Supporting information

Photoenhanced cytosolic protein delivery based on a photocleavable group-modified dendrimer

Yafei Li,† Yang Zhou,† Tianyi Wang, Kaiqi Long, Yaming Zhang, Weiping Wang*

State Key Laboratory of Pharmaceutical Biotechnology & Department of Pharmacology and Pharmacy, Li Ka Shing Faculty of Medicine & Dr. Li Dak-Sum Research Centre

The University of Hong Kong

Hong Kong SAR, China.

†These authors contributed equally to this work.

* Corresponding Author. Email: wangwp@hku.hk

Materials

DEACM was purchased from INDOFINE Chemical Company (New Jersey, USA). Ethylenediamine-cored and amine-terminated generation-five PAMAM dendrimer (MW: 28826 Da, 5 % (w/w) in methanol) was purchased from Sigma-Aldrich (St. Louis, MO). Before the preparation procedures, methanol was evaporated under vacuum, which gave the dendrimer as transparent gels. Rhodamine B isothiocyanate (RBITC) were obtained from Macklin (China). Bovine serum albumin (BSA) was obtained from Sigma-Aldrich (St. Louis, MO). Glucose oxidase (GOx), horseradish peroxidase (HRP), and 3,3',5,5'-Tetramethylbenzidine (TMB) were purchased from J&K Co. (Beijing, China). Ethylisopropylamiloride (EIPA), Genistein, Chlorpromazine

(CPZ), and Methyl-β-cyclodextrin (M-β-CD) were purchased from Dieckmann (Shenzhen, China). LysoTracker® Green DND-26 and Pierce™ Protein Transfection Reagent were obtained from Thermo Fisher (HK, China). All other chemicals were purchased from J&K Scientific Co. Ltd and used without further purification. Deionized water was used for all aqueous systems.

Synthesis and characterization of DEACM-conjugated PAMAM (PAMAM-DEACM)

DEACM was activated with 4-nitrophenyl chloroformate (4-NPC) according to a reported procedure.[1] Generally, 170 mg DEACM (0.6878 mmol) and 1.38 g 4-NPC (6.88 mmol) were dissolved in 10 mL dry dichloromethane. N, N-Diisopropylethylamine (DIPEA, 6.85 mmol, 1.2 mL) was added slowly on an ice bath. After 15 min, the mixture was stirred at room temperature for another 6 h in the darkness. Then the reaction solution was washed with 100 mL 0.01 M hydrochloride twice. The organic layer was collected and dried over anhydrous magnesium sulfate. The crude product was purified by flash chromatography (CombiFlash® system, Teledyne ISCO, Nebraska, USA) using DCM and 2% methanol as mobile phase. Yield: 239.9 mg, 84.8%.

The activated DEACM was then mixed with PAMAM in anhydrous DCM: DMSO (1:1, v/v) solution with a trace amount of DIPEA for 24 h in the darkness. Then the product was dialyzed (Cutoff MW: 3500 Da) against DMSO until there was no obvious DEACM fluorescence in the outer fluid. For conjugation of DEACM with different mole ratios, different amount of DEACM was used. Synthesized products were additionally dialyzed against water to remove DMSO and then freeze-dried for ^{1}H NMR characterization (Bruker DX 500 spectrometer at 400 Hz) and UV-Vis spectrum analysis (SpectraMax M4, Mollecular Devices).

To measure the photo-triggered release of DEACM, PD0.4 was dissolved in a mixture of methanol and water (1:1, v/v) and exposed to 420 nm light at 50 mW/cm^2 (Light source: Mightex

LED) for different time periods. The product was then analyzed with high-performance liquid chromatography (HPLC, Agilent Technologies, 1260 Infinity II).

Synthesis of fluorescent dye-labeled proteins:

BSA or GOx was dissolved in phosphate-buffered saline (PBS, pH 7.4) to get 10 mg/mL protein solutions. The solutions were mixed with RBITC at a RBTIC/protein mass ratio of 1:10. The mixed solutions were stirred overnight at room temperature in the darkness. The labeled proteins were purified by dialysis (Cutoff MW: 3500 Da) against PBS and then deionized water. The purified products (noted as rBSA and rGOx, respectively) were lyophilized for UV-Vis characterization and further experiments.

Preparation and characterization of PAMAM-DEACM/protein complex

The complexes were prepared through flash nanoprecipitation.[2] Briefly, 20 μg PAMAM-DEACM was added into protein solutions (3-12 μg) dropwise under vigorous stirring. The formed complex was noted as PD/protein. To further increase the stability, hyaluronic acid (HA, 16 μg) and BSA (200 μg) were subsequently added under stirring to form protective coating layers on the surface of the complex. Then the mixture was incubated at room temperature for 30 min. The stable complexes (noted as BH-PD/protein) were diluted with water or medium for further characterizations.

