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Probing Protease Activity by Single-Fluorescent-Protein Nanocapsules

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Materials. All chemicals were purchased from Sigma-Aldrich unless otherwise specified, and were used as received. *N*-(3-Aminopropyl) methacrylamide hydrochloride was purchased from Polymer Science, Inc. 4-((4- (dimethylamino)phenyl)azo)benzoic acid (DABCYL), succinimidyl ester, peptides Ac-DEVD-pNA and Ac-DEVD-CHO were purchased from Anaspec, Inc. CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS) reagent was purchased from Promega Corporation. Sulfo-SMCC (Sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate) was purchased from Pierce. The deionized water was prepared by a Millipore NanoPure purification system (resistivity higher than 18.2 $M\Omega \cdot cm^{-1}$).

Instruments. Absorbance for the Bradford assay was measured on a Thermo Scientific GENESYS 20 spectrometer while dynamic absorbances for *in vitro* activity assays of CP3/nanocapsules were measured using a Beckman Coulter DU® 520 spectrometer. TEM images of nanocapsules were obtained on a Philips EM-120 TEM instrument. Zeta potential and particle size distribution were measured on the Malvern particle sizer Nano-ZS. LC-MS was conducted with a Shimadzu 2010 EV Liquid Chromatography Mass Spectrometer using positive electrospray ionization and a Phenomenex Luna 5μ 2.0 × 100 mm C18 reverse-phase column. Samples were separated over a linear gradient of 5% to 95% CH_3CN (vol/vol) for 30 min and 95% CH_3CN (vol/vol) for an additional 30 min in H_2O supplemented with 0.1 % (vol/vol) trifluoroacetic acid at a flow rate of 0.1 mL/min at room temperature. Fluorescent images of cells were obtained with either a Zeiss Axio Observer Z1 Inverted Microscope or a Yokogawa spinning-disk confocal microscope (Solamere Technology Group, Salt Lake City, UT) on a Nikon eclipse Ti-E Microscope equipped with a 60 × 1.49 Apo TIRF oil objective and a Cascade II: 512 EMCCD camera (Photometrics). Flow cytometry analysis was performed on FACScan and FACSort (BD Bioscience). UV spectra were obtained by BeckmanCoulter DU640 UV-Vis spectrometer. Fluorescence spectra were recorded on Spectrofluorimeter-SPEX. Fluorescence intensity of 96-well plate was measured by Spectra MAX GEMINI XS (Molecular Devices). The photo-irradiation was carried out by a UV light source from Spot Light Curing, Omnicure Series 1000 (100 W, 365 nm).

Protein Expression and Purification. The wild-type CP3 expression plasmid pHC332 was a generous gift from Dr. A. Clay Clark (North Carolina State University). *Escherichia coli* BL21(DE3) *LysS* cells were transformed with plasmid pHC332. Transformed cells were inoculated overnight at 37 ºC with shaking in Luria-Bertani medium containing 100 μg/mL ampicillin. Overnight cultures were diluted 1:200 and grown in Fernbach flasks containing 1 liter of LB medium with 100 μg/mL ampicillin at 37 ^{o}C with shaking at 270 rpm. When the cultures reached an absorbance *A*600 ~1.2, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM to induce protein expression, and the cells were incubated at 16 ^{o}C overnight. The cells were harvested by centrifugation (2,000g, 4 ^{o}C, 15 min), resuspended in 30 mL Buffer A (50 mM Tris-HCl, pH 8.0, 2 mM DTT, 2 mM EDTA), and lysed by sonication. Cell debris and insoluble proteins were removed by centrifugation (20,000g, 4^{o}C, 30 min). After centrifugation, the cleared cell lysate was incubated with 0.5 mL of Ni-NTA resin (Qiagen) for 3 hours at 4 ºC. The protein was then purified using a step gradient of Buffer A with increasing concentrations of imidazole (10, 20, and 250 mM). CP3 was eluted with 5 mL Buffer A containing 250 mM imidazole. The protein concentration was qualitatively assessed by SDS-PAGE and quantitatively determined by the Bradford protein assay using bovine serum albumin (BSA) as the standard. Likewise, His-tagged eGFP was expressed from transformed *Escherichia coli* BL21 and purified using Nickel-resin affinity column.

Synthesis of Photolabile Peptide Linker GVDEVD$_m$TC. Photolabile modified aspartic acid (Fmoc-Asp(ODMNB)-OH) was synthesized by modifying a previously reported procedure[1]. Briefly, 2.06 g (5.0 mmol) of *N*-α-Fmoc-L-aspartic acid-α-*t*-butyl ester was dissolved in 20 mL of dichloromethane (DCM). Then, 1.08 g of 4,5-dimethoxy-2-nitrobenzyl alcohol (DMNBA, 5.1 mmol) dissolved in 40 mL of DCM and 30 mg of 4-dimethylaminopyridine (DMAP, 0.21 mmol) were added. 1,3-dicyclohexylcarbodiimide (DCC, 1.03 g, 5.0 mmol) dissolved in 25 mL of DCM was then added dropwise over 2 minutes. The reaction mixture was stirred at room temperature for 3 h. After filtration, the crude product was washed three times by aqueous 2.5% $NaHCO_3$. The organic phase was separated and evaporated yielding a

yellow oil, which was dissolved in 100 mL TFA:water (19:1) and stirred for 90 min to remove the α-carboxyl protecting group (tBu). The solvent was removed by vacuum and the crude product was dissolved in 50 mL DCM. Following an aqueous wash, the organic layer was isolated, evaporated and dried to give 2.22 g (~90% yield) of pure Fmoc-Asp(ODMNB)-OH, as confirmed by LC-MS. Peptide GVDEVD$_m$TC with photolabile aspartic acid D$_m$ was assembled using standard solid-phase peptide synthesis procedures for Fmoc-chemistry on a custom-designed semiautomatic multireactor synthesizer. Wang Rink Amide MBHA resin was used as the solid support, and each coupling step was monitored using a ninhydrin test. Coupling of amino acids to the resin backbone was accomplished by a 0.9 equivalent of HBTU activation and 2 equivalents of DIEA. The Fmoc group was removed by 20% piperidine in DMF. Cleavage from the resin was achieved with a mixture of TFA/phenol/water/TIPS (88/5/5/2) for 2 h. The crude product was precipitated and washed with diethyl ether and further purified by HPLC equipped with a reverse-phase 10μ 150 × 21.2 mm C18 column (Jupiter), using a linear gradient of 5% to 95% CH_3CN in H_2O (vol/vol) over 30 min and 95% CH_3CN (vol/vol) for an additional 20 min supplemented with 0.1% (vol/vol) trifluoroacetic acid at a flow rate of 5 mL/min. The LC-MS analysis of GVDEVD$_m$TC is shown in Fig. S3.

Synthesis of Photolabile Peptide Linker *q*-GVDEVD$_m$TC. 30 mg of GVDEVD$_m$TC was dissolved in 2 mL DMF. Then 18 mg of 4-((4- (dimethylamino)phenyl)azo)benzoic acid (DABCYL), succinimidyl ester dissolved in 50 μL DMF with 25 μL DIPEA was added dropwis in a nitrogen atmosphere. The reaction mixture was stirred at room temperature for 3 h. The crude product was precipitated and washed with diethyl ether. After lyophilization, the crude powder was dissolved in methanol and further purified by HPLC (~70% yield). The LC-MS analysis of *q*-GVDEVD$_m$TC is shown in Fig. S4.

