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A light-up imaging protocol for neutral pH-enhanced fluorescent detection of lysosomal neuraminidase activity in living cells

Lei Bao,‡ Lin Ding,‡ Jingjing Hui and Huangxian Ju*

State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210023, P.R. China

Experimental

Materials and reagents. Graphene oxide suspension (GO, 4 mg mL^{-1}) was purchased from JCNANO Tech (Nanjing, China). 1-Pyrenebutyric acid N-hydroxysuccinimide ester (PASE), 3-aminophenylboronic acid (APBA), branched poly(ethyleneimine) solution (BPEI, average M_n ~1200, average M_w ~1300 by LS, 50 wt.% in H_2O), 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate (4MUNA), N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (NADNA), sodium butyrate (NaBu) and neuraminidase (Neu) from *Clostridium perfringens (C. welchii)* were purchased from Sigma-Aldrich Inc. (USA). LysoTracker Green was obtained from Invitrogen (USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from KeyGen Biotech (Nanjing, China). Citric acid-sodium citrate buffer (0.1 M, pH 5.0) contained 42.3 mM citric acid and 57.7 mM sodium citrate. Glycine-NaOH buffer (0.05 M, pH 10.0) contained 50 mM glycine and 32 mM NaOH. Phosphate buffer saline (PBS, 0.01M, pH 7.4) contained 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4 and 1.41 mM KH_2PO_4. All other reagents were of analytical grade. All aqueous solutions were prepared using ultrapure water (≥ 18 MΩ, Milli-Q, Millipore).

Apparatus. The fluorescence spectra were obtained on a RF-5301PC spectrofluorophotometer (Shimadzu, Japan). The UV-vis absorption spectra were obtained with a UV-vis spectrophotometer (Nanodrop-2000C, Nanodrop, USA). The cell images were gained on confocal laser scanning microscope (CLSM, Leica TCS SP5, Germany) and inverted fluorescent

microscope (Olympus IX71, Japan). The morphology of nanocomposites was examined by atomic force microscopy (AFM) in tapping mode under ambient conditions using an Agilent 5500 AFM/SPM system with Picoscan, v5.3.3. The Zeta potential measurements of nanocomposites were performed at 25 °C on a Zetasizer (Nano-Z, Malvern, UK). Infrared spectra were recorded on a Fourier transform infrared (FT-IR) spectrometer (Nicolet iS10, Thermo, USA). MALDI-TOF mass spectrometry was performed using an Applied Biosystems 4800 proteomics analyzer (Applied Biosystems) equipped with a Nd:YAG laser operating at 355 nm, a repetition rate of 200 Hz and an acceleration voltage of 20 kV. α-Cyano-4-hydroxycinnamic acid (CHCA) was used as MALDI matrix. Flow cytometric analysis was performed on MoFlo XDP flow cytometer (Beckman-Coulter).

Cell culture. Human colon tumor (HCT-15) cells were purchased from KeyGen Biotech (Nanjing, China), and cultured in a flask in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U mL^{-1}, Gibco), and streptomycin (100 μg mL^{-1}, Gibco), i.e. complete medium, at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. Cell number was determined using a Petroff-Hausser counting chamber.

The Neu activity of living cells was inhibited by culturing cells in NADNA-containing (1 mM) complete medium for 24-48 h in the presence of 0.5% (v/v) Lipofectamine 2000 (Invitrogen). The cell apoptosis was induced by culturing cells in NaBu-containing (5 mM) complete medium or FBS-depleted medium for 24-48 h, respectively.

Preparation of Linker/BPEI/GO nanocomposite. 12.5 μL GO (4 mg mL^{-1}) was diluted with 812.5 μL methanol and mixed with 50 μL PASE solution (1 mM in methanol) for 2 h while stirring. After addition of 100 μL APBA solution (5 mM in methanol), the mixture was stirred for another 4 h. Subsequently, 25 μL BPEI solution (4 mM in methanol) was added into the mixture slowly. After 2 h stirring, 1.5-fold volume of ultrapure water was added. The unbound excess PASE, APBA and BPEI were removed by ultrafiltration through a 100 kDa MWCO centrifugal filter (Amicon Ultra) at 4000×g and washed with 40% methanol and ultrapure water twice, respectively. 0.05 mg of the obtained Linker/BPEI/GO was then resuspended in 1.0 mL ultrapure water.

Preparation of nanoprobe. The nanoprobe was prepared by incubating Linker/BPEI/GO (0.05 mg mL^{-1}, 1 mL) with 4MUNA solution (2 mM, 50 μL) for 4 h. The unbound excess 4MUNA was removed by ultrafiltration through a 100 kDa MWCO centrifugal filter at 4000×g and washed twice. The obtained nanoprobe was resuspended in 1.0 mL ultrapure water and stored at 4 °C. For AFM characterization of nanoprobe and GO, the samples were prepared via directly casting aqueous dispersions onto freshly cleaved mica sheets and dried overnight at room temperature.

