Electronic Supplementary Information

A near-infrared fluorescent nanoprobe for senescence-associated β-galactosidase sensing in living cells

Ye Zhao,[a] Shan Sun,*[b] Yan Lyu,[a] Mengyao Gao,[a] Hengwei Lin *[ab] and Cheng Yang *[a]

[a] *Key Laboratory of Synthetic and Biological Colloids, Ministry of Education, School of Chemical and Material Engineering, Jiangnan University, Wuxi 214122, China*

[b] *International Joint Research Center for Photo-responsive Molecules and Materials, School of Chemical and Material Engineering, Jiangnan University, Wuxi 214122, China*

* Corresponding authors.

E-mail addresses: sunshan@jiangnan.edu.cn (S. Sun), linhengwei@jiangnan.edu.cn (H. Lin), cyang@jiangnan.edu.cn (C. Yang).

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1. Experimental section

1.1 Instruments

The transmission electron microscopy (TEM) images were taken on JEM-2100 plus (JEOL, Japan). Hydrodynamic diameters (DLS) and Zeta potentials were recorded by Zeta PALS (Brookhaven, USA). Fourier transform infrared (FT-IR) spectra were measured on Nicolet iS50 FT-IR (Thermo Fisher Scientific, USA). ^{1}H NMR spectrometer was obtained with AVANCE Ⅲ HD 400 MHz (Bruker, Switzerland). Ultraviolet-visible (UV−vis) absorption spectra were acquired from UV-1800 (AOE instrument, China). Fluorescence excitation spectra were measured by CARY Eclipse (Varian, USA). Cell images were acquired from confocal laser fluorescence microscope (TCS SP8, Leica, Germany). MTT assay was conducted via a microplate reader (SpectraMax M2, Molecular devices, USA).

1.2 Materials and Reagents

N,N'-Carbonyldiimidazole (CDI), anhydrous acetonitrile (ACN), ethylenediamine (EDA) were acquired from Adamas Co., Ltd. Poloxamer 407 (F127), phosphate buffered saline (PBS) were purchased from Shanghai Sangon Co., Ltd. Poly[2,1,3-benzothiadiazole-4,7-diyl[4,4-bis(2-ethylhexyl)-4H-cyclopenta[2,1-b:3,4-b'] dithiophene-2,6-diyl]] (PCPDTBT) was bought from Sigma Co., Ltd. Tetrahydrofuran (THF), $MgCl_2$, dimethyl sulfoxide (DMSO), N,N-dimethylformamide (DMF) were purchased from Sinopharm Chemical Reagent Co., Ltd. *P*-nitrophenol (*p*-NP) was acquired from J&K Scientific Ltd. *P*-nitrophenyl-β-D-galactopyranoside (PNPG), β-galactosidase, ɒ-galactal were purchased from Shanghai yuanye Bio-Technology Co., Ltd. HeLa cells were bought from ATCC. Fetal bovine serum (FBS), dulbecco's modified eagle medium (DMEM), antibiotics (10,000 U/mL penicillin and 10,000 μg/mL streptomycin) were acquired from Thermo Fisher Scientific Inc. 4% paraformaldehyde, 5-Bromo-4-chloro-3-indole β-D-galactoside (X-gal), lysosome green fluorescent probe (Lyso-Tracker Green) were purchased from Beyotime Biotechnology Co., Ltd. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), doxorubicin hydrochloride (DOX) were

purchased from Aladdin Industries Inc. Potassium ferricyanide ($K_3Fe(CN)_6$) and potassium hexacyanoferrate ($K_4Fe(CN)_6$) were bought from Macklin Biochemical Co., Ltd.

1.3 Synthesis of F127-NH_2

F127-NH_2 was synthesized according to the previous report[1]. Firstly, N,N'-carbonyldiimidazole (CDI) was dissolved in anhydrous acetonitrile (ACN) and stirred continuously under N_2 atmosphere at room temperature. Next, F127 was dissolved in anhydrous ACN and followed by addition into the CDI dispersion at the molar ratio of 1:10 (n(F127):n(CDI)). After reacting for 4 h, ethylenediamine (EDA) was injected into the mixed solution (n(F127):n(EDA)=1:150) and the reaction was kept overnight. The as-prepared sample was dialyzed against deionized water for 3 days for purification, and the final product was obtained via lyophilization.

1.4 Synthesis of SPN-NH_2

PCPDTBT (250 μg) and F127-NH_2 (20 mg) were firstly dissolved in THF, followed by injection into the mixture of 1 mL of THF and 9 mL of ultrapure water. After 5 min of sonication, THF was removed under a stream of N_2. The crude SPN-NH_2 was filtered through 0.22 μm membrane filter and washed 3 times via centrifugation (3500 rpm,15 min) by ultrafiltration tubes (50 kDa). The obtained SPN-NH_2 was stored at 4 °C in dark for further use.

