Supporting Information

A light-activated polymer with excellent serum tolerance for intracellular protein delivery

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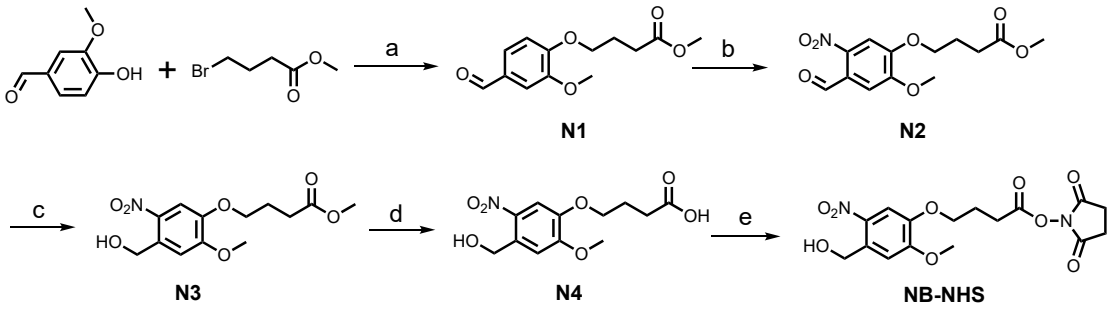
EXPERIMENTAL SECTION

Materials. Generation 5 (G5) polyamidoamine dendrimer was purchased from Dendritech (USA). Cytochalasin D (Cyt D), chlorpromazine (CPZ), methyl-β-cyclodextrin (MβCD) and genistein (GEN) were purchased from Sigma-Aldrich (USA). β-Gal was obtained from J&K Scientific (China). Hoechst 33342, β-gal in situ staining and reporter gene assay kit were obtained from Beyotime (China). Lysotracker Red was from Life technologies (USA). Resorufin-β-D-gal was from Aladdin (China). 5-Flu-O-β-gal was purchased from Biopike (China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Sangon Biotech. (China). Trypan blue was obtained from Yesen (China). TransExcellent™ (TransEx) was gifted from Cenji Biotech. (China). PULSin™ was from Polyplus-transfection (France).

Synthesis of NB-NHS. NB-NHS was synthesized by following procedures (Scheme S1).



Scheme S1. Synthesis route for NB-NHS. Reagents and conditions: a) K_2CO_3, acetonitrile, 90℃. b) 70% nitric acid, 0℃. c) $NaBH_4$, CH_3OH, 0℃. d) K_2CO_3, CH_3OH, rt. e) NHS, dichloromethane (DCM), rt.

Compound N1: The solution of vanilline (5.0 g, 32.9 mmol, 1.0 eq.) in 120 mL acetonitrile was sequentially added methyl 4-bromobutanoate (7.14g, 39.5 mmol, 1.2 eq.) and K_2CO_3 (6.81g, 49.3 mmol, 1.5 eq.) at nitrogen atmosphere. After 10 h stirring at 90°C, the mixture was vacuum dried for removal of acetonitrile. Then, the leftover sample was diluted by 500 mL DCM and 200 mL water. The aqueous layer was separated and extracted by 300 mL DCM for 3 times. We collected all the organic phases and dried them with anhydrous Na_2SO_4. Through vacuum evaporation to remove volatile, the raw product was obtained and readied for further purification via silica gel column chromatography with a mobile phase of DCM/CH_3OH mixed solvent (50:1, v/v) to afford N1 (7.1 g, yield 85.6%).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.81 (s, 1H), 7.34-7.49 (m, 2H), 6.95 (d, *J* = 8.1 Hz, 1H), 4.13 (t, *J* = 6.3 Hz, 2H), 3.89 (s, 3H), 3.66 (s, 3H), 2.54 (t, *J* = 7.2 Hz, 2H), 2.15-2.20 (m, 2H). (Fig. S1)

^{13}C NMR (101 MHz, Chloroform-*d*) δ 191.0, 173.5, 153.8, 149.9, 130.2, 126.8, 111.6, 109.3, 67.9, 56.0, 51.7, 30.3, 24.3. (Fig. S2)

MS (ESI):m/z Cal. for $C_{13}H_{16}O_5$ $[M+H]^+$: 252.1. Found: 252.1.

Compound N2: The solution of N1 (7.1 g, 28.2 mmol, 1.0 eq.) and 70% nitric acid (2.2 mL, 33.8 mmol, 1.2 eq.) were prepared by 10 mL and 120 mL acetic anhydride, respectively. Then, the N1 solution was dropwise added into nitric acid solution at 0°C. The reaction solution was stirred at 0°C for 30 min and precipitated by ice water. Through filtration, 20 mL ethanol washing and 200 mL ethanol recrystallization stepwisely, the obtained product was purified to afford N2 (5.7 g, yield 67.9%) as light yellow solid.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.43 (s, 1H), 7.60 (s, 1H), 7.39 (s, 1H), 4.21 (t, *J* = 6.2 Hz, 2H), 3.99 (s, 3H), 3.70 (s, 3H), 2.57 (t, *J* = 7.2 Hz, 2H), 2.25-2.19 (m, 2H). (Fig. S3)

^{13}C NMR (101 MHz, Chloroform-*d*) δ 187.8, 173.2, 153.5, 151.7, 143.8, 125.6, 109.9, 108.1, 68.7, 56.7, 51.9, 30.2, 24.1. (Fig. S4)

MS (ESI): m/z Cal. for $C_{13}H_{16}NO_7$ $[M+H]^+$: 298.1. Found: 298.1.

Compound N3: The compound N2 (5.7 g, 19.2 mmol, 1.0 eq.) solution was prepared in 100 mL MeOH/THF mixed solvent (10:1, v/v). Sodium borohydride (1.5 g, 39.7 mmol, 2.0 eq.) was slowly added into N2 solution at 0°C for 3 h. Then, the reaction was quenched by 30 mL water and vacuum evaporated to remove MeOH and THF. The residual material was further diluted with 300 mL DCM and 100 mL water. After twice extraction of the aqueous layer by 200 mL DCM, the organic layers were combined, followed by brine washing, Na_2SO_4 drying and vacuum evaporation to remove volatile. Finally, the purified compound N3 (4.8 g, yield 83.8%) was obtained from silica gel column chromatography (DCM: MeOH = 50:1, v/v).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.70 (s, 1H), 7.18 (s, 1H), 4.96 (s, 2H), 4.13 (t, J = 6.2 Hz, 2H), 3.98 (s, 3H), 3.71 (s, 3H), 2.57 (t, *J* = 7.2 Hz, 2H), 2.22-2.18 (m, 2H). (Fig. S5)

^{13}C NMR (101 MHz, Chloroform-*d*) δ 173.4, 154.3, 147.1, 139.6, 132.5, 111.1, 109.5, 68.3, 62.8, 56.4, 51.8, 30.4, 24.3. (Fig. S6)

MS (ESI): m/z Cal. for $C_{13}H_{17}NO_7Na$ $[M+Na]^+$: 322.1. Found: 322.1.