Preparation of eGFP-NGs. Single eGFP encapsulated nanogels were prepared according to our previously reported procedure with modifications. Briefly, a volume of 10 mg eGFP in 3.8 mL of pH 8.5, 50 mM sodium carbonate buffer was reacted with 4 mg *N*-acryloxysuccinimide in 40 μL dimethyl

sulphoxide (DMSO) for 2 h at room temperature. Buffer exchange with 1×PBS was then carried out for three times (Amicon Ultra-15 10K devices, Millipore Corp.). The degree of modification was ~ 17 vinyl groups per protein, measured using matrix-assisted laser desorption/ionization-time of flight (MALDITOF) mass spectra. Modified eGFP was diluted to 1 mg/mL with 10 mM pH 8.5 sodium bicarbonate buffer. Then acrylamide (AAm) monomer, prepared in a 200 mg/mL aqueous solution, was added to 3 mL of protein solution with stirring for 10 min at 4 ^{o}C. Subsequently, the other positively charged monomer *N*-(3-Aminopropyl) methacrylamide (APMAAm) was added. Afterwards, crosslinker *N*,*N*'-methylene bisacrylamide was added. The molar ratio of AAm/APMAAm/crosslinker was adjusted to 6/4/1. The polymerization was immediately initiated by adding 3 mg of ammonium persulfate (APS) dissolved in 30 μL of deoxygenated and deionized water and 3 μL of *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED). The polymerization was allowed to proceed for 90 min in a nitrogen atmosphere at room temperature. Finally, buffer exchange with 1×PBS was performed to remove unreacted monomers and initiators. As-synthesized eGFP nanocapsules show similar fluorescent spectra to those of native eGFP (data are not shown here). The yield of the protein nanocapsules was higher than 95%. The unmodified EGFP was removed using size-exclusion chromatography.

Preparation of eGFP-NG-Q. eGFP-NG-Q was prepared by conjugating *q*-GVDEVD$_m$TC to eGFP-NGs. Briefly, fresh eGFP-NG was diluted to a final concentration of 0.1 mg/mL in 1×PBS (pH=7.4). Then 0.1 mg of sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate)acrylamide (SMCC) in 5 μL DMSO was added to 1 mL of nanogel solution with stirring for 45 min at room temperature. Buffer exchange with 1×PBS was then immediately performed to remove excess SMCC. In a separate microtube, 0.1 mg *q*-GVDEVD$_m$TC was dissolved in 200 μL methanol and then added dropwise to the SMCC modified eGFP-NG solution (pH=7.4), with desirable molar ratio of *q*-GVDEVD$_m$TC to eGFP-NG. Covered with aluminum foil, the reaction was allowed to stand under nitrogen for 2.5 h at room temperature. The final product was washed by a buffer exchange with 1×PBS for three times (Amicon

Ultra-15 30K devices, Millipore Corp.). The obtained eGFP-NG-Q solution was covered with aluminum foil and stored at 4°C.

Characterization of Protein NGs. The protein content in NGs was determined by the bicinchoninic acid (BCA) colorimetric protein assay[2] Briefly, a tartrate buffer (pH 11.25) containing 25 mM BCA, 3.2 mM $CuSO_4$, and appropriately diluted protein/NCs was incubated at 60 °C for 30 min. After the solution was cooled to room temperature, absorbance readings at 562 nm were determined with a UV-Vis spectrometer (Thermo Scientific GENESYS 20). BSA solutions with known concentrations were used as standards. Far-UV circular dichroism (CD) spectra of the native protein and protein NGs were performed at 20 ^oC in a buffer containing 100 mM KH_2PO_4/K_2HPO_4, pH 7.4, with 20 μM of protein (JASCO J-715 Circular Dichroism Spectrometer). FTIR spectra of the native protein and protein NGs were recorded on a FTIR spectrometer (Nicolet 560) using KBr pellets. The hydrodynamic size of eGFP and NGs were measured by dynamic light scattering (Malvern particle sizer Nano-ZS, Fig. S9). The mean hydrodynamic size and zeta potential of native proteins and protein NGs are listed in Table S1. For TEM images of protein NGs, samples (0.05 mg/mL) were negatively stained for 2 minutes with 2% uranyl acetate in alcoholic solution (50 % ethanol) of on carbon-coated electron microscopy grids (Ted Pella, Inc.) and images were obtained with Philips EM-120 TEM operated at 80 kV.

Cell Culture. HeLa cells (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% bovine growth serum (BGS, Hyclone, Logan, UT), 1.5 g/L sodium bicarbonate, 100 μg/mL streptomycin and 100 U/mL penicillin. The cells were cultured at 37 ^oC, in 98% humidity and 5% CO_2. Cells were regularly subcultured using Trypsin-EDTA.

Cytotoxicity Study Using MTS Assay. Cells were seeded into 96-well plates at a density of 5,000 cells per well and cultivated in 100 μL of DMEM with 10 % BGS. The plates were then incubated in 5% CO_2 and at 37 ^oC for 12 h to reach 70-80% confluency before addition of protein/nanocapsules. After

incubation with NGs, the cells were washed with PBS solution and incubated with 100 μL fresh DMEM and 20μL MTS solution (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Invitrogen). The plates were incubated for an additional 3 h. The absorbance of the plates was read at 550 nm and a reference wavelength of 690 nm using a microplate reader (PowerWave X, Bio-tek Instruments, USA).

Inhibition of Specific Endocytotic Pathways. HeLa cells were seeded in a six-well culture dish at a density of 100,000 cells/well the day before the administration. Before the experiment, the medium was replaced with 0.5 mL of fresh DMEM with 5mM amiloride (inhibitor for macropinocytosis), 20 μg/mL chloroproamzine (CPZ, inhibitor for clathrin-mediated endocytosis), or 5 mM β-cyclodextrin (β-CD, inhibitor for caveolae-mediated endocytosis). After 30 min, 50 nM eGFP nanocapsules (eGFP-NCs) were added into cell medium and incubated at 37°C for 2 h. After washing with PBS, the cells were trypsinized, centrifuged, re-suspended in PBS and analyzed via FACS. HeLa cells incubated in medium without endocytosis inhibitors were used as a control. After the medium was removed, 1 mL of fresh medium containing eGFP NCs (1.5 nM) was added and incubated for 2 h at 4 ^{o}C or 37 ^{o}C in 5% CO_2. The cells were washed with fresh medium, trypsinized and re-suspended in PBS buffer. Flow cytometry was performed with a 488 nm argon laser. The signal from the FL1 bandpass emission (530/30) was used for eGFP. HeLa cells incubated in medium without endocytosis inhibitors were used as a control. For the experiment using an endocytosis inhibitor, before the experiment, the medium was replaced with 1mL of fresh medium with 2mM amiloride (inhibitor for macropinocytosis), 40 μM chloroproamzine (CPZ, inhibitor for clathrin-mediated endocytosis), or 10 mM β-cyclodextrin (β-CD, inhibitor for caveolae-mediated endocytosis) respectively. After 30 min, 1.5 nM eGFP-NCs were added into cell medium and incubated at 37°C for 2 h. After washing with PBS, the cells were trypsinized, centrifuged, re-suspended in PBS and analyzed via FACS.

Cellular Internalization Trafficking. For trafficking studies with different endocytic markers, eGFP NCs (50 nM) were added to HeLa cells at 4ºC for 30 min. The cells were shifted to 37 ºC for various

incubation periods, fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100, and immunostained with antibodies against early endosomes and late endosomes (rabbit anti-EEA antibody EEA1 and CI-MPR, Cell Signaling Technology, Inc.). Texas red-conjugated antimouse IgG and Cy5-conjugated goat anti-rabbit IgG antibodies were used as the secondary antibodies. Nuclei were stained by DAPI Nucleic Acid Stain (Invitrogen). Colocalization coefficients were calculated using the Manders' overlap coefficient by viewing more than 10 cells at each tim points. Manders overlap coefficient was generated using the Nikon NIS-Elements software.

Colorimetric Caspase-3 Activity Assay for Cisplatin Treatment. HeLa cells were seeded in six-well culture dishes at a density of 500,000 cells/well the day before the administration. For the induction of apoptosis, cisplatin with a final concentration of 100 μM was incubated with cells at 37 °C for the time indicated in Fig. S15. HeLa cells were then washed with PBS, resuspended in CHAPS {3[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate} lysis buffer (20 mM Tris (pH 7.2), 0.1 M NaCl, 1 mM EDTA, 10 mM dithiothreitol (DTT), 0.5% CHAPS, 10% sucrose) and incubated on ice for 30 min. Cell lysates were analyzed for caspase-3 activity with the colorimetric peptide substrate Ac-DEVD-pNA (acetyl-DEVD-para-nitroanilide). Assay mixtures (0.1 mL) contained 20 μg of total protein from samples and 0.2 mM pNA substrate (final concentration). The release of pNA was monitored at 409 nm with a microplate reader (PowerWave X, Bio-tek Instruments, USA).