1.5 Fluorometric assay of β-galactosidase

Time-dependent quenching performance of SPN-NH_2 was explored firstly. The working solution was prepared via mixing of SPN-NH_2 (10 μg/mL), PNPG (0.1 M), $MgCl_2$ (1.6 M) and β-gal (1 U) in phosphate buffer (10 mM, pH=4.5). The fluorescence emission spectra of working solution were recorded at different time points under the excitation wavelength of 680 nm. Concentration-dependent quenching performance of SPN-NH_2 was subsequently investigated. The working solution was prepared following the above mention process except for the concentration of β-gal was varied from 0 to 0.500 U and the mixing time was set as 1 h. The fluorescence signal was recorded

under the above-mentioned condition.

The limit of detection (LOD) of the fluorescent probe was calculated according to the following equation[2]:

$LOD = 3\sigma/k$

where σ is the standard deviation of blank measurements, k is the slope of the linear regression equation obtained from Fig. 3e.

Selectivity of SPN-NH_2 probe towards β-gal was investigated as follows. Biological species including cations (*Fe^{3+}, Cu^{2+}, K^{+}, Ca^{2+}, Na^{+}, Mg^{2+}*, 3.3 mM), amino acids (*serine (Ser), Valine (Val), phenylalanine (Phe)*, 10 mM), enzymes (*Acid proteases (Ala), Dispase, Pepsin, Trypsin, xanthine oxidase (XOD)*, 10 U), H_2O_2 (10 mM) and mixture of them were added into the solution of SPN-NH_2 probe (10 μg/mL), PNPG (0.1 M) and $MgCl_2$ (1.6 M). After 20 min incubation at 37 °C, the corresponding fluorescence signal of these samples were recorded.

1.6 Quenching mechanism

To gain further insight into the FL quenching mechanism, the temperature-dependent quenching behavior was investigated. In brief, 10 μg/mL of SPN-NH_2 was incubated with different concentrations of *p*-NP (0.0, 8.3, 16.6, 25.0 and 33.3 mM) at 25, 35 and 45 °C, respectively. The classical Stern-Volmer model was introduced here to analysis the FL quenching process based on the following equation:

$F_0/F = 1 + K[Q]$

In which F_0 and F represent the FL intensities in the absence and presence of quencher respectively; K is the Stern–Volmer quenching constant; [Q] is the concentration of the quencher.

1.7 Cell culture

HeLa cells were obtained from Shanghai Institute of Biochemistry and Cell Biology. Cells were cultured in dulbecco's modified eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % antibiotics (10,000 U/mL penicillin and 10,000 μg/mL streptomycin) in a 5% CO_2 incubator at 37 °C.

1.8 Cytotoxicity assay

The cytotoxicity of SPN-NH_2 and PNPG was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, 100 μL of HeLa cells were seeded in a 96-well plate at a density of 1×10^4 cells per well and allowed to adhere for 24 h. Then the culture medium was removed, cells were incubated with different concentrations of SPN-NH_2 (0, 25, 50, 75, 100 μg/mL) or PNPG (from 1 to 5 μmol) dispersed in fresh medium. After 24 h incubation, culture medium was discarded, and 100 μL of MTT (0.5 mg/mL) dispersed in fresh medium was added into each well and incubated for 4 h at 37 °C. Subsequently, MTT was removed, 150 μL of DMSO was added into each well followed by shaking for 10 min. Finally, the sample absorbance was recorded at 490 nm using a Microplate reader.

1.9 Co-staining experiment

For lysosomal colocalization experiment, HeLa cells were seeded in glass-bottom dishes with a density of 8×10^4 cells per mL and allowed to adhere overnight. Then HeLa cells were incubated with SPN-NH_2 probe (50 μg/mL) for 24 h. Afterward, cells were stained with Lyso-Tracker Green fluorescent probe (50 nM) dispersed in fresh medium for 20 min (SPN-NH_2 probe, λ_{ex} = 638 nm, λ_{em} = 648-792 nm; Lyso-Tracker probe, λ_{ex} = 488 nm, λ_{em} = 498-550 nm). Cells were washed twice with PBS before imaging.

1.10 Cellular senescence induction

HeLa cells were seeded in 24-well plate at a density of 4×10^4 cells per well and allowed to adhere overnight. Next, cells were incubated with DOX (0.5 μM) dissolved in fresh medium for 72 h. For X-gal staining, cells were pre-treated with 4% of paraformaldehyde for 20 min, followed by staining with working solution including X-gal (1 mg/mL), $K_3Fe(CN)_6$ (5 mM), $K_4Fe(CN)_6$ (5 mM), $MgCl_2$ (1 mM) and 1×PBS (pH=6.0) for 24 h at 37 °C. After washing twice with PBS, the staining images were captured by inverted microscope.