The hydrodynamic diameter and zeta potential of these complexes were characterized by dynamic light scattering (DLS) using Malvern Zetasizer (Nano ZS 90, Malvern, UK). The stability test was conducted in complete DMEM medium with 10% (v/v) fetal bovine serum (FBS) for 48 h at 37 ^{o}C. The morphology of the nanocomplexes was observed by transmission electron

microscope (TEM, Philips CM100). Förster resonance energy transfer (FRET) assay was used to determine the interaction between PD0.4 and rBSA. Rhodamine B on BSA was fluorescence acceptor to quench the fluorescence from DEACM. The fluorescence spectra were recorded at excitation and emission wavelengths of λ_{ex} = 405 nm and λ_{em} = 450-650 nm.

The binding efficiency was conducted to determine the binding ability of PAMAM-DEACM to proteins. Generally, BH-PD/rBSA complexes were prepared with PD0.1, PD0.2, PD0.3, PD0.4, and PD0.6, separately. Complexes and rBSA were centrifuged at 14,000 rpm for 20 min and the absorbance at 560 nm of supernatant was measured by a microplate reader. The concentration of unbound rBSA was calculated according to the standard curve of rBSA (C= 0.0017Ab + 0.0125, in which C is the concentration of rBSA, μg/mL, Ab is the absorbance at 560 nm). The protein binding efficiency of PAMAM-DEACM with different grafting ratios were calculated according to Equation (1):

$$Protein\ binding\ efficiency = \frac{C_{rBSA} - C_{BH-PD/rBSA}}{C_{rBSA}} \times 100\% \quad (1)$$

The binding constants of PAMAM-DEACM conjugates with rBSA were determined by measuring the fluorescence intensities of PD/rBSA nanocomplexes at different molar ratios (λ_{ex} = 548 nm, λ_{em} = 586 nm). The binding constant K_b was obtained by Equation 2:

$$\log\left(\frac{F_0 - F_c}{F_c}\right) = \log K_b + n\log C \quad (2)^3$$

F_0 and F_C are the fluorescence intensity of free rBSA and PD/rBSA mixtures at different conjugate concentrations, respectively. n is the number of binding sites. C is the concentration of conjugates.

Cell culture and cytosolic delivery of BSA

The commercial A549 human lung carcinoma cell line (American Type Culture Collection, ATCC) was used in this study. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (all from Gibco-Invitrogen Corp.) at 100% humidity and 37 ^{o}C in an incubator under 5% CO_2 condition.

To investigate cytosolic delivery of BSA, A549 cells were seeded in 24-well plates in complete DMEM medium for 24 h. Then BH-PD/rBSA nanocomplexes or free rBSA (5 μg) were added. Light-treated groups were immediately irradiated by a 420 nm LED (50 mW/cm^2, 2 min). After incubation for 4 h, culture medium was removed, and cells were washed with PBS and collected with trypsin for cellular uptake measurement. The fluorescence intensity of cells was measured by flow cytometry (ACEA NovoCyte Quanteon, CH). Experiments for each group was repeated three times.

To visualize cellular uptake, cells were seeded on 8-well Nunc Lab-Tek chambered slides for 24 h and then cells were treated with BH-PD/rBSA nanocomplexes or free rBSA (2 μg) for different time periods. The light-treated groups were irradiated (420 nm, 50 mW/cm^2, 2 min) immediately after addition of the nanocomplexes. Then cells were washed and fixed with 2.5% paraformaldehyde for 20 min and observed with a confocal microscope (Carl Zeiss LSM 900). Other control groups were treated with protein mixture with PAMAM (equivalent molar ratio to PD0.4) or a commercial Pierce^{TM} Protein Transfection Reagent Kit following the manufacturer's instruction (1 μL kit solution per 2 μg protein). For subcellular localization analysis, cells were seeded onto confocal dishes, additionally treated with 75 nM Lysotracker^{TM} green DND-26 for 90 min, and then observed without fixation.

To study light induced endosomal escape, cells were incubated with BH-PD/rBSA nanocomplexes and 50 nM LysoTracker® Deep Red for 2 h, then medium was removed, and light irradiation (420 nm, 50 mW/cm^2, 2 min) was applied. Cell images were measured immediately with a confocal microscope.

To investigate HA targeting abilities and endocytosis pathways of the nanocomplexes, A549 cells were pretreated with HA (5 mg/mL) or four endocytosis inhibitors including EIPA (20 μM), genistein (400 μM), chlorpromazine (20 μM) and M-β-CD (10 mM) for 2 h. Then, the cells were incubated with the BH-PD/rBSA nanocomplexes for 2 h before measuring the fluorescence intensity through flow cytometry.

