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A novel ratiometric fluorescent probe for highly sensitive and selective detection of β-galactosidase in living cells

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Summary of main observation and conclusion β-galactosidase, a glycoside hydrolase enzyme, has been proved to be an important biomarker of cell senescence and primary ovarian cancer. Effective detection of β-galactosidase has attracted widely attention. Herein, one ratiometric fluorescent probe has been successfully synthesized for detecting the β-galactosidase in living cells. The as-prepared probe exhibits two emission peaks at 490 nm and 530 nm, respectively, and the ratio of fluorescence intensities from the two emission peaks could be utilized to monitor the β-galactosidase. This present ratiometric fluorescent probe is, therefore, very promising for effective, sensitive, and selective detection of the β-galactosidase in living cells.

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Introduction

As a lysosomal exoglycosidase, β-galactosidase (β-Gal) plays a vital role in the human body for removing galactose residues from various substrates, such as gangliosides, sphingolipids, glycoproteins, and keratin sulfate [1]. A variety of diseases (e.g. ovarian cancer, β-galactosialidosis, and morquio B syndrome) was implicated in the alteration of the β-galactosidase concentration [2]. At the cellular level, the β-galactosidase can also be employed as a reporter to examine the transcription and transfection efficiencies, or as a marker for targeted the expression of gene or destruction in cells [3]. It is also a vital biomarker for cell aging [4]. So, monitoring the β-galactosidase activity within living cells or tissues has aroused considerable interests. Some methods have been explored for detecting the β-galactosidase, such as magnetic resonance, positron emission tomography, and single photoemission computed tomography et al [5]. However, these methods are hampered by the high cost, laborious manipulation, modest sensitivity, and invalid monitor of β-galactosidase in living cells [6]. Due to the advantage of fluorescent probes, such as advanced sensitivity, convenient handling procedures, less expensive instruments, and strong talent of bioimaging, the fluorescent probes have received considerable attentions for monitoring the β-galactosidase activity in living cells [7].

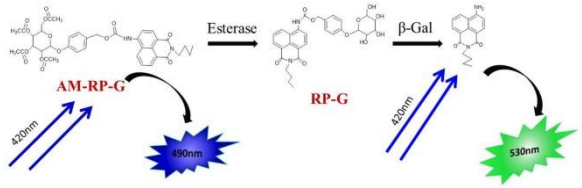
As the enzymatic hydrolysis could produce a blue dimerized chromophore for fluorescent observation, the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) has been used as one common fluorescent probe for β-galactosidase [8]. However, if the X-gal was used to detect the β-galactosidase in cell, the cells must be fixed and usually the detection efficacy of β-galactosidase is manually counted [9]. The fluorescein di-β-D-galactopyranoside (FDG) as another common fluorescent probe exhibits a turn-on fluorescent response after it was hydrolyzed [10]. However, its poor ability to be loaded in cell would restrict the FDG for the observation of the cellular β-galactosidase [11]. On the other hand, the fluorescent intensity of these probes were directly utilized for detecting the β-galactosidase [12]. However, the measurement for fluorescence with this strategy would be influenced by many factors, such as various environment conditions (pH, polarity, temperature, etc), the localization of the probes in cells, collection efficiency of emission, and excitation intensity [13]. To eliminate the influence of these factors, the emission ratiometric probe could provide a selective measurement mechanism for the detection of β-galactosidase [14, 19].

In the present work, we designed an intramolecular-charge-transfer (ICT) based ratiometric probe (AM-RP-G) composed of 4-amino-1, 8-napthalic anhydride derivative as the fluorophore [7, 14], esterified α-D-galactopyranosyl as the β-galactosidase hydrolytic site, and 4-hydroxybenzaldehyde as the linkage group, respectively, for β-galactosidase detection. The hydrolysis by the β-galactosidase would make the emission wavelength of AM-RP-G shift from 490 nm to 530 nm. Moreover, as the increase of the β-galactosidase concentration, the emission intensity of unhydrolyzed probe at 490 nm would be decreased. Meanwhile, the emission intensity of the hydrolyzed probe at 530 nm would be increased simultaneously. So the ratio of the fluorescence intensity at 530 nm and 490 nm would sensitively depend on the concentration of β-galactosidases. It was found that the fluorescence intensity ratio of the probe showed good linear dependence on the concentration of the β-galactosidase in the range of 0-0.07 U/ml, and a detection limit of 1.4×10^{-3} U/mL was achieved. Moreover, this probe can also be applied to monitor the β-galactosidase activity in cells, which opens opportunities for biomedical research, containing researches for cell aging, cells or tissues imaging even targeted tumor imaging [15].