1.11 Detection of SA-β-galactosidase in living cells

HeLa cells were seeded in glass-bottom dishes with a density of 8×10^4 cells per mL

and waited to adhere overnight. Cells treated with or without 0.5 μM of DOX for 72 h was set as senescent and normal group, respectively. Subsequently, cells were incubated with SPN-NH_2 (50 μg/mL) for 24 h, and followed by incubation without or with PNPG (40 mM) for 1 h. Finally, cells were fixed by 4% of paraformaldehyde and washed twice with PBS before imaging via confocal microscopy. The quantitative analysis was performed by ImageJ.

2. Schematic illustration for the preparation of SPN-NH_2

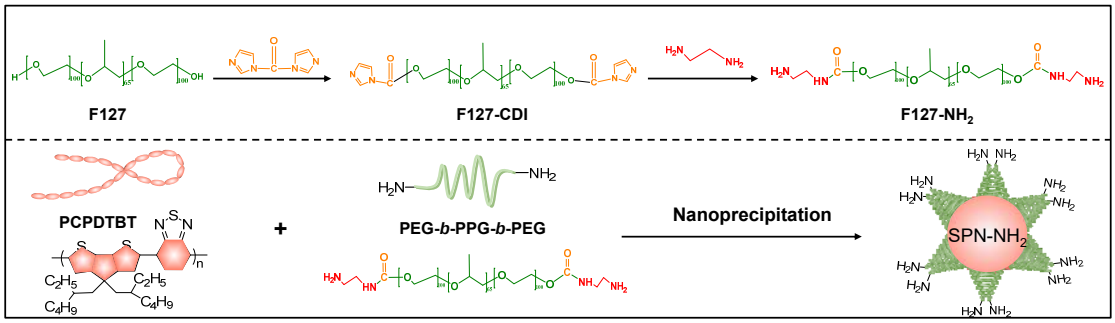

Fig. S1 Schematic diagram of the synthetic route of SPN-NH_2.

3. Characterization of SPN-NH_2

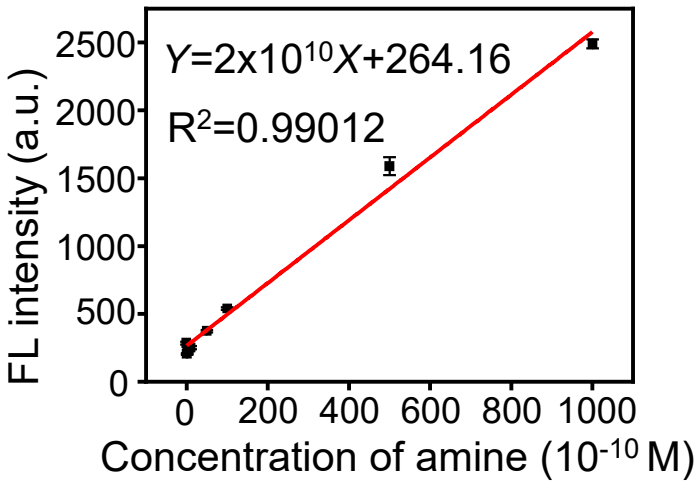

Fig. S2 The relationship between the florescence intensity of n-butylamine added fluorescamine at 478nm and the concentration of n-butylamine.

Tab. S1 The amine content analysis results of F127-NH_2 (10 mg).

	FL intensity (a.u.)	**Content of amine (M)**
1st	792.666	$2.64x10^{-8}$
2nd	734.023	$2.35x10^{-8}$
3rd	713.261	$2.25X10^{-8}$
Average	**746.65**	**$2.42x10^{-8}$**

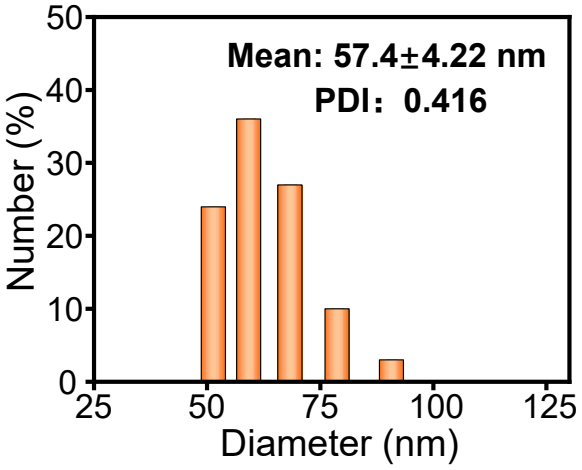

Fig. S3 DLS profile of SPN-NH_2 in aqueous solution.

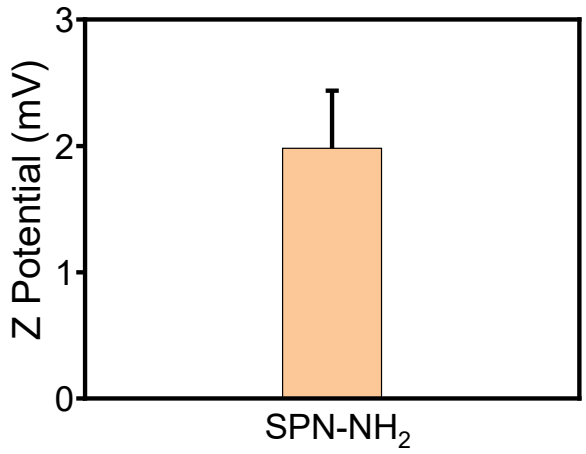

Fig. S4 Zeta potential of SPN-NH_2 in aqueous solution.

