Supporting Information

Nanogel-facilitated Protein Intracellular Specific Degradation through Trim-Away

Binglin Sui, Mingming Wang, Chen Cheng, Quanguang Zhang, Jiajia Zhang, Daping Fan, and Peisheng Xu,*

Materials and Methods

Materials

2-Mercaptoethanol (BME), glacial acetic acid, ethylenediamine, L-glutathione (GSH), bovine serum albumin (BSA), and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Triethylamine (TEA), pyridine, tris(2-carboxyethyl)phosphine (TCEP), 4-nitrophenyl chloroformate (NPC), and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Tokyo Chemical Industry Co., Ltd (Portland, OR, USA). Anti-GFP antibody (Cat#: RT0265) was purchased Bio X cell. Inc. β-actin antibody (A1978) was purchased from Sigma. Anti-COPZ1 antibody (H-12) (cat#: sc-398219) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Ubiquitin antibody (Cat#: 3933S) and Anti-rabbit IgG HRP-linked antibody (7074S) were purchase from Cell Signaling Tech. Plasmid pmCherry-C1-mTrim21, originally created by Schuh et al.,[1] was purchased from Addgene (Watertown, MA, USA). Cyanine3 NHS ester (Cy3-NHS) and Cyanine5 NHS ester (Cy5-NHS) were purchased from Lumiprobe Co. (Cockeysville, MD, USA). Cyclic Arg-Gly-Asp-D-Phe-Cys peptide (RGD) was

purchased from GL Biochem (Shanghai) Ltd. (Shanghai, China). Gibco™ Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (PS), trypsin-EDTA, Pierce™ BCA Protein Assay Kit, Lipofectamine® 3000 Transfection Kit, anti-β-actin antibody, and Invitrogen™ Hoechst 33342 were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All the other solvents used in this research were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and directly used without further purification.

Synthesis of PDA-PEG-BME

PDA-PEG-BME polymer was prepared following our previously reported procedure through a thiol-disulfide exchange reaction between polymer PDA-PEG and 2-mercaptoethanol (BME).[2] Briefly, PDA-PEG polymer, synthesized according to our published method with the PDA:PEG at the ratio of 1:1 and charaterized by ^{1}H NMR and gel permeation chromatography (Mw: 21.5 kDa, PDI: 1.3),[2] was first dissolved in DCM supplemented with glacial acetic acid. After that, BME dissolved in DCM was added dropwise under stirring at room temperature. The reaction was stopped after being processed in the dark overnight. The reaction mixture was purified by precipitating in ice-cold diethyl ether for three times.

Synthesis of PDA-PEG-NPC

30 mg PDA-PEG-BME and 7.2 mg 4-nitrophenyl chloroformate (NPC) were dissolved in 500 μL dichloromethane (DCM). After cooled in an ice bath for 30 min, 20 μL pyridine was added dropwise, and the reaction solution was stirred for 24 h at room temperature in the

dark. The produced polymer was purified through dialysis of the reaction mixture towards DMSO using Spectra/Por® dialysis tube (regenerated cellulose, MWCO: 8 kDa). The desired product PDA-PEG-NPC was collected through precipitation with ice-cold diethyl ether. Further removal of DMSO residue was performed twice via DCM/ice-cold diethyl ether precipitation. After *in vacuo* dryness in the dark for 48 h (28 mg, 87.5%), the polymer was analyzed by ^{1}H-NMR (Varian Mercury Bruker Avance/VX400 system) in CDCl3 using TMS as an internal standard to confirm its chemical structure.

Synthesis of PDA-PEG-Cy3

The PDA-PEG-Cy3 polymer was synthesized following our previously reported procedures.[3] Briefly, cysteamine (0.84 mg in 500 µL DMSO) was added into PDA-PEG (20 mg in 500 µL DSMO) dropwise. After overnight reaction, Cy3 NHS ester (0.142 mg in DMSO) was added into the above mixture and reacted for 2 h. The raw product was purified by dialysis towards DMSO in Spectra/Por® dialysis tube (regenerated cellulose, MWCO: 8 kDa) to remove free Cy3.

Preparation of nanogels

To a pre-cooled solution of 33 mg polymer PDA-PEG-NPC in 1 mL DMSO, 11 mg protein/antibody dissolved in 1 mL PBS buffer (pH 8.5) was added dropwise at 4 °C under vigorous stirring, and the resulted solution was stirred for 48 h at 4 °C in the dark. The process of reaction was monitored by measuring the absorbance of released side product 4-nitrophenol at 400 nm using UV-Vis spectroscopy as reported in literature.[4] When the reaction was completed, 2.6 mg TCEP and 1.1 mg ethylenediamine dissolved in 0.2 mL pre-

cooled deionized water was added for crosslinking and the solution was stirred for 24 h at 4 °C. Then the produced nanogels were purified through dialysis in Spectra/Por® dialysis tube (regenerated cellulose, MWCO: 100 kDa) against PBS buffer for 48 h at 4 °C. The final nanogels were stored in PBS (pH 7.4) at 4 °C for use. For the nanogels modified with Cy3, polymer PDA-PEG-Cy3 was mixed into the reaction solution before the crosslinking step. The concentration of Cy3 was measured by a microplate reader (λex = 555 nm, λem = 570 nm). For the nanogels post-decorated with RGD peptide, the nanogel dispersion in PBS buffer (pH 7.4) was added RGD solution (1 mg/mL) in PBS buffer at the ratio of 1:200 for RGD:nanogel and then stirred overnight at 4 °C.[5] The RGD-modified nanogels were purified through dialysis in Spectra/Por® dialysis tube (MWCO: 8 kDa) against PBS buffer for 48 h at 4 °C to remove the unconjugated RGD peptide. The particle size, size distribution, and zeta potential of the nanogels were determined by dynamic light scattering (DLS), recorded on Zetasizer (Zetasizer Nano ZS, Malvern Instruments Ltd, Malvern, UK). The increased zeta potential for RGD modified nanogels shown in Figure S3 proved the success of RGD modification. The physical morphology was observed using Hitachi HT7800 transmission electron microscopy (TEM, Hitachi High-Technologies Corporation, Tokyo, Japan) using a formvar-carbon coated Copper Grids (Electron Microscopy Science).

The amount of protein contained in nanogels was analyzed using the Pierce™ BCA Protein Assay Kit, following the reported method.[4] The loading content (LC) and loading efficiency (LE) of Protein/antibody were calculated by the following equations.

$$\text{LC (\%)} = \frac{\text{mass of protein in nanogel}}{\text{mass of nanogel}} \times 100\%$$

$$\text{LE (\%)} = \frac{\text{mass of protein in nanogel}}{\text{mass of protein fed}} \times 100\%$$

Protein/antibody release

The release of protein/antibody from the nanogels was analyzed with SDS-PAGE gel electrophoresis. 12 μL of different samples were mixed with 10 μL loading buffer, and 20 μL of each sample was loaded onto the gel. For the redox-responsive release test, the nanogels were treated with 10 mM GSH for 4 h prior to gel electrophoresis.

Fluorescence labeling of protein

For cellular uptake studies of protein/antibody and nanogels, bovine serum albumin (BSA) was labeled with fluorescent dye Cyanine5 (Cy5) to form BSA-Cy5. In brief, 5 mg of BSA dissolved in 1 mL $NaHCO_3$ buffer (0.1 M, pH = 8.5) was cooled in an ice bath for 30 min, and then 50 μL freshly prepared Cy5-NHS solution (10 mg/mL in DMSO) was added dropwise in the dark. The reaction mixture was protected from light and stirred at room temperature overnight. The produced BSA-Cy5 was purified by size exclusion chromatography using Sephadex G-25 (GE Healthcare).

Cell culture

Human breast cancer MCF-7 cells, green fluorescence protein (GFP) expressed MCF-7/GFP cells, and mouse embryonic fibroblast NIH-3T3 cells were cultured in GibcoTM DMEM

supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 75 mL culture flasks under a humidified atmosphere of 5% CO_2. Cells were sub-cultured when the cell confluence reached ~80%.

