

Electronic Supplementary Information

Mesoporous Nanocontainer Gated by Stimuli-Responsive Peptide for Selective Triggering of Intracellular Drug Release

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Materials. Cetyltrimethylammonium bromide (CTAB), tetraethylorthosilicate (TEOS), 3-aminopropyltriethoxysilane, propargyl bromide, copper (II) sulfate, trifluoroacetic acid (TFA), ninhydrin, thioanisole, 1,8-dioxa-1,8-octane-dithiol, N,N-dimethylformamide (DMF), piperidine, doxorubicin (DOX), glutathione (GSH), acetic anhydride, and sodium ascorbate were obtained from Aldrich and used as received. Buthionine sulfoximine (BSO), an inhibitor of gamma-glutamyl cysteine synthetase (gamma-GCS), and glutathione monoethyl ester (GSH-OEt), a membrane/lipid-permeable derivative of GSH, were purchased from Aldrich. N,N-diisopropylcarbodiimide (DIPC), 1-hydroxybenzotriazole (HOBT), and Rink Amide MBHA resin were purchased from Advanced Chem. Tech. Fmoc-L-Dap(N₃)-OH was purchased from IRIS Biotech GmbH. Antibodies against PARP, cleaved caspase-3, and β -actin were obtained from Cell Signaling Technology (Beverly, MA, USA). N- α -Fmoc-protected amino acids from Novabiochem were also used as received. All solvents were purified using a published procedure.¹

Synthesis of Si-NP. A MCM-41-type mesoporous silica nanoparticle (Si-NP) with pore size of 2.5 nm was prepared as previously described.² Briefly, an aqueous solution (192 mL) of CTAB (0.43 g) was added into a NaOH solution (2 M, 1.4 mL). After stirring at 80 °C for 5 min, TEOS (2 mL) was added and the mixture was stirred for 2 h. The resulting solid was washed thoroughly with water by centrifugation and then dried in vacuo at 60 °C overnight.

Synthesis of Si-NH₂. 3-Aminopropyltriethoxysilane (3.5 mL) was added into an ethanol solution (20 mL) of Si-NP (50 mg), which was then allowed to react overnight at 60 °C. The resulting solid was washed thoroughly with ethanol.

Synthesis of Si-alkyne. An ethanol solution (10 mL) of Si-NH₂ (50 mg) was allowed to react with propargyl bromide (1.75 mL) overnight at 50 °C. The resulting solid was washed thoroughly with ethanol.

Peptide synthesis. All peptides were synthesized using Fmoc-chemistry in solid-phase peptide synthesis.³ Fmoc-protected amino acids were assembled on Rink Amide MBHA resin (Fig. S9). The coupling reaction for each amino acid was carried out using a 3-molar excess of the corresponding Fmoc amino acid and coupling reagents in DMF. DIPC (40 mg, 0.3 mmol), HOBT (40 mg, 0.3 mmol) and Fmoc-protected amino acid (0.3 mmol) in DMF (3 ml) were added to Rink amide resin (200 mg, 0.1 mmol), and the resulting solution was stirred for 4 h at room temperature. After filtration, the resin was washed three times with DMF (4 ml) and methanol (4 ml). The coupling reaction was repeated until no color change was observed in the ninhydrin test. After completion of the solid-phase synthesis, the peptide was deprotected and cleaved from the resin by treatment with a mixture of TFA/thioanisole/1,8-dioxo-1,8-octane-dithiol/H₂O (87.5:2.5:5:5, v/v) at room temperature for 3–4 h. After cleavage of the peptide from the resin, the resin was filtered and excess TFA in solution was removed, after which the peptide was obtained by precipitation into cold diethylether at -20 °C. The successful synthesis and high purity of the peptide were confirmed by HPLC and ESI-mass spectroscopy analyses (Fig. S10-S12). The peptide masses (WCGKC-N₃: calcd 748.29 [M+H]⁺, obsd 749.3 [M+H]⁺; GCGKC-N₃: calcd 619.23, obsd 620.3 [M+H]⁺) were characterized by mass spectrometry.

