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# A fluorescent light-up probe based on AIE and ESIPT processes for β-galactosidase activity detection and visualization in living cells

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A novel fluorescent probe **SA-βGal** is reported here with light-up response to β-galactosidase. **SA-βGal** possesses the β-galactopyranoside group to react with β-galactosidase and releases the fluorescent salicylaldehyde azine with both aggregation induced emission (AIE) and excited-state intramolecular proton transfer (ESIPT) characteristics. The linear fluorescent response enables in vitro quantification of β-galactosidase activity in a range of 0−0.1 U/mL with a detection limit of 0.014 U/mL. The probe exhibits significant advantages, such as no self-quenching at high concentrations, large Stokes shift (190 nm) and high specificity to β-galactosidase with an excellent light-up ratio of 820 fold. Moreover, thanks to its good retention in living cells, the application of **SA-βGal** for the imaging of cellular β-galactosidase was also achieved with high contrast.

## 1. Introduction

Glycosidases are important reporter genemarkers. Among them, β-galactosidase, which is over expressed in primary ovarian cancers, is a molecular target for visualizing peritoneal metastases from ovarian cancers[1]. The enzymatic activities of β-galactosidase are reporetd to be enhanced in primary ovarian cancers compared with normal ovaries[2]. In addition, abnormally accumulated β-galactosidase activity has long been reported in senescent cells, allowing β-galactosidase to be an important biomarker for senescence.[3, 4] The sensitive detection of β-galactosidase is thus of great importance.

The fluorescent probes for various analyts have received much interests because of the advantages such as simplicity, low cost, good sensitivity, and capability of real-time detection, which enable effective in-vitro and in-vivo studies.[5-16] Several probes for β-galactosidase activity assay and visualization in living cells have been reported, but most suffer from various disadvantages, such as poor cellular permeability,[17] easy diffusion away from living cells,[18] small Stokes shifts (<40 nm)[19, 20] and self-quenching when accumulated in cells[4, 21]. For example, Y. Urano and coworkers have constructed a fluorescent probe consists of the Tokyo-Green scaffold, which is a fluorescein dye, capable of monitoring β-galactosidase activity in living cells.[18] However, the fluorescence images of β-galactosidase in cells provided by this probe is not clear, mainly because the intracellularly fluorescent product generated by the probe tends to diffuse across the cell membrane, which results in a poorly defined signal. To solve this problem, they replaced TokyoGreen with rhodol as the core fluorophore because rhodol is likely to favor intracellular accumulation in living cells and they clearly visualized β-galactosidase activity in cultured cells.[19] However, rhodol undergoes aggregation-caused quenching (ACQ)[22, 23] when accumlated in cells, which makes the fluorescence in aggregate state much weaker as compared to that in solution[24, 25]. Therefore, it is highly desirable to develop β-galactosidase probes, which can accumulate in living cells without ACQ.

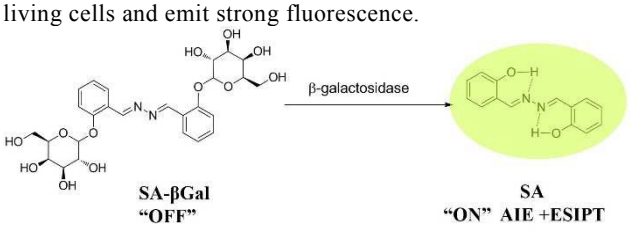
Contrary to many fluorescent dyes, such as fluorescein or rhodamine, which show bright fluorescence in dilute solution, but little or no fluorescence in aggregate state because of the ACQ effect, fluorogens with aggregation induced emission (AIEgens)[26-31] are a new series of molecules emitting strong fluorescence in their aggregate states. Heretofore, many AIEgen systems based on hydrocarbons, heteroatoms or organometallics have been constructed as fluorescent materials for biomedical applications.[32-41] Usually these AIEgens show large Stokes shifts with almost no overlap between their excitation and emission spectra. Besides, they are normally non-fluorescent in solution as a result of the non-radiative decay of the excited states caused by intramolecular motions, while in aggregates, this process is restricted to produce strong fluorescence.[29,35]

Previously, it has been found that a class of salicylaldehyde azines (SAs) are AIE-active[29, 36]. Besides their AIE property, they are also characterized with the excited-state intramolecular proton transfer (ESIPT)[37-40]. Usually, compounds with ESIPT characteristics are found to have high quantum efficiency at high concentrations or in aggregated state due to their large Stokes shift, which greatly reduces self quenching of fluorescence.[41] As a result, SA derivatives that largely benefit from both AIE and ESIPT characteristics can find their potential applications as promising fluorescent materials.