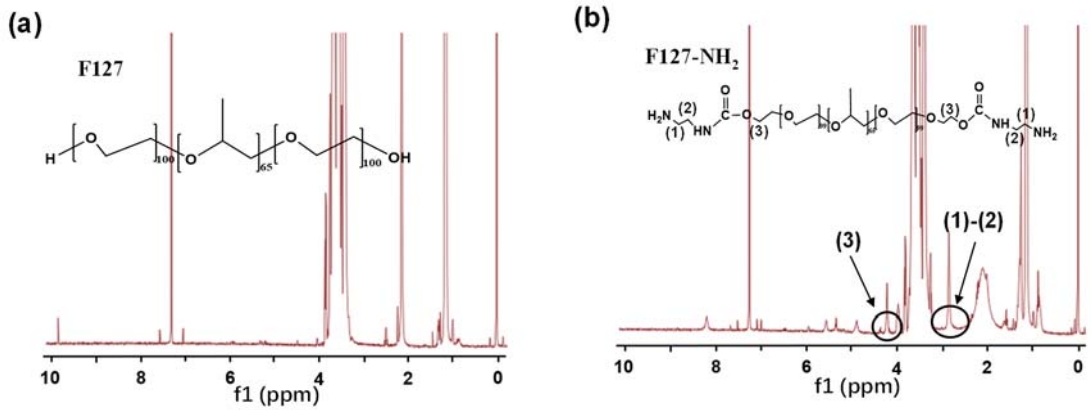

Fig. S5 ^1H NMR spectra of (a) F127 and, (b) F127-NH_2 dispersed in $CDCl_3$.

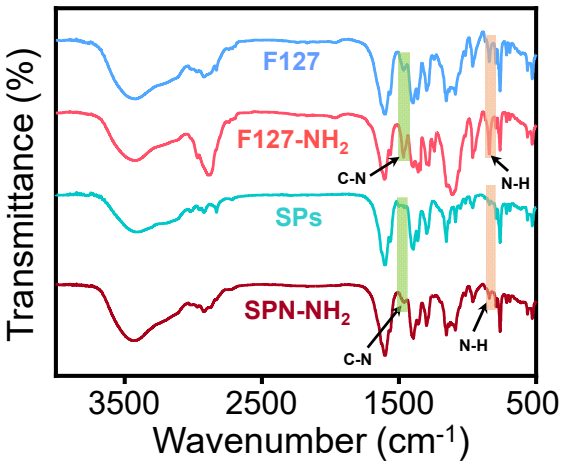

Fig. S6 FT-IR spectra of F127, F127-NH_2, SPs and SPN-NH_2.

4. Optical properties of SPs

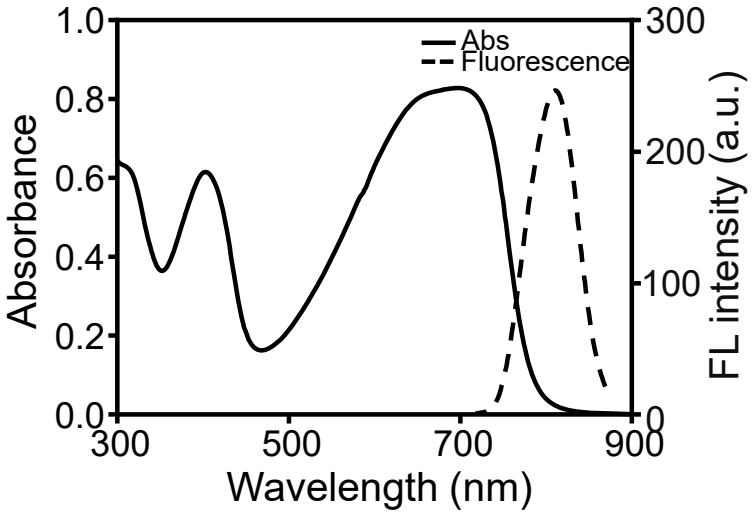

Fig. S7 UV-visible absorption (solid line) and fluorescence emission spectra (dash line) of SPs (10 μg/mL) in THF solution.