Enzymatic cleavage of 4MU from nanoprobe by commercial Neu. 20 μL of nanoprobe (0.05 mg mL^{-1}, 7.9 μM 4MUNA equiv.) was diluted to 200 μL with citric acid-sodium citrate buffer (0.1 M, pH 5.0) in the presence of Neu (0.01 mU mL^{-1}). After incubation for different times (from 10 min to 2 h, 37 ^{o}C), aliquots of the reaction solutions were mixed with 4-fold volume of glycine-NaOH buffer (0.05 M, pH 10.0), which were adjusted to pH 7.4 to inhibit the reaction, and immediately analyzed with a spectrofluorophotometer.

Specificity of nanoprobe to commercial Neu. 10 μL of nanoprobe (0.05 mg mL^{-1}, 7.9 μM 4MUNA equiv.) or Linker/BPEI/GO (0.05 mg mL^{-1}) was added to 90 μL citric acid-sodium citrate buffer (0.1 M, pH 5.0) in the presence and absence of commercial Neu (0.1 U mL^{-1}), respectively. After incubation at 37 ^{o}C for 2 h under stirring, the reaction solution was mixed with 400 μL of glycine-NaOH buffer (0.05 M, pH 10.0) and adjusted to pH 7.4 for fluorescence detection. Since one unit of Neu activity was the amount required to liberate 1 μmol of 4MU per minute (37 °C, pH 5.0), the 4MUNA was completely hydrolyzed after 2 h incubation in the presence of Neu.

For inhibition experiments, 10 μL of nanoprobe (0.05 mg mL^{-1}, 7.9 μM 4MUNA equiv.) was added to 90 μL citric acid-sodium citrate buffer (0.1 mM, pH 5.0) in the presence of commercial Neu (0.1 U mL^{-1}) and NADNA (1 mM).

Preparation of crude lysosomal lysate. HCT-15 cells at 90% confluency after trypsinized and suspended in DMEM with 10% FBS were centrifuged at 600×g for 5 min. The following procedure was performed at 2-8 ^{o}C to obtain the crude lysosomal lysate. The collected cells (5×10^{7}) were washed in ice-cold PBS by centrifuging at 600×g for 5 min and broken by glass homogenizer after suspended in 1 mL PBS containing protease inhibitors, phosphatase inhibitors

and 1 mM phenylmethyl sulfonyl fluoride (PMSF) (KeyGen Biotech, Nanjing, China). After the homogenate was centrifuged at 1000×g for 10 min, the pellet containing nuclei and other cell debris was discarded, and the supernatant was centrifuged at 20000×g for 20 min. The obtained supernatant containing membrane fraction was discarded, and the pellet (crude lysosomal fraction) was finally resuspended in 200 μL lysis buffer (KeyGen Biotech, Nanjing, China) containing protease inhibitors, phosphatase inhibitors and 1 mM PMSF and ultra-sonicated on ice to obtain the suspension (1 mL corresponded to 2.5×10^8 cells) for Lyso-Neu activity measurement.

Colocalization of internalized nanoprobe. The Linker/BPEI/GO prepared with high PASE concentration was used as the tracker for the colocalization of internalized nanoprobe. The tracker was prepared by assembling PASE of 0.5 mM and BPEI of 0.1 mM on GO, on which the fluorescence of excess PASE was collected for colocalization experiment.

HCT-15 cells were seeded into 35-mm confocal dishes (glass bottom dish) at a density of 1×10^4 per dish and incubated for 24 h at 37 °C. The complete medium was then replaced with DMEM (without glucose, Gibco) containing the tracker (10 μg mL^{-1}) to incubate at 37 °C for 4 h. Before imaging, the cells were rinsed with PBS and further incubated with 1.0 μM LysoTracker Green for 15 min and then kept in DMEM. The lysosomes and the tracker in the cells were visualized with CLSM. LysoTracker Green and the tracker were excited at 488 nm and 405 nm, respectively. The emission was collected from 505 to 535 nm and 450 to 480 nm, respectively.

Fluorescent imaging of nanoprobe transfected living cells. After HCT-15 cells (1×10^4 per dish) were cultured in complete medium for 24 h at 37 °C in glass bottom dish, the medium was replaced with DMEM (without glucose) containing nanoprobe (10 μg mL^{-1}, 1.58 μM 4MUNA equiv.) to incubate at 37 °C for 4 h. For colocalization of 4MU, the nanoprobe-incubated cells were also stained with LysoTracker Green. After the cells were rinsed with PBS, the fluorescent imaging of the released 4MU and LysoTracker Green in HCT-15 cells were performed on inverted fluorescent microscope with 377±25 nm and 475±25 nm excitation, respectively. The images were collected at 447±30 nm and 540±20 nm, respectively.