As for AIE-active SA derivatives, the hydroxyl groups at the ortho-position on the benzene ring of these compounds are responsible for ESIPT and necessary for their AIE fluorescence.[36, 42] Therefore, the fluorescence of SA, which is AIE-active via ESIPT, can be quenched if the the hydroxyl groups are blocked and can be subsequently activated after they are unblocked. Recently, we have developped several fluorescent probes based on this strategy for detection of thiols[43], lysosomal esterase[44], palladium cation[45], perborate[45] and UV light[45, 46], demonstrating the versatility of the

platform. In addition, the diameters of the nanoaggrates of the AIEgens are usually in a range of tens to hundreds nanometers, which are suitable for cellular uptake through endocytosis and could be well retained inside the cells.

Herein, we report a fluorescent probe **SA-βGal** with light-up response to β-galactosidase. **SA-βGal** emits very weak fluorescence in aqueous buffer. In the presence of β-galactosidase, the β-galactopyranoside group on **SA-βGal** is cleaved[19], resulting in the formation of strong greenish yellow fluorescent product (**SA**). The probe shows large Stokes shift, high light-up ratio and high selectivity towards β-galactosidase. More importantly, it could be well retrained in living cells and emit strong fluorescence.




**Scheme 1** The fluorescent light-up probe **SA-βGal** for β-galactosidase detection.

## 2. Experiment

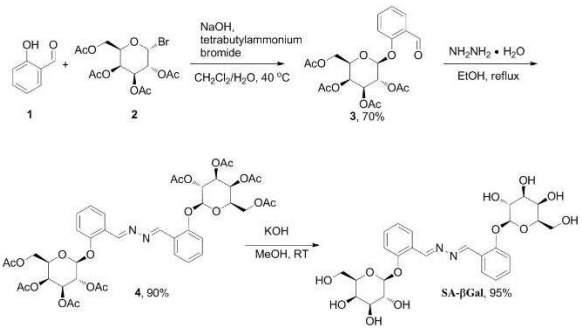
### 2.1 Materials and instruments

All chemicals were purchased from Sigma-Aldrich (St. Louis, USA) and used without further purification.

Absorption spectra were measured on a UV visible spectrometer (Shimadzu UV-1700). All fluorescence spectra were determined on a fluorescence spectrometer (Perkin Elmer LS-55, United States). The determination of fluorescence quantum yield was carried out on a combined steady state and fluorescence lifetime spectrometer (Edinburgh FLS 920, United Kingdom). All NMR spectra were recorded using a NMR spectrometer (Bruker ARX 400, Germany). The mass spectra were obtained on an ion trap time-of-fight mass spectrometry (Shimadzu MS-IT-TOF, Japan). The pH measurement was performed with a pH meter (Sartorius PB-10 basic, Germany). Fluorescence imaging was carried out on a fluorescence microscope (Olympus FV-1000 confocal microscope, Japan).

### 2.2 Synthesis of compound SA-βGal (Scheme 2)

The synthetic route to **SA-βGal** is depicted in Scheme 2. The reaction between 2-hydroxybenzaldehyde **1** and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **2** firstly formed compound **3** in 70% yield. Then compound **3** further reacted with hydrazine hydrate and compound **4** was obtained in 90% yield. Compound **4** further reacted with KOH and the compound **SA-βGal** was produced with 95% yield. The molecular structures were confirmed to be right by the NMR spectroscopy (details can be found in Supplementary Information).




**Scheme 2** Synthetic route to the compound **SA-βGal.**

### 2.3 Analytical procedures

The stock solution ($1.0\times10^{-2}$ M) of **SA-βGal** was prepared in DMSO. Stock solutions of β-galactosidase (100 U/mL) and other analytes ($MgCl_2$, $CaCl_2$, Vitamin C, Lysozyme, Capthsin B, Esterase and β-glucuronidase) were prepared in distilled deionized water, respectively. In a typical detection, 10 μL of **SA-βGal** stock solution was added into a test tube, which was diluted to 1 mL with PBS buffer (10 mM, pH 7.4). Subsequently, a proper amount of β-galactosidase stock solution was added. The fluorescence spectra were recorded before and after the addition of β-galactosidase at different time.