Compound N4: The compound N3 (4.8 g, 16.0 mmol, 1.0 eq.) was dissolved in 100 mL MeOH and added potassium carbonate (3.3 g, 23.9 mmol, 1.5 eq.) at room temperature, followed by 10 h stirring. Then, the residual material was treated as mentioned above, such as mixed solvent dilution and aqueous phase extraction twice. After brine washing and anhydrous Na_2SO_4 drying, the obtained product was vacuum evaporated to afford N4 (3.6 g, yield 78.8%) without further purification.

Compound NB-NHS: The compound N4 (3.6 g, 12.6 mmol, 1.0 eq.) in 100 mL dichloromethane was added EDCl (3.6 g, 18.9 mmol, 1.5 eq.) and NHS (2.2 g, 18.9 mmol, 1.5 eq.) and stirred at room temperature for 4 h. The crude product was vacuum evaporated to remove dichloromethane, followed by further purification using silica gel column chromatography (DCM: MeOH =25:1, v/v) to afford NB-NHS (3.1 g, yield 64.5%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.69 (s, 1H), 7.41 (s, 1H), 5.59 (t, J = 5.5 Hz, 1H), 4.92-4.68 (m, 2H), 4.14 (t, J = 6.4 Hz, 2H), 3.93 (s, 3H), 2.87 (t, J = 7.4 Hz, 2H), 2.82 (s, 4H), 2.10 (p, J = 6.9 Hz, 2H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 170.2, 168.6, 153.8, 145.8, 138.3, 134.5, 109.8, 109.1, 67.2, 60.1, 56.1, 26.9, 25.4, 23.9.

HRMS (ESI): m/z Cal. for $C_{16}H_{18}N_2O_9Na$ $[M+Na]^+$: 405.0910. Found: 405.0911.

Synthesis of ND polymer. The solution of G5 dendrimer in anhydrous methanol was added NB-NHS in DMSO at a G5 dendrimer/NB-NHS molar ratio of 1:20. After 8 h stirring at room temperature, the solution was intensively dialysed against DMSO and deionized water. After lyophilization, the prepared ND polymer was characterized by ^{1}H NMR (Bruker, 500MHz).

Photoactivity of NB-NHS and ND polymer. 2 mg of NB-NHS dissolved in DMSO-d_6 was irradiated by a 365 nm ultraviolet (UV) flashlight using UPF100 (Uvata, China) at 10 mW·cm^{-2} for 300 s. After that, the solution was characterized by ^{1}H NMR. The sample without UV irradiation was also characterized as the control. For investigation the photoactivatable property of ND polymer, ND with or without 20 s UV irradiation was diluted at 80 μg/mL and detected by a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, USA).

Preparation and characterization of ND/GFP complexes. ND and GFP solutions were mixed and incubated for 5 min, followed by UV irradiation using a 365 nm laser at 10

$mW \cdot cm^{-2}$ for 20 s. Afterwards, the complexes were diluted by solutions such as deionized water, buffer and culture media at pH 7.4 or 5.0 for next step of measurement. When ND and GFP were diluted at the concentration of 4 and 2 μg/mL, respectively, the complex solutions were characterized by DLS using a Zetasizer Nano ZS 90 instrument (Malvern, UK) and TEM (H-7700, Hitachi, Japan) at an acceleration voltage of 100.0 kV. The ND/GFP complexes without UV irradiation were set as controls. The ND/GFP complexes were also characterized by a fluorescence spectroscopy (Hitachi F-4500, Japan). As to quantitatively measure the GFP loading efficiency of ND polymer, the prepared complexes in different solvents (ND, 8 μg/mL; GFP, 4 μg/mL) were centrifuged at 13000 rpm for 20 min. we collected the supernatant to measure the contained free GFP content by fluorescence spectroscopy. The standard curves of GFP in deionized water, PBS and opti-MEM medium, respectively were prepared ($Y_{water}=1283*X_{water}+487.6$, $R^2=0.989$; $Y_{PBS}=1214*X_{PBS}+233.6$, $R^2=0.997$; $Y_{Medium}=1211*X_{Medium}+292.5$, $R^2=0.994$; where Y is the fluorescence intensity of GFP solutions, X is the GFP concentration, μg/mL). To verify the serum tolerance of ND/GFP complexes after LAC, the samples were incubated with 10%, 20%, 30%, 40% and 50% FBS-containing deionized water, PBS or opti-MEM medium, and then analyzed as described above.

Agarose gel electrophoresis. We conducted agarose gel electrophoresis to investigate the responsive GFP release from photoactivated ND/GFP complexes. After LAC treatment, the complexes were diluted by 50 μL acetate buffer (0.1M, pH 7.4 or 6.0) with or without 10% serum. Glycerol-water solution (50%, v/v) was used as 10× loading buffer. 1× TAE buffer was used for 2% (w/v) agarose gel preparation and electrophoresis. The samples loaded on gel were run at 100 V for 30 min. Then, we photographed the gels via a UV light-irradiated Tanon 2500 gel image system (China).

Cell culture and protein delivery. 143B, HeLa, MEF and MDA-MB-453 cells were cultured at 10% fetal bovine serum (FBS, Gemini) contained Dulbecco's modified Eagle's medium (DMEM, GIBCO). BAT and iWAT cells were incubated at 20% FBS-contained DMEM. Cells were cultured with 5% CO_2 atmosphere at 37℃.

For protein delivery experiments, the cells were pre-seeded at 48-well plates overnight until 70% confluence. ND/GFP complexes were prepared and further diluted

by different contents of FBS-contained medium, and then incubated with the cells for specific incubation time. Before flow cytometry (BD LSRFortessa, USA) measurement, the cells were rinsed by PBS and 0.04% (w/w) trypan blue solution to quench extracellular GFP fluorescence, then collected by trypsin digestion. For confocal imaging, the cells were pre-seeded on confocal dishes until 70% confluence. The concentrations of ND polymer and GFP were equal to those used in 48-well plates. After treatment by PBS washing and trypan blue quenching, the cells were imaged by laser scanning confocal microscopy (LSCM, Leica SP8, Germany). Commercial reagents PULSin and TransEx were used as controls. In addition, the cells were treated with LysoTracker Red (Life tech.) and Hoechst 33342 (Beyotime) to stain the acidic organelles and nuclei, respectively to study the intracellular localization.