5. Sensing properties of SPN-NH_2

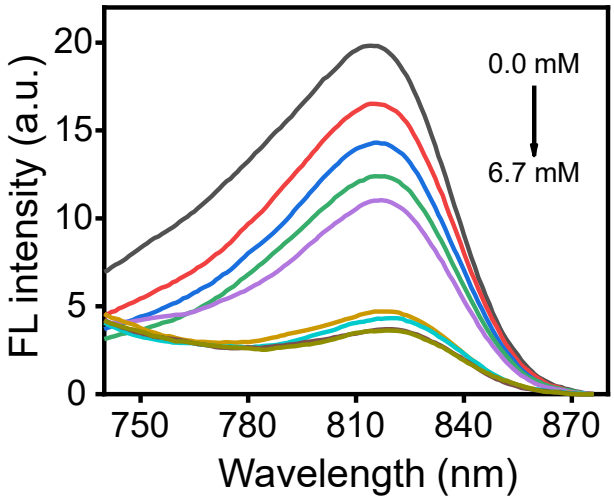

Fig. S8 Fluorescence spectra of SPN-NH_2 (10 μg/mL) with the addition of *p*-NP at different concentrations (from 0 to 6.7 mM).

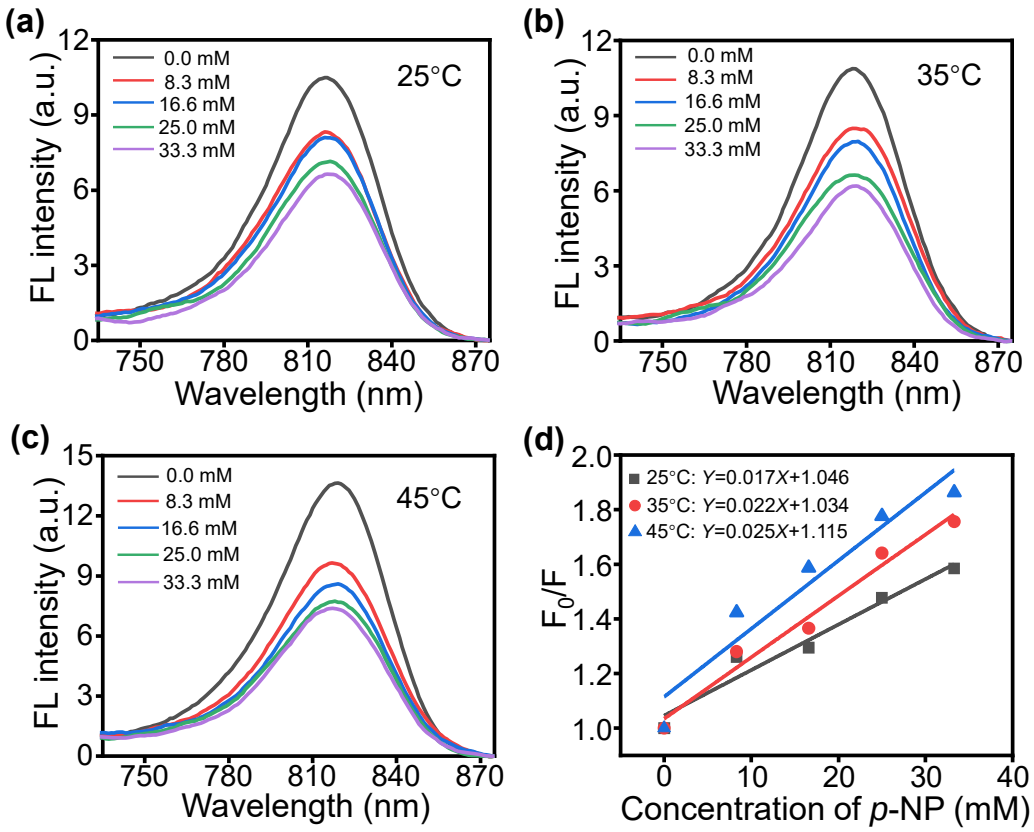

Fig. S9 Fluorescence quenching spectra of SPN-NH_2 (10 μg/mL) incubated with different concentration of *p*-NP for 3 min at (a) 25°C, (b) 35°C, (c) 45°C, and (d) Stern–Volmer plots for the quenching of SPN-NH_2 by *p*-NP at different temperatures.

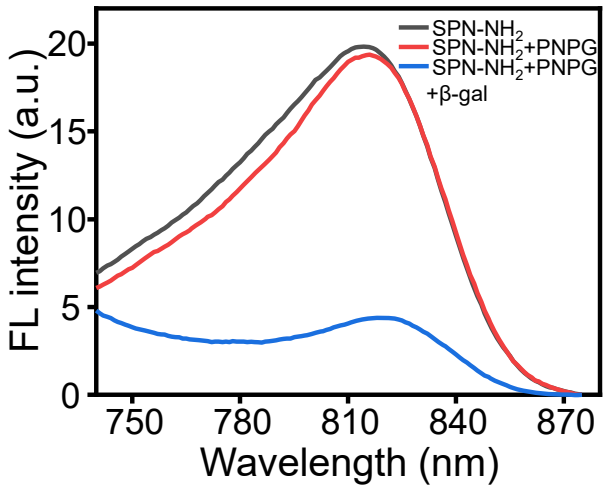

Fig. S10 Fluorescence emission spectra of SPN-NH_2 (10 μg/mL) treated without and with PNPG (0.1 M), and the mixture of SPN-NH_2 (10 μg/mL), PNPG (0.1 M) and β-gal (1 U).