UV Treatment and Real-Time Fluorescence intensity Record. The irradiation was performed through a UV light source from Spot Light Curing, Omnicure Series 1000 (100 W, 365 nm). For caspase-3 activity detection in live cells, HeLa cells were seeded in 96-well culture dishes at a density of 250, 000 cells/mL the day before the administration. For induction of apoptosis, cisplatin with a final concentration of 100 μM was added at various time points to guarantee the final duration (at the time point for UV irradiation) indicated in Fig. 3b. eGFP-NG-Q with a final concentration of 800 nM was incubated with cells for 3 h. After the removal of eGFP-NG-Q, the same concentration cisplatin in fresh medium was

replaced for further incubation for 2 h. HeLa cells were then thoroughly washed with PBS and incubated with PBS (100 μL/well). A self-made support box equipped with an ice bath and an aluminum foil mask was used for the localized treatment of the 96-well plate. The distance between the light source and the plate bottom was fixed as 2.5 cm. Fluorescence intensity of 96-well plate was measured by Spectra MAX GEMINI XS (read type: fluorescence; excitation wavelength: 480 nm; cut off wavelength: 495 nm; monitored wavelength: 510 nm).

Table S1. The mean hydrodynamic size and zeta potential of native eGFP and eGFP-NGs in 1×PBS (pH=7.4).

	Native eGFP	eGFP-NG	eGFP-NG-Q
size (nm)	4.6	12.2	13.5
zeta potential (mV)	-6.40±0.49	8.46±0.24	6.51±0.59

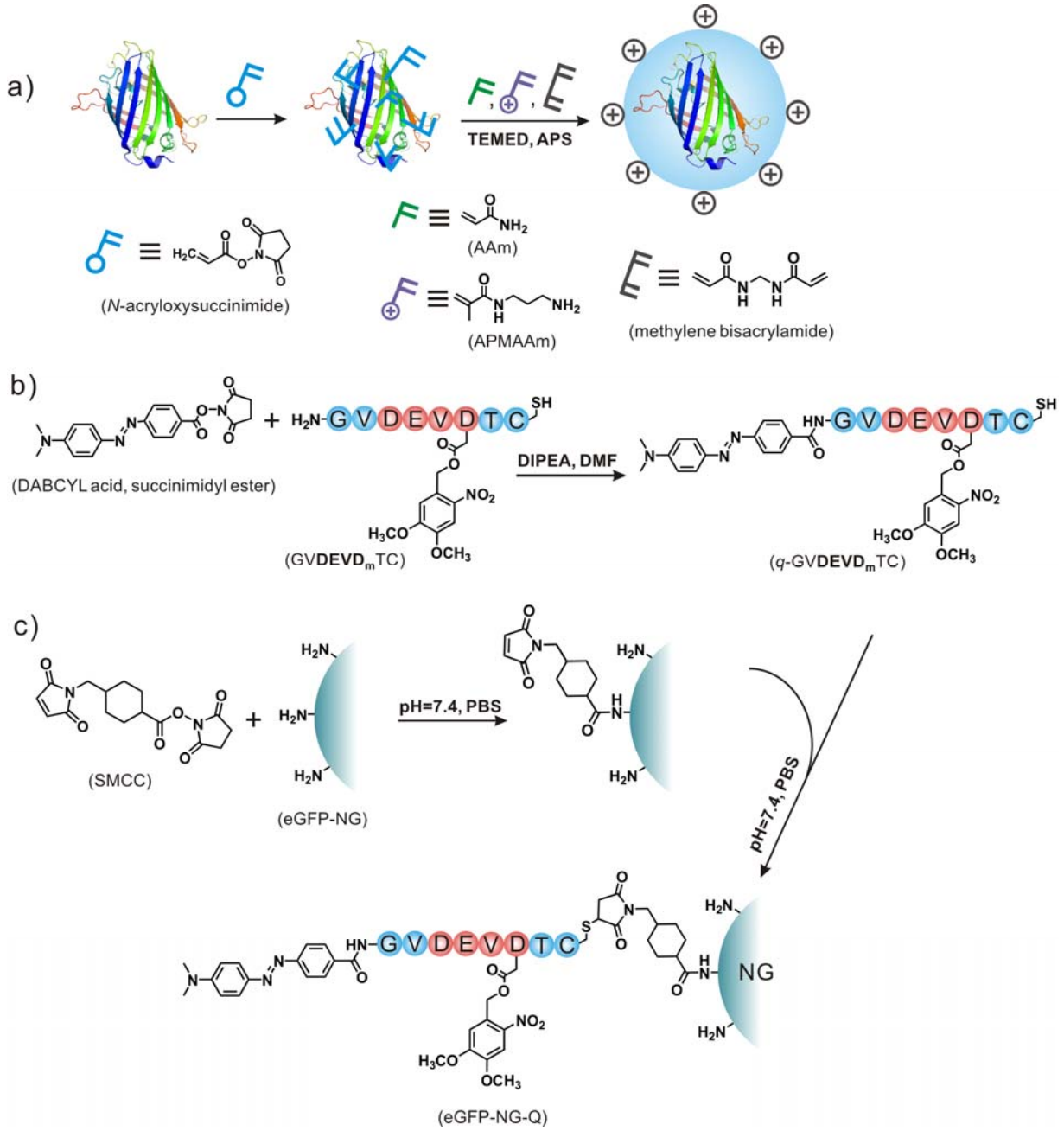

Figure S1. Schematic diagram of synthesis of eGFP-NG (a), *q*-GVDEVD$_m$TC (b) and eGFP-NG-Q (c).

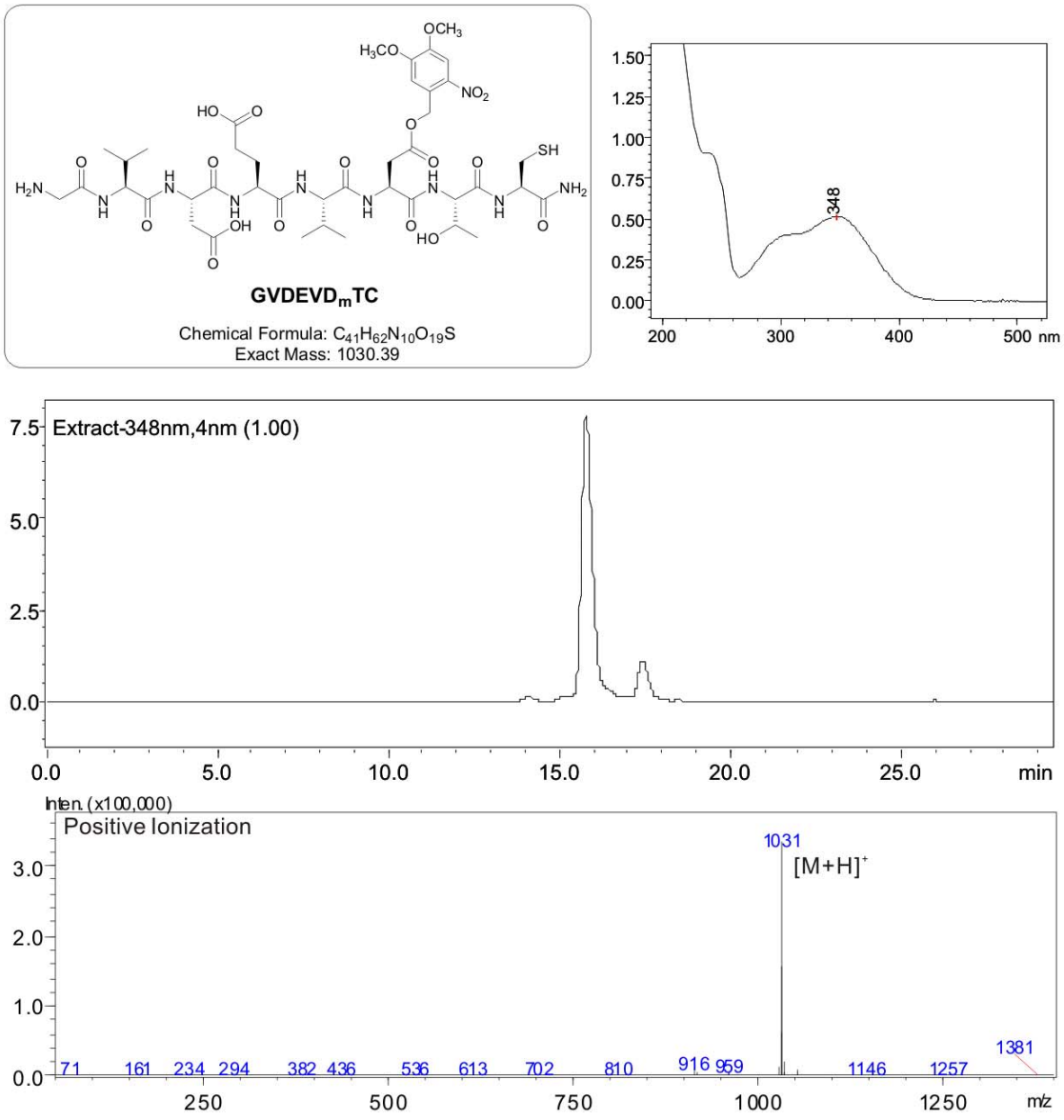

Figure S2. Structure, UV spectrum and LC-MS analysis of GVDEVD$_m$TC.