To study the cellular uptake pathway of ND/GFP complexes, 143B cells seeded at 48-well plate were pre-treated with specific inhibitors (10 μM Cyt D, 700 μM GEN, 20 μM CPZ, 10 mM MβCD) for 1 h, and then incubated with ND/GFP complexes after LAC for 4 h at 37℃. The cells incubated with photo-activated ND/GFP complexes at 4℃ were also tested to investigate the energy-dependent internalization pathway. The cells without inhibitor treatment at 37℃ were tested as the control. After incubation with complexes, the cells were treated as the above procedures and measured by flow cytometry.

Confocal microscopy. LAC-treated ND/GFP complexes incubated with 0-50% FBS contained opti-MEM medium were observed by a LSCM (Leica SP8) with the 488 nm laser. For the cellular imaging, cells cultured at a 35mm confocal dish were dispersed in PBS solution, and then scanned by LSCM (Leica SP8) with the 488 nm and/or 561 nm laser at room temperature.

Cell viability assay. The viability of ND polymers with or without UV light irradiation at concentrations ranging from 16 to 48 μg/mL was evaluated on 143B cells. When the cells seeded at a 96-well plate were growing up to 70% confluence, they were incubated with polymers for 4 h. Afterwards, the culture medium was removed for the addition of complete medium. After another 20 h incubation, a standard MTT assay was applied for analyzing the viability of 143B cells treated with ND polymer.

***In vitro* β-gal enzymatic activity assay.** ND/β-gal complexes were incubated in different medium (pH7.4 or pH 5.0) with or without serum, followed by the addition of X-gal-contained working solutions that referrred to β-gal staining kit Beyotime) at

37 ℃ for 10 min. Then the absorbance at 633 nm was measured by a microplate reader. Free β-gal in each of tested medium was used as control.

Intracellular β-gal delivery. 143B cells were pre-seeded at 48-well plate overnight until 70% confluence. The ND/β-gal complexes (ND, 32 μg/mL; β-gal, 12 μg/mL) were incubated for 5 min, followed by 365 nm UV irradiation at 10 mW/cm^2 for 20 s. Then, the complexes were diluted by culture media containing different FBS concentrations, and incubated with cells for 4 h. We added fresh complete medium to renew the culture medium and incubated for another 20 h. After PBS washing for 3 times, a working solution containing 5% 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-gal) was added for overnight incubation at 37℃ without CO_2 according to the instruction of β-gal in situ staining assay kit (Beyotime). The treated cells were photographed by an Olympus optical microscope (Japan). The complexes without UV light activation were tested as controls.

For quantitative measurement of β-gal enzyme activity after intracellular delivery, we used a β-gal reporter gene assay kit (Beyotime) to test the treated cells. Briefly, the ND/β-gal complexes treated cells in each well were received 150 μL cell lysis buffer. Each of 50 μL lysate and 50 μL detection reagent were sequentially added into a 96-well plate. After 30 min, 150 μL terminating buffer was added to terminate the β-gal catalytic reactions. The sample absorbances at 420 nm were recorded via a Multiskan GO microplate reader (Thermo scientific, USA).

The substrate resorufin-β-D-gal was used to investigate the intracellular β-gal enzyme activity. ND/β-gal complexes treated 143B cells were stained by a working solution containing resorufin-β-D-gal instead of X-gal according to the protocol of in situ β-gal staining kit. After 20 min incubation, the samples were photographed by an optical camera. 20 μL of the supernatant solution in each well was given and diluted by 500 μL PBS. Then the fluorescence intensity was detected by a Hitachi F-4500 fluorescence spectroscopy (Excitation: 485 nm; Emission: 515-700 nm). The standard curve of β-gal acting on the substrate resorufin-β-D-gal was worked out ($F=932.2*C-211.3$, $R^2=0.9556$; where F and C represent the fluorescence intensity and β-gal concentration, respectively).

5-Flu-O-β-gal was another substrate for investigating intracellular β-gal delivery.

ND/β-gal complexes treated with or without LAC were incubated with 143B cells at a 96-well plate for 4 h. Then, the culture media were replaced with complete media. After 20 h incubation, 100 μM 5-flu-O-β-gal was treated with the cells for another 24 h. We determined the cell viability via CCK-8 assay referring to the manufacturer's indication. PULSin and TransEx were applied as positive controls.

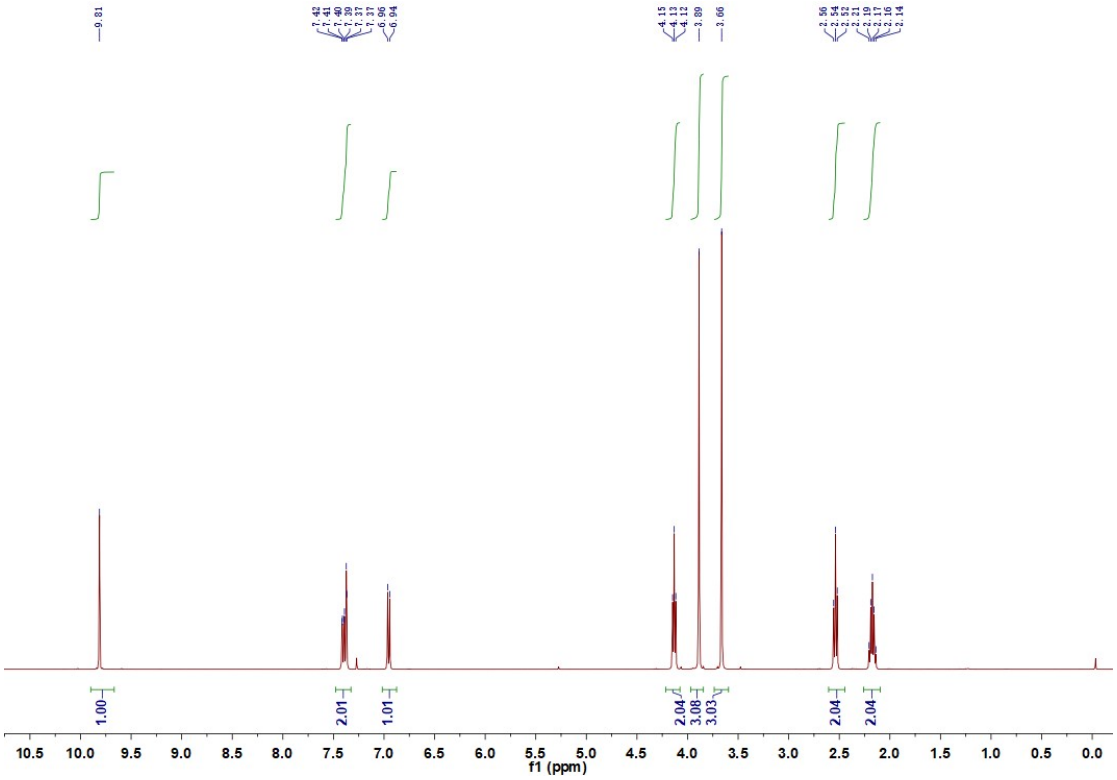


Figure S1. ^{1}H NMR spectrum of N1.