Results and Discussion

Owing to the high photo stability and large Stokes shifts of the polycyclic aromatic skeleton, the fluorophore 4-amino-1, 8-napthalic anhydride derivative was chosen as the fluorophore [14, 15, 20]. Then α-D-galactopyranosyl was used as identifying component and 4-hydroxybenzaldehyde was selected as the linker. For improving the accumulation of the probe in cells, the α-D-galactopyranosyl as a part of the probe was esterified. All the three parts make up the probe AM-RP-G. Once the probe AM-RP-G entered into the cells, the ester moiety of the probe would react with the ubiquitous intracellular esterases and the RP-G was resultantly cleaved from AM-RP-G [10]. Then, the identifying component α-D-galactopyranosyl of the RP-G was recognized by the target β-galactosidase. The β-galactosidase-mediated hydrolysis would induce a self-immolative reaction in RP-G. Consequently, the amino group was freed [7]. The amino group as a strong electron donor could enhance the ICT in the fluorophore. As a result, the wavelength of maximal emissions from fluorophore would shift from 490 nm to 530 nm (SI, Figure. S1). The mechanism was illuminated in scheme 1.

Scheme 1. The principle of AM-RP-G for β-galactosidase detection.



To investigate the response of the AM-RP-G to β-galactosidase in aqueous solution, the AM-RP-G was firstly incubated with esterase to produce RP-G. The stock solution of the AM-RP-G was diluted with 0.05M PBS to form a solution of 4.5×10^{-5} M AM-RP-G (the system containing 9 U/mL esterase, pH 7.4, 1% DMSO, 37 ℃). As shown in SI, Figure.S2, insignificant change was observed from the fluorescence when the AM-RP-G was treated with the esterase of 9 U/mL for 0.5 h. However, when the β-galactosidase was subsequently added into the system, the fluorescence intensities at 530 nm gradually increased accompanying the gradual decrease of the fluorescence intensities at 490 nm (Figure. 1), indicating the AM-RP-G has an effective response to β-galactosidase.

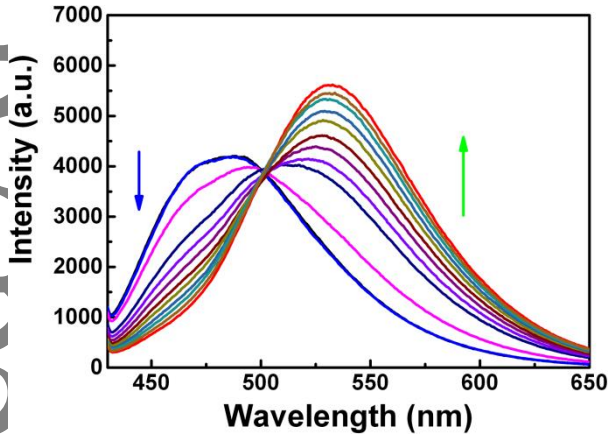


Figure.1. Fluorescence spectra of AM-RP-G with time after addition of 0.06 U/L β-galactosidase in 4.5×10^{-5} M AM-RP-G solution in 50 mM PBS buffer (pH 7.4, containing, 9 U/L esterase,, 1% DMSO , 37 °C).