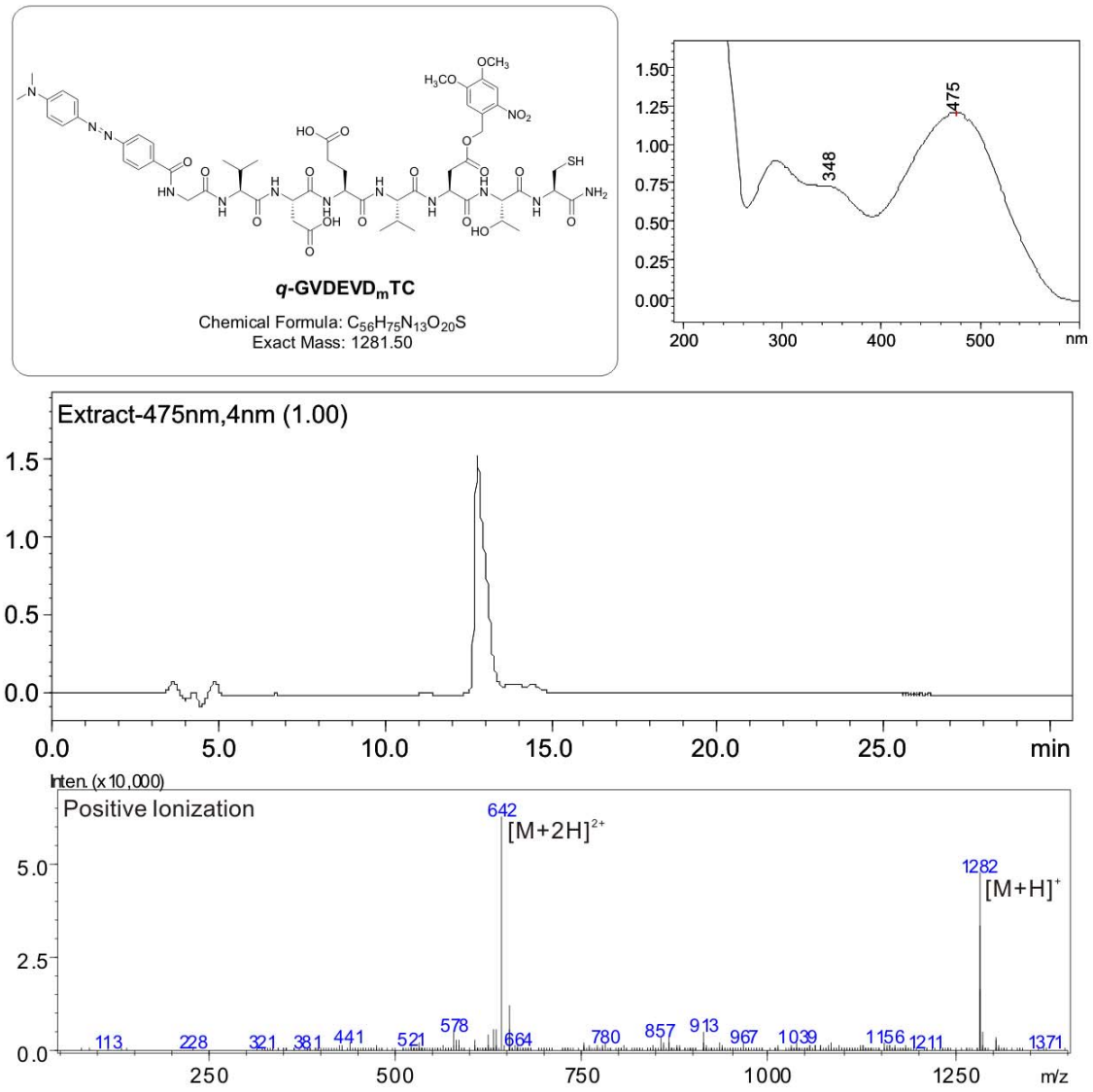

Figure S3. Structure, UV spectrum and LC-MS analysis of *q*-GVDEVD$_m$TC.

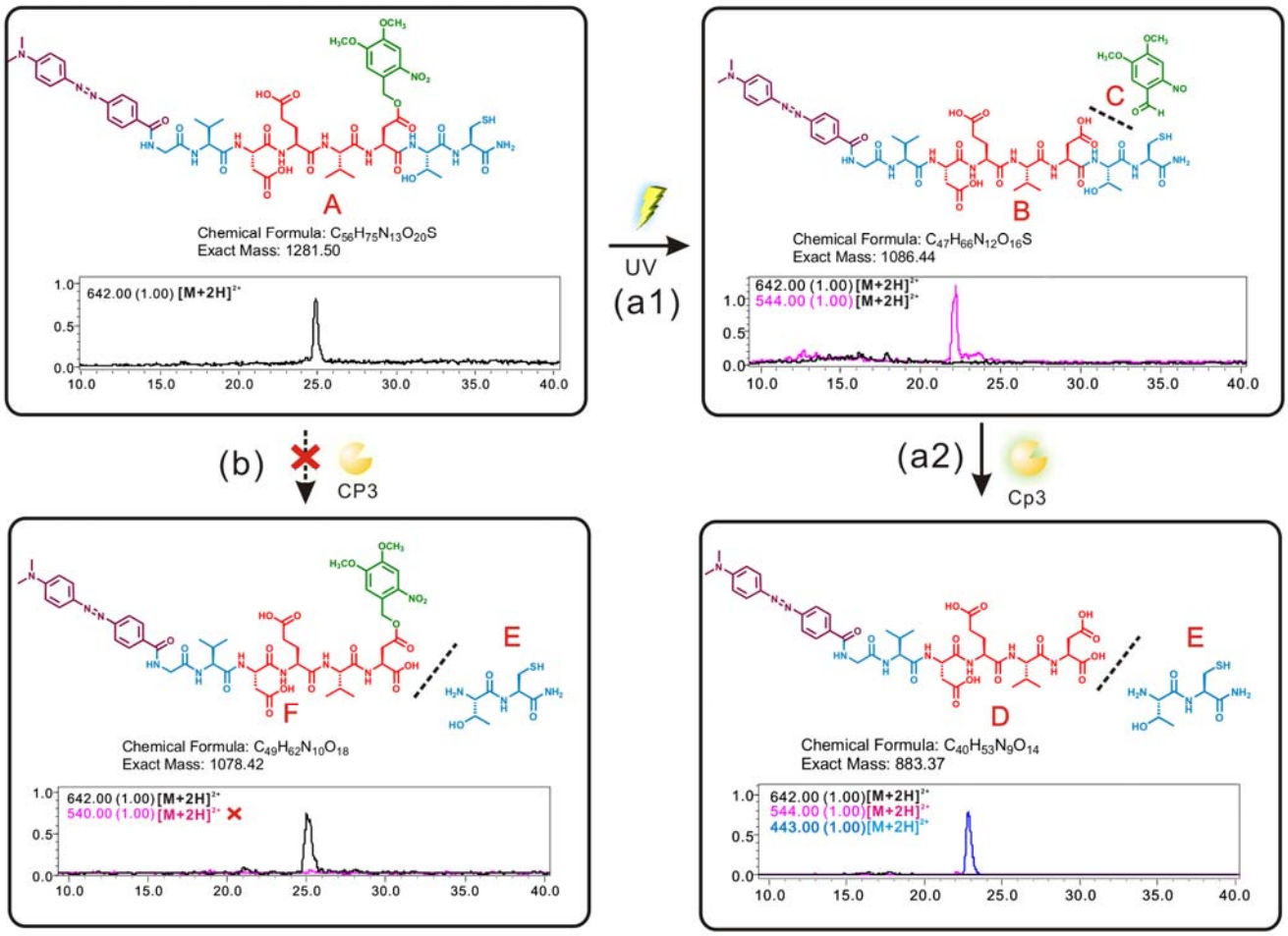

Figure S4. LC-MS analysis of the synergetic cleavage process of *q*-GVDEVD$_m$TC. a1) 50 μg (1 mg/mL) compound A ($[M+2H]^{2+}$=642) in PBS buffer was treated with UV for 40 s to produce compound B, the mass signal of which can be detected ($[M+2H]^{2+}$=544); a2) compound B was further digested by 10 μg CP3 (1 mg/mL) for 60 min to produce compound D, the mass signal of which ($[M+2H]^{2+}$=443) can be detected; b) 50 μg (1 mg/mL) compound A in PBS buffer was incubated with 10 μg CP3 (1 mg/mL) for 60 min to assume to produce compound F, the mass signal of which ($[M+2H]^{2+}$=540) cannot be detected.