Internal disulfide bond formation in WCGKC-N₃ and GCGKC-N₃. Air was bubbled overnight into a solution of WCGKC-N₃ in acetonitrile/water (80:20, v/v). WCGKC-SS-N₃ was obtained after drying in vacuo. Successful intramolecular disulfide bond formation was confirmed by mass spectrometry as shown in Fig. S13 and S14 (WCGKC-SS-N₃: calcd 746.27, obsd 747.3 [M+H⁺]⁺; GCGKC-SS-N₃: calcd 617.22, obsd 618.3 [M+H⁺]⁺).

Synthesis of silica nanoparticles with peptide gatekeepers. Surfactant was removed from silica nanoparticles by stirring Si-alkyne (50 mg) in 25 mL of ethanol with 60 mg of ammonium nitrate at 60 °C for 30 min. The resulting solid was washed thoroughly with ethanol. For cargo loading, surfactant-cleared Si-alkyne (30 mg) was soaked in a DMF solution (0.8 mL) of DOX (3 mg) and stirred overnight. Then, WCGKC-SS-N₃ (15 mg) in 2.5 mL of DMF, copper (II) sulfate (11.24 mg) in 100 µL of water, sodium ascorbate (17.83 mg) in 100 µL of water, and 500 µL of t-butanol were added. The click reaction for conjugation of the peptide onto the surface of the silica nanoparticle was performed by microwave (150 W) exposure (90 °C) with stirring for 10 min. The resulting solid was washed thoroughly with DMF and dried in vacuo to yield WCGKC-SS-Si. Other peptides were conjugated onto silica nanoparticles using the same method. To prepare WCGKC-Zn-Si, Zn(II) ion was added immediately after the click reaction with WCGKC-N₃ instead of WCGKC-SS-N₃ and then washed. The conjugation of each peptide onto silica nanoparticles was confirmed by FT-IR (Fig. S3).

Synthesis of PEG-WCGKC-SS-Si. For surface functionalization of WCGKC-SS-Si with PEG, a DMF solution of methoxy poly (ethylene glycol) (MW 2000, 1 g, 0.5 mmol) was allowed to react with hexamethylene diisocyanate (0.5 mmol) containing ferric acetylacetonate (10 µmol) for 3 h at 50 °C. The resulting mixture was added to a DMF

solution of WCGKC-SS-Si (50 mg) and stirred for 2 d. The resulting product was washed thoroughly with DMF and distilled water by centrifugation.

Transmission electron microscopy. TEM images were obtained using a Philips CM 200 instrument operated at an acceleration voltage of 120 kV. TEM samples were prepared by placing a drop of dispersed sample in distilled water ($100 \text{ mg}\cdot\text{L}^{-1}$) onto a 300-mesh copper grid coated with carbon film. About 2 min after deposition, a filter paper was touched to the grid to remove surface water. The samples were dried before measurement.

Fluorescence measurements. All fluorescence measurements were performed using a Shimadzu RF-5301PC spectrofluorophotometer with an excitation wavelength of 485 nm (absorption maximum wavelength of DOX). Emission and excitation slit widths were set at 3 nm.

Fourier transform infrared spectroscopy. FT-IR spectra were obtained using a VERTEX 80V vacuum spectrometer.

Zeta-potential analysis. Zeta-potential values were obtained using an OTSUKA Particle Size Analyzer ELS-Z2 using samples dispersed in distilled water.

Circular dichroism. CD spectra were recorded between 190 and 250 nm at room temperature on a J-815 spectropolarimeter (Jasco, Tokyo, Japan) using a quartz cell with a 10-mm path length. The concentration of peptide was $45 \mu\text{M}$ in 10 mM PBS (pH 7.0) containing 40% CH_3CN . Four scans, obtained at a scan speed of 10 nm/min, were averaged for each measurement. CD spectra were expressed as the mean residue ellipticity.

Cell lines and culture conditions. A549 human lung cancer cells and CCD normal human lung cells were purchased from ATCC (Manassas, VA, USA). All cells were maintained in 75-cm² plastic tissue culture flasks with Dulbecco's Modified Eagle's Medium (DMEM; Hyclone Laboratories Inc., Logan, Utah, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc.) and 1% penicillin/streptomycin (Hyclone Laboratories Inc.) in a humidified 5% CO₂/95% air incubator at 37 °C.