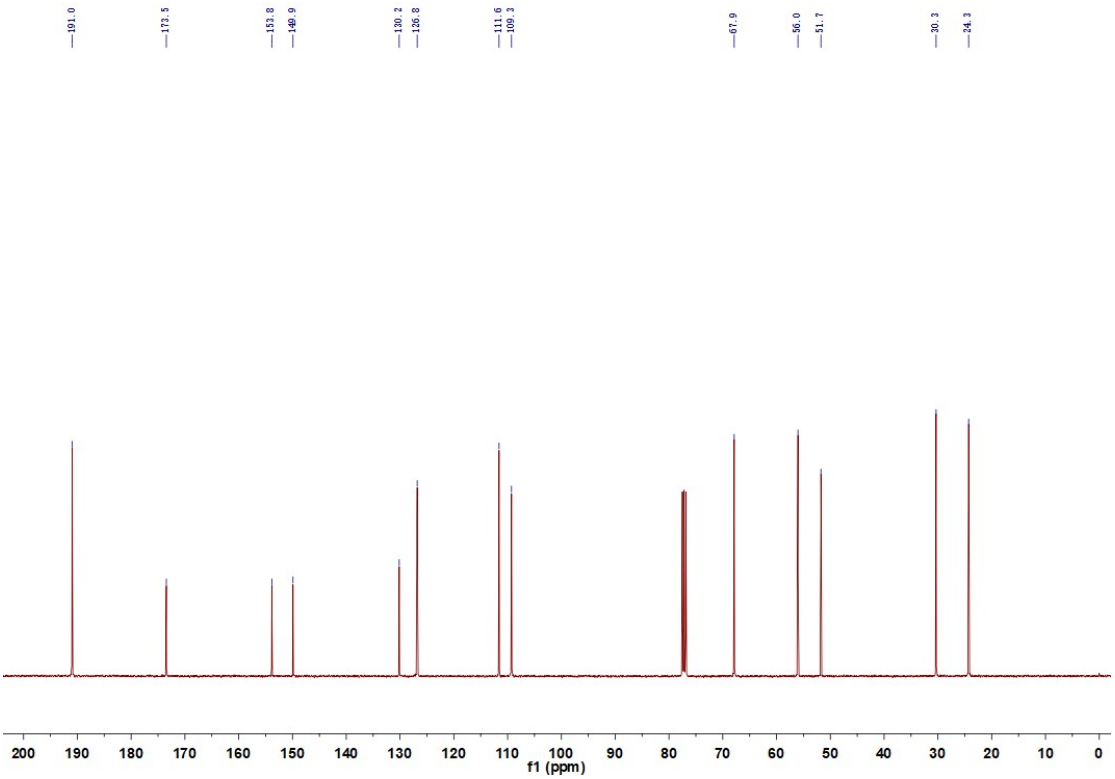


Figure S2. ^{13}C NMR spectrum of N1.

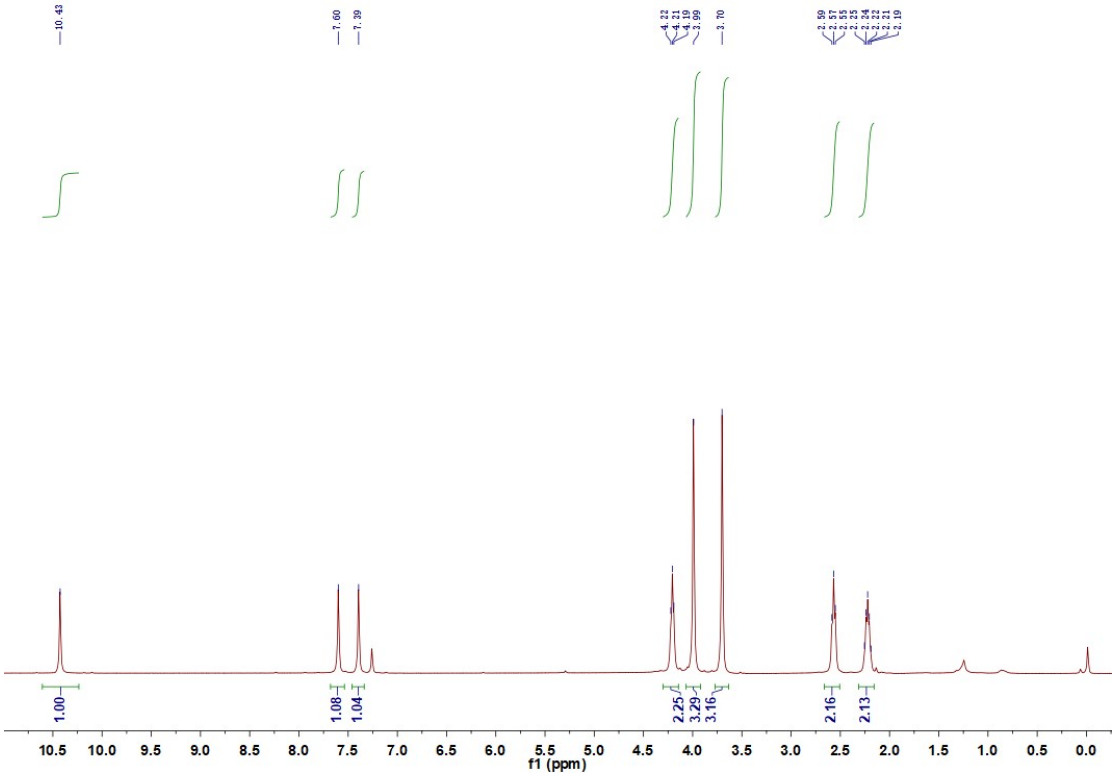


Figure S3. ^{1}H NMR spectrum of N2.

