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Self-immolative trigger-initiated polydiacetylene probe for β-glucuronidase activity†

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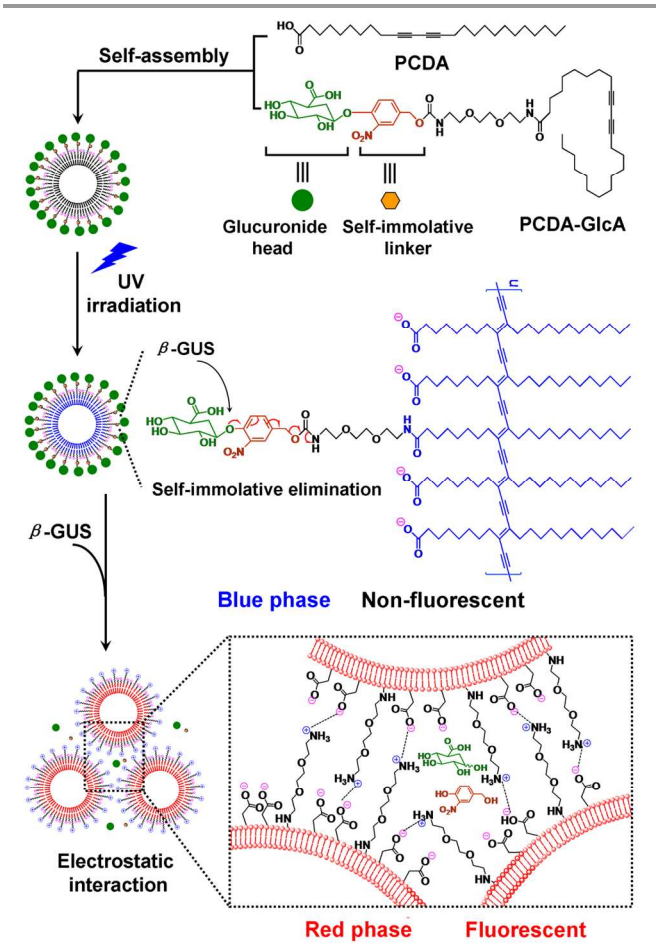
We report a novel probe for β-glucuronidase activity based on the colorimetric and fluorescent responses of polydiacetylene liposomes.

β-Glucuronidase (*β*-GUS) that exists in organisms catalyzes the cleavage of glucuronic acid moieties from various glycoconjugates.[1] In recent years, this enzyme has gained considerable attention as a tumor marker,[2] a converting enzyme for cancer pro-drug therapies,[3] and a report gene marker for tracing gene delivery vectors.[4] *β*-GUS is also involved in many metabolic diseases such as rheumatoid arthritis, leprosy, myocardial infarction, bacterial or virus infection and cancer.[5] Accordingly, monitoring *β*-GUS activity and screening its potential inhibitors are of great importance for the early diagnosis and therapy of *β*-GUS related diseases. Some known fluorogenic substrates and radiochemistry- or magnetic resonance-based techniques have been developed for *β*-GUS activity analysis.[6,7] However, the requirements of specific instrumentation and well-trained personnel always make these methods complex, time-consuming and expensive. Therefore, a simple, rapid and reliable detection system for *β*-GUS is highly desirable. Particularly, equipment-free sensor systems such as colorimetric detection by the naked eye would be among the best and most practically useful methods.[8]

Polydiacetylenes (PDAs), a family of the most intensively researched conjugated polymers, have been demonstrated to have very effective sensing properties.[9] PDAs display an intense blue color and the blue PDAs can undergo a color shift to a red phase upon environmental stimulation. The stimulus-induced blue-to-red transition and fluorescence enhancement of PDAs have made these polymers ideal materials for the development of various chemosensors.[10]