Enzymatic activity evaluation of HAPD/GOx

The enzymatic activity of BH-PD/GOx nanocomplexes was measured following a reported TMB protocol with modification.[4] In general, 10 mU/mL BH-PD/GOx nanocomplex or GOx were incubated with 0.5 mg/mL glucose and 0.2 mg/mL heparin at 37 ^{o}C for 10 min. Then 15 mU/mL HRP and 0.05 mg/mL TMB were added. The absorption at 370 nm was recorded by a microplate reader at 10-s intervals for 10 min. To measure the photo-triggered protein release, nanocomplexes in PBS were irradiated with 420 nm light at 50 mW/cm^2 for 2 min before measurement. To measure the protein stability, nanocomplexes were preincubated at 37 ^{o}C for a certain period before measurement. High concentration of negatively charged heparin was used to compete with materials for protein binding, leading to the dissociation of nanocomplexes and release of proteins.[5]

Cytosolic delivery of GOx

Cytosolic delivery of RBITC labeled GOx (rGOx) was measured through flow cytometry and confocal microscope, using the same methods for the evaluation of rBSA delivery. For GOx delivery, glucose-free medium was used during the incubation.

Cell viability evaluation of GOx nanocomplexes

BH-PD/GOx nanocomplex or free GOx were diluted with glucose-free medium and added to the 96-well plates with A549 cells at different concentrations. Light-treated groups were immediately irradiated with 420 nm light at 50 mW/cm^2 for 2 min. After 4 h incubation, cells were washed with PBS to remove extracellular GOx and incubated in complete medium containing glucose for another 20 h. Then cell viability was evaluated by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with absorbance at 570 nm.

Statistical analysis

Statistically significant difference was analyzed by Student's t test. $P < 0.05$ was considered significant. ns, not significant, $P>0.05$, *$P < 0.05$; **$P < 0.01$, and ***$P < 0.001$.

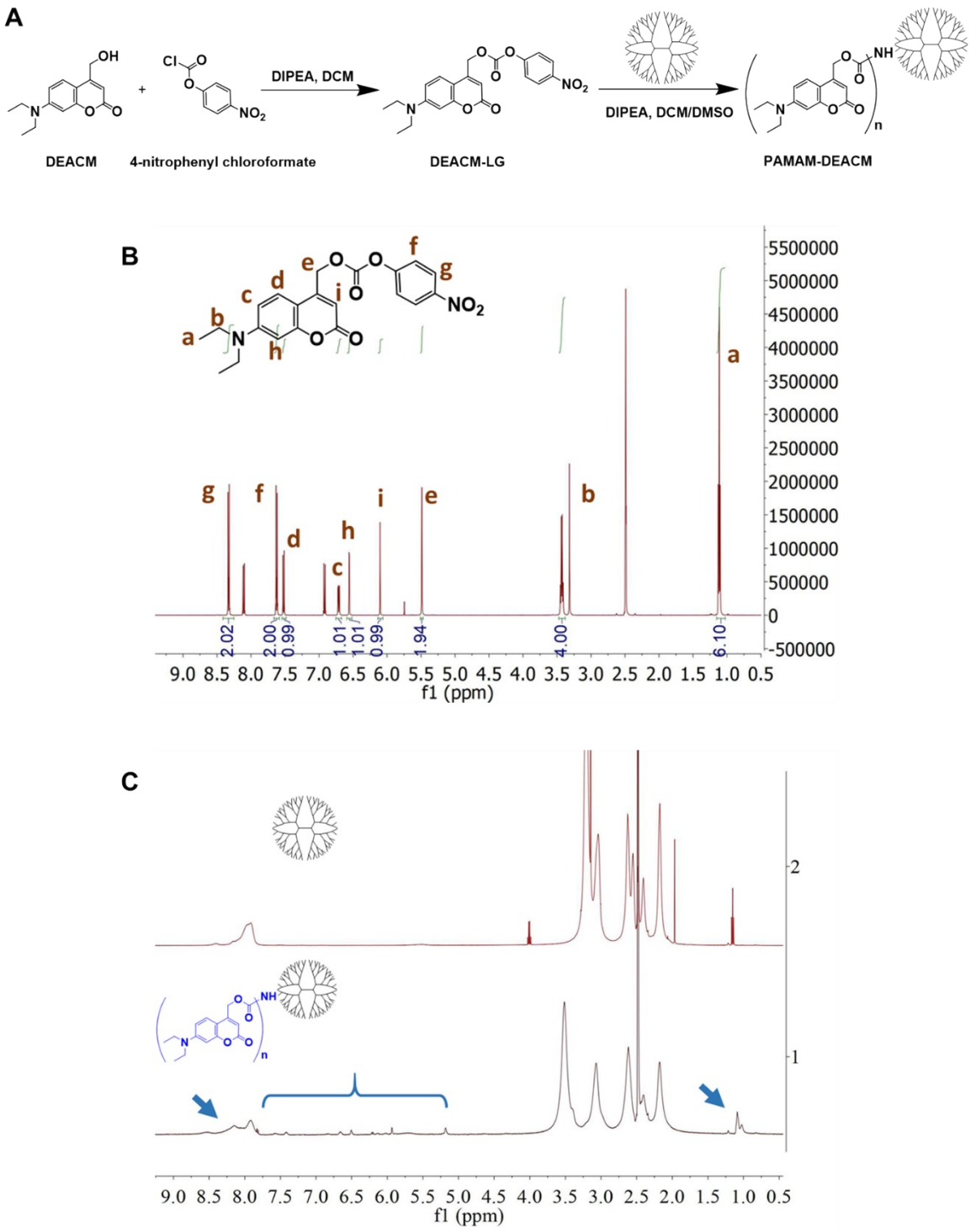
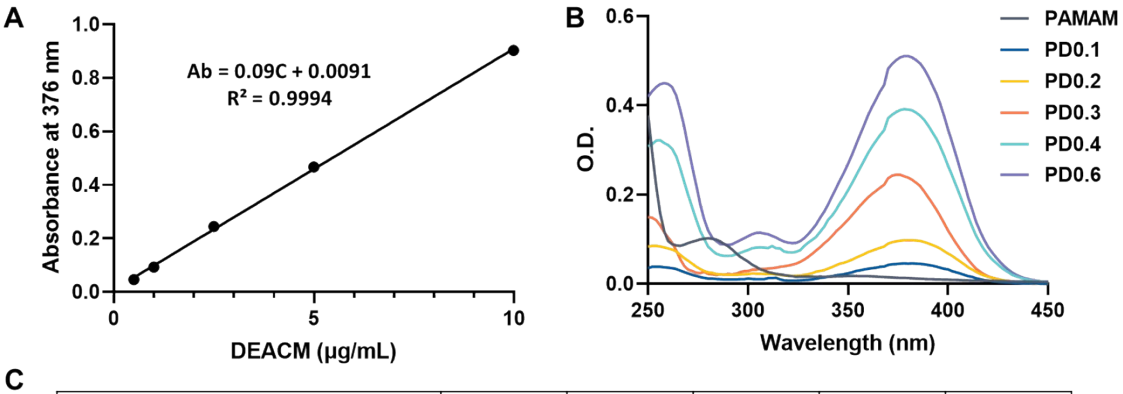