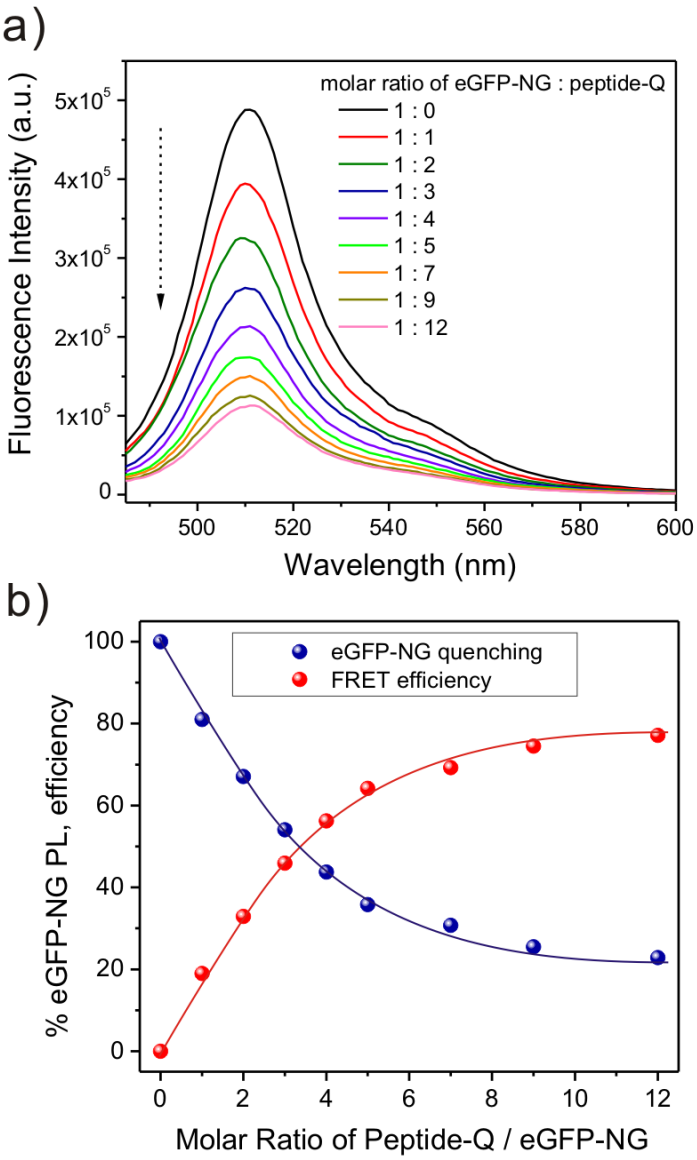

Figure S5. a) Fluorescence spectra of eGFP NGs prepared with different molar ratio of eGFP to Peptide-Q. Excitation wavelength: 475 nm. b) FRET efficiency (red) and eGFP-NG quenching (blue) plots vs reaction molar ratio of eGFP to Peptide-Q.

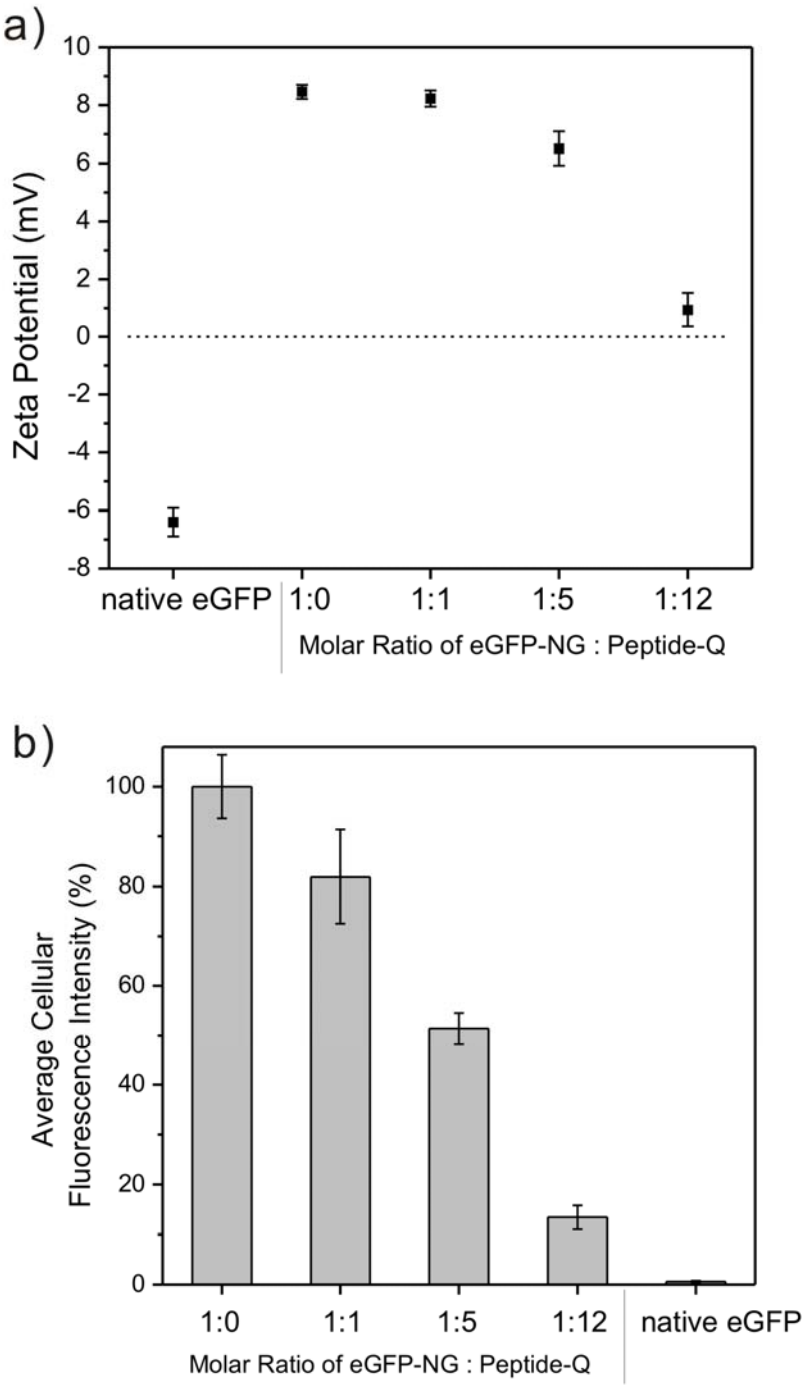

Figure S6. Summary of Zeta potential (a) and average cellular fluorescence intensity after uptake of the native eGFP and NGs (b) prepared with different molar ratio of eGFP to Peptide-Q. HeLa cells were co-cultured with 2 nM eGFP or nanogels for 3 h at 37 °C. The error bars indicate s.d.