Here, we report a novel PDA probe for *β*-GUS, which displays fluorescence enhancement and colorimetric changes in a self-immolative manner upon the addition of *β*-GUS (Scheme 1). To construct the probe system, we first design and synthesize a new diacetylene monomer PCDA-GlcA that contains a *β*-D-glucuronide head and a self-immolative linker 4-hydroxy-3-nitrobenzyl alcohol (Scheme 1). After PCDA-GlcA and 10,12-pentacosadiynoic acid (PCDA) liposome formation in aqueous solution and subsequent polymerization under 254 nm UV-light irradiation, the resulting liposomes exhibit a grossly evident blue phase, as well as a non-fluorescent feature. Upon the addition of *β*-GUS, the *β*-D-glucuronide moieties are specifically cleaved by *β*-GUS and trigger a



Scheme 1. Schematic illustration of the PDA liposomes designed for *β*-GUS activity detection.

self-immolative elimination[11] along the 4-hydroxy-3-nitrobenzyl alcohol backbone. Thus, a perturbation on the liposome surface occurs. Simultaneously, the exposed primary amine groups promote liposome aggregation through electrostatic interactions. As a result, these molecular interactions induce a conformational transition of the conjugated backbone,[12] which produces a blue to red color change and fluorescence enhancement.

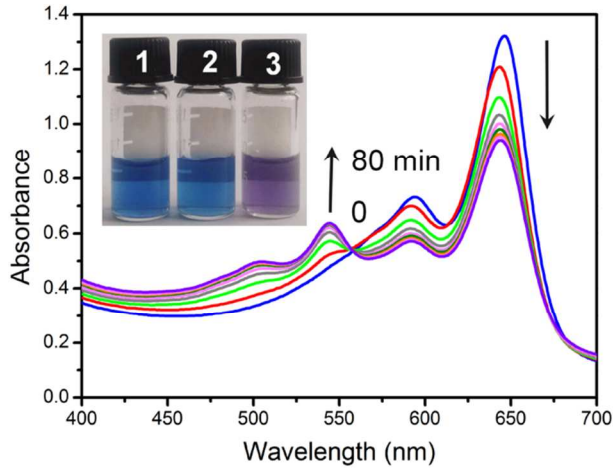


Figure 1. UV-vis absorbance spectra of PDA liposomes (250 μM) prepared from 30% PCDA-GlcA and 70% PCDA (molar percent) as a function of reaction time upon incubation with β-GUS (1.2 μM). The spectra were measured every 10 min. Inset: Color changes in PDA liposome 1 prepared from 30% PCDA-GlcA and 70% PCDA without β-GUS, PDA liposome 2 prepared from pure PCDA (250 μM) with β-GUS, and PDA liposome 3 prepared from 30% PCDA-GlcA and 70% PCDA with β-GUS after 40 min incubation in PBS (50 mM, pH 7.4) at 35 °C.

To demonstrate this concept, PDA liposomes containing 30% PCDA-GlcA and 70% PCDA (molar percent) were first prepared. These liposomes showed an intense blue color after photo-polymerization (Figure 1, inset). The reason for the use of the PCDA-GlcA to PCDA ratio can be found in the Supplementary Materials (Figures S1 and S2, ESI†). As expected, the liposome solution exhibited a blue to red color change when β-GUS was added. However, no color change was observed for the liposome solution prepared using pure PCDA. These phenomena imply that the self-immolative elimination triggered by β-GUS occurs on the PDA liposome surface, leading to perturbations of the PDA backbone and further causing distinct color changes. The β-GUS-induced color change of PDA liposomes was further monitored by UV-vis spectroscopy (Figure 1). Pristine blue-phase PDA liposome solution showed a maximum absorption at 645 nm. β-GUS addition caused a dramatic decrease of this absorption intensity, as well as a remarkable increase in absorption at 545 nm with increasing time. Considering that the PDA color change is often accompanied by fluorescence enhancement, the enzymatic assays were also detected using fluorescence spectroscopy (Figure 2). Gradual fluorescence enhancement appeared as the incubation time increased. Further analysis shows that the color change and fluorescence enhancement also depend on β-GUS concentration (Figure S3, ESI†). An increase in β-GUS concentration resulted in a rapid colorimetric response (CR, %)[12] and fluorescence enhancement at the same reaction time (Figure S4, ESI†). The limit of detection (LOD) of this assay, as determined using the fluorescence enhancement within 50 min at S/N = 3, was 0.05 μM.[13] All of the results thus far demonstrate that the constructed probe system can been employed to detect β-GUS activity and monitor the process of the enzymatic hydrolysis in a simple and visible manner.