Figure S1. Synthesis route and ^{1}H NMR characterization of PAMAM-DEACM. (A) Synthetic route of PAMAM-DEACM. ^{1}H NMR spectra of (B) DEACM-LG, (C) PAMAM (up), and PD0.1 (down) in d6-DMSO.



C

	PD0.1	PD0.2	PD0.3	PD0.4	PD0.6
Feeding DEACM per PAMAM	12.8	25.6	38.4	51.2	76.8
Calculated DEACM per PAMAM	5.41	6.40	15.06	24.50	32.19

Figure S2. UV-Vis absorption of PAMAM-DEACM. (A) Absorbance of DEACM at 376 nm in methanol. (B) Absorption spectra of PAMAM and PAMAM-DEACM in methanol. (C) Feeding and calculated molar ratio of DEACM to amino groups on PAMAM.

Table S1. Diameter of BSA complexes with different materials

	PAMAM/BSA	PD0.1/BSA	PD0.2/BSA	PD0.3/BSA	PD0.4/BSA	PD0.6/BSA
Diameter (nm)	887.9	136.0	147.8	163.8	126.5	145.5
Polydispersity index	0.849	0.150	0.206	0.203	0.179	0.168

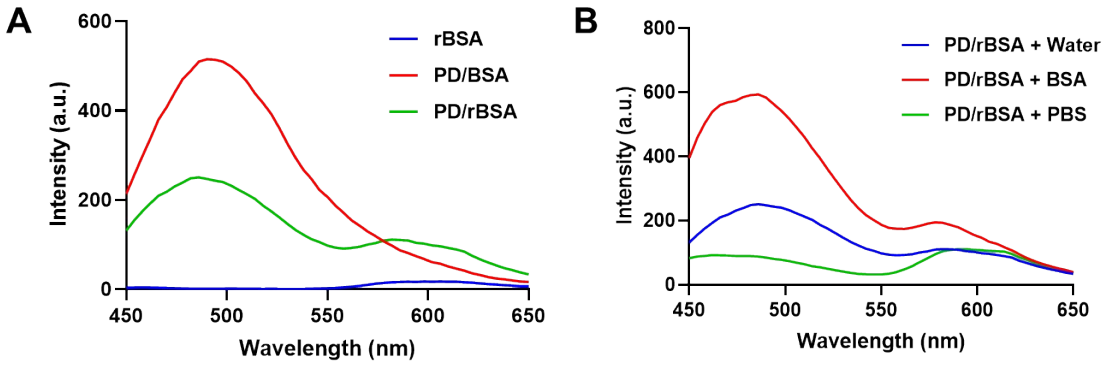


Figure S3. FRET analysis for the interaction of PD0.4 with rBSA. (A) Fluorescence spectra of rBSA, PD/BSA and PD/rBSA in water. (B) Fluorescence spectra of PD/rBSA in water, water containing 5% BSA and PBS. λ_{ex} = 405 nm.

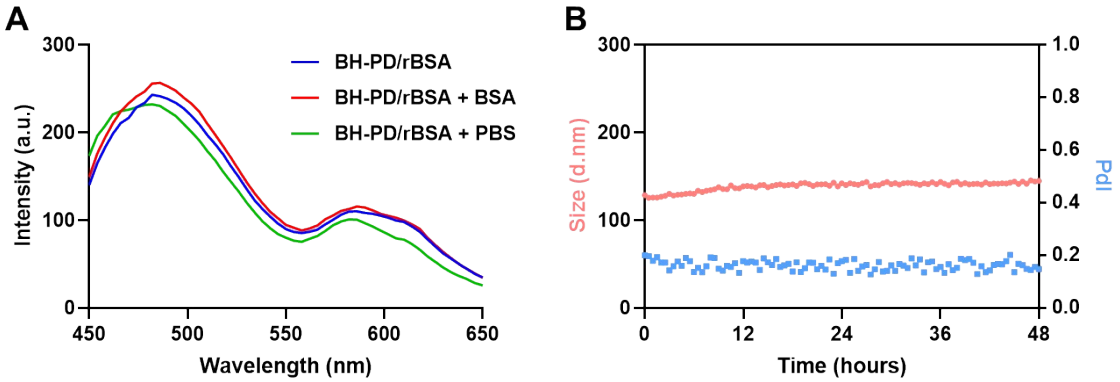


Figure S4. Stability analysis of protein nanocomplexes. (A) Fluorescence spectra of BH-PD/rBSA in water, 5% BSA and PBS. λ_{ex} =405 nm. (B) Size of BH-PD/BSA nanocomplexes in complete medium at 37 °C for 48 h.