Cellular internalization and intracellular colocalization

The cellular internalization of nanogels was examined by confocal fluorescence microscopy in MCF-7 cells. Cells were seeded in 35 mm^2 Petri dish with a glass window at a density of 200,000 cells per dish for 24 h. Then cells were incubated with Cy3 and BSA-Cy5 co-loaded nanogel NG-BSA-Cy5-Cy3 (5 μg/mL for Cy5, 4.1 μg/mL for Cy3) at the Cy3:Cy5 ratio of 1:1 for 3 h. Cells without any incubation were utilized as a negative control. In the positive control group, cells were co-incubated with nanogels NG-Cy3 and NG-BSA-Cy5. Then the medium was replaced with a fresh medium, and cells were fixed with 4% paraformaldehyde in PBS for 10 min at predetermined time points. After removal of paraformaldehyde, the nuclei of cells were stained with Hoechst 33342 for 10 min, and then cells were imaged under a confocal fluorescence microscope (LSM 700, Carl-Zeiss Inc.). To investigate the subcellular localization of the nangel after entering the cells, lysotracker green was added to the medium 3 h after the addition of NG-BSA-Cy5. The cells were imaged under a confocal microscope as described above.

Flow cytometry assay

The uptake of protein/antibody loaded nanogel by NIH3T3 cells was also quantitatively determined by flow cytometry. Cells were seeded in 6-well plates at a density of 300,000 cells/well for 24 h at 37 °C with 5% CO_2. Then cells were washed with PBS (pH 7.4) and

incubated with NG-aGFP or NG-aGFP-R with an equivalent concentration of Cy3 for 3h. Cells with no incubation were utilized as a control. After 3 h of incubation, cells were washed with PBS, trypsinized with trypsin-EDTA, and collected through centrifuging (2000 rpm, 3 min). Cells were suspended into PBS and then centrifuged for two more times. Finally, collected cells were re-suspended into PBS for analysis. Intracellular fluorescence intensity was quantified by flow cytometer (BD Accuri C6, BD Biosciences) at λ_{ex} 488 and λ_{em} 560 nm.

Cell viability assay

The cytotoxicity of free antibody and nanogels was evaluated by MTT assay. Cells were seeded in 96-well plates at a density of 5,000 cells per well for 24 h at 37 °C with 5% CO_2. Then cells were incubated with free antibody and nanogels in fresh medium for 48 h. In the control group, cells were allowed to grow without any treatment. After that, the medium was replaced with fresh medium containing MTT reagent (final concentration 1 mg/mL) and cells were further incubated for 4 h. The purple MTT crystal was dissolved with MTT stop solution (10% SDS in 0.01 M HCl) and the optical density at 595 nm was recorded on a microplate reader (SpectraMax i3x, Molecular Devices, LLC.).

Protein expression

For the expression of protein Trim21, cells were transfected with pmCherry-C1-mTrim21 plasmid as reported in the literature.[1] Plasmids were transfected using Lipofectamine® 3000 Transfection Kit according to the manufacturer's instructions 12 h prior to Trim-Away assay. Successful transfection was indicated by the observed cherry fluorescence emitted from cells.

Protein specific degradation assay

The Trim-Away of protein GFP was conducted in TRIM21-transfected MCF-7/GFP cells. For fluorescence imaging, cells seeded in 35 mm^2 Petri dish with a glass window were incubated with free anti-GFP antibody and anti-GFP loaded nanogels. Cells with no incubation were utilized as control. At predetermined post-incubation time points, cells were imaged by fluorescence microscopy. To quantitatively analyze the Trim-Away efficiency, the fluorescence of cells seeded in 96-well plates after incubation with anti-GFP and nanogels was measured by a microplate reader at different time points (λex = 485 nm, λem = 525 nm).

For the Trim-Away of protein COPZ1, TRIM21-transfected cells were incubated with free anti-COPZ1 antibody and anti-COPZ1 loaded nanogels at different concentrations for 48 h. Cells in the control group were allowed to grow with no incubation. Then the medium was replaced with fresh medium containing MTT reagent (final concentration 1 mg/mL) and cells were further incubated for 4 h. The purple MTT crystal was dissolved with MTT stop solution and the optical density at 595 nm was recorded by a microplate reader.

Proteasome inhibitory assay

TRIM21-transfected MCF-7/GFP cells were cultured in 6-well plates at the density of 25,000 cells/well. After overnight incubation, cells were treated with blank, free Anti-GFP (40 μg/mL), NG-aGFP-R (40 μg/mL), blank + bortezomib (100 nM), free Anti-GFP (40 μg/mL) + bortezomib (100 nM), or NG-aGFP-R (40 μg/mL) + bortezomib (100 nM) for 8 h. Cells were washed with 1×PBS twice before being collected for western blotting assay. .

Western blotting assay

TRIM21-transfected MCF-7 cells were incubated with free anti-COPZ1, NG-aCOPZ1, and NG-aCOPZ1-R nanogels (equivalent to 40 μg/mL anti-COPZ1) for 10 h and then lysed for 30 min at 4 °C. The protein concentration of each sample was measured by Bradford assay. The cell lysates were separated by SDS-PAGE and transferred to a PVDF membrane, which was activated by methanol for 30 min prior to use. The transferred membrane was blocked for 1.5 h at room temperature with 5% BSA, washed with Tris-buffered saline with Tween-20 (TBST buffer), and incubated overnight at 4 °C with anti-COPZ1 (1:500), anti-ubiquitin (1:500), anti-GFP (1:500), or anti-β-actin (1:1000). Bands were visualized with the help of Western Lightning Plus ECL detection reagent (Perkin Elmer, Waltham, MA, USA) and recorded with a ChemiDoc Touch™ (Bio-Rad). The blot bands were analyzed and quantified with ImageJ software.

Statistical Analysis

GraphPad Prism 6.0 software (La Jolla, CA) was used to conduct statistical analysis. Data were expressed as Mean ± SD. The differences between groups were examined using Student's t-test or ANOVA with Tukey's multiple comparison tests. When the p-value was less than 0.05, the differences were considered significant (*$p < 0.05$, $^{\#}p<0.01$).

References

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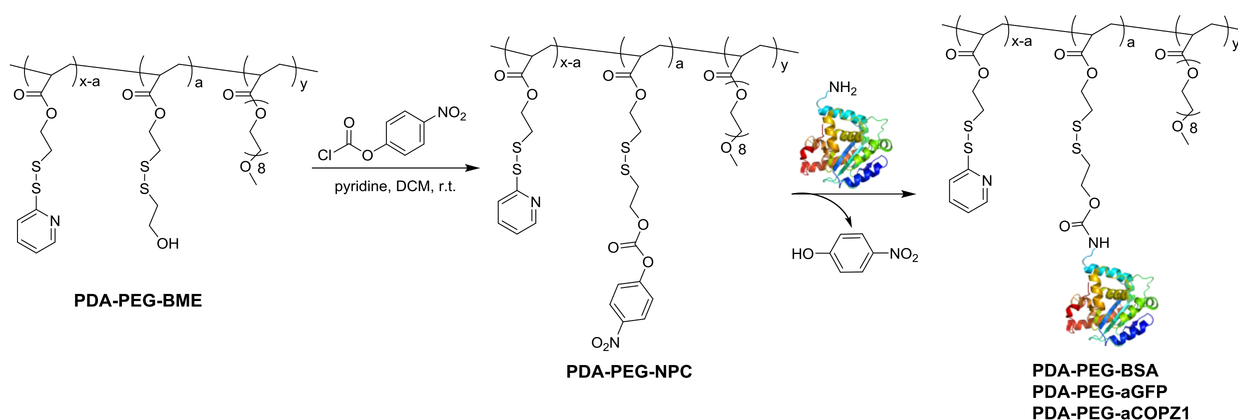
4. Dutta, K.; Hu, D.; Zhao, B.; Ribbe, A. E.; Zhuang, J.; Thayumanavan, S. Templated self-assembly of a covalent polymer network for intracellular protein delivery and traceless release. *J. Am. Chem. Soc*. **2017**, *139*, 5676-5679.

5. R. Bahadur K. C, P. Xu, Advanced Materials 2012, 24, 6479



Scheme S1. Synthesis of protein/antibody conjugated polymers.