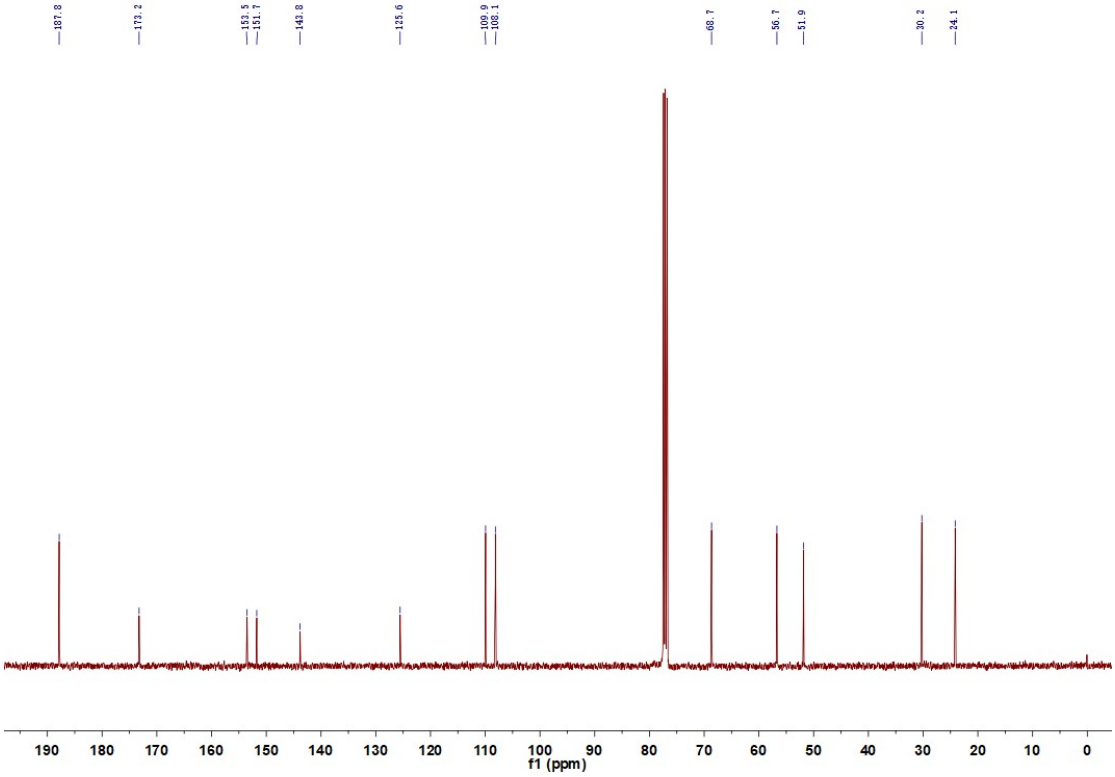


Figure S4. ^{13}C NMR spectrum of N2.

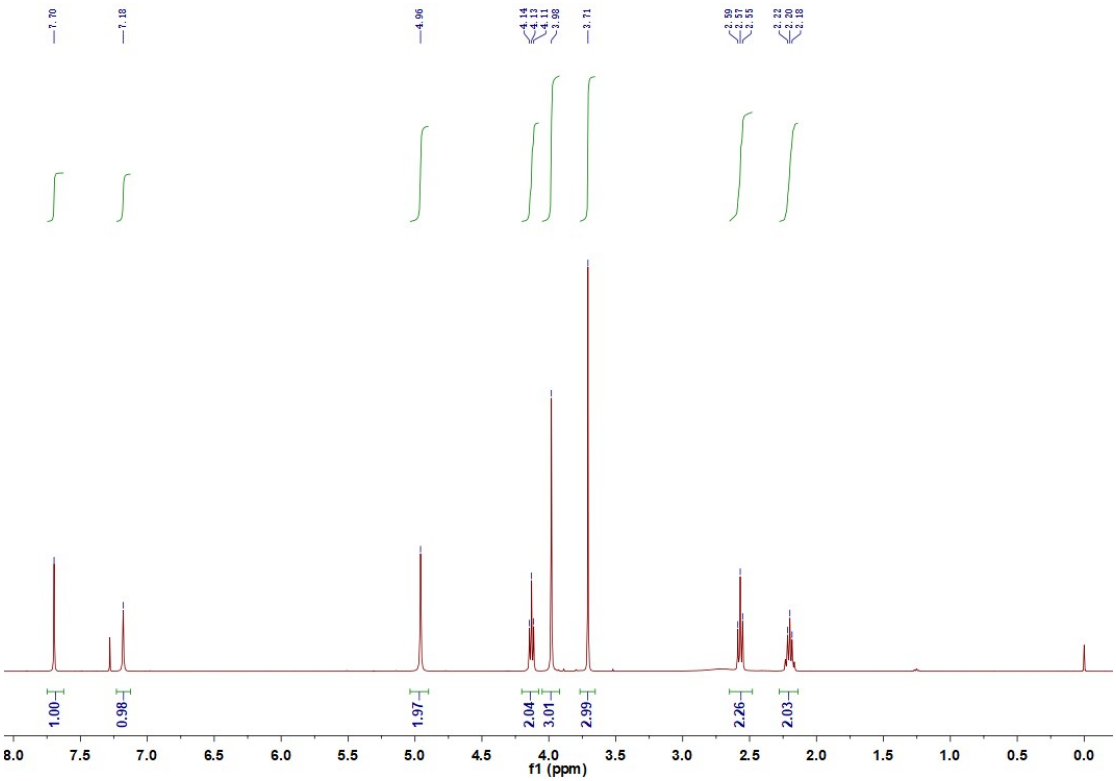


Figure S5. ^{1}H NMR spectrum of N3.