For monitoring the dynamic change of Lyso-Neu activity, after cultured in complete medium for 24 h, the cells were treated by NaBu, FBS-depletion and NADNA for 24-48 h respectively before being subjected to imaging.

Determination of cell viability. The cytotoxicity of nanoprobe as well as Linker/BPEI/GO prepared with varying BPEI concentration was tested by MTT assay. HCT-15 cells (1×10^4 per well) were seeded into a 96-well plate in 200 μL complete medium and cultured at 37 ^{o}C for 24 h. After rinsing with PBS, HCT-15 cells were incubated with 200 μL DMEM (without glucose) containing nanoprobe (5, 10 and 15 μg mL^{-1}) or Linker/BPEI/GO (10 μg mL^{-1}) for different times. Then the incubation medium was replaced with 300 μL DMEM containing 100 μL of MTT (5 mg mL^{-1}) to incubate for 4 h. After the supernatant was removed, 300 μl DMSO was added to every well to dissolve the formazan for absorbance measurement at 490 nm on a microplate reader (Bio-Rad 680, USA). The cell viability was calculated with the equation: cell viability (%) = (mean OD value of treated cells - background noise) / (mean OD value of control cells - background noise) × 100%.

Flow cytometric analysis. HCT-15 cells (1×10^5 per dish) were cultured in 35-mm dishes with or without the pretreatment by NaBu, FBS-depletion or NADNA at 37 °C for different times. Then the cells were trypsinized, washed and subject to flow cytometric assay. The cells were assayed by MoFlo XDP flow cytometer equipped with 365-nm excitation.

Supporting figures

FT-IR, Zeta potential and UV-vis absorption characterization of nanoprobe.

FT-IR spectrum of GO revealed the existence of –OH (3430 cm^{-1}), C=O (1715 cm^{-1}), C–OH (1380 cm^{-1}), C=C (1640 cm^{-1}), and C–O (1060 cm^{-1}), and PASE/GO presented the C=O stretching vibration (1741 and 1773 cm^{-1}) of succinimidyl ester in PASE (Fig. S1A and S1B). After binding APBA to PASE/GO, a shoulder band corresponding to B-O stretching emerged at around 1350 cm^{-1} (Fig. S1A and S1B). The BPEI coating on Linker/GO was confirmed by the C-H vibration at 2961 cm^{-1} (Fig. S1A and S1B), which significantly increased the zeta potential from -30 mV to +46 mV (Fig. S1c). The FT-IR spectrum of the nanoprobe showed the characteristic peaks of 4MUNA at 1700 and 1560 cm^{-1} (Fig. S1A and S1B), suggesting the successful linking of 4MUNA with Linker/BPEI/GO. The UV-vis absorption spectrum of 4MUNA displayed a strong peak at 315 nm with a molar extinction coefficient (ε) of 13300 M^{-1} cm^{-1} (Fig. S1D),[S1] with which the loading amount of 4MUNA could be determined after subtracting the absorbance of Linker/BPEI/GO at the same wavelength.