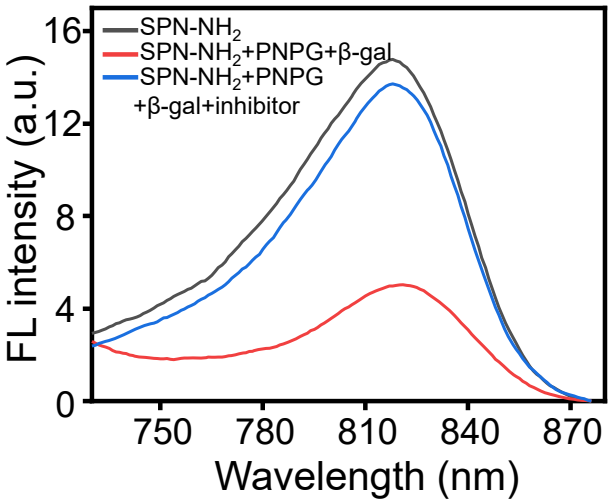

Fig. S11 Fluorescence emission spectra of SPN-NH_2 (10 μg/mL) and the mixture of SPN-NH_2 (10 μg/mL), PNPG (0.1 M) and β-gal (1 U) with and without the addition of inhibitor (ɒ-galactal,33 mM).

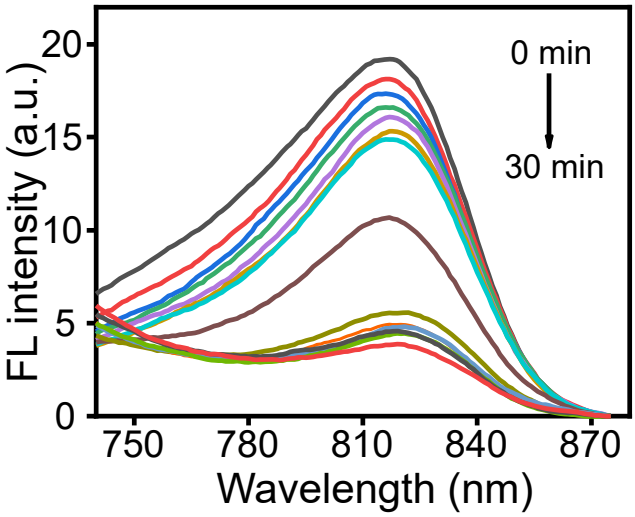

Fig. S12 Fluorescence spectra of SPN-NH_2 (10 μg/mL) incubated with β-gal (1 U) and PNPG (0.1 M) for different times (0 to 30 min) in PB buffer (10 mM, pH=4.5) at 37 °C.

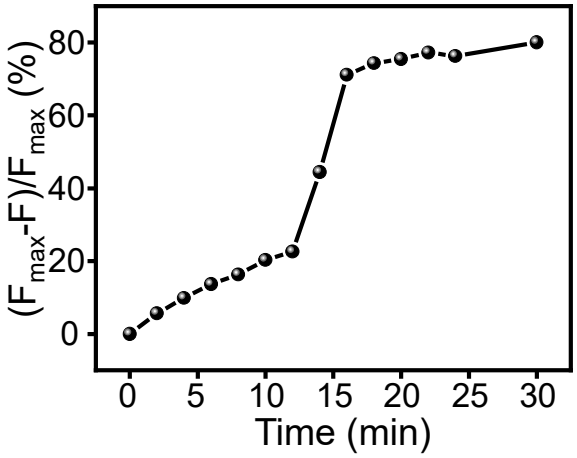

Fig. S13 Inhibition rate of SPN-NH_2 fluorescence intensity at 817 nm as a function of incubation time.