According to our original hypothesis, the enzyme-induced color change of the PDA liposomes may be caused by two main reasons: (1) surface perturbation caused by self-immolative elimination along the 4-hydroxy-3-nitrobenzyl alcohol backbone after the β-D-glucuronide moiety is specifically cleaved by β-GUS, and (2) electrostatic interactions between protonated amines produced by the self-immolative reaction and the PCDA terminal carboxyl groups.

4-Hydroxy-3-nitrobenzyl alcohol has maximum absorbance at 420 nm in the UV-vis absorption spectrum.[7] Therefore, the β-GUS induced elimination reaction can be monitored by the formation of 4-hydroxy-3-nitrobenzyl alcohol in the reaction system. To confirm this elimination, we dissolved pure PCDA-GlcA in water to obtain a clear solution. When the solution was incubated with β-GUS, a gradual but apparent increase in the absorbance band at 420 nm was observed within 35 min incubation (Figure S5, ESI†). Electrospray ionization mass spectroscopy analysis (Figure S8, ESI†) also showed that PCDA residues are present in the reaction mixture. These results indicate that the self-immolative reaction induced by β-GUS actually occurs in the substrate.

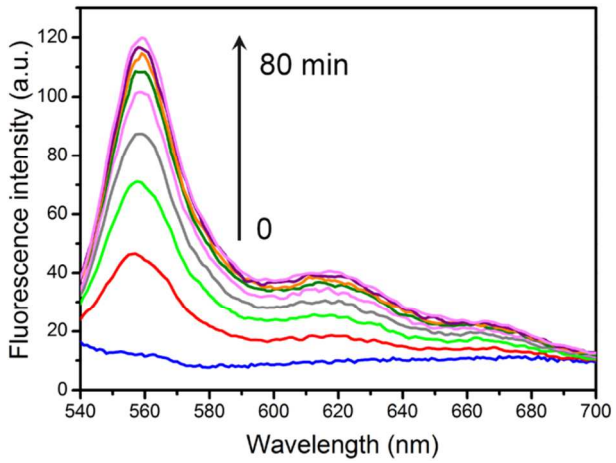


Figure 2. Fluorescence emission spectra of PDA liposomes (250 μM) prepared from 30% PCDA-GlcA and 70% PCDA (molar percent) as a function of reaction time upon incubation with β-GUS (1.2 μM). The spectra were measured every 10 min.

To verify the presence of electrostatic interaction in the post-reaction system, we first investigated changes in the size, morphology and zeta-potential (ζ) of the PDA liposomes before and after incubation with β-GUS. In the absence of the enzyme, the PDA liposomes are almost spherical and well separated (approximately 177 nm mean size) (Figure S9, ESI†). However, addition of β-GUS to the PDA liposomes caused a dramatic morphological change, i.e. extensive aggregation (mean size ~316 nm) occurred. These results suggest that electrostatic attraction between liposomes occurs after the enzymatic hydrolysis reaction. Zeta-potential (ζ) analysis showed that the ζ value for the PDA liposomes was -12.4 mV. In the presence of β-GUS, the ζ increased to 1.35 mV. Such a change is attributed to the exposed amine groups as well as electrostatic interactions. To verify the validity of this hypothesis, we used carboxyl-protected PCDA (compound 7, ESI†) instead of PCDA to prepare liposome solution. The carboxyl-protected PCDA cannot provide anions in aqueous solution. Incubation of the solution with β-GUS resulted in insignificant changes in the fluorescence and absorption spectra with CR values below 10% (Figure S6, ESI†). In addition, liposomes prepared with pure compound 7 showed no response to β-GUS (Figure S7, ESI†). These results confirm that electrostatic interactions between the protonated amine and the terminal carboxyl group of PCDA promote the β-GUS-induced color

change of the PDA liposomes. Also, the results provide supportive evidence that the self-immolative elimination can affect the optical property of PDA liposomes.