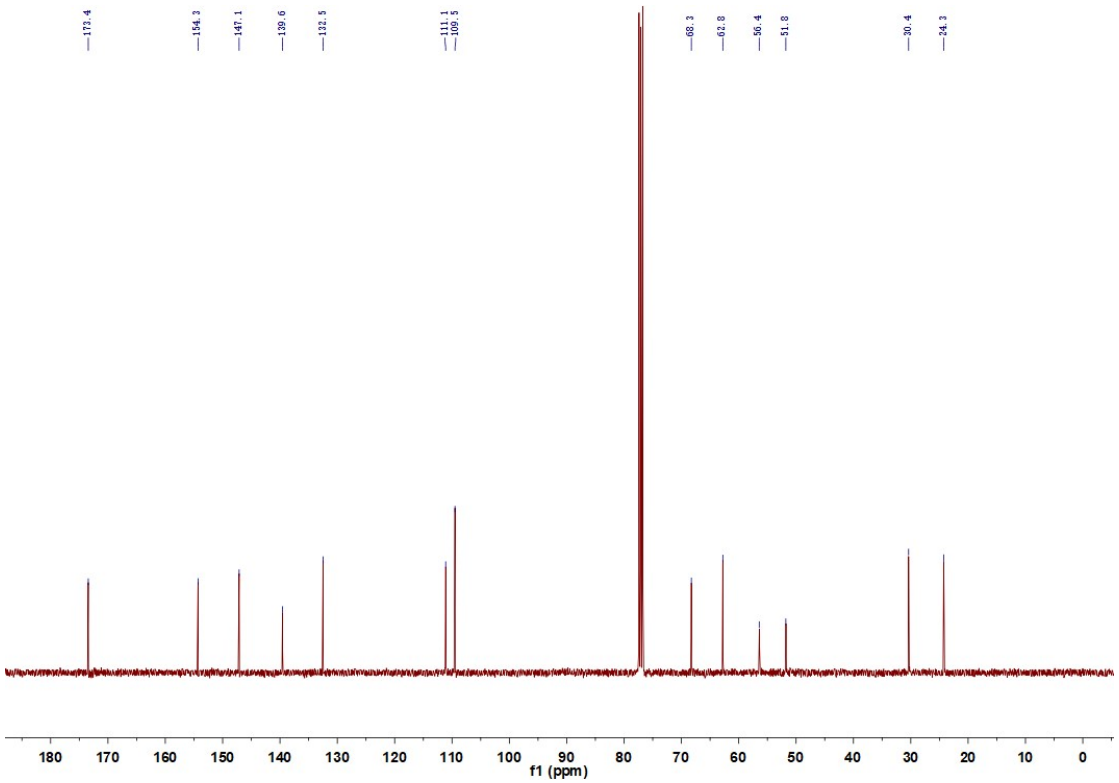


Figure S6. ^{13}C NMR spectrum of N3.

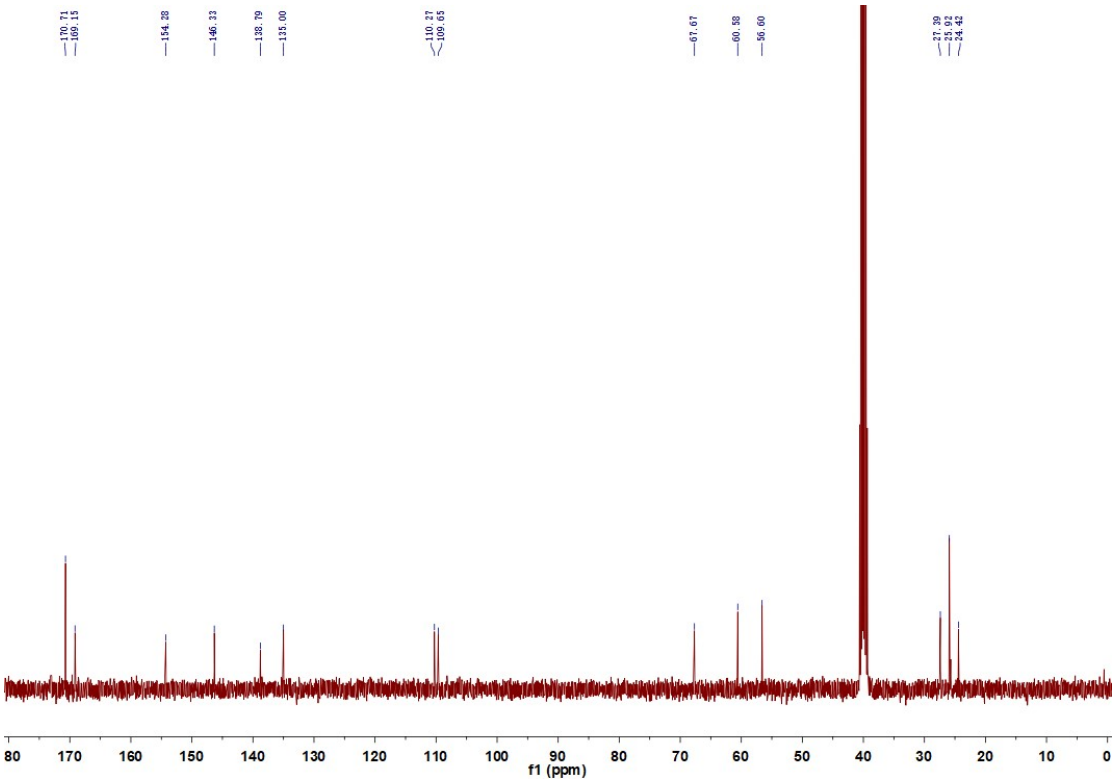


Figure S7. ^{13}C NMR spectrum of NB-NHS.