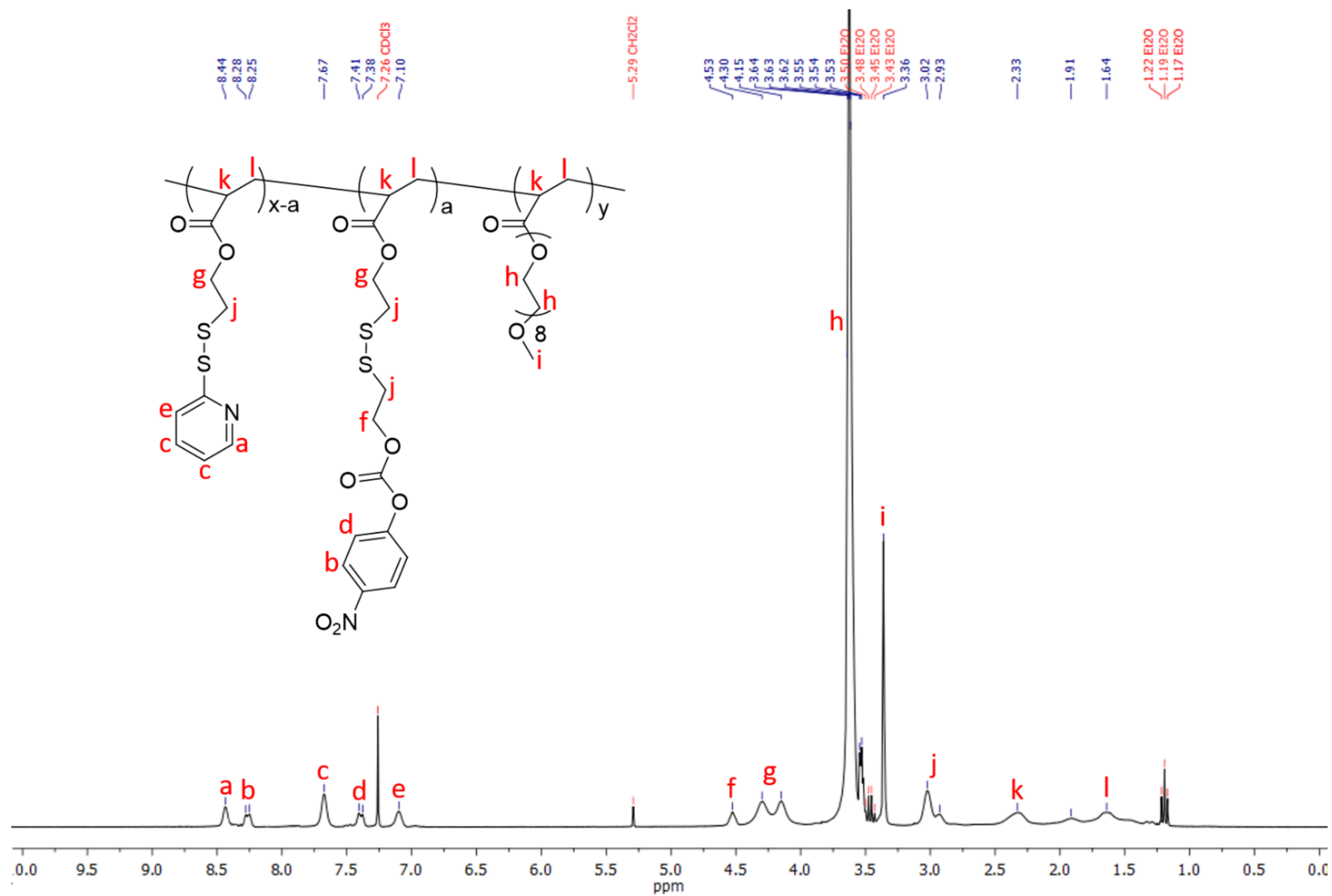


Figure S1. ^{1}H NMR spectrum of PDA-PEG-NPC.

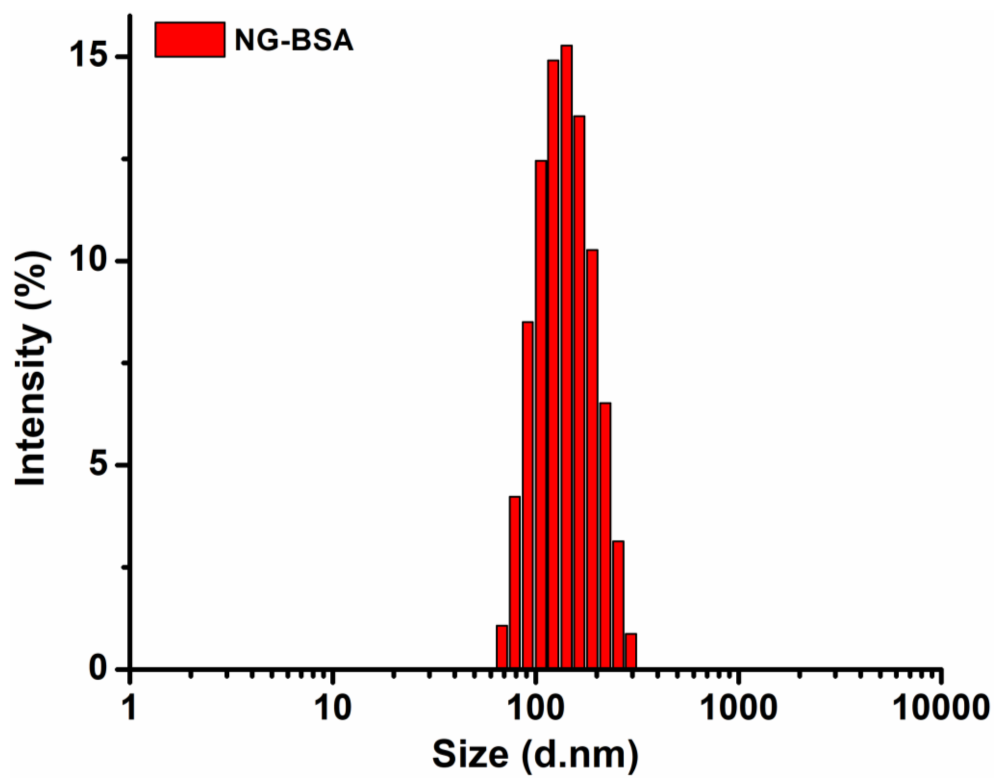


Figure S2. Size distribution of nanogel NG-BSA.

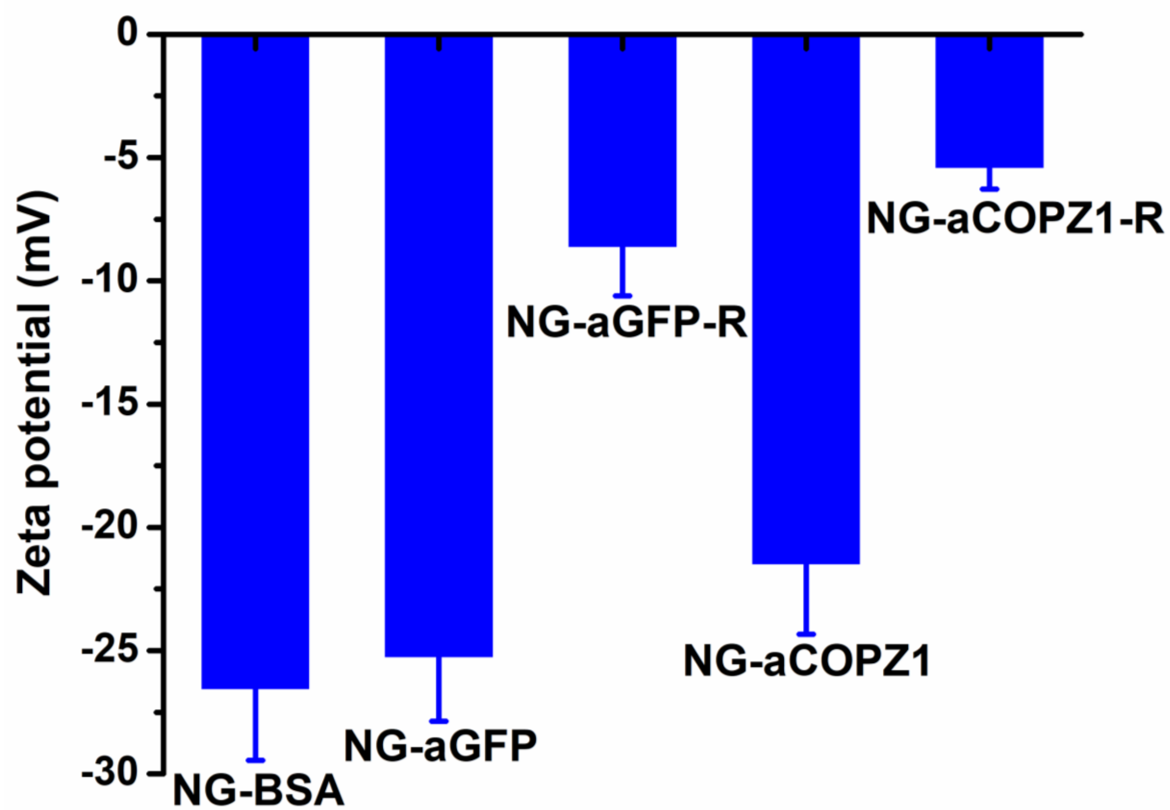


Figure S3. Zeta potential of nanogels NG-BSA, NG-aGFP, NG-aGFP-R, NG-aCOPZ1, and NG-aCOPZ1-R.

