mL^{-1}), cellulase (5 U mL^{-1}), alkaline phosphatase (ALP) (5 U mL^{-1}), γ -glutathione (GSH) (100 $\mu\text{g mL}^{-1}$), ascorbate (100 $\mu\text{g mL}^{-1}$), bovine serum albumin (BSA) (100 $\mu\text{g mL}^{-1}$) and a β -glucosidase ($\beta\text{-Glc}$) (5 U mL^{-1}). (b) Plotting the ratios of the maximum fluorescence emission intensity of Gal-TPE-BT (10 μM) of the different analytes. All measurements were performed in a solvent mixture of phosphate buffered saline (PBS) (0.01 M, pH 7.4)/DMSO (7 : 3, v/v) with an excitation of 360 nm.

mined to be 27 μM and 0.04 $\mu\text{M s}^{-1}$, respectively, from the Lineweaver–Burk plots (Fig. S2c and d†). We also measured the K_m and V_{max} of a commercial fluorescent $\beta\text{-Gal}$ probe, 4-methylumbelliferyl- β -D-galactoside (4-MU- $\beta\text{-Gal}$) (Fig. S3†). The \sim twofold smaller K_m of Gal-TPE-BT (27 μM) than that of 4-MU- $\beta\text{-Gal}$ (48 μM) suggests a higher affinity of our probe for the enzyme.

Next, we studied the morphological changes of Gal-TPE-BT before and after the enzymatic reaction by high-resolution transmission electron microscopy (HRTEM). In its representative TEM images (Fig. S4†), we observed tube-like structures of Gal-TPE-BT, and after addition of $\beta\text{-Gal}$, aggregated particles began to emerge. In addition, DLS (dynamic light scattering) used showed that the hydrodynamic parameter of the probe after reaction with $\beta\text{-Gal}$ was much larger than that of Gal-TPE-BT without the treatment of $\beta\text{-Gal}$ (Fig. S5a†). These results corroborate the SSLE property of the probe before and after treatment of the enzyme. The critical micelle concentration (CMC) of the probe was determined to be 5.2 μM (Fig. S5b†), and subsequent DLS and fluorescence analyses by continuously incubating the probe in PBS for 60 h suggest the good colloidal stability of Gal-TPE-BT (Fig. S5c and d†).

Finally, we evaluated the selectivity of Gal-TPE-BT with a range of different biological species (Fig. 4a and b). We determined that the presence of unselective enzymes including lysozyme, ribonuclease A (RNase A), cellulase and alkaline phosphatase (ALP), and biologically relevant species including γ -glutathione (GSH), vitamin C (VC) and bovine serum albumin (BSA) did not cause the fluorescence emission of the probe to change. More importantly, the treatment of Gal-TPE-BT with a β -glucosidase ($\beta\text{-Glc}$) that hydrolyzes glucose, which is the C4-epimer of galactose similarly caused minimal fluctuation in fluorescence of the probe. These results suggest the good selectivity of Gal-TPE-BT for $\beta\text{-Gal}$ sensing.

To conclude, the incorporation of both ESIPT and SSLE mechanisms into a single molecular probe led us to achieve the ratiometric detection of $\beta\text{-Gal}$ activity over a range of other

enzymes. The large Stokes shift associated with the probe makes it a promising tool for the unbiased analysis of glycosidase activities in diagnostic and drug-screening assays. This research also paves the way for the design of sensitive fluorescent probes for the detection of other enzymatic activities based on mixed photophysical mechanisms.^{37–41}

Conflicts of interest

The authors have no conflicts of interest to declare.

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