In order to further verify that the β-galactosidase indeed caused the change of the emission wavelength of the AM-RP-G, the inhibitor experiments were conducted. The D-galactose, a competitive inhibitor for β-galactosidase [16] was selected, with the β-galactosidase were added into the 4.5×10^{-5} M AM-RP-G solution in 50 mM PBS buffer (pH 7.4, containing 9 U/L esterase, 1% DMSO, 37 °C), incubated for 110 min. The results are showed in Figure.2. From Figure.2a, it can be found little change of the emission spectra was observed from AM-RP-G after adding D-galactose and β-galactosidase (Figure. 2a). But the emission spectra from AM-RP-G increased gradually at 530 nm with incidental decrease at 490 nm after adding only β-galactosidase (Figure.1). Figure.2b indicated the ratio of the fluorescence at 530 nm and 490 nm from Figure.1 and Figure.2a. Obviously, the ratio change of the fluorescence with D-galactose could be ignored and the ratio change of the fluorescence without D-galactose was significant. These results illuminated that the change of the emission from the AM-RP-G were caused by the β-galactosidase.

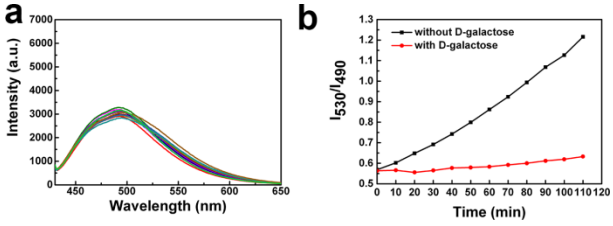
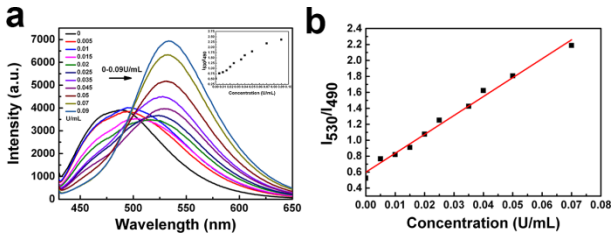


Figure.2. (a).The fluorescence spectra of AM-RP-G with 2.8×10^{-5} D-galactose with time after addition of 0.06 U/L β-galactosidase in 4.5×10^{-5} M AM-RP-G solution in 50 mM PBS buffer (pH 7.4, 9 U/L esterase, 1% DMSO, 37 °C); (b) The fluorescence ratio I_{530}/I_{490} of AM-RP-G with time in Figure.1 and Figure.2a (red line and black line represent with and without D-galactose, respectively).

The response of the AM-RP-G to β-galactosidase with various concentrations was investigated for qualitatively detection of β-galactosidase. Different concentrations of β-galactosidase (0 U/mL to 0.09 U/mL) in 4.5×10^{-5} M AM-RP-G solution in 50 mM PBS buffer (containing 9 U/mL esterase, 1% DMSO, pH 7.4, 37 °C) were incubated. As shown in Figure. 3a, the emission at 490 nm was gradually decreased as the concentration of β-galactosidase was increased, while, the emission at 530 nm was enhanced gradually.

Figure.3. (a) The fluorescence emission spectra of AM-RP-G at



different concentrations of β-galactosidase after reaction for 110 min. The inset plots in (a) shows the ratio of I_{530}/I_{490} at different concentrations of β-galactosidase after reaction for 110 min. (b) The linear dependence of the ratio of I_{530}/I_{490} on β-galactosidase. The probe concentration is 4.5×10^{-5} M, in PBS buffer (0.05 M, pH 7.4, containing 9 U/L esterase, 1% DMSO, 37 °C).

In addition, the intensity ratio of I_{530}/I_{490} was improved almost four times when the concentration of the β-galactosidase increased from 0 U/mL to 0.09 U/mL. The reaction nearly reached equilibrium with 0.07 U/mL β-galactosidase (Figure. 3a inset). A linear dependence of I_{530}/I_{490} on the concentrations of β-galactosidase from 0 U/mL to 0.07 U/mL can be obtained. A detection limit of 1.4×10^{-3} U/mL was achieved according to LOD = 3σ/s, where σ was the standard deviation of ten blank signals and s was the slope value [17], as shown in Figure. 3b.

Next, the ability of AM-RP-G to monitor the β-galactosidase in real time was examined. The change of I_{530}/I_{490} with the incubation time was analyzed at various β-galactosidase concentrations from 0 U/mL to 0.09 U/mL. The fluorescent intensities ratios were measured with the time interval of 10 min and the results were shown in Figure. 4.