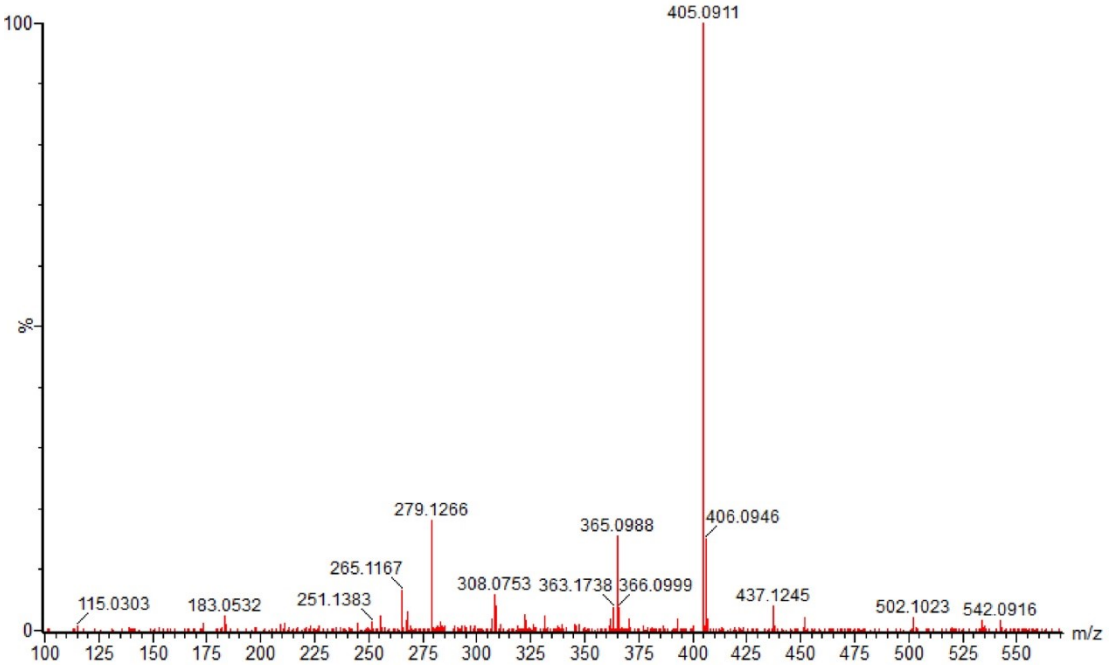


Figure S8. ESI-MS spectrum of NB-NHS.

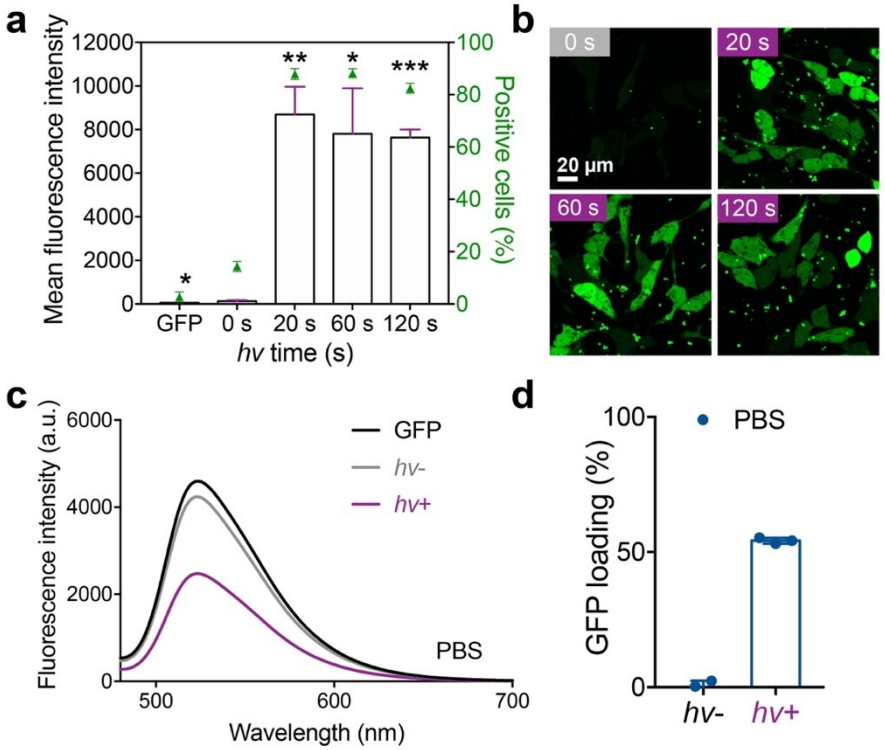
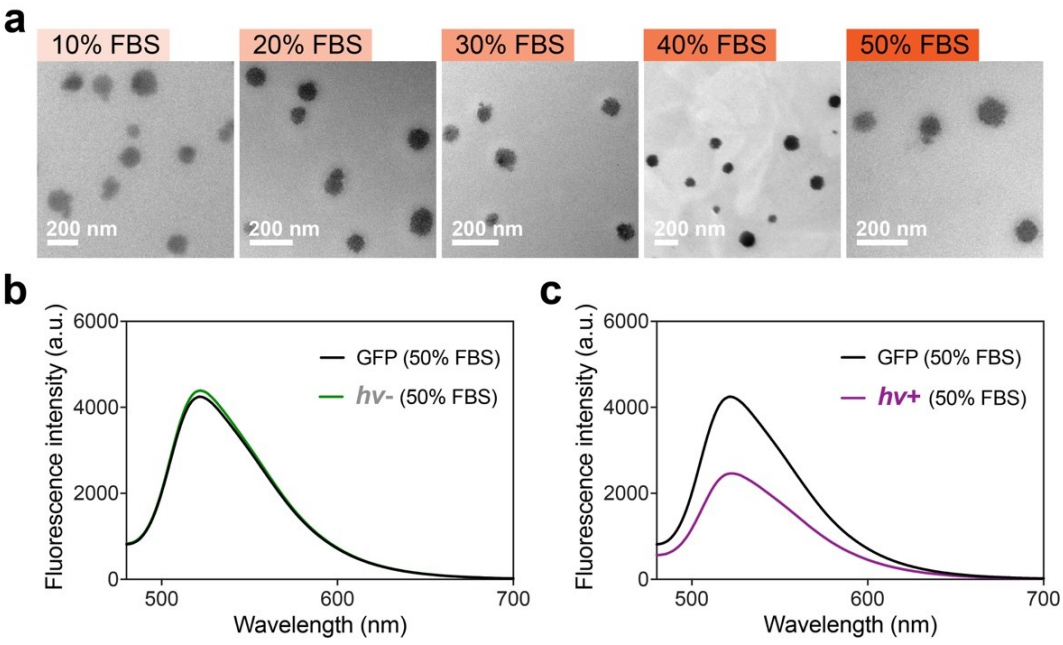


Figure S9. Flow cytometry (a) and confocal images (b) of 143B cells treated with ND/GFP complexes irradiated by a 365 nm UV light for 0, 20, 60 and 120 s. The concentrations of GFP and ND polymer were 16 and 32 μg/mL, respectively. Native GFP was used as a control. (c) Fluorescence spectra and (d) GFP loading efficiency of the ND/GFP complexes before and after UV light activation in PBS buffer. The doses of GFP and ND polymer were 4 and 8 μg/mL, respectively.

Figure S10. (a) TEM images of ND/GFP complexes after UV light irradiation in culture media containing different concentrations of FBS. Fluorescence intensity of ND/GFP complexes without (b) and with (c) UV light irradiation in DI water containing 50% FBS. Free GFP was tested as a control.