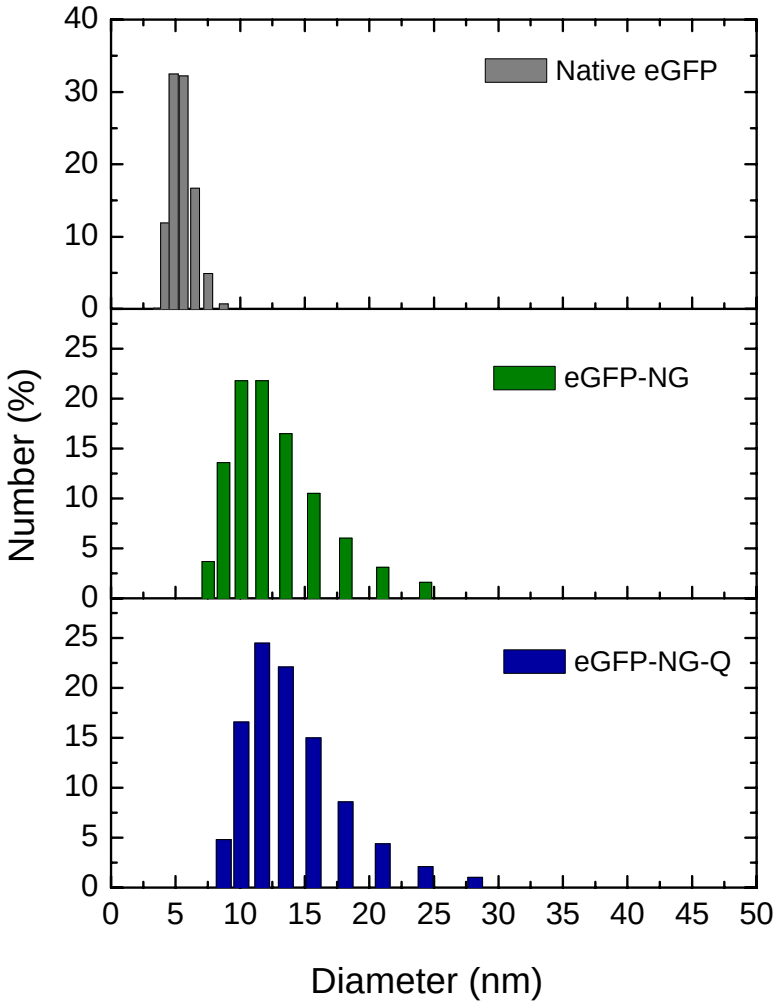

Figure S7. The hydrodynamic size of the native eGFP (gray), eGFP-NG (green) and eGFP-NG-Q (blue) measured by dynamic light scattering.

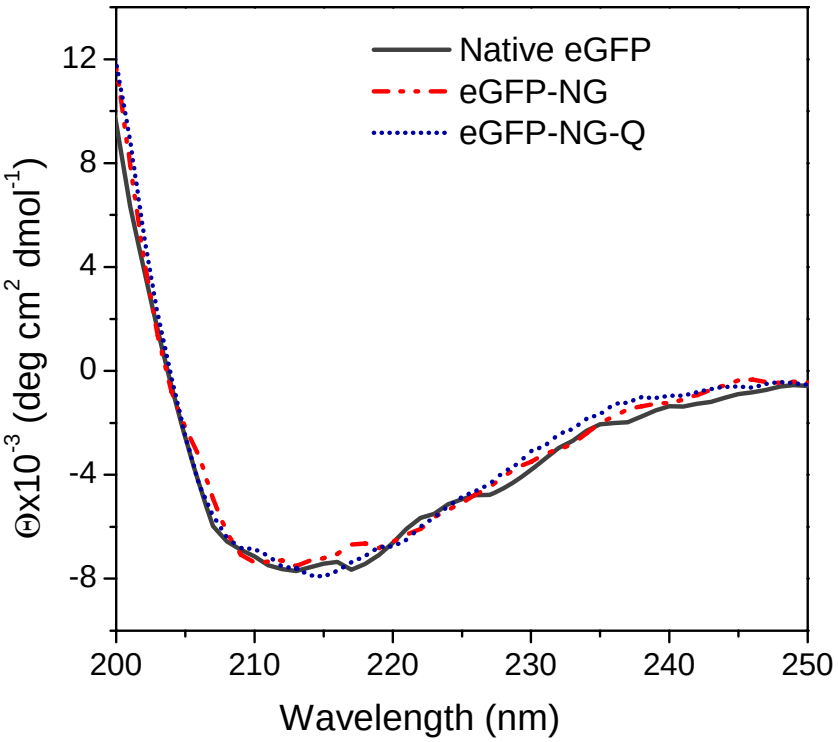

Figure S8. Far-UV circular dichroism (CD) spectra of the native eGFP (gray line), eGFP-NG (red dash-dot line) and eGFP-NG-Q (blue dot line).

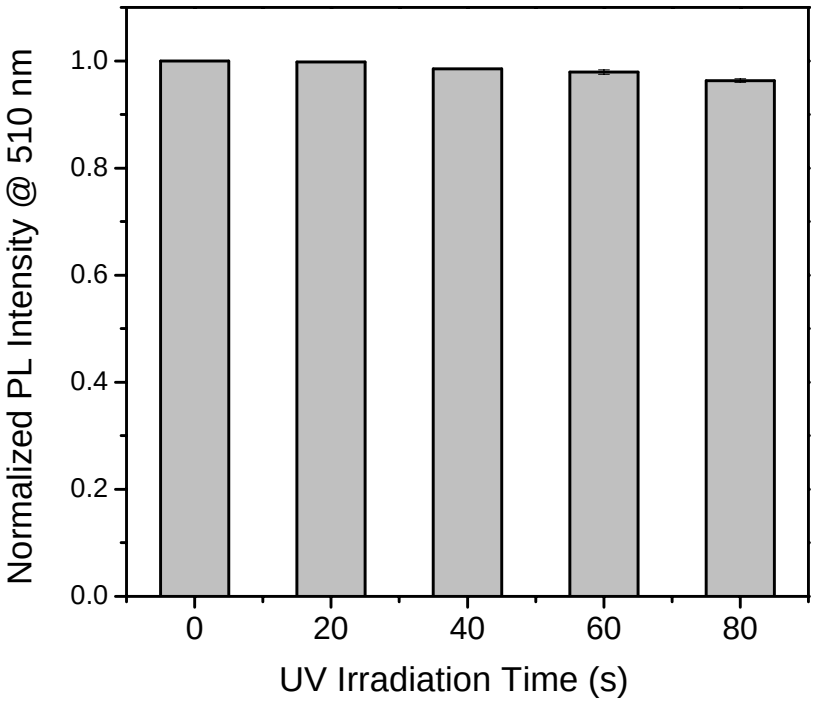

Figure S9. Normalized fluorescence intensity of the eGFP-NG-Q at 510 nm in response to UV irradiation over time. The error bars indicate s.d.

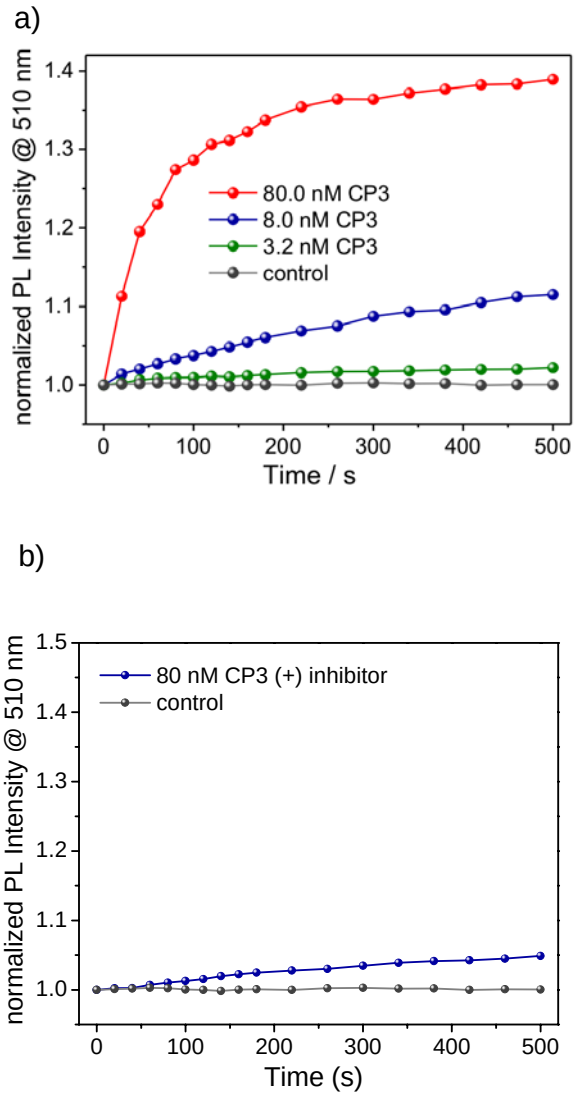

Figure S10. a) Sensitivity of eGFP-NG-Q towards CP3 after triggering by UV irradiation for 60 seconds. Assays were performed at 25 °C. b) Activity assays of eGFP-NG-Q towards 80 nM caspase-3 with 400 nM inhibitor (Ac-DEVD-CHO).