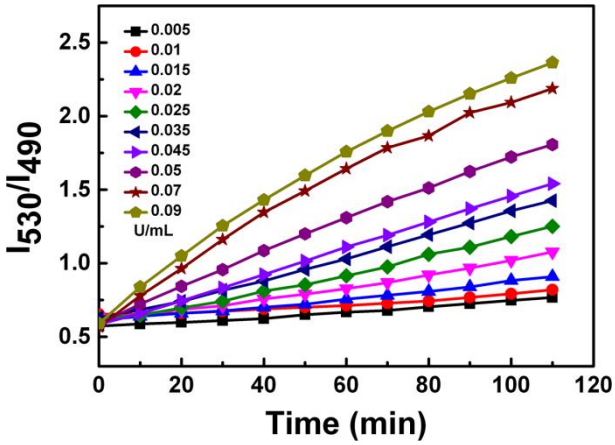


Figure.4. Fluorescence ratio I_{530}/I_{490} of AM-RP-G recorded every 10 min under varying β-galactosidase concentrations. The probe concentration is 4.5×10^{-5} M, in PBS buffer (0.05 M, pH 7.4, containing 9 U/L esterase, 1% DMSO, 37 °C).

It can be found that the ratio of I_{530}/I_{490} gradually increased with either the increase of the β-galactosidase concentrations or

duration of incubation time, which are in accord with the enzymatic reaction kinetics.

The selectivity of the probe to β-galactosidase was further investigated. Various co-existing enzymes in cells were added into the 4.5×10^{-5} M AM-RP-G system (0.05 M PBS, containing 9 U/L esterase, 1% DMSO, pH=7.4, 37 ℃) and incubated for 110 min. The emission wavelengths of the systems showed little change except the β-galactosidase. (Figure.5a).

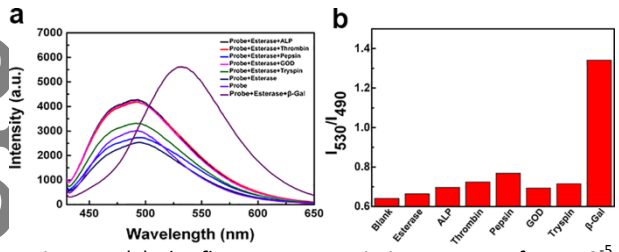


Figure.5. (a) The fluorescence emission spectra of 4.5×10^{-5} M AM-RP-G incubated with different enzymes. The fluorescence ratio I_{530}/I_{490} of AM-RP-G after incubated with different enzymes. The concentration of esterase is 9 U/mL, ALP is 3U/mL, Thrombin is 3 U/mL, Pepsin is 3U/mL, GOD (Glucose Oxidase) is 3 U/mL, Tryspin is 3 U/mL, β-galactosidase is 0. 35 U/mL. (0.05 M PBS , 1% DMSO , 37℃, 110 min).

As shown in Figure.5b, the ratio of I_{530}/I_{490} almost unchanged with the addition of other enzymes. These results indicated that the present AM-RP-G had favorable selectivity to β-galactosidase.

Moreover, to study the stability of AM-RP-G for detection of β-galactosidase at various pH, the AM-RP-G was dissolved in solutions with different pH at 37 ℃ for 10 min and then the fluorescence of the AM-RP-G was monitored. Varying the pH from 3 to 10, the ratio of I_{530}/I_{490} almost exhibited little change (Figure. 6a), indicating the AM-RP-G owns intrinsic stability under various pH conditions. The stability and performance of AM-RP-G in a complex system was also detected. The AM-RP-G was dissolved in a solution containing 10% BSA incubated at 37 ℃ for 120 min. The ratio of I530/I490 almost exhibited little change (Figure. 6b) in the BSA solution at 120 min. Then 0.05U β-galactosidase was added and the ratio changed obviously after the addition of β-galactosidase for 120 min. This result manifested that the AM-RP-G can be used to detect β-galactosidase activity at physiological environment.