Assessment of DOX release by confocal laser scanning microscopy. Intracellular release of DOX from PEG-WCGKC-SS-Si was assessed by first seeding cells in an 8-well chamber slide (Nunc, Roskilde, Denmark). DOX fluorescence was imaged for 24 h after the addition of DOX (5 μM)-loaded PEG-WCGKC-SS-Si into cultures using an inverted Nikon C1-Plus laser-scanning TE2000E confocal microscope (Nikon, Tokyo, Japan). Single-plane confocal image sequences were taken every 2 h.

Assessment of localization of DOX by confocal laser scanning microscopy. Intracellular release of DOX from PEG-WCGKC-SS-Si and the accumulation of DOX in nuclei were assessed microscopically in cells seeded on coverslips coated with 0.2% gelatin. After treating with PEG-WCGKC-SS-Si loaded with 5 μM DOX for 24 h, coverslip-mounted cells were fixed with 3.7% paraformaldehyde (PFA) for 5 min. The coverslips were washed three times with PBS, and cell nuclei were stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) for 2 min. Coverslips were washed three times with PBS and mounted onto slides using a mounting reagent (Invitrogen, Carlsbad, CA, USA). The fluorescence intensities of DOX and DAPI-stained nuclei were analyzed using a laser-scanning TE2000E confocal microscope (Nikon, Tokyo, Japan).

Clonogenic cell survival. Cells were seeded in 6-well plates and incubated overnight in a humidified 5% CO₂/95% air incubator at 37 °C. Cells were then treated with DOX, PEG-WCGKC-SS-Si without DOX, or PEG-WCGKC-SS-Si loaded with 5 μM DOX. After a 6-h treatment, cells were gently washed three times with PBS and cultured for an additional 14 days. Colonies that formed were fixed with methanol and stained with crystal violet (0.1% in methanol); the number of colonies containing more than 50 cells was counted. The surviving cell fractions of treated groups were calculated by expressing the plating efficiency of treated cells relative to that of untreated control cells.

TUNEL assay. Cells were seeded in an 8-well chamber slide and incubated overnight in a humidified 5% CO₂/95% air incubator at 37°C. Cells were then treated with 5 μM DOX, PEG-WCGKC-SS-Si w/o or PEG-WCGKC-SS-Si loaded with 5 μM DOX. After a 10-h treatment, cells were fixed with 4% (v/v) paraformaldehyde for 15 min, washed with PBS containing 1% (w/v) bovine serum albumin, permeabilized with 0.1% (v/v) Triton X-100, washed with PBS, and incubated for 1 h at 37°C in the dark with an apoptosis detection solution (Apoptosis Detection System kit; Roche Molecular Biochemicals, Mannheim, Germany). *In situ*-labeled nuclei were observed and photographed using a Nikon C1-Plus laser-scanning TE2000E confocal microscope.

Immunoblot analysis. Proteins in whole-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with 2% (v/v) nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20, and then were incubated with primary antibodies against the proteins of interest. Immunoreactive proteins were detected with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse IgG, and visualized using chemiluminescence reagents (Pierce, Rockford, IL, USA).

Intracellular GSH concentration. GSH concentration was measured using a glutathione assay kit (Sigma-Aldrich) following the manufacturer's instructions. Briefly, cells were seeded in 6-well plates and either left untreated or treated with 50 μ M BSO or 100 μ M GSH-OEt before assaying for GSH. Cell lysates were treated with 5% 5-sulfosalicylic acid and centrifuged to remove protein precipitates. The supernatants were then treated with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB). GSH reduces DTNB to generate TNB, and the total TNB formed was determined by measuring absorption at 412 nm in a Beckman DU640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

Statistical analysis

All grouped data are presented as means \pm S.E.M. Differences between groups were analyzed using analysis of variance (ANOVA) or Student's t-test, as appropriate, using GraphPad Prism software (GraphPad Software, Inc, La Jolla, CA, USA). All experiments were repeated in at least duplicate with triplicate technical replicates.