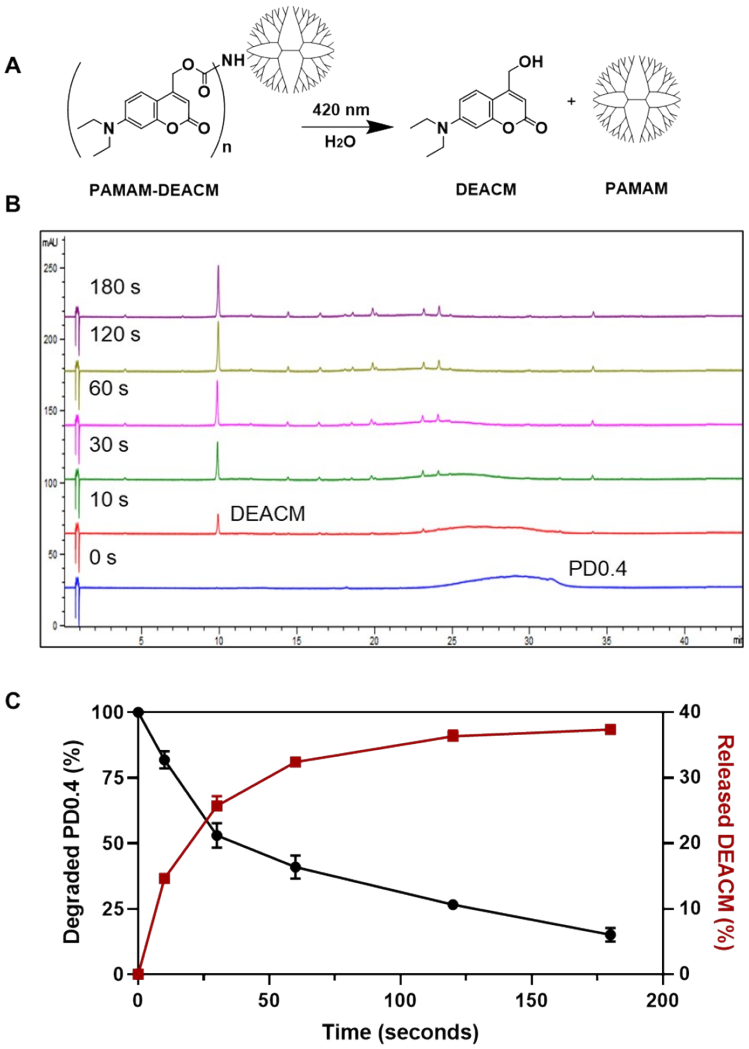


Figure S5. HPLC analysis of PD0.4 degradation under 420 nm light irradiation (50 mW/cm^2) for different time periods. (A) Photocleavage reaction of PAMAM-DEACM. (B) HPLC spectra. Detection wavelength: 380 nm. (C) The quantified data from (B). Data analysis: n=3.

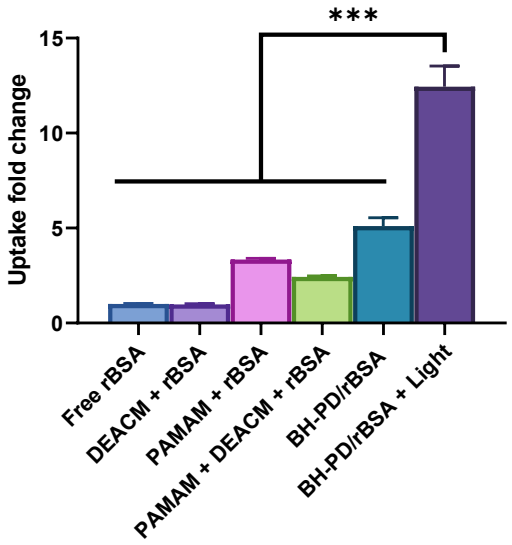


Figure S6. Cellular uptake of rBSA, rBSA mixture, or nanocomplexes. All groups were mixed with the equal amount of HA and BSA as used in the preparation of BH-PD/rBSA nanocomplexes. Light irradiation condition: 420 nm, 50 mW/cm^2, 2 min. Data analysis: n=3. ***p<0.001.

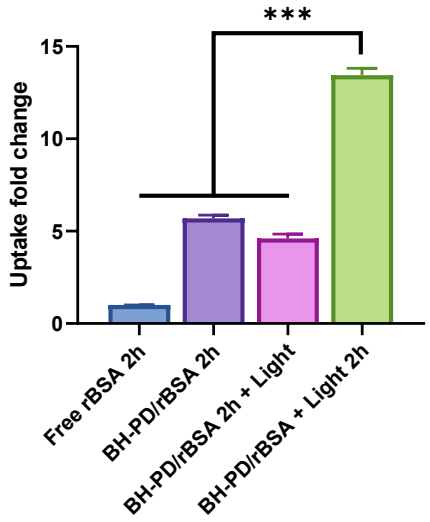


Figure S7. Cellular uptake of rBSA and nanocomplexes with different light irradiation treatments. Cells were incubated with rBSA or BH-PD/rBSA nanocomplexes with or without light irradiation for 2 h. Then cells were washed with fresh medium. In the third group, cells were incubated with BH-PD/rBSA for 2h, and then washed with fresh medium. The light irradiation was applied. All groups were incubated for 1 h more before collection for flow cytometry analysis. Light irradiation condition: 420 nm, 50 mW/cm^2, 2 min. Data analysis: n=3. ***$p<0.001$.