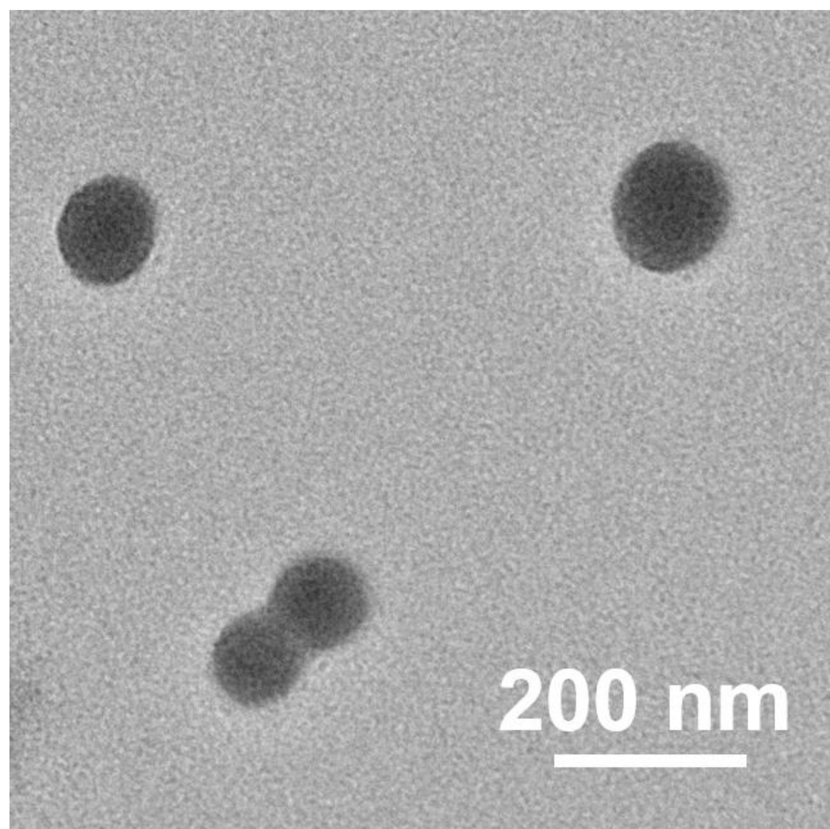


Figure S4. TEM image of nanogel NG-BSA.

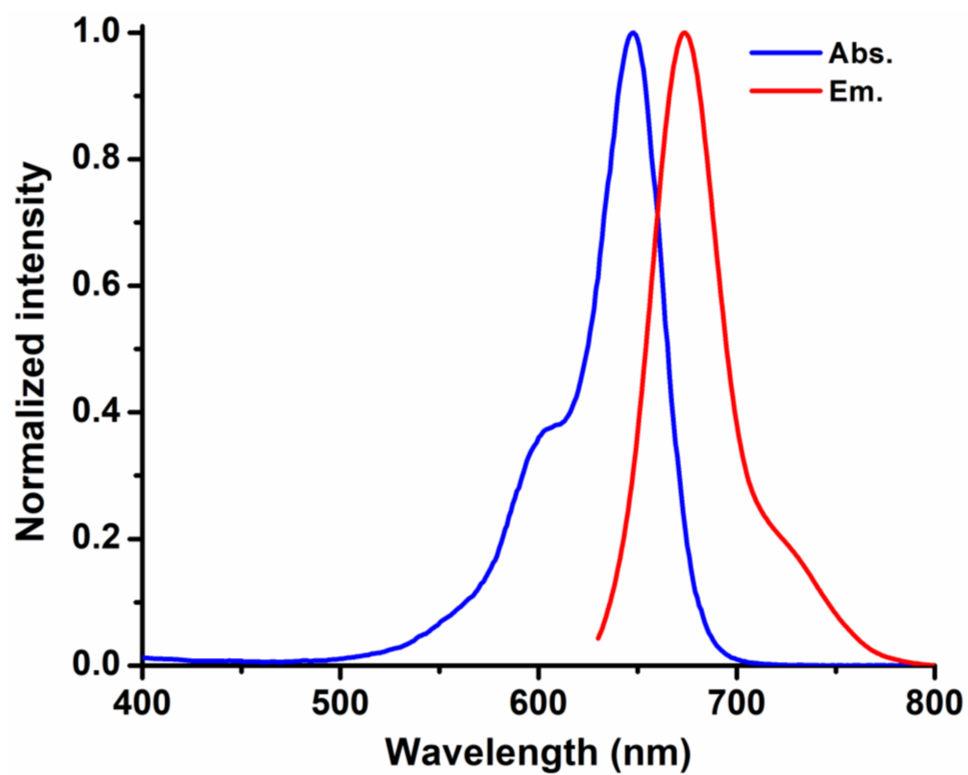


Figure S5. Absorption and fluorescent emission spectra of BSA-Cy5.

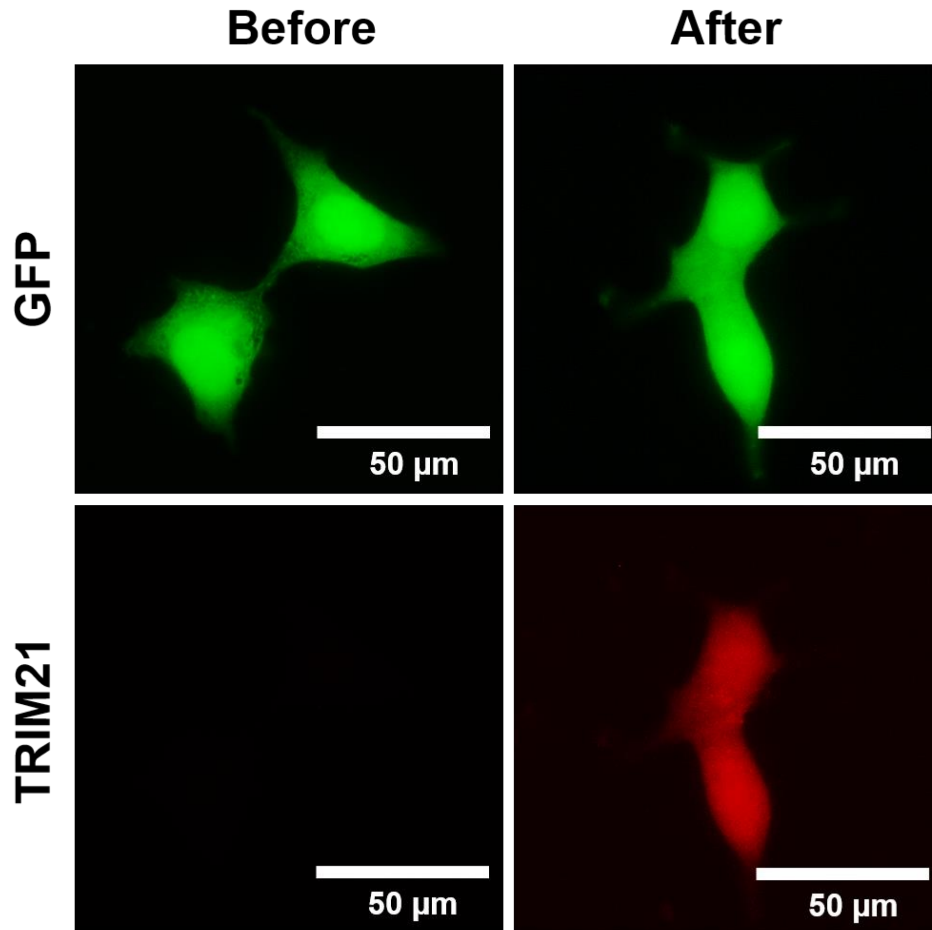


Figure S6. Fluorescence of GFP (green) and TRIM21 (red) emitted from MCF-7/GFP cells before and after transfection with pmCherry-C1-mTRIM21 plasmid.