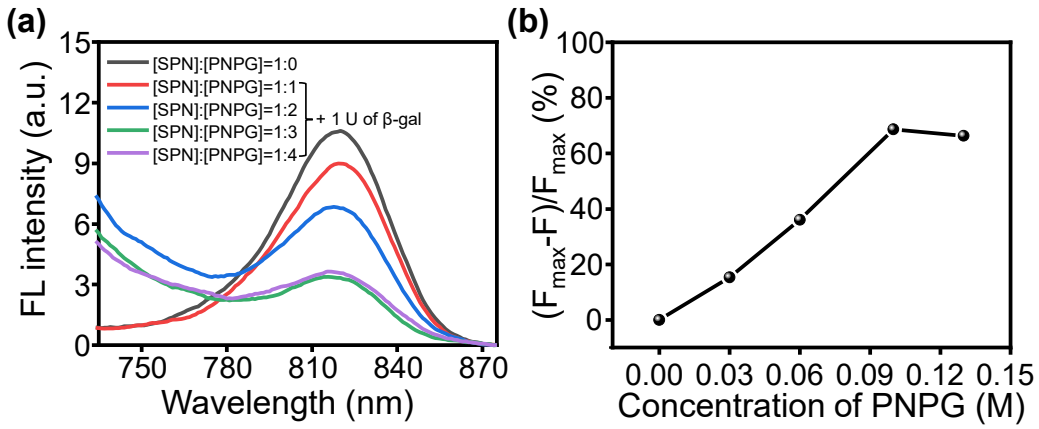

Fig. S14 a) Fluorescence spectra of SPN-NH_2 incubated with PNPG at different concentrations and the equal amount of β-gal (1 U) and, b) corresponding inhibition rate of SPN-NH_2 fluorescence intensity at 817 nm.

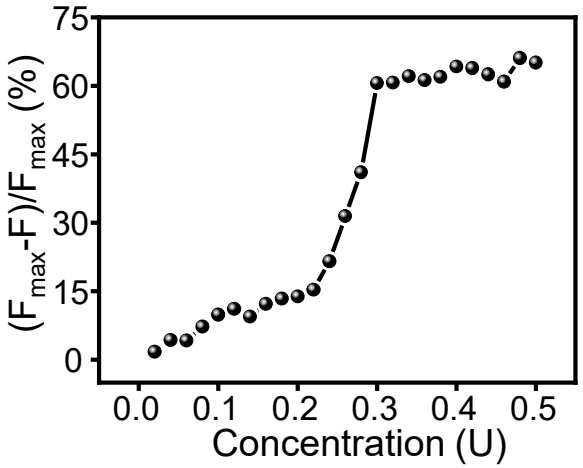

Fig. S15 Inhibition rate of SPN-NH_2 fluorescence intensity at 817 nm as a function of β-gal concentration.

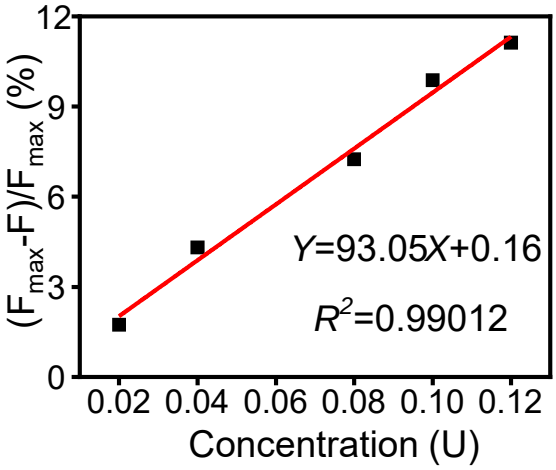

Fig. S16 Fitting curve between the inhibition rate of fluorescence intensity and the concentration of β-gal from 0 to 0.120 U. F max and F represent the fluorescence intensity of SPN-NH_2 at 817 nm without and with corresponding treatment.

6. Cytotoxicity of SPN-NH_2 and PNPG

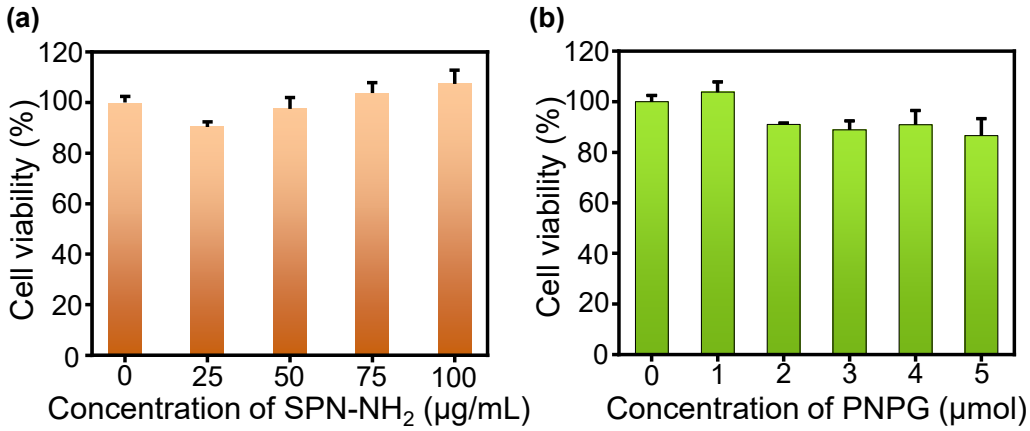

Fig. S17 Cytotoxicity of (a) SPN-NH_2 and, (b) PNPG towards HeLa cells at different concentrations.