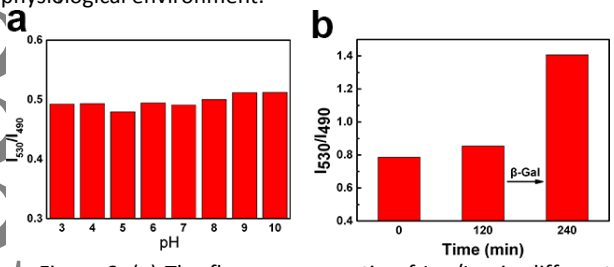


Figure.6. (a) The fluorescence ratio of I_{530}/I_{490} in different pH reaction environment. (b) The fluorescence ratio of I_{530}/I_{490} in 10% BSA solution. The probe concentration is 4.5×10^{-5} M, 1% DMSO, 37 °C.

For further application of AM-RP-G in cells, the cytotoxicity of AM-RP-G was investigated with the MTT method and the results were shown in Figure S3. The viability of HeLa cells was 80% after incubation for 12h with 1.0×10^{-6} M AM-RP-G. Then, the AM-RP-G was used to detect exogenous β-galactosidase in live HeLa cells. Before taking the fluorescence image, the cells were incubated with AM-RP-G for 5 min in cell culture medium and subsequently washed with PBS solution for five times to remove the extracellular AM-RP-G. Then the exogenous β-galactosidase were added to the dishes and incubated for different times. The fluorescence images of the HeLa cells were captured with a Nikon A1 confocal microscopy with an oil 100× objective lens and excitation of 405 nm laser. As shown in Figure. 7a, the HeLa cells only exhibited blue fluorescence emission in 0 min. However, when the cells were incubated with β-galactosidase for 1 h （Figure.7b-merged）and 2 h （Figure.7c-merged）, the blue emission from HeLa cells was gradually weakening (Figure. 7b-blue and c-blue) and the HeLa cells simultaneously exhibited remarkable green (Figure. 7b-green and c-green) emission. At the same time, the florescence changes of HeLa cells along the yellow arrows in Figure. 7 (a, b, c) were collected in different incubation times of 0 min, 60 min and 120 min. (Figure. 7d, e, f). It can be observed from Figure.7e-f that the intensities of green emission were gradually enhanced.

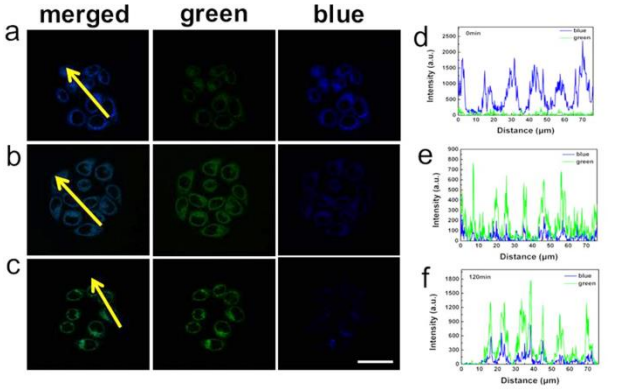


Figure.7. Confocal fluorescence microscopy images of HeLa cells. HeLa cells sequentially treated with 0.005 U/ml β-galactosidase for 0 min (a), 60 min (b), 120 min (c), (d, e, f) the fluorescence intensity profile within the regions, yellow arrows of (a, b, c). The scale bar is 20 μm. The green line indicates the green emission and the blue line indicates the blue emission. Excited at 405 nm; blue channel: 430 nm-490 nm; green channel: 510 nm-580 nm.