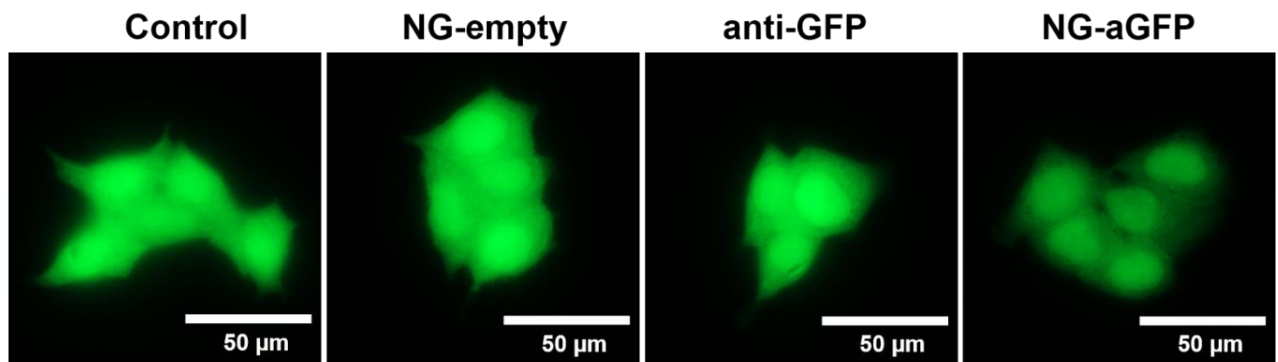


Figure S7. Fluorescence images of MCF-7/GFP cells without TRIM21-transfection after incubation with NG-empty, free anti-GFP, and NG-aGFP nanogel at an anti-GFP equivalent concentration of 100 μg/mL for 6 h.

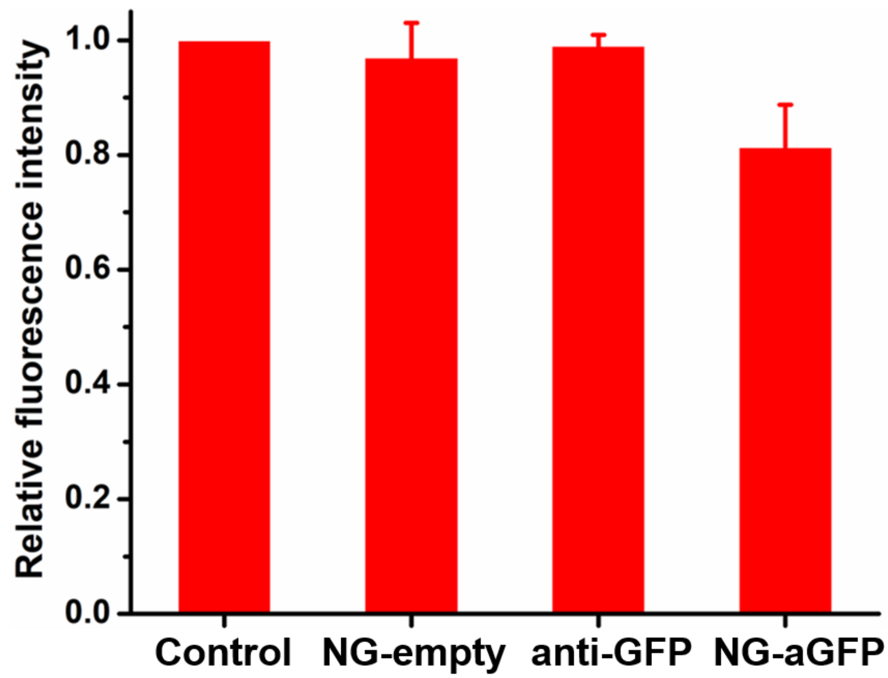


Figure S8. Relative fluorescence intensity of MCF-7/GFP cells without TRIM21-transfection after incubation with NG-empty, free anti-GFP, and NG-aGFP nanogel at an anti-GFP equivalent concentration of 100 μg/mL for 6 h.

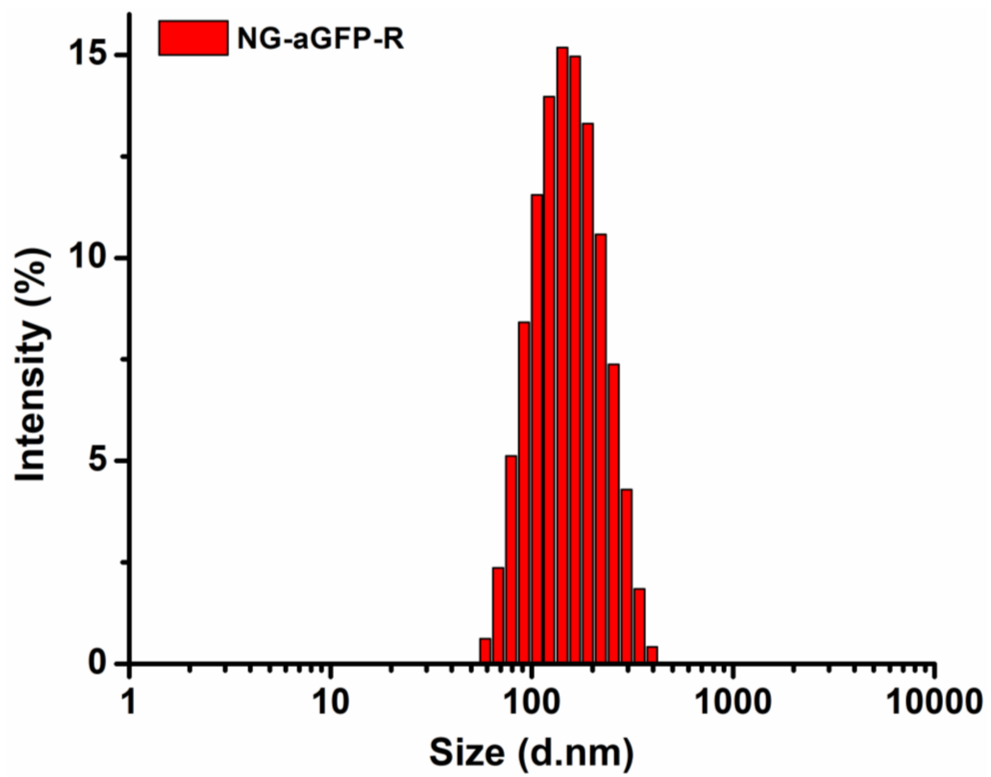


Figure S9. Size distribution of nanogel NG-aGFP-R.

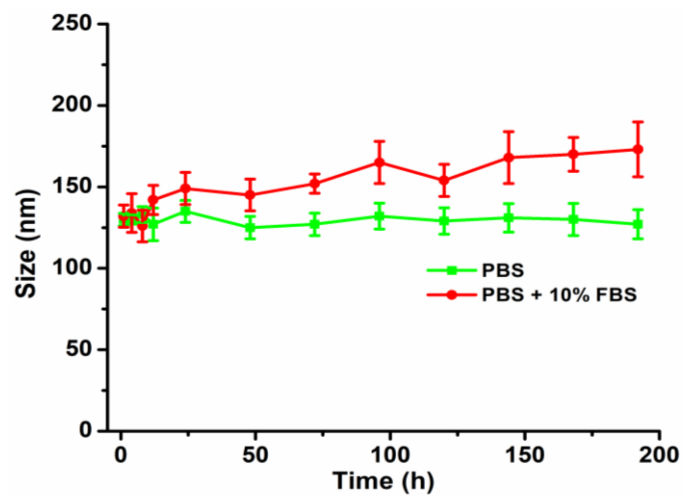


Figure S10. Size stability of NG-aGFP-R in PBS and PBS supplemented with 10% FBS.