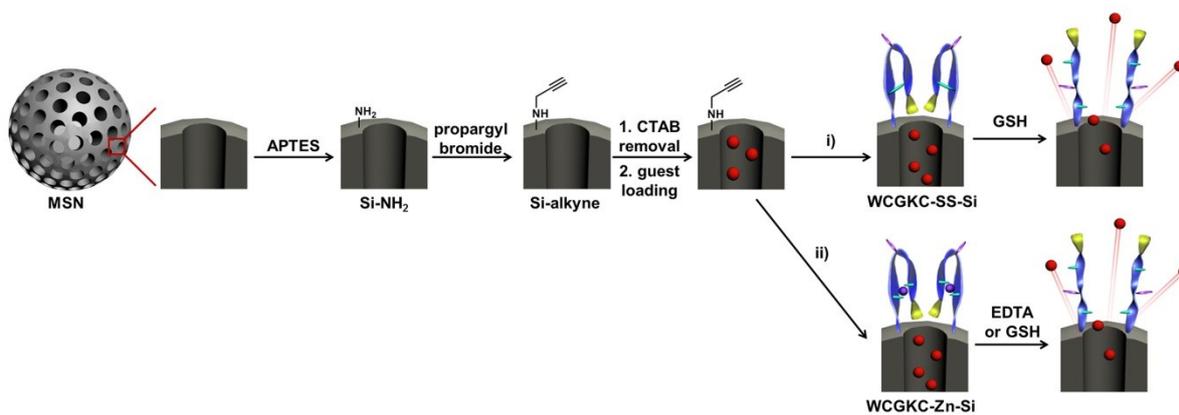


Fig. S1 Synthetic route to WCGKC-SS-Si and WCGKC-Zn-Si. Conditions: i) WCGKC-SS-N₃, sodium ascorbate, copper(II) sulfate; ii) WCGKC-N₃, sodium ascorbate, copper(II) sulfate, zinc(II) perchlorate.

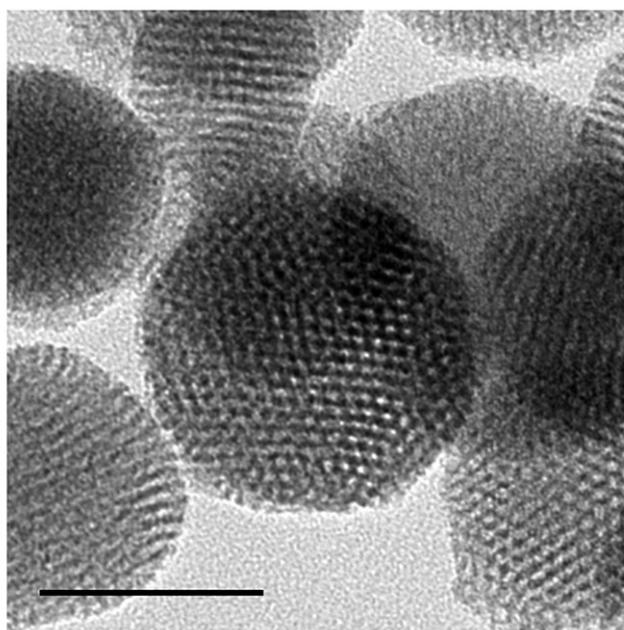


Fig. S2 TEM image of MSN. Scale bar = 50 nm.