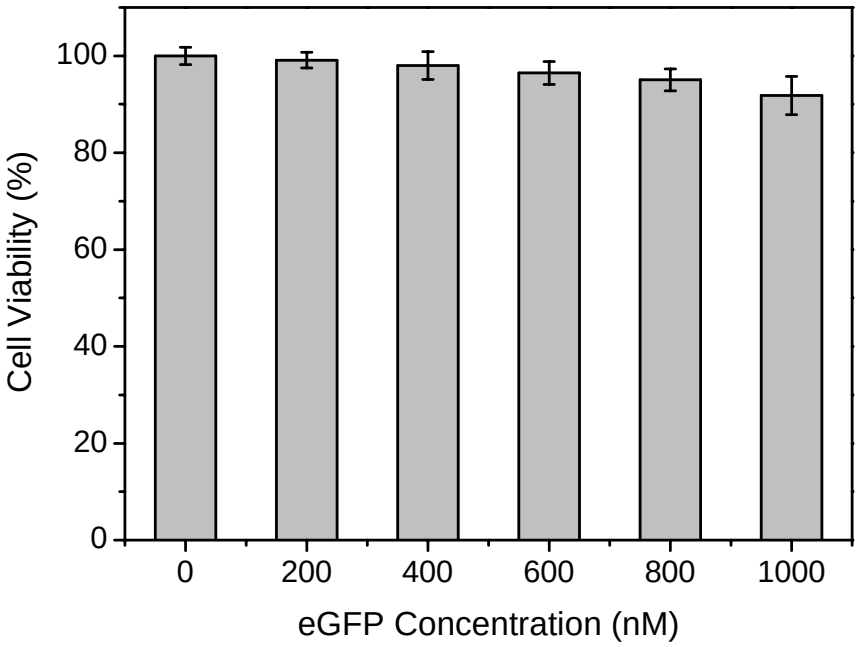

Figure S11. Cytotoxicity of eGFP-NG-Q towards HeLa cells, determined by the MTS assay. Cells were incubated with eGFP-NG-Q at the indicated concentrations for 3 h, followed with a thorough wash by PBS and incubated with fresh medium for another 24 h. The error bars indicate s.d.

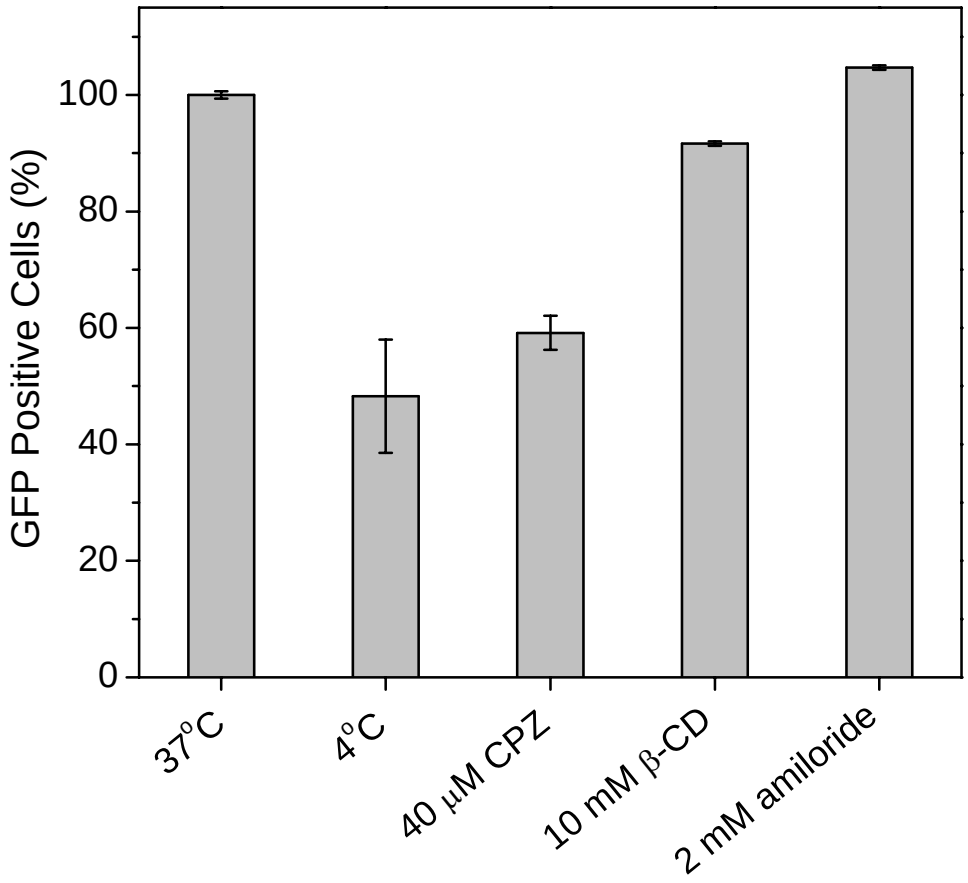

Figure S12. Inhibition of the cellular internalization of eGFP-NCs (5.0 nM) by HeLa cells. The mean fluorescence of cells was measured by flow cytometry and is represented as the percentage of fluorescence in untreated cells for 2 h at 37 °C: 1) low temperature (4 °C); 2) 40 μM chloroproamzine (CPZ, inhibitor for clathrin-mediated endocytosis); 3) 10 mM β-cyclodextrin (β-CD, inhibitor for caveolae-mediated endocytosis) and 4) 5mM amiloride (inhibitor for macropinocytosis). The error bars indicate s.d.