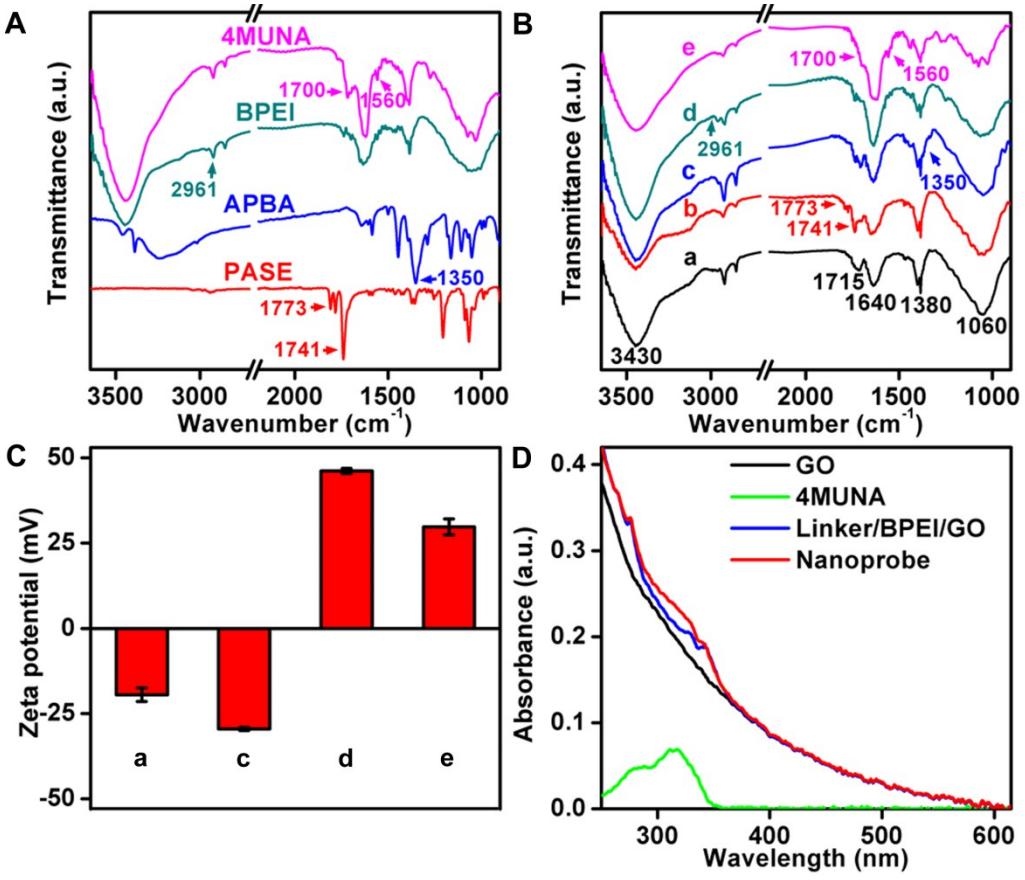

Fig. S1 FT-IR spectra of (A) PASE, APBA, BPEI and 4MUNA, and (B) GO (a), PASE/GO (b), Linker/GO (c), Linker/BPEI/GO (d) and nanoprobe (e). (C) Zeta potential of a, c, d and e. Data are means ± SD (n = 3). (D) UV-vis spectra of GO (0.1 mg mL^{-1}), 4MUNA (0.05 mM), Liker/BPEI/GO (0.1 mg mL^{-1}) and nanoprobe (0.1 mg mL^{-1}).

Fluorescent quenching of PASE by GO.

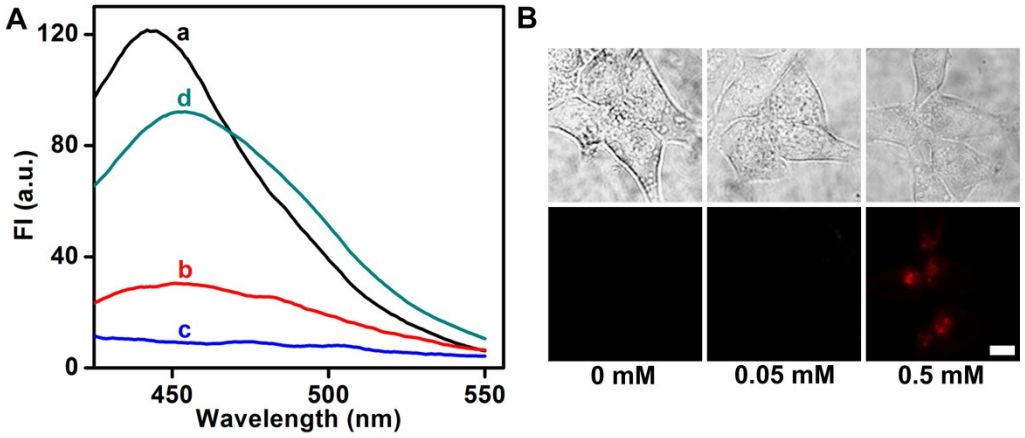

Fig. S2 (A) Fluorescence intensity (FI) of the mixture containing PASE (0.05 mM), APBA (0.5 mM) and BPEI (0.1 mM) in the (a) absence and (b) presence of GO (50 μg mL^{-1}), and the solutions of Linker/BPEI/GO (50 μg mL^{-1}) prepared with (c) 0.05 and (d) 0.5 mM PASE (λ_{ex} = 365 nm). (B) Bright field and fluorescent images of HCT-15 cells after 4-h incubation with 10 μg mL^{-1} Linker/BPEI/GO constructed with marked PASE concentrations. Scale bar: 5 μm.

Optimization of BPEI concentration for nanoprobe preparation.