7. X-Gal staining images of normal and senescent cells

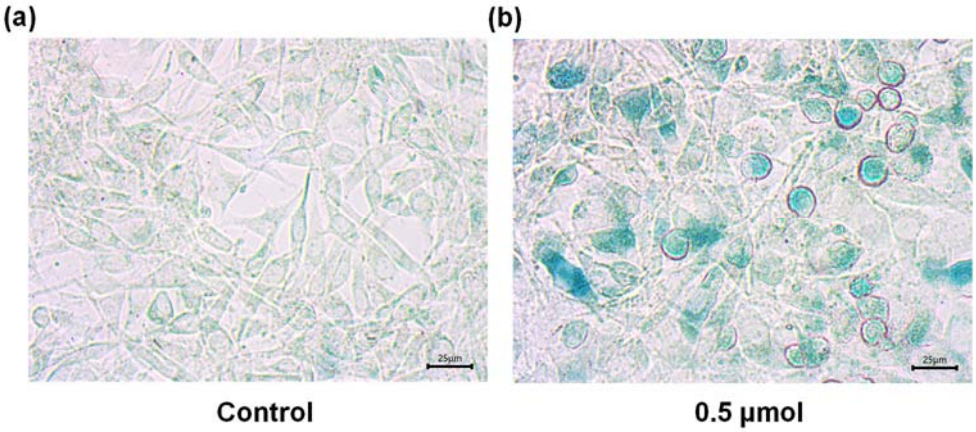

Fig. S18 X-Gal staining images of HeLa cells treated (a) without and, (b) with DOX (0.5 μM, 72 h).

8. Cellular fluorescence images of SPN-NH_2 added with β-gal

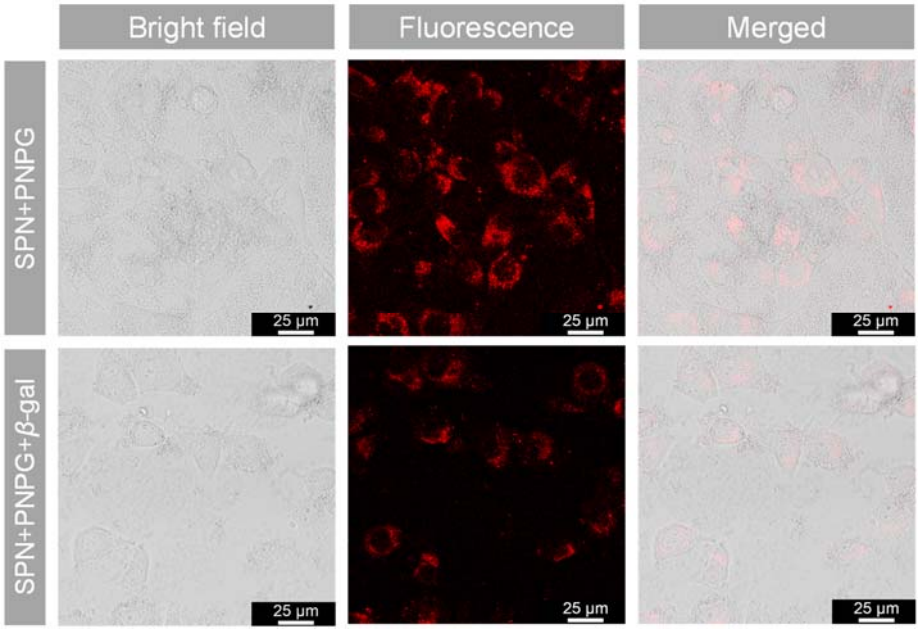

Fig. S19 Confocal fluorescence images of normal HeLa cells incubated with SPN-NH_2 (50 μg/mL) for 24 h, and then PNPG (40 mM) and the mixture of PNPG and β-gal (20 U) were added, respectively (SPN-NH_2 channel: λ_{ex} = 638 nm, λ_{em} = 648-792 nm).

9. The comparison of β-gal detection probes

Tab. S2 The comparison of basic performance between SPN-NH_2 and representative FL probe

Probe	Probe type	λ_{em}(nm)	LOD	Locating feature	Imaging applications	Ref
TPh-PyBz-β-gal	Molecular	606	0.22 U/mL	/	HepG2 cells	3
DCM-CHO-βgal	Molecular	665	3.15×10^{-5} U/mL	/	HepG2/SKOV3 cells and zebrafish	4
TZ-Br	Molecular	570/670	1.9×10^{-3} U/mL	Lysosome	OVCAR-3 cells and zebrafish	5
HBT-gal	Molecular	492	0.19×10^{-3} U/mL	/	HepG2 cells	6
Si NPs	Nanoprobe	450	1.36×10^{-3} U/mL	/	/	7
Probe 1	Molecular	444/374	5.4×10^{-3} U/mL	/	/	8
ESIPT-gal	Molecular	552	3.9×10^{-5} U/mL	/	Hela and OVCAR-3 cells	9
SiNPs-Rho6G	Nanoprobe	415/550	0.72×10^{-3} U/mL	/	/	10
SPN-NH_2	**Nanoprobe**	**817**	**0.046 U/mL**	**Lysosome**	**Hela cells**	**This work**

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