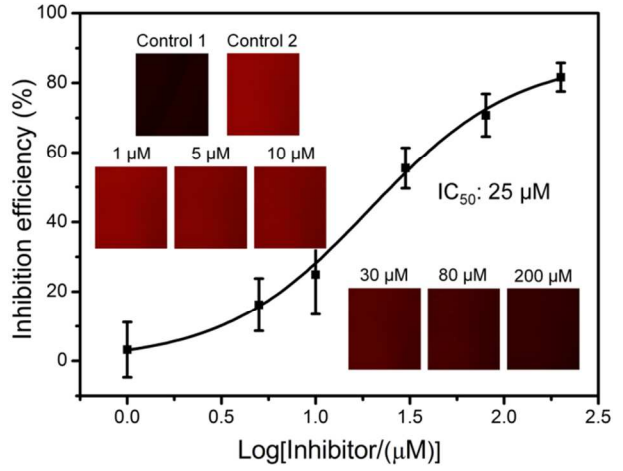


Figure 3. Plot of inhibition efficiency of D-glucaric acid-1,4-lactone for β-GUS versus the concentration of the inhibitor determined by fluorescence intensity. Inset: Visualization of β-GUS inhibition using PDA liposomes (250 μM) prepared from 30% PCDA-GlcA and 70% PCDA in different concentrations of D-glucaric acid-1,4-lactone (concentrations: 1, 5, 10, 30, 80, and 200 μM). Control groups are set as control 1 (without the enzyme and the inhibitor) and control 2 (without the inhibitor).

In terms of simplicity and visualization, the proposed colorimetric and fluorometric turn-on probe also provides a convenient solution for β-GUS inhibitor screening. D-glucaric acid-1,4-lactone, a known β-GUS inhibitor,[5b] was chosen to demonstrate the applicability of our probe system to the screening of enzyme inhibitors. Different concentrations of the inhibitor were mixed with β-GUS and incubated with PDA liposomes for 1 h. As shown in Figure 3, D-glucaric acid-1,4-lactone exhibited potent inhibition of β-GUS activity. The assay solution without β-GUS and inhibitor showed almost no fluorescence (Figure 3, inset). By contrast, when the solution with β-GUS was incubated, a strong red fluorescence was noted. Addition of the inhibitor decreased the fluorescence intensity. Furthermore, as the concentration of the inhibitor increased, the fluorescence became increasingly weaker. The inhibition effect can also be monitored via color changes (Figure S10, ESI†). The color change of the PDA liposome solutions became slowly in the presence of the inhibitor. As the concentration of inhibitor increased, the color of the solution approached the original blue color. The color remained nearly constant when the D-glucaric acid-1,4-lactone concentration reached 200 μM, which indicates complete inhibition of β-GUS activity. These results reveal that the inhibitor decreases the activity of β-GUS and reduces the hydrolysis rate of β-D-glucuronide from PDA liposomes. Therefore, less perturbation occurred and fewer amines were produced on the surface of PDA liposomes, resulting in limited aggregation of PDA liposomes. Based on the inhibition efficiency curves (Figure 3 and Figure S10 in ESI†)) obtained from the absorbance at 545 and 645 nm, as well as the fluorescence intensity of the solutions, the half maximal inhibitory concentrations (IC_{50}) of D-glucaric acid-1,4-lactone for β-GUS were found to be 25 μM (from fluorescence intensity) and 33 μM (from absorbance at 545 and 645 nm). These values are close to the previously reported value.[5b] The results suggest that the colorimetric and fluorometric screening method for enzyme inhibitors may provide a promising alternative for clinical assays.

In summary, we have developed a novel probe for β-GUS activity based on the colorimetric and fluorescent responses of PDA liposomes. Using this probe system, we can simply detect β-GUS activity with the naked eye without complex instrumentation or experimental procedures. Potential enzyme inhibitor screening can be also determined in a high-throughput manner. By changing the sugar moieties on the liposome surface, this detection system may be made suitable for other enzymes (such as β-galactosidase and β-glucosidase) as a general method for enzymatic detection.

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† Electronic Supplementary Information (ESI) available: Experimental details and supplementary figures. See DOI: 10.1039/c000000x/

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