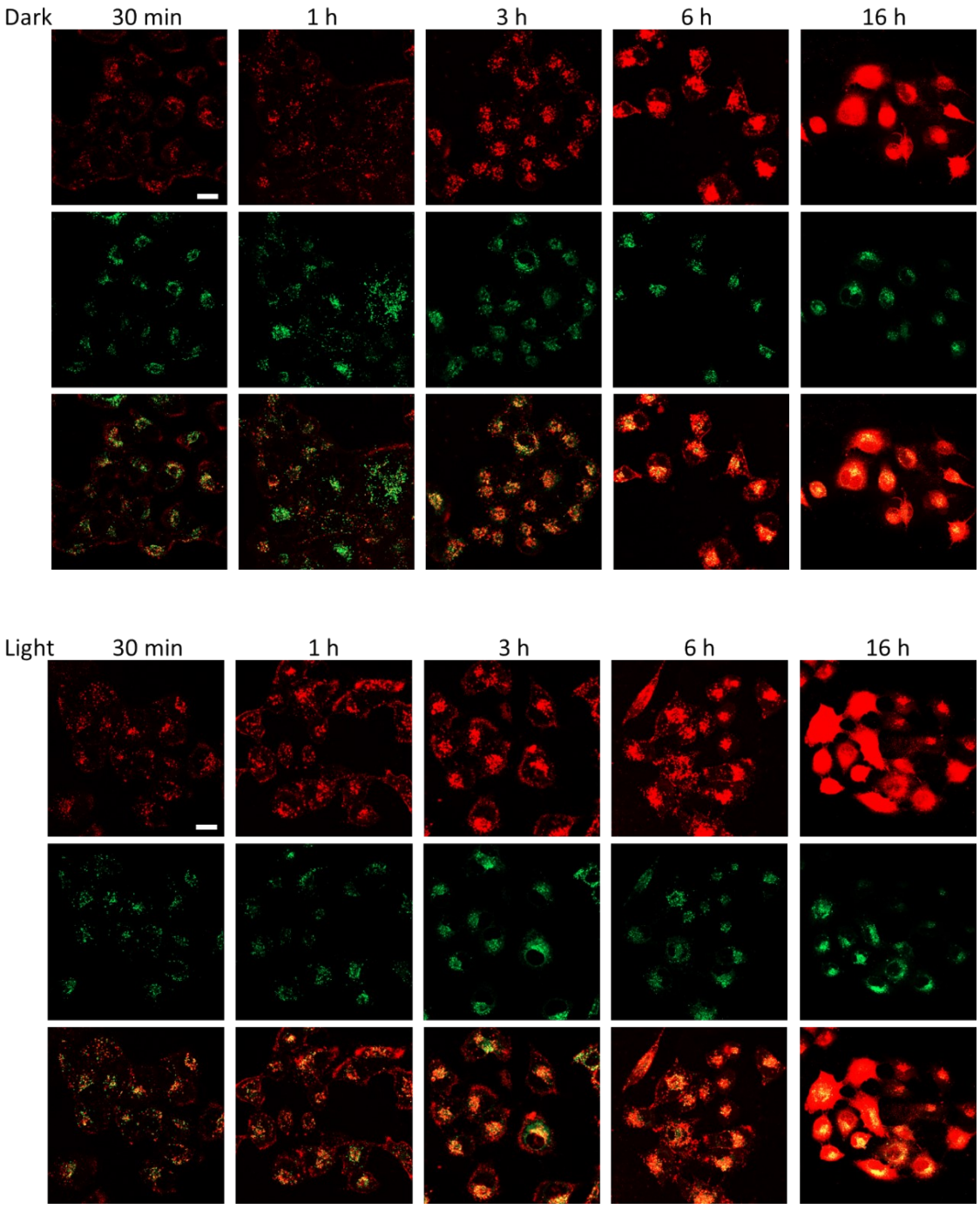


Figure S8. Confocal images of A549 cells treated with BH-PD/rBSA nanocomplexes for different time periods. Cells were incubated for 30 min, 1 h, 3 h, 6 h, and 16 h, respectively. Endosomes were stained with LysoTracker Green DND-26. Light irradiation condition: 420 nm, 50 mW/cm^2, 2 min.

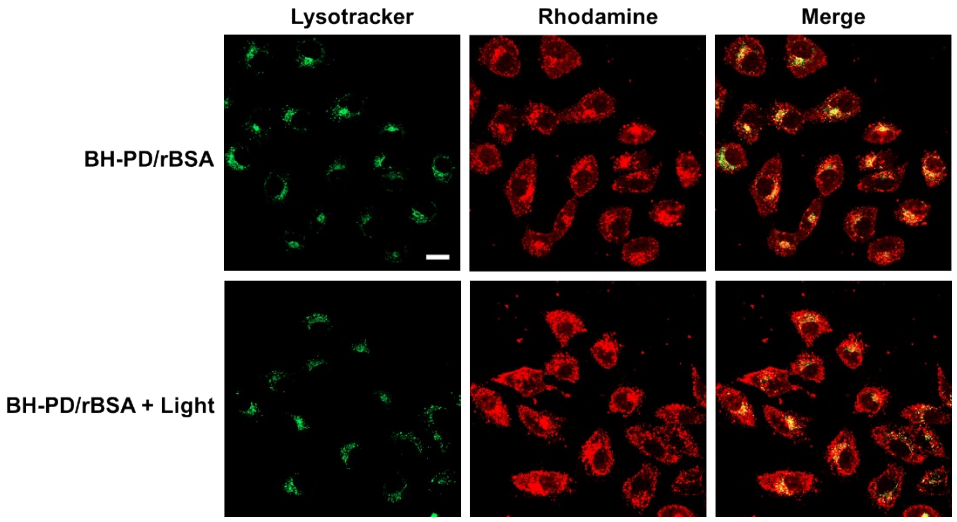


Figure S9. Confocal images of A549 cells treated with BH-PD/rBSA nanocomplexes for 1 h in serum-free DMEM medium. Endosomes were stained with LysoTracker Green DND-26. Light irradiation condition: 420 nm, 50 mW/cm^2, 2 min.