### 2.4 Cell imaging

C6/LacZ or HeLa cells were seeded at a density of $2\times10^4$/well in the chamber (LAB-TEK, Chambered Cover glass System) and grown for 18 h at 37°C in a humidified 5% $CO_2$ incubator. The loading solution of **SA-βGal** was prepared by adding 5 μL of its DMSO stock solution (10 mM) into a microtube. Subsequently 995 μL DMEM was added with vigorous mixing. This loading solution of **SA-βGal** (50 μM) was added to the cells and the cells were incubated for 2 h at 37 °C. The fluorescence imaging of the cells was then implemented. The images were collected upon excitation at 405 nm, and the signals were collected using an emission filter at 525-625 nm.

## 3. Results and discussion

### 3.1 Probe Design

As shown in Scheme 1, after the hydroxyl groups at the ortho-position on the benzene ring of salicylaldehydeazine (**SA**) are blocked by β-galactopyranoside, no ESIPT is possible in the molecule (**SA-βGal**) because there are no more hydroxyl protons at the ortho-position. Therefore, this blocked compound (**SA-βGal**) should be non-fluorescent. Upon the addition of β-galactosidase to remove the blocking groups, the hydroxyl and ESIPT in the yielded **SA** can be recovered to regain the AIE fluorescence.

### 3.2 The AIE characteristics of SA

The AIE characteristic of **SA** was investigated in water/DMSO co-solvents with different water volume fraction ($f_w$= 0-90 vol%), and the results are shown in Fig. 1 and Fig. S1 in the Supplementary information. **SA** dissolves well in DMSO but is not soluble in water. In a good solvent ($f_w$= 0 vol%), **SA** disperses

well and exhibits weak fluorescence. Nevertheless, in a poor solvent ($f_w$= 90 vol%), an intense fluorescence band at 545 nm can be observed. The AIE effect of **SA** occurs when the water volume fraction in the co-solvent is more than 50%. The AIE fluorescence intensity is also found to be enhanced by a higher water volume fraction, which correlates well with the increased aggregates formation in poorer solvents.

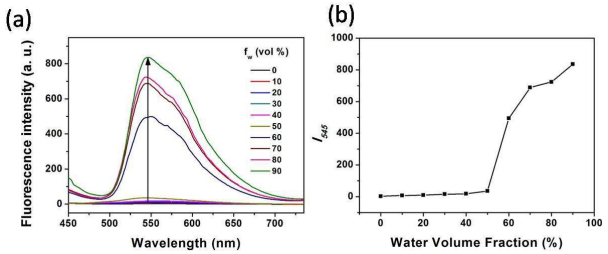



**Fig.1** (a) Fluorescence spectra of 100 μM **SA** in co-solvent mixtures containing 0~90 % PBS buffer (10 mM, pH 7.4) and 100~10% DMSO, as well as (b) the corresponding fluorescence intensity ($I_{545}$) change. Excitation is performed at 387 nm.

### 3.3 Study of β-galactosidase activities in solution

The fluorescence of **SA-βGal** in the absence and presence of β-galactosidase was first studied to prove our design principle above (Scheme 1). As shown in Fig. S2 (Supplementary information), **SA-βGal** (100 μM) formed colloidal aggregates in PBS buffer (10 mM, pH 7.4) containing 1% DMSO, which was confirmed by dynamic light scattering (DLS) analysis, indicating that the average particle diameter is 173 nm. The blocked compound **SA-βGal** displays very weak fluorescence (quantum yield $\Phi$<0.001) in the colloid solution, suggesting successful blocking effect of β-galactopyranoside group. Upon addition of β-galactosidase, the fluorescence of **SA-βGal** enhanced gradually until strong greenish yellow fluorescence (545 nm) was observed (quantum yield $\Phi$=0.246). The time-dependent fluorescence intensity change of **SA-βGal** in the colloid solutions was recorded without and with the addition of β-galactosidase (Fig. 2b and Fig. S3), showing that the fluorescent light-up effect only occurs in the presence of β-galactosidase and the reaction could be fully completed. Thanks to the low background fluorescence of the blocked compound **SA-βGal,** the light-up ratio could be as high as 820 fold, which is comparable to that reported by Nagano (986 fold)[19] and better than that reported by Tung (110 fold)[21]. As shown in Fig. 2d and Fig. S4, a good linearity could be found in the β-galactosidase concentration range of 0-0.10 and 0.1-1.0 U/mL. The detection limit was calculated as 0.014 U/mL based on the definition by IUPAC ($C_{DL}$ =3$S_b$/$m$) from 10 blank solutions, which is similar to that reported by Urano[19].