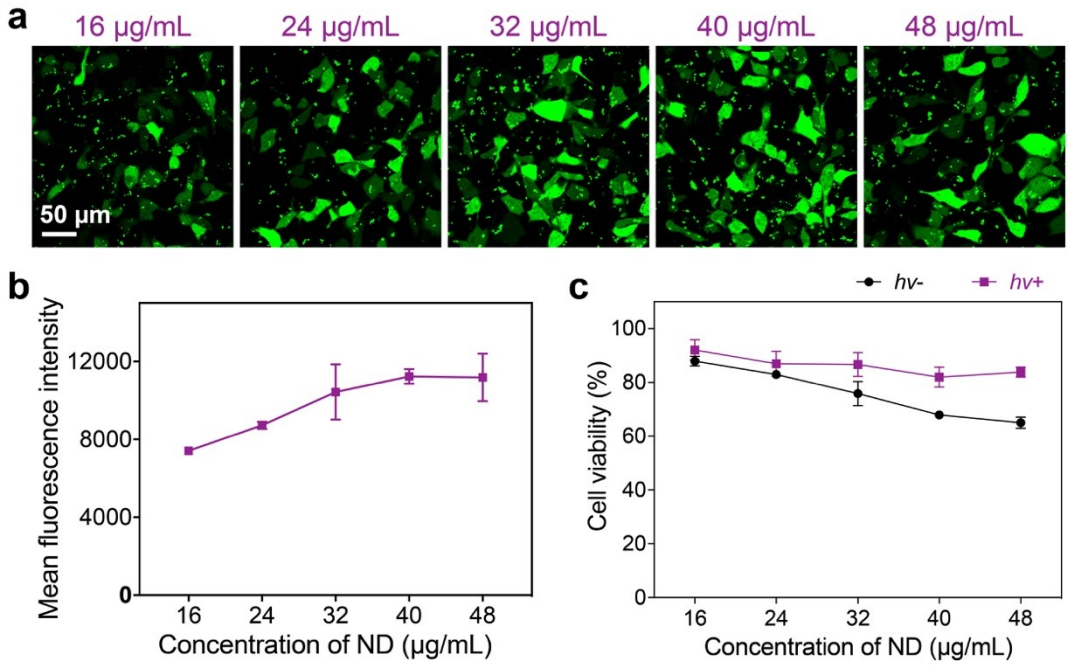


Figure S11. Confocal images (a) and fluorescence intensity (b) of 143B cells treated with ND/GFP complexes (GFP, 16 μg/mL; ND, 16-48 μg/mL) after LAC. (c) Viability of 143B cells treated with ND polymer with or without LAC at different concentrations.

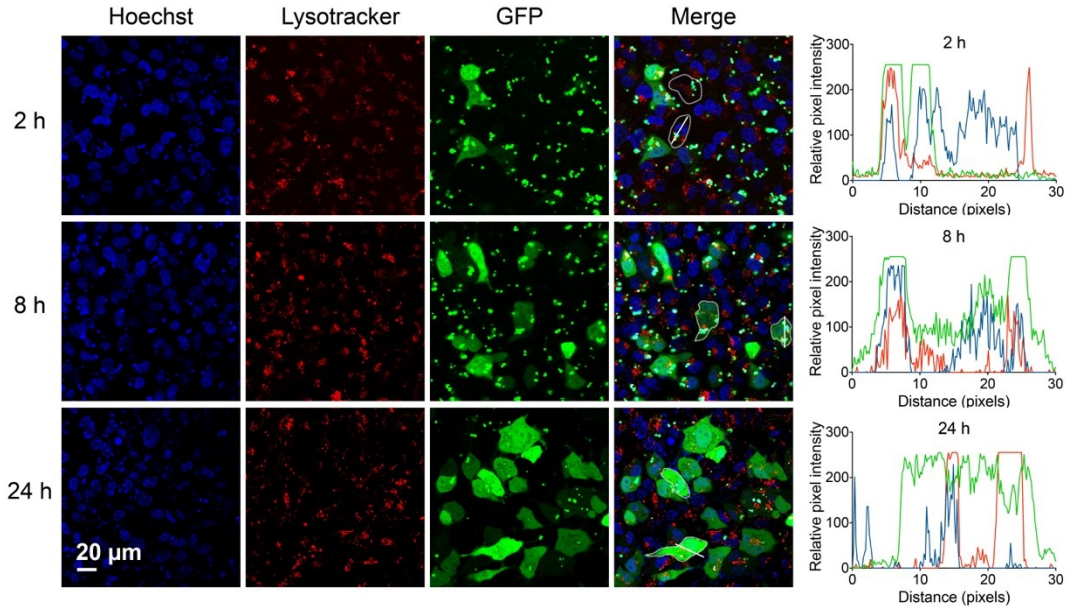


Figure S12. Confocal images of 143B cells treated with ND/GFP complexes after UV light irradiation for different incubation time. The cell nuclei and acidic organelles were dyed with Hoechst 33342 and Lysotracker Red, respectively. The representative cells were marked with white dotted circles. Quantitative analysis of the fluorescence intensity across a cell at each time point were shown on right.

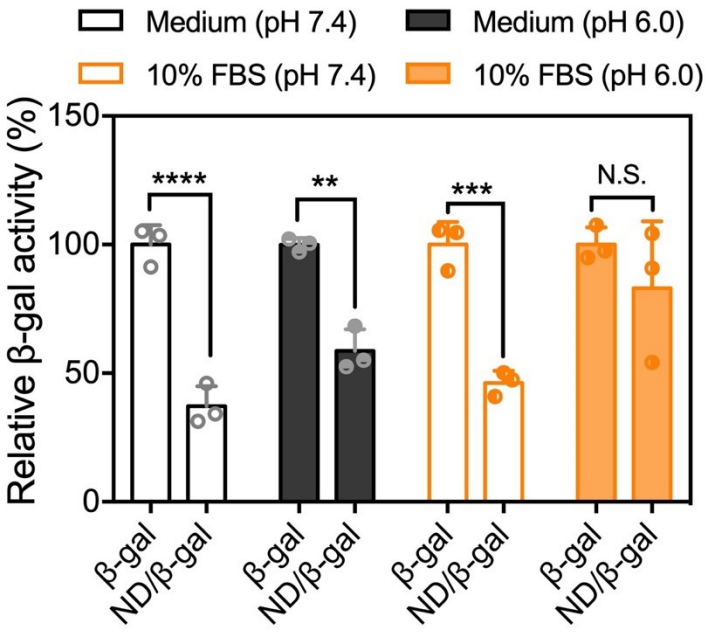


Figure S13. *In vitro* enzymatic activity of β-gal or ND/β-gal complex in different culture medium. Data were shown as mean ± s.d. (n=3). $^{N.S.}p > 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ were calculated by one-way ANOVA.