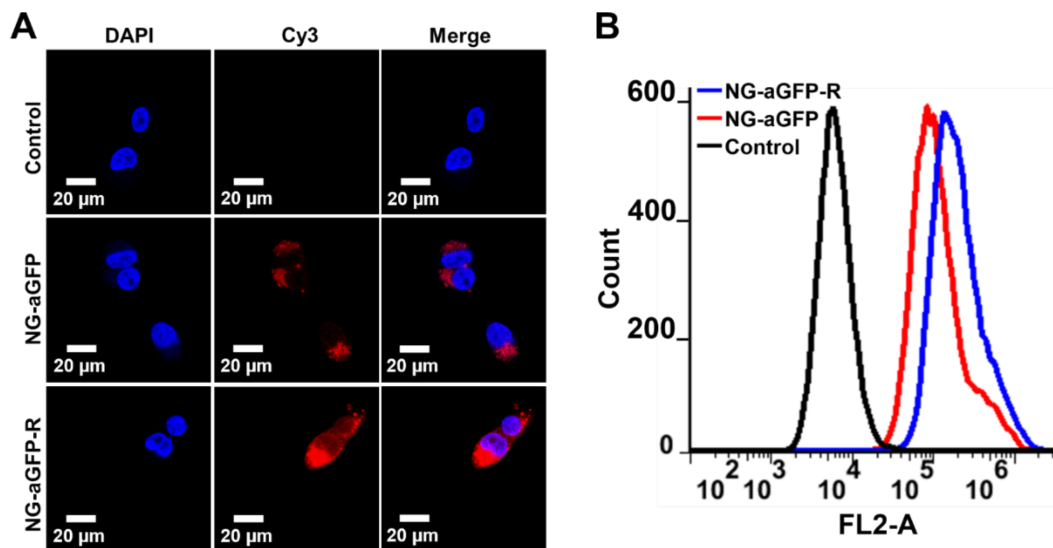
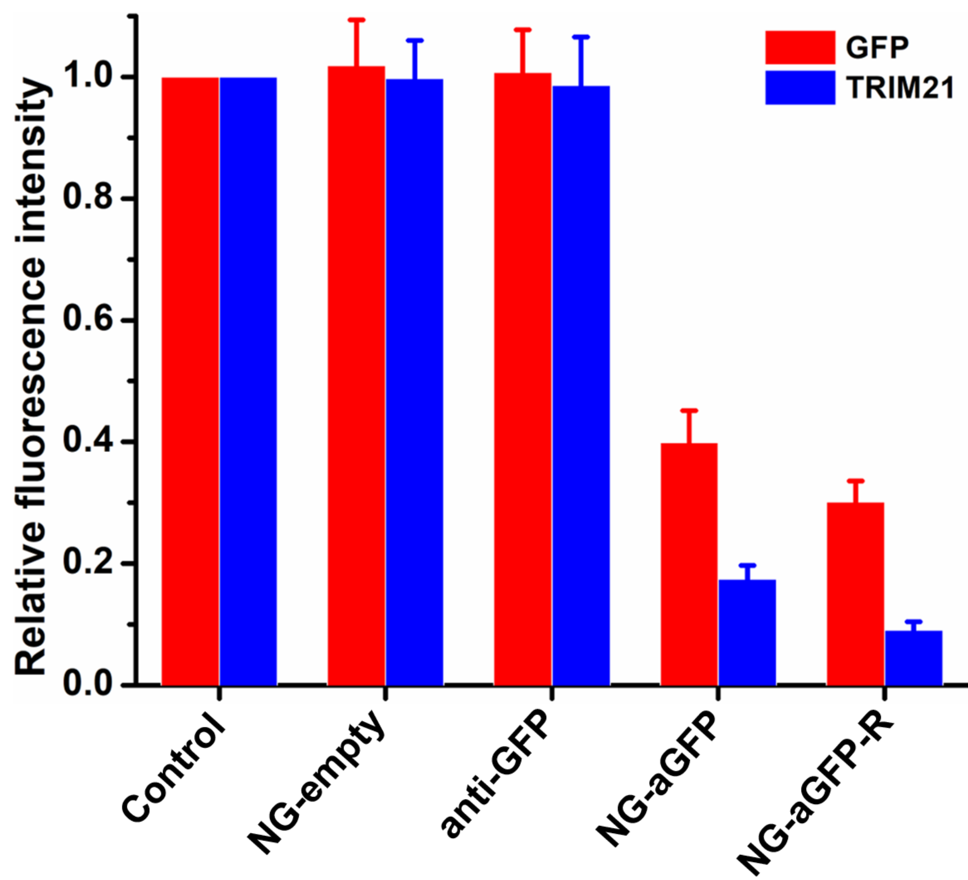


Figure S11. Cellular uptake of the NG-aGFP and NG-aGFP-R. (A) The confocal images of cells after being treated with different NGs for 3 h. (B) Flow cytometry spectra of the cells after being treated with different NGs for 3 h.



GFP
TRIM21
Relative fluorescence intensity
0.0
0.2
0.4
0.6
0.8
1.0
Control
NG-empty
anti-GFP
NG-aGFP
NG-aGFP-R

Figure S12. The relative intensity of both GFP and TRIM21 fluorescence in TRIM21-transfected MCF-7/GFP cells after incubation with free anti-GFP and nanogels at an anti-GFP equivalent concentration of 20 μg/mL for 9 h.

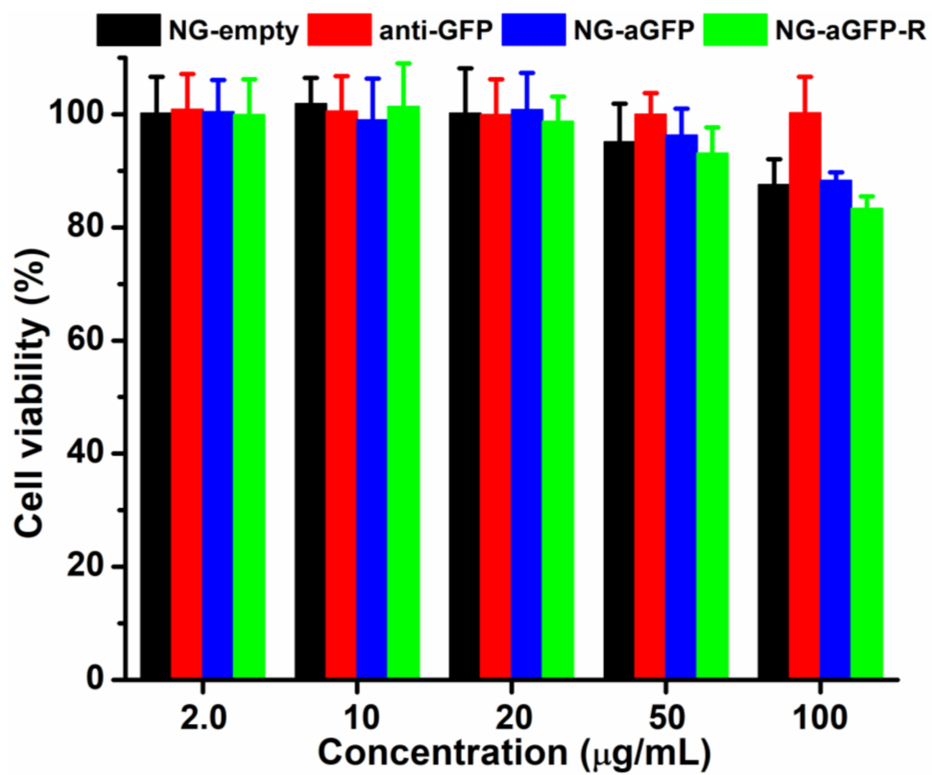


Figure S13. Cell viability of TRIM21-transfected MCF-7/GFP cells after incubation with NG-empty, free anti-GFP, NG-aGFP, and NG-aGFP-R nanogels at different anti-GFP equivalent concentrations.