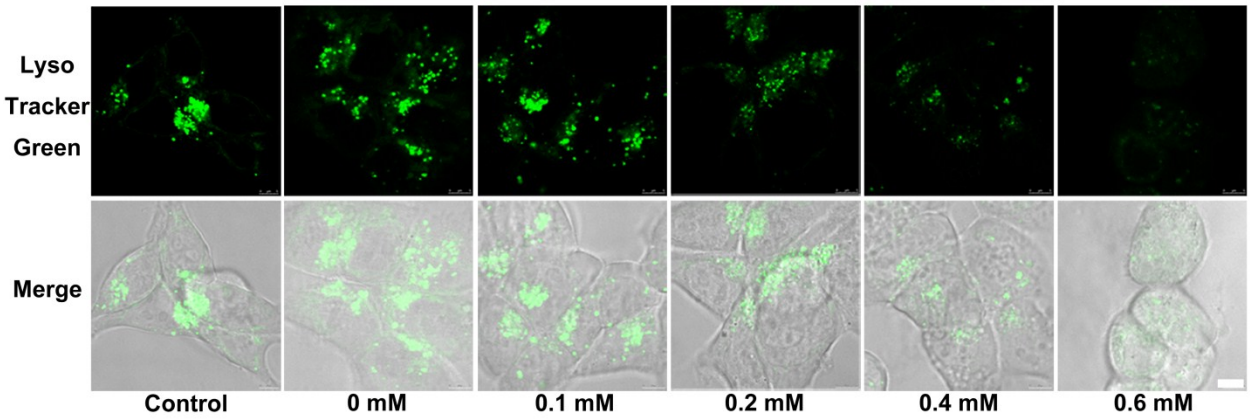

Fig. S3 Fluorescent and bright field merged images of HCT-15 cells after incubation with Linker/BPEI/GO (10 μg mL^{-1}) prepared with different BPEI concentrations for 4 h followed by LysoTracker Green staining. Scale bar: 5 μm.

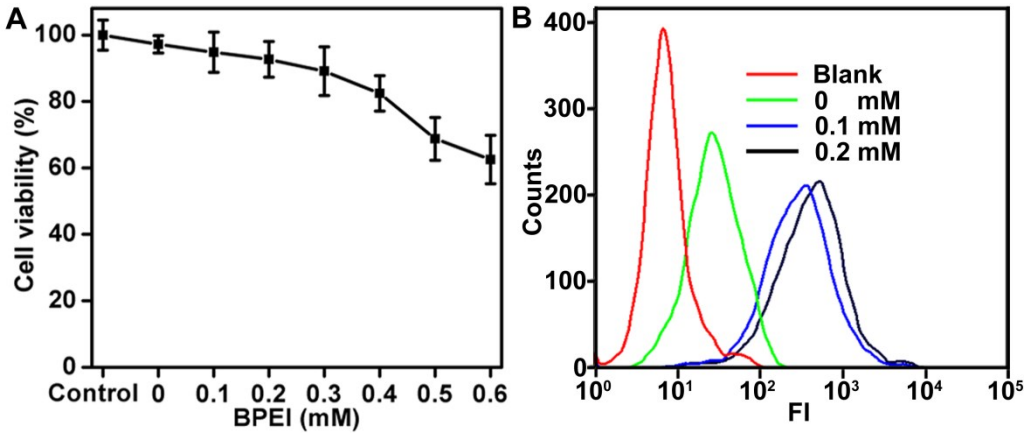

Fig. S4 (A) MTT assays for HCT-15 cells after 4-h incubation with Linker/BPEI/GO (10 μg mL^{-1}) prepared with different BPEI concentrations. Data are means ± SD (n = 3). (B) Flow cytometric detection of HCT-15 cells after incubation with Linker/BPEI/GO (10 μg mL^{-1}) prepared with 0.5 mM PASE and marked BPEI concentrations for 4 h. Cells without treatment serve as blank.

Neu cleavage of 4MUNA to generate 4MU with pH-dependent fluorescence.

After 4MUNA (5 μL, 2 mM) and Neu (5 μL, 2 U mL^{-1}) were added into 90 μL citric acid-sodium citrate buffer (0.1 M, pH 5.0) to react at 37 °C for 2 h under stirring, the 4MUNA was totally hydrolyzed. The reaction solution was then mixed with 400 μL of glycine-NaOH buffer (0.05 M, pH 10.0) and adjusted to pH 7.4. The fluorescence of the released 4MU was detected with 365-nm excitation and 445-nm emission.

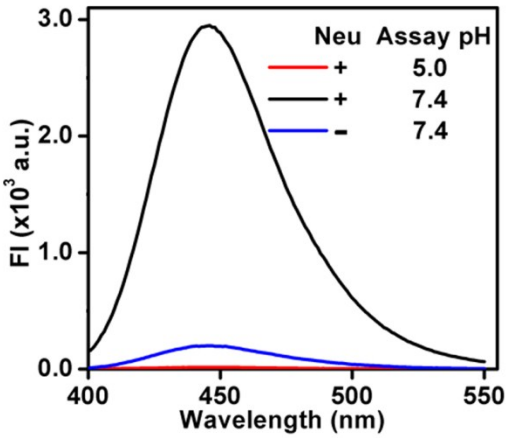

Fig. S5 Fluorescence spectra of 20 μM 4MUNA after incubation with Neu at pH 5.0 for 2 h to warrant complete hydrolysis, tested at pH 5.0 (red) and pH 7.4 (black), and the spectrum of 4MUNA at pH 7.4 without Neu incubation (blue).