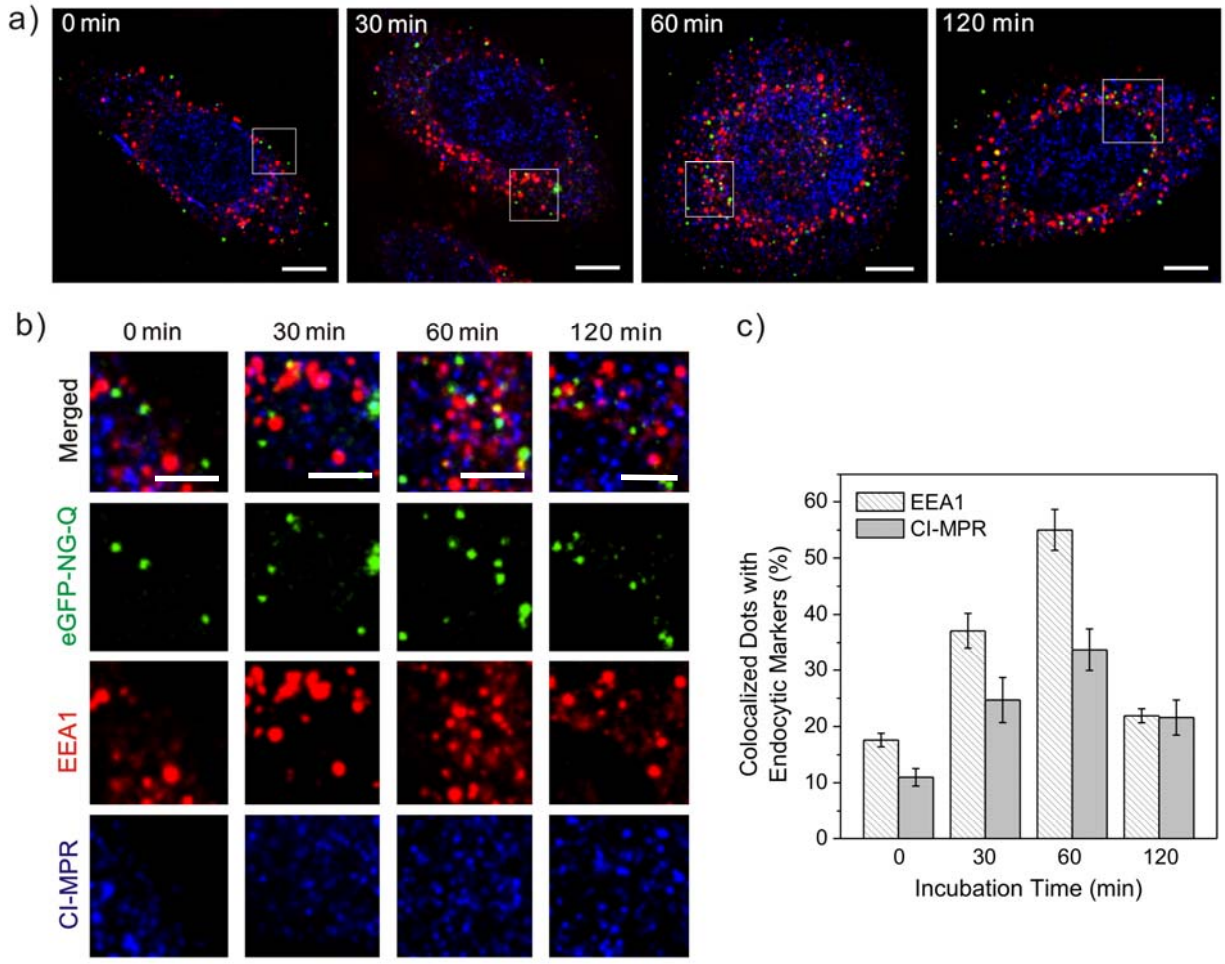

Figure S13. The trafficking of eGFP NCs through endosomes. (a) Early and late endosomes were detected by early endosome antigen 1 (EEA1, red) and cation-independent mannose-6-phosphate receptor (CI-MPR, blue), respectively (the scale bar is 10 μm) and zoom-in images (the scale bar is 5 μm) (b). HeLa cells were incubated with 200 nM eGFP-NG-Qs at 37 ^{o}C for various time points of 0, 30, 60 and 120 min and then fixed.. (c) quantification of eGFP-NG-Qs colocalized with EEA1^{+} (pattern) or CI-MPR^{+} (gray) endosomes at different incubation times. The error bars indicate s.d.

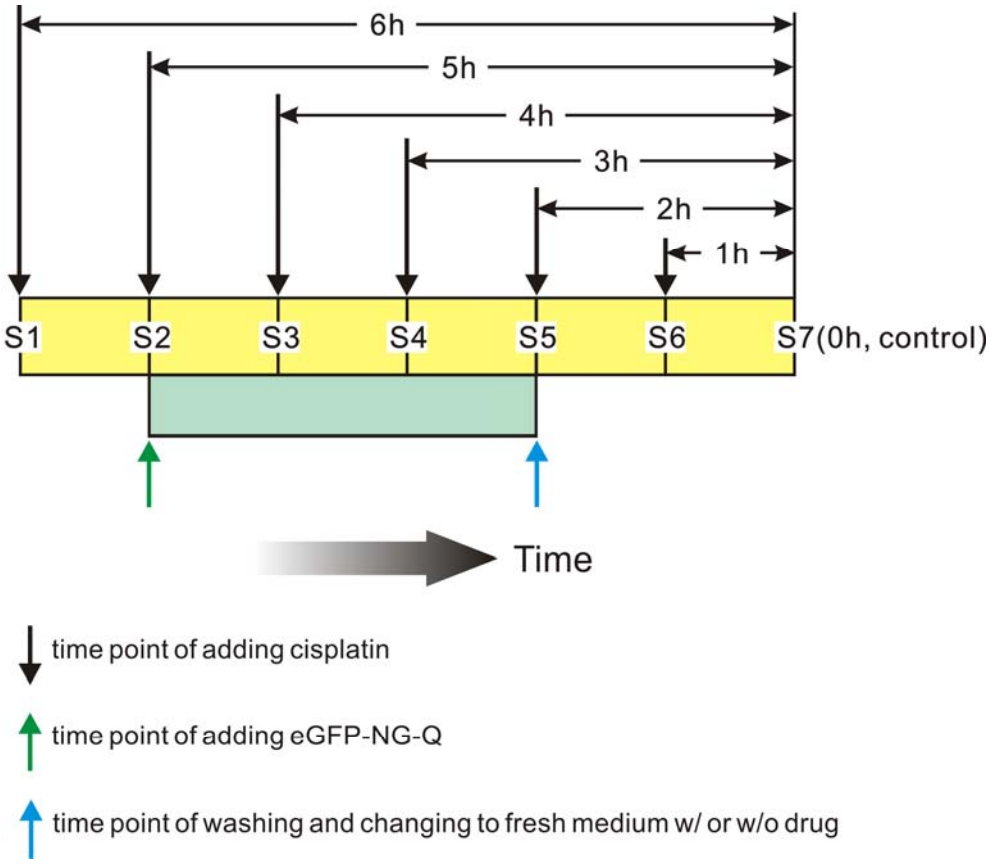

Figure S14. Schematic time course of the drug treatment with cisplatin and incubation with eGFP-NG-Q. For each sample, cellular uptake of eGFP-NG-Q was allowed to take place for continuous three hours, followed by replacement of fresh medium to remove excess probes. After the cells were treated with cisplatin for the desired periods, the cells were washed with PBS and the PL intensity data were scanned by a fluorescence plate reader. To measure CP3 activity in each well, UV irradiation was first simultaneously performed for 60 seconds on all wells to decage the nanoprobes. 10 minutes later, the fluorescence plate reader was used again to record the PL intensity of each sample.

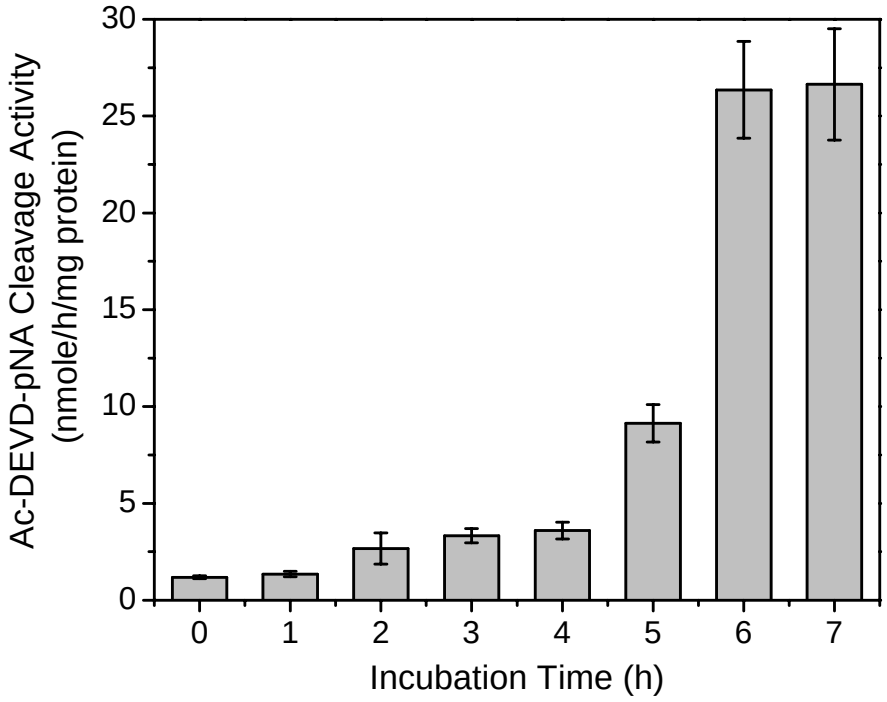

Figure S15. Colorimetric caspase-3 activity assays. HeLa cell lysates (20 μg of protein) were incubated in the presence of 0.2 mM Ac-DEVD-pNA for 2 h at 37°C. Release of pNA was analyzed by optical density at 409 nm, and caspase activity is displayed as the number of nanomoles of pNA released per hour per total milligram of protein as calculated from a standard curve by using free pNA. The error bars indicate s.d.

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