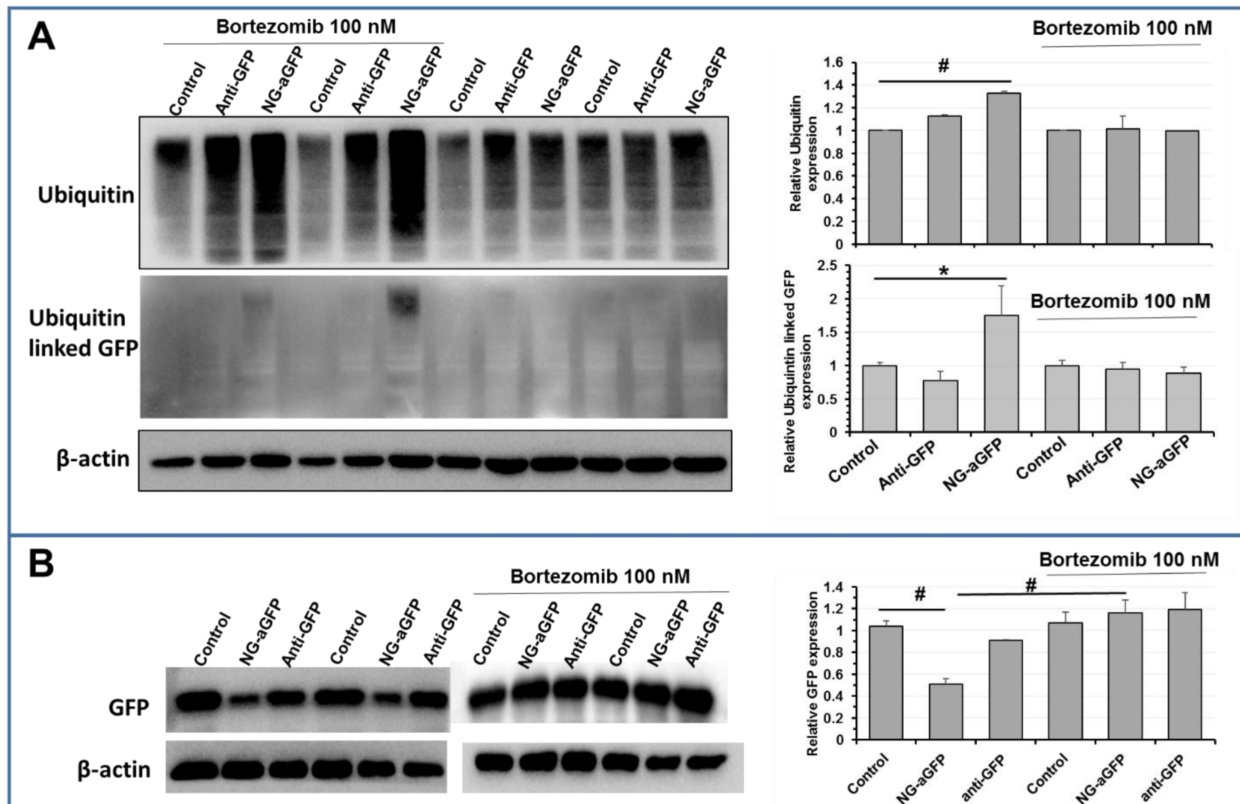


Figure S14. The effect of proteasome inhibitor on the function of Nano-ERASER. (A) Western blot images of ubiquitin and ubiquitin linked GFP in cells with or without receiving bortezomib treatment. (B) Western blot images of GFP in cells with or without receiving bortezomib treatment. Data represent the means ± SD, n=2. $^{*}P<0.05$, $^{\#}P<0.01$.

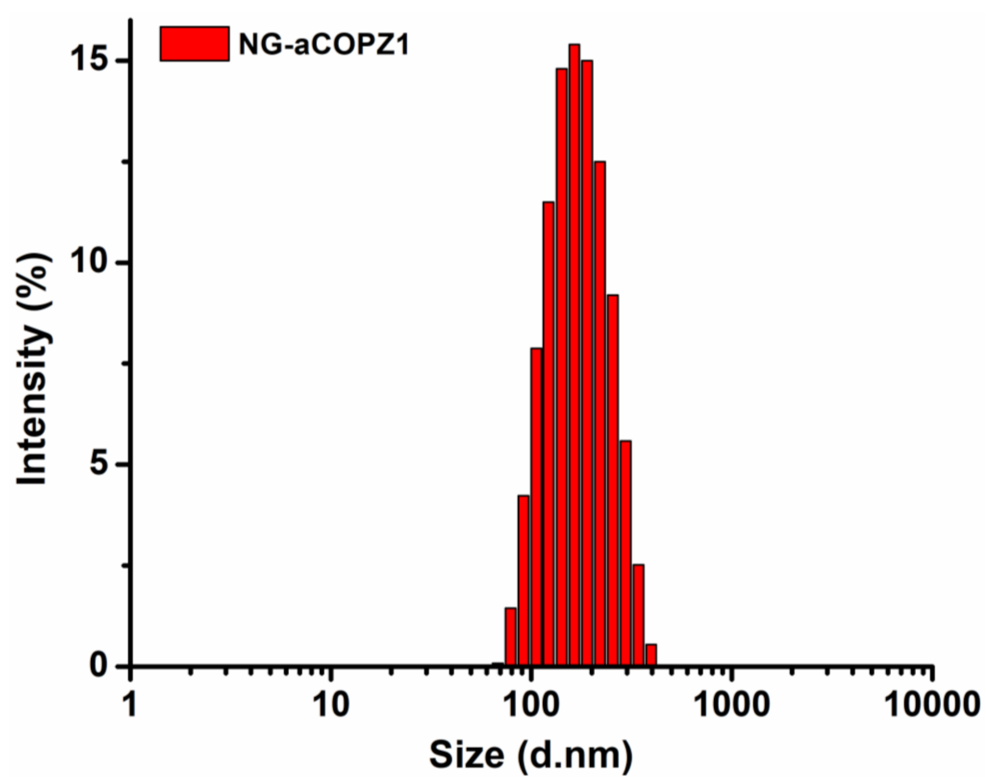


Figure S15. Size distribution of nanogel NG-aCOPZ1.

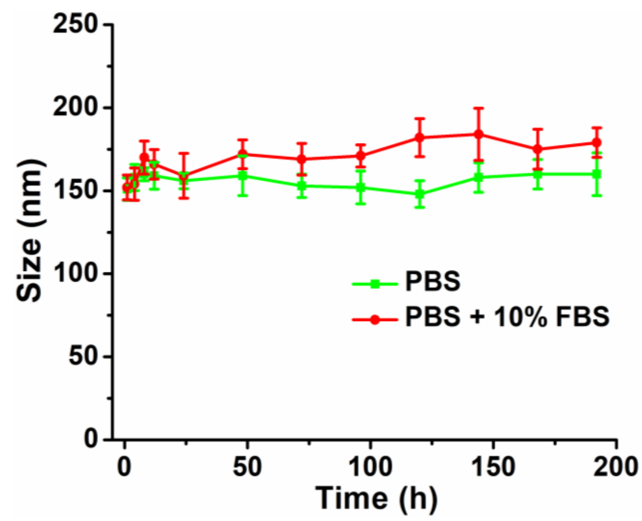


Figure S16. Size stability of NG-aCOPZ1-R in in PBS and PBS supplemented with 10% FBS.

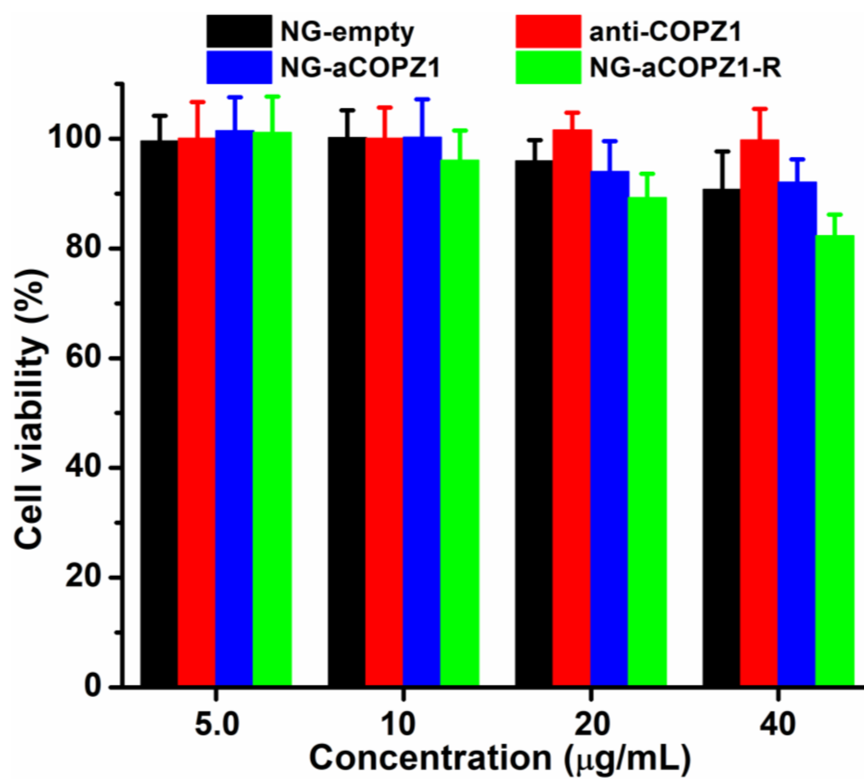


Figure S17. Cell viability of MCF-7 cells after incubation with NG-empty, free anti-COPZ1, NG-aCOPZ1, and NG-aCOPZ1-R nanogels at different anti-COPZ1 equivalent concentrations.