Effect of APBA conjugation on Neu-catalyzed hydrolysis of 4MUNA.

4MUNA (5 μL, 2 mM) and APBA (10 mM) of different volumes were mixed with 95 μL of citric acid-sodium citrate buffer (0.1 M, pH 5.0). After incubation for 2 h to form 4MUNA-APBA compound, the mixture was mixed with Neu (5 μL, 2 U mL^{-1}) to incubate for 2 h at 37 ^{o}C under stirring. After addition of 400 μL of glycine-NaOH buffer (0.05 M, pH 10.0) and adjusting the pH to 7.4, the fluorescence of released 4MU was detected.

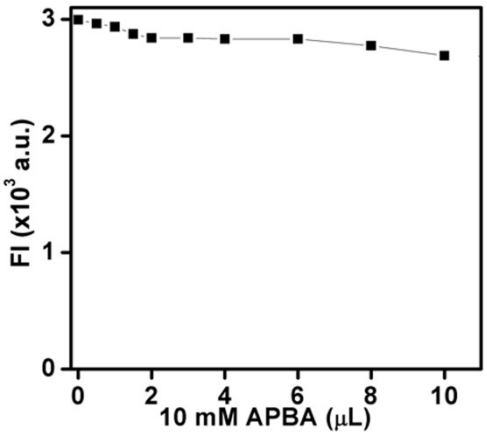

Fig. S6 Effect of APBA conjugation to 4MUNA on the cleavage activity of Neu.

Effect of Linker/BPEI/GO on released 4MU.

After 20 μL of 4MUNA (7.9 μM) was mixed with 180 μL of citric acid-sodium citrate buffer (0.1 M, pH 5.0) containing 100 mU mL^{-1} to incubate for 2 h, 100 μL of the solution was mixed with 400 μL glycine-NaOH buffer (0.05 M, pH 10.0) and adjusted to pH 7.4 for 3 h. In the presence and absence of Linker/BPEI/GO (1 μg mL^{-1}, final concentration) in the buffer, the fluorescence of released 4MU was detected.

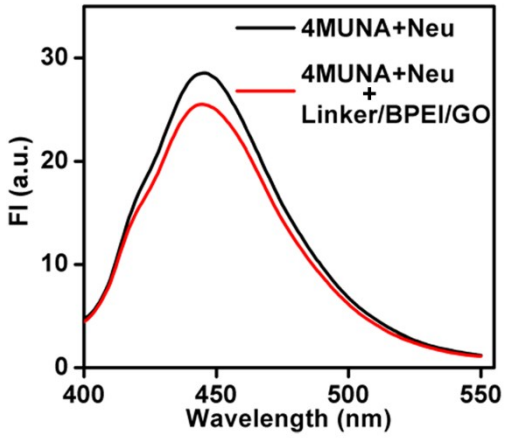

Fig. S7 Fluorescence spectra of 4MUNA (158 nM, final concentration) pre-hydrolyzed with Neu completely by incubation for 2 h and then mixed with glycine-NaOH buffer in absence and presence of Linker/BPEI/GO (1 μg mL^{-1}, final concentration) at pH 7.4 for 3 h.

MALDI-TOF-MS characterization of Neu-triggered hydrolysis product of nanoprobe.

After 10 μL of nanoprobe (0.05 mg mL^{-1}, 7.9 μM 4MUNA equiv.) was added into 90 μL citric acid-sodium citrate buffer (0.1 M, pH 5.0) containing commercial Neu (0.1 U mL^{-1}) to incubate for 2 h at 37 °C, the mixture was washed by ultrafiltration through a 100 kDa MWCO centrifugal filter at 4000×g. The obtained pellet containing the hydrolysis product was resuspended in water and subjected to MALDI-TOF-MS detection.

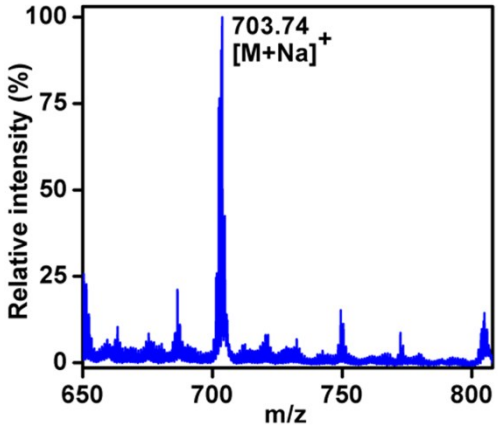

Fig. S8 MALDI-TOF mass spectrum of hydrolysis product on the nanoprobe.