It has been reported by Urano's group[19] and others[4,21] that β-galactopyranoside can be cleaved by β-galactosidase to release hydroxyl group. To verify this, the fluorescent product after reaction between **SA-βGal** and the enzyme was isolated by filtration and subsequently characterized. The results in Fig. S6 clearly suggest the formation of **SA** from **SA-βGal**. In addition, the fluorescence spectrum of **SA-βGal** after reaction with β-galactosidase and that of the reference compound **SA** are almost identical (see Fig. S7), supporting the hypothesis in our design (Scheme 1).

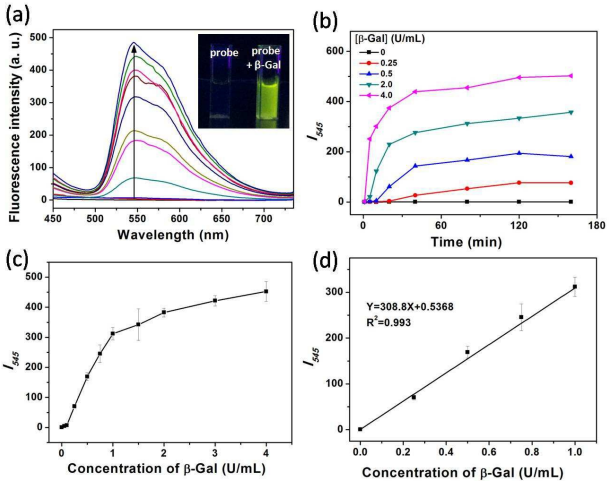



**Fig.2** (a) Fluorescence spectra of **SA-βGal (**100 μM) in the presence of various concentrations of β-galactosidase (0-4.0 U/mL) in PBS buffer solution (10 mM, pH 7.4, 37 ℃). Insets from left to right: photographs of **SA-βGal** (100 μM) without or with β-Gal (4.0 U/mL) under UV light (365 nm). (b) The corresponding fluorescence intensity ($I_{545}$) change over time and (c) concentration. (d) Calibration curve of the fluorescence intensities ($I_{545}$) versus β-galactosidase concentrations.

The selectivity of **SA-βGal** towards β-galactosidase was also studied (Fig. 3). Upon the addition of β-galactosidase, the fluorescence intensity of **SA-βGal** was greatly enhanced. However, no change was observed with some commonly used inorganic salts or biomolecules, such as $MgCl_2$, $CaCl_2$, Vitamin C, Lysozyme, Capthsin B, Esterase and β-glucuronidase. Thus, **SA-βGal** shows good selectivity to β-galactosidase.

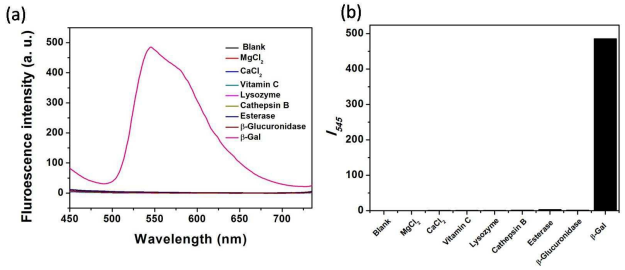



**Fig.3** (a) Fluorescence spectra of **SA-βGal (**100 μM) upon addition of various species: $MgCl_2$ (1 mM), $CaCl_2$ (1 mM), vitamin C (1 mM), and enzymes (lysozyme, cathepsin B, esterase, β-glucuronidase and β-galactosidase, each enzyme is 4.0 U/mL) in 10 mM PBS buffer ( pH 7.4, 37 ℃). (b) The corresponding fluorescence intensity ($I_{545}$).