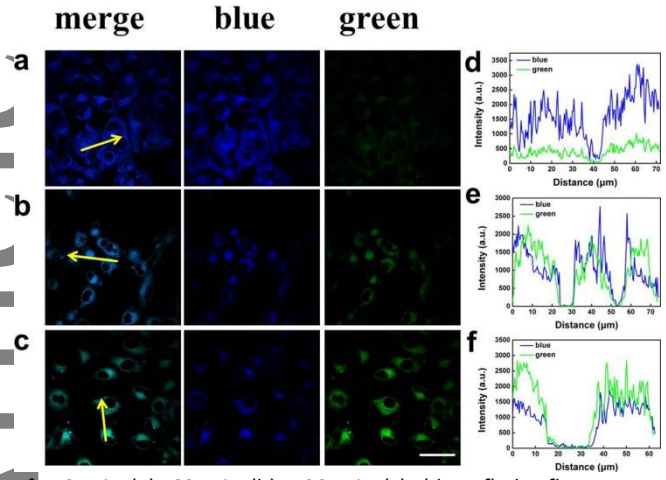
The change of fluorescence emission intensity in HeLa cells at different times were calculated, as shown in Figure.S4, the ratio of green emission and blue emission had an obvious change. And the change of fluorescence emission from HeLa cells was accordant with the change of the fluorescence spectra shown in Figure. 1. All results confirmed that AM-RP-G could be used as a novel ratiometric fluorescence probe for an effective detection of β-galactosidase in cells.

To apply this probe to detect endogenous β-galactosidase, live SKOV3 cells in which the concentration of β-galactosidase is high than normal cells were incubated with AM-RP-G for 8 mins in cell culture medium and subsequently washed with PBS solution for three times to remove the extracellular AM-RP-G, before the fluorescence images were captured. The fluorescence images of the SKOV3 cells were captured with a Nikon A1 confocal microscopy with an oil 60× objective lens and excitation of 405 nm laser. As shown in Figure. 8a, the SKOV3 cells only exhibited blue fluorescence emission in 0 min. However, when the cells were incubated with cells for 1 h（Figure.8b-merged）and 2 h （Figure.8c-merged）, the blue emission from SKOV3 cells was gradually weakening (Figure. 8b-blue and c-blue) and the SKOV3 cells simultaneously exhibited remarkable green (Figure. 8b-green and c-green) emission. At the same time, the florescence changes of SKOV3 cells along the yellow arrows in Figure. 8 (a, b, c) were collected in different incubation times of 0 min, 60 min and 120 min. (Figure. 8d, e, f). It can be observed from Figure.8e-f that the intensities of green emission were gradually enhanced. And the change of fluorescence intensity in SKOV3 cells at different times were calculated, as shown in Figure.S5 the ratio of green emission and blue emission had an obvious change. For verify that the fluorescence change was caused by the β-galactosidase in live SKOV3 cells, another dish full of SKOV3 cells was incubated with inhibitor (D-galactose) before the AM-RP-G was dropped in, the

fluorescence change had almost no change in 120 min, Figure.S6.

The results confirmed that AM-RP-G could be used as a ratiometric fluorescence probe for an effective detection and imaging of β-galactosidase in cells.

Figure.8. Confocal fluorescence microscopy images of SKOV3 cells. SKOV3 cells sequentially treated with 5.5×10^{-6} M AM-RP-G



for 0 min (a), 60 min (b), 120 min (c), (d, e, f) the fluorescence intensity profile within the regions, yellow arrows of (a, b, c). The scale bar is 20 μm. The green line indicates the green emission and the blue line indicates the blue emission. Excited at 405 nm; blue channel: 430 nm-490 nm; green channel: 510 nm-580 nm

Conclusions

In summary, we have designed and fabricated the AM-RP-G composed of 4-amino-1, 8-napthalic anhydride derivative as fluorescence group and α-D-galactopyranosyl as the identifying component for accurate detection of the β-galactosidase in aqueous solution and cells. The probe showed a linear dependence on the concentration of the β-galactosidase between 0-0.07 U/ml with a detection limit of 1.4×10^{-3} U/mL. Moreover, the probe also exhibited high selectivity toward the β-galactosidase. Present probe may have a potential for the monitor of the β-galactosidase in living cells to evaluate the efficacy of cancer chemotherapy.

Experimental

Material and apparatus

4-hydroxybenzaldehyde, 4-amino-1, 8-napthalic anhydride and N-butylamine were purchased from Sigma-Aldrich. 2, 3, 4, 6-Tetra-O-acetyl-α-D-galactopyranosylbromide and triphosgene were purchased from Aladdin. Other reagents and solvents were purchased from Beijing Chemical Regent Co. Hela cells were purchased from the center of cells, Peking Union Medical College. All the reagents and solvents were used without further purification.