Detection of Neu activity in crude lysosomal lysate.

A standard addition method was used for quantifying the Lyso-Neu activity in HCT-15 cells. After 10 μL crude lysosomal lysate was mixed with 50 μL of citric acid-sodium citrate buffer (0.1 M, pH 5.0) containing commercial Neu at different concentrations, 40 μL of nanoprobe (0.05 mg mL^{-1}, 7.9 μM 4MUNA equiv.) was added in the mixtures to incubate at 37 °C for 10 min. The reaction mixtures were then mixed with 4-fold volume of glycine-NaOH buffer (0.05 M, pH 10.0) to stop the reaction and immediately analyzed with spectrofluorophotometer.

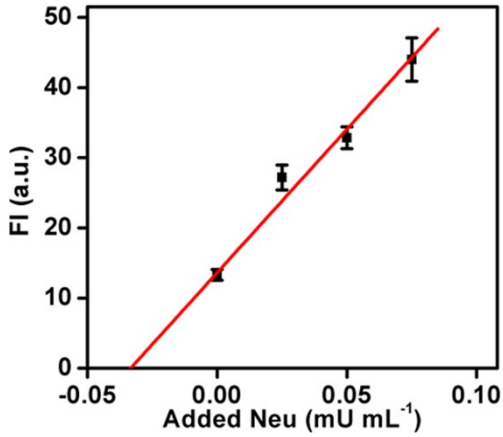

Fig. S9 Analysis of Neu activity in crude lysosomal lysate by standard addition method. Data are means ± SD (n = 3).

Stability of nanoprobe in complex intracellular environment.

The stability of the nanoprobe was also investigated by soaking the nanoprobe (10 μg mL^{-1}) in DMEM containing 0.05 mM glucose and 10% FBS at pH 5.0 and 7.4 to simulate complex lysosomal and cytosol environment, respectively.[S2] After 12-h incubation at 37 °C, the nanoprobe was condensed and purified by ultrafiltration through a 100 kDa MWCO centrifugal filter at 4000×g and washed with H_2O. The nanoprobe (5 μg mL^{-1}, 0.79 μM 4MUNA equiv.) was then incubated with Neu (0.1 U mL^{-1}) in 100 μL citric acid-sodium citrate buffer (0.1 M, pH 5.0) at 37 °C for 2 h under stirring, the reaction solutions were mixed with 400 μL of glycine-NaOH buffer (0.05 M, pH 10.0) and adjusted to pH 7.4 for fluorescence detection.

After the same Neu treatment, the nanoprobe pre-incubated at pH 5.0 and 7.4 for 12 h maintained 92% and 89% fluorescent signal, respectively, demonstrating good stability of the nanoprobe in complex lysosomal and physiological environments in living cell, and the 4MUNA on the nanoprobe could not be displaced by glucose of low concentration inside living cells.[S3]

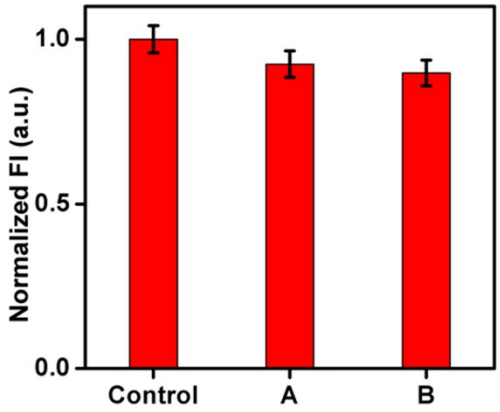

Fig. S10 Fluorescence responses of nanoprobes after stored in DMEM containing 10% FBS and 0.05 mM glucose at pH 5.0 (A) and 7.4 (B) for 12 h and then incubated with Neu. The nanoprobe stored in water serves as control. Data are means ± SD (n = 3).

Fluorescent imaging of NADNA-pretreated cells using the proposed nanoprobe.