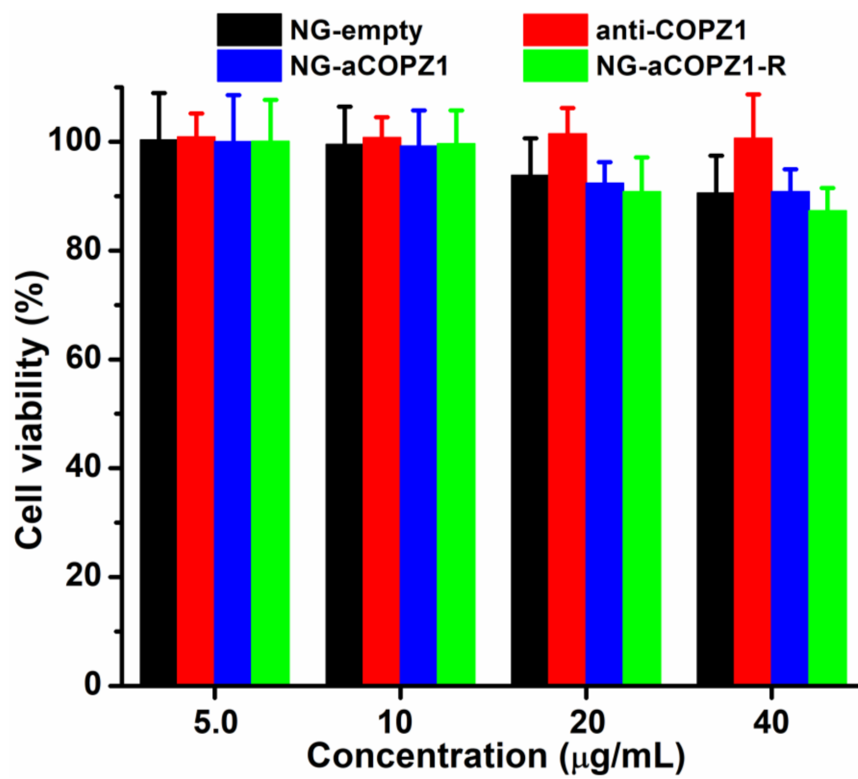


Figure S18. Cell viability of TRIM21-transfected NIH-3T3 cells after incubation with free anti-COPZ1, NG-empty, NG-aCOPZ1, and NG-aCOPZ1-R at different anti-COPZ1 equivalent concentrations.

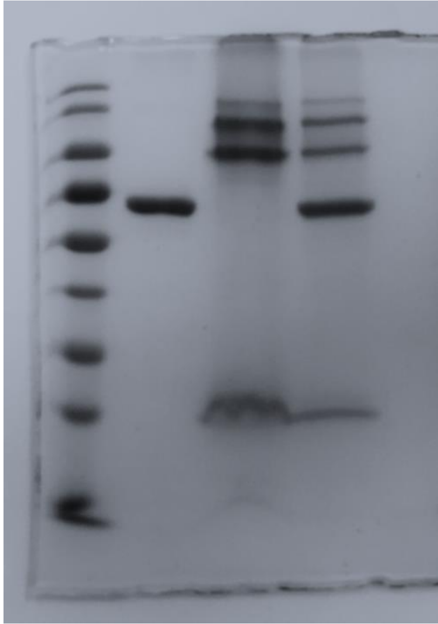


Figure S19. The original agarose gel image of Figure 2A.

Figure S20. The original western blot image of figure 4D for the expression of COPZ1 in the cells treated with different antibody formulations.

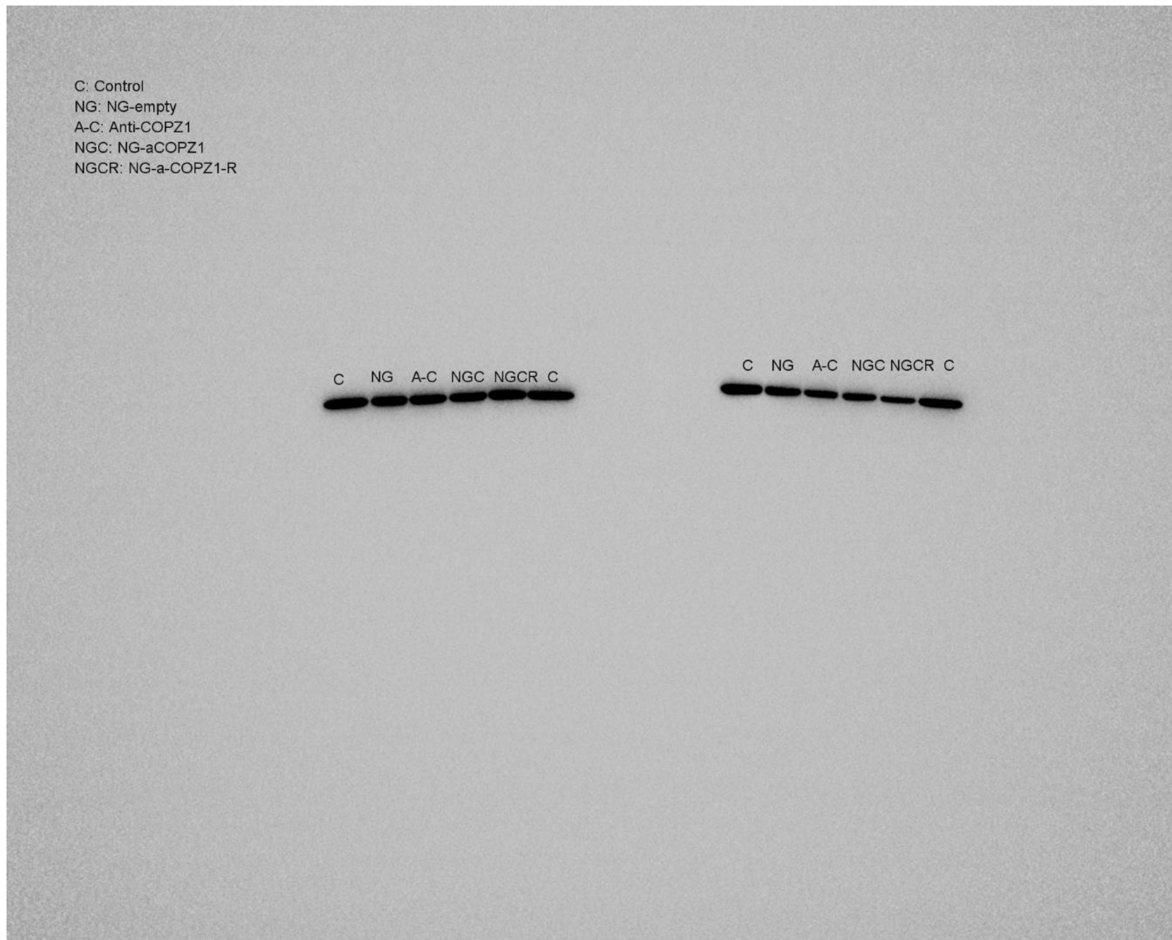


Figure S21. The original western blot image of figure 4D for the expression of β-actin in the cells treated with different antibody formulations.