### 3.4 Cellular imaging

The use of **SA-βGal** in its colloid solutions also has been investigated to visualize β-galactosidase activity in living cells. The results of DLS analysis (Fig. S2, Supplementary information) supported the formation of colloids of **SA-βGal** in aqueous solutions. The average diameter of the particles of **SA-βGal** in colloid solutions is about 173 nm, which is suitable to be uptaken by cells through endocytosis and expected to be well retained in cells. It is reported that β-galactosidase is over expressed in rat glial tumor C6/LacZ cells.[21] Therefore, we applied probe **SA-βGal** for imaging β-galactosidase activity in C6/LacZ cells. After incubating C6/LacZ cells with the colloid solution of **SA-βGal** for 2 h at 37 °C, strong fluorescence was observed (Fig. 4a). In

order to demonstrate the specificity of **SA-βGal** towards β-galactosidase activity, we undertook a control experiment (Fig. 4b). HeLa cells do not express β-galactosidase,[47] thus we expect that there would be no fluorescence observed when HeLa cells are incubated with **SA-βGal**. As can be seen in Fig. 4b, almost no fluorescence could be observed in HeLa cells, which confirms the specificity of probe **SA-βGal** for β-galactosidase activity studies.

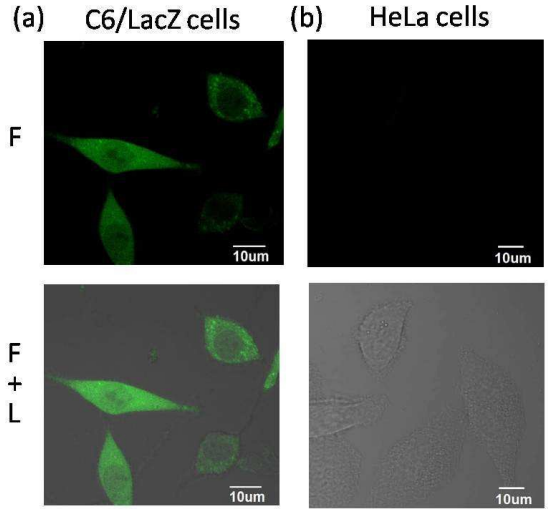



**Fig. 4** Imaging β-galactosidase activity in cells. Images of probe **SA-βGal** (50 μM) in (a) C6/LacZ cells and (b) HeLa cells for 2 h at 37 ℃, ("F", fluorescence image; "F+L", overaly image). The excitation was set at 405 nm.

## 4. Conclusions

In summary, we have developed a blocked salicylaldehyde azine derivative showing light-up fluorescence response to β-galactosidase activity. Upon the addition of β-galactosidase, the blocking groups at the hydroxyl moieties of the probe **SA-βGal** were removed, thus recovering ESIPT and strong fluorescence in the aggregate states. Unlike other β-galactosidase probes sufferring from easy diffusion away from living cells and self-quenching when accumulated in cells, the fluorescent hydrolysis product of **SA-βGal** was well retained inside the living cells and displayed strong emission when accumulated in living cells due to its AIE characteristic, making **SA-βGal** more preferable for imaging intracellular β-galactosidase activity. **SA-βGal** also exhibits other advantages such as high light-up ratio (820 fold) and large Stokes shift (190 nm). This "AIE + ESIPT" fluorescent light-up probe design strategy provides opportunities for specific detection and cellular imaging of various enzymes simply through the relative cleavable reaction.

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## Notes and references

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†Electronic Supplementary Information (ESI) available: Characterization of **SA-βGal** and supplementary Figures. This material is available free on line. See DOI: 10.1039/b000000x/
‡ These authors contributed equally to this work

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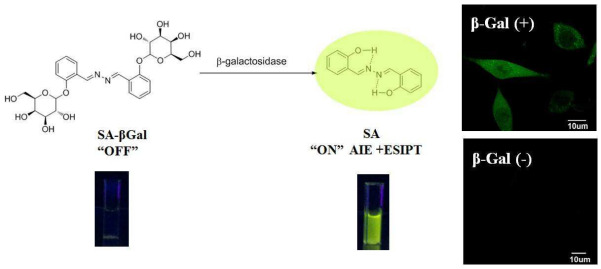
Graphical abstract

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