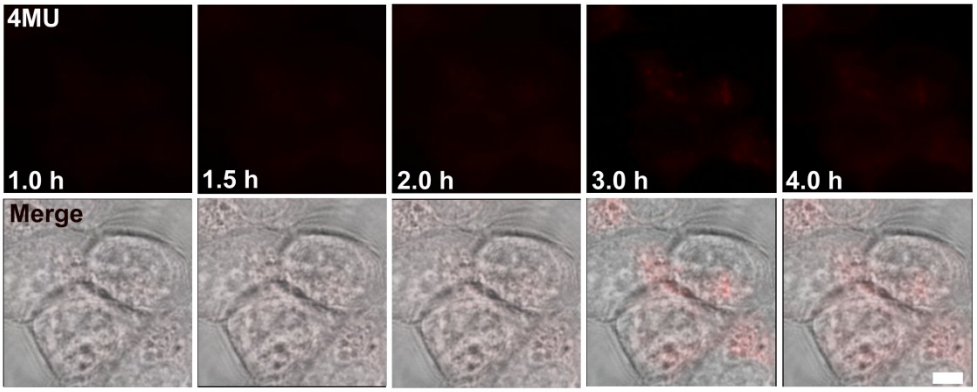

Fig. S11 Fluorescent and bright field merged images of HCT-15 cells after NADNA treatment for 48 h and then incubation with 10 μg mL^{-1} nanoprobe for marked times. Scale bar: 5 μm.

Cell viability after incubation with nanoprobe.

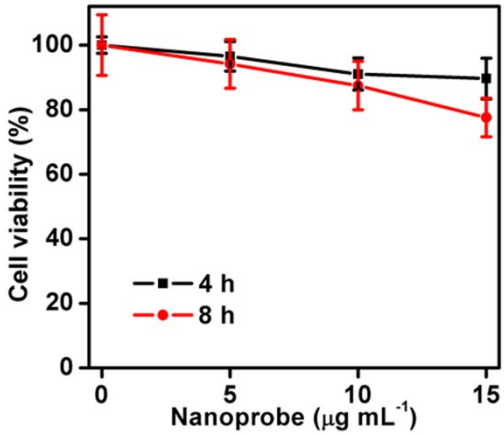

Fig. S12 MTT assays for HCT-15 cells after incubation with nanoprobe of marked concentrations for 4 or 8 h. Data are means ± SD (n = 3).

Detection of Neu activity in crude lysosomal lysates extracted from treated cells.

After the crude lysosomal lysates extracted from HCT-15 cells pretreated with NaBu, FBS-depletion DMEM or NADNA for 24 or 48 h were incubated with the nanoprobe at 37 ^{o}C for 10 min, the fluorescent assay was performed. The fluorescence intensities, normalized with the fluorescent signal of lysosomal lysate from untreated HCT-15 cells, were recorded. The cell apoptosis led to increasing Lyso-Neu activity. After HCT-15 cells were cultured in FBS-free condition for 24 and 48 h, the Lyso-Neu activity increased 1.3 and 1.7 times, while NaBu-treated HCT-15 cells showed 1.2- and 1.8-times activity increase, respectively. NADNA-treated HCT-15 cells presented suppressed Lyso-Neu activity, which decreased to 51 % and 30 % for 24- and 48-h incubation, respectively.

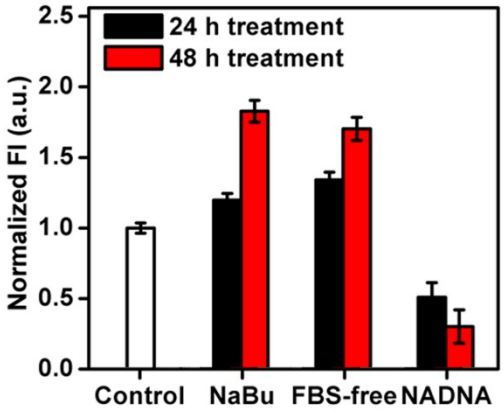

Fig. S13 Normalized fluorescence intensities of crude lysosomal lysates from HCT-15 cells pretreated with FBS-free, NaBu and NADNA for 24 or 48 h after incubated with nanoprobe at 37 ^{o}C for 10 min. The cells without pretreatment (Fig. S9) serve as the control. Data are means ± SD (n = 3).

Flow cytometric analysis of control samples.

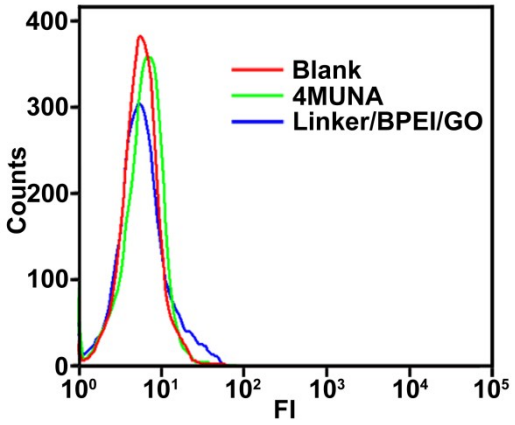

Fig. S14 Flow cytometric analysis of HCT-15 cells after incubation with 4MUNA (1.58 μM) and Linker/BPEI/GO (10 μg mL^{-1}) for 4 h. The cells without incubation serve as the blank.

Supporting references

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