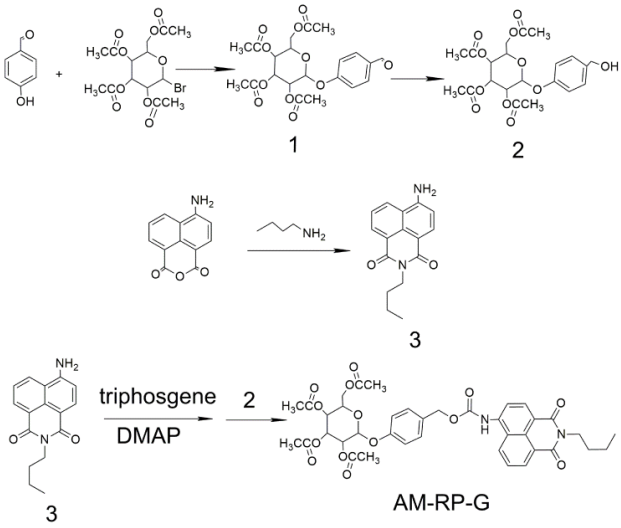
^{1}H NMR spectra were recorded on a Bruker Advance 400 spectrometer with tetramethylsilane as an internal standard. Electrosprayionization high resolution mass spectra (ESI-HRMS) were recorded on a Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS, Bruker Apex IV). ^{13}C NMR (100 Hz) spectra were obtained on a Bruker Avance 400. Fluorescence spectra were got from a Hitachi 4600 spectrophotometer with excitation of 420 nm. Absorption spectra were got from a Hitachi, U-3900 spectrophotometer. Samples for fluorescence emission measurements were contained in 1 cm × 1 cm quartz cuvette. Fluorescent images of the cells were got from a confocal laser scanning microscopy Nikon A1 with a 100× oil-immersion objective lens, excited at 405 nm, collected in blue and green channels.

Cell culture

Before taking the fluorescence image, Hela cells and SKOV3 cells were cultured in confocal microscope dishes in Dulbecco's Modified Eagle Medium (DMEM) media at 5% CO_2 air atmosphere at 37 °C in a humidified incubator of Panasonic MCO-5AC for 48 h for cell attachment.

Synthesis of AM-RP-G

The synthetic routes for the AM-RP-G were outlined in scheme 2.



Scheme 2. Synthesis of AM-RP-G.

At first, the molecule 1 and 2 were synthesized following a previous work in supporting information [16]. And then, molecule 3 was synthesized as follow: 4-amino-1, 8-napthalic anhydride (150 mg, 0.7 mmol) was dissolved in 25 mL ultra-dry ethanol ethanol under nitrogen atmosphere and heated to 90 ℃ in dark. Then, n-butylamine (0.3 mL, 3 mmol) was added into the ethanol and refluxed for 20 hours [17]. After cooling, the solvent was removed under reduced pressure, and the crude materials was purified by chromatography on silica gel (20:1 DCM: EtAc) to obtain the compound 3 as a yellow solid in yield of 93.5 %.^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61 (d, J = 7.4 Hz, 1H), 8.43 (d, J = 7.9 Hz, 1H), 7.52(s,2H),7.49 – 7.38 (m, 1H), 7.00 (d, 1H), 6.89 (d, J = 8.3 Hz, 1H), 4.15 (t, J = 7.4 Hz, 2H), 1.71 – 1.67 (m,2H), 1.67 – 1.09 (m, 2H), 0.97 (t, J = 7.4 Hz, 3H). ^{13}C NMR (CDCl3) δ, 164.6, 164.1, 148.9, 133.7, 129.8, 125.0, 123.3, 120.2, 112.4, 109.6, 40.1, 30.3, 20.4, 13.9. ESI-HRMS (m/z): calculated for $C_{16}H_{16}N_2O_2$ [M -H] $^-$: 267.1279, found: 267.1141.