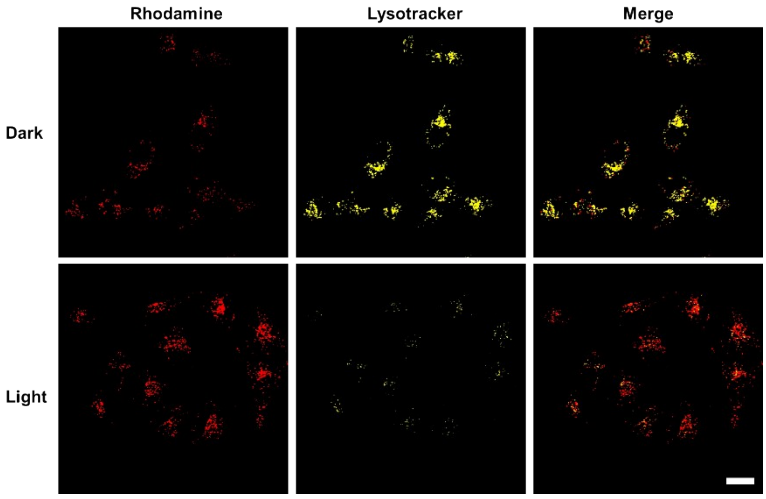


Figure S10. Endosomal escape induced by light irradiation. Cells were incubated with BH-PD/rBSA nanocomplexes and 50 nM LysoTracker® Deep Red for 2 h, then the medium was removed, and light irradiation (420 nm, 50 mW/cm^2, 2 min) was applied. Cell images were measured immediately with a confocal microscope.

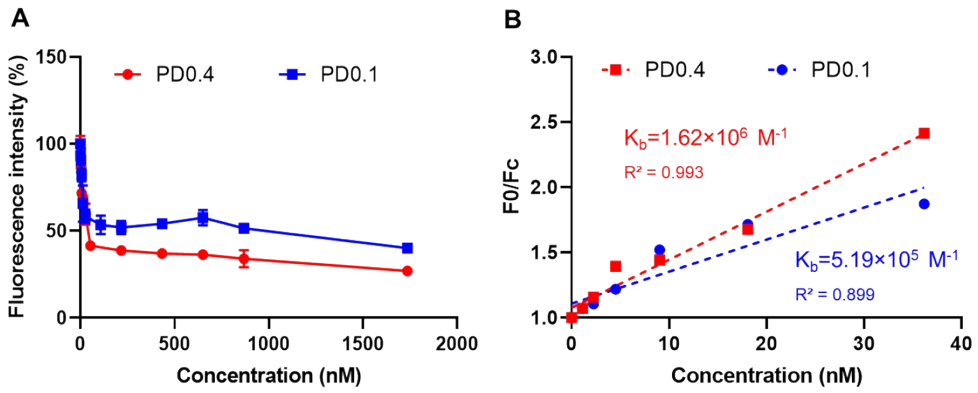


Figure S11. **Binding ability of PAMAM-DEACM with GOx.** (A) Fluorescence change of rGOx (100 nM) with different concentrations of PAMAM-DEACM. (B) The Stern-Volmer curve for the interaction of PAMAM-DEACM with rGOx (100 nM). F0 is the fluorescence intensity of rGOx, Fc is the fluorescence intensity of PD/rGOx. λ_{ex}=548 nm, λ_{em}=588 nm.

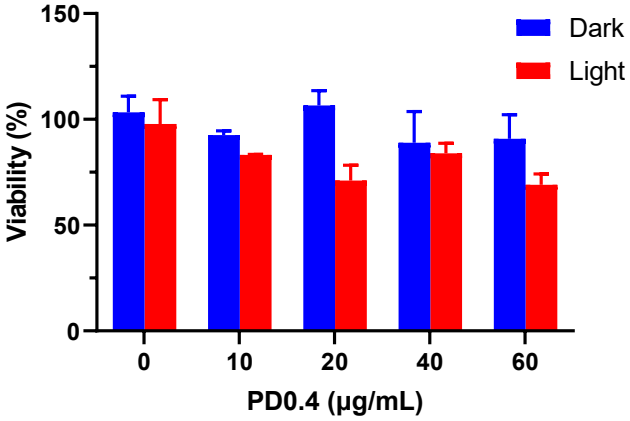


Figure S12. Cytotoxicity evaluation of PD0.4. Cells were treated with PD0.4 for 24 h. Light treated groups were irradiated right after the addition of the material. Irradiation condition: 420 nm, 50 mW/cm^2, 2 min, n=4.

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