Next, a solution of triphosgene (61 mg, 0.2 mmol) in toluene (3 mL) was added dropwise to the mixture of compound 3 (53.6 mg, 0.2 mmol) and DMAP（4-dimethylaminopyridine） (74 mg, 0.6 mmol) in toluene (4 mL). The solution was heated at 100 ℃ for 3.5 hours. After the solution was cooled to room temperature, the reaction mixture was diluted and filtered with dichloromethane (6 mL) and the filtrate was obtained without further purification. Then, the compound 2 (90.8 mg, 0.2 mmol) was added into above filtrate and the solution was stirred at room temperature for 4 hours [18]. After that, the solution was concentrated and purified by column chromatography (silica gel, 1:10 EtOAc/CH_2Cl_2) and obtained AM-RP-G as yellow solid (49.7

mg, 0.07 mmol, 35% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 – 8.47 (m, 2H), 8.32 (d, J = 8.0 Hz, 1H), 8.06 (d, J = 8.5 Hz, 1H), 7.69 (t, J = 7.9 Hz, 1H), 7.34 (d, J = 8.0 Hz, 2H), 7.02 – 6.88 (m, 2H), 5.48 – 5.38 (s, 2H), 5.28 (d, 1H), 5.18 (s, 1H), 5.02 (dd, 8.2 Hz, 2H), 4.3（t, 1H）, 4.17(t, 2H), 4.15(t, 1H), 4.10(d, 2H), 2.15(s, 3H) 2.02 (s, 3H), 1.98 (s, 3H), 1.95 (s, 3H), 1.68-1.62 (m, 2H), 1.43-1.32 (m, 2H), 0.90 (dd, 3H). ^{13}C NMR ($CDCl_3$) δ, 170.3, 169.5, 164.2, 163.8, 157.4, 153.1, 138.9, 132.6, 131.4, 130.6, 130.4, 129.1, 126.8, 125.8, 123.7, 123.0, 118.2, 117.1, 99.7, 77.4, 71.3, 70.9, 68.8, 67.6, 70.0, 61.4, 40.4, 30.4, 29.8, 20.8, 20.7, 20.5, 14.0. ESI-HRMS (m/z): calculated for $C_{38}H_{40}N_2O_{14}$ $[M+Na]^+$ 771.25, found 771.2373. The stock solution of the AM-RP-G (4.5×10^{-3}M) were prepared in dimethyl sulphoxide (DMSO).

Cytotoxicity assay of AM-RP-G

HeLa cells were firstly seeded to a 96-well plate with the density of 5×10^3 per well and cultured in the Dulbecco's modified eagle medium (DMEM) at 37 ° C for 24 h. Then, AM-RP-G with different concentrations were added to each well, which were incubated with the cells for 12 h. After the incubation, the cells were washed thrice with PBS. Then, 10% volume ratio (v/v) MTT dye solution (10 mg/mL) were added to obtain the value of cell viability. All experiments were performed in triplicate.

Acknowledgement

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A Novel Ratiometric Fluorescent Probe for Highly Sensitive and Selective Detection of β-galactosidase in Living Cells

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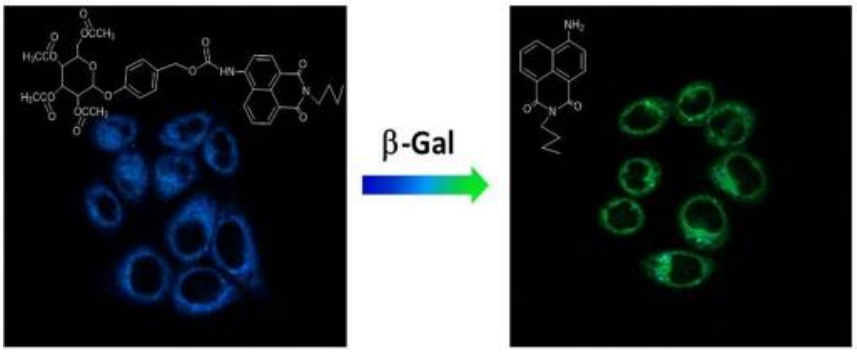

A novel ratiometric fluorescent probe for β-galactosidase detection in living cells was designed and synthesized.