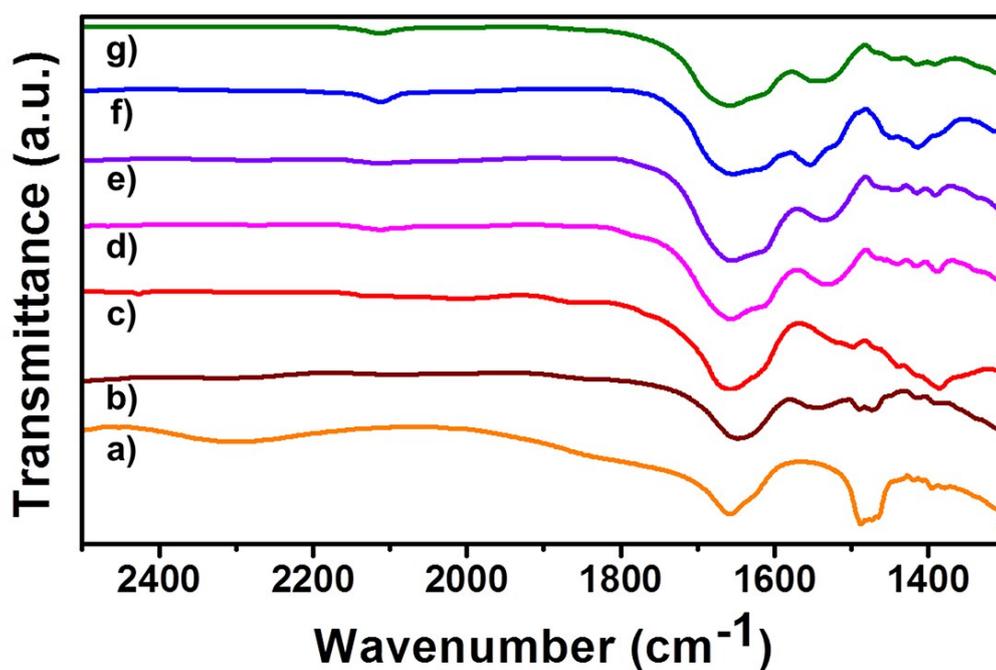


Fig. S3 FT-IR spectra of Si-NP (a), Si-NH₂ (b), Si-alkyne (c), WCGKC-Si (d), WCGKC-SS-Si (e), WCGKC-Zn-Si (f) and GCGKC-SS-Si (g).

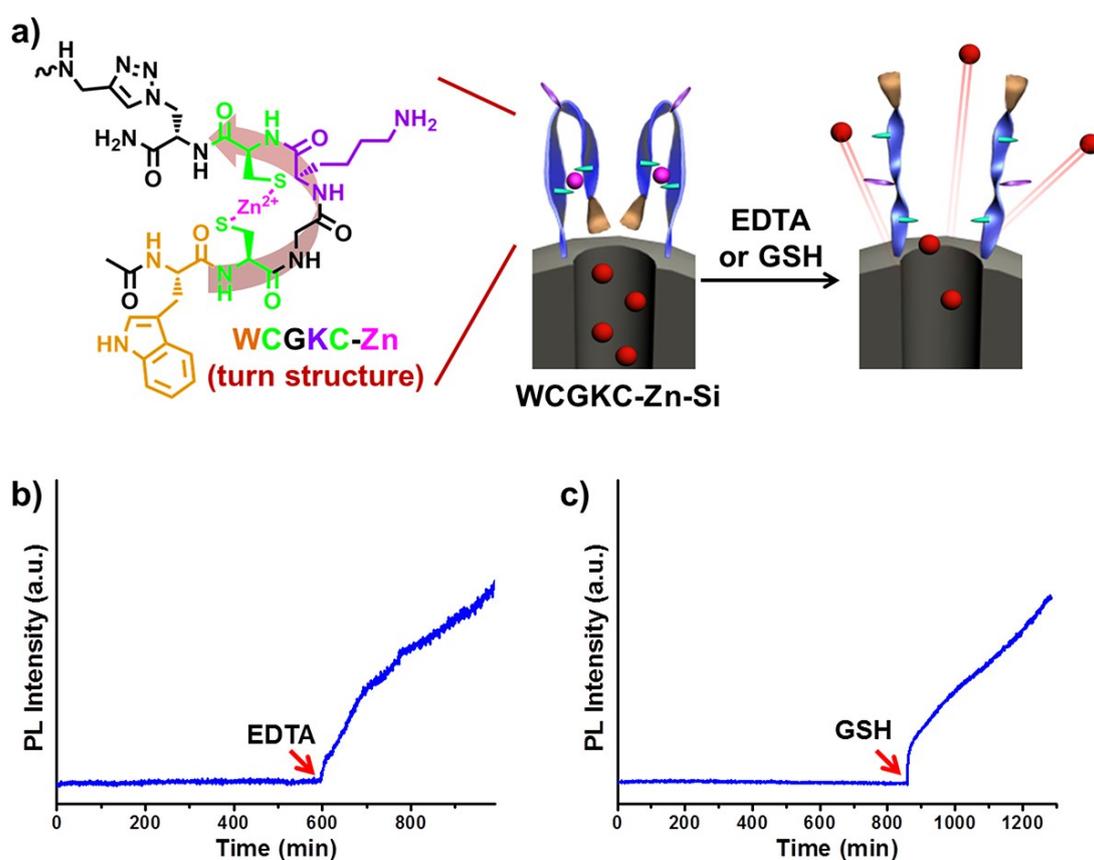


Fig. S4 a) Schematic representation of WCGKC-Zn-Si. b and c) Release profile of WCGKC-Zn-Si with EDTA (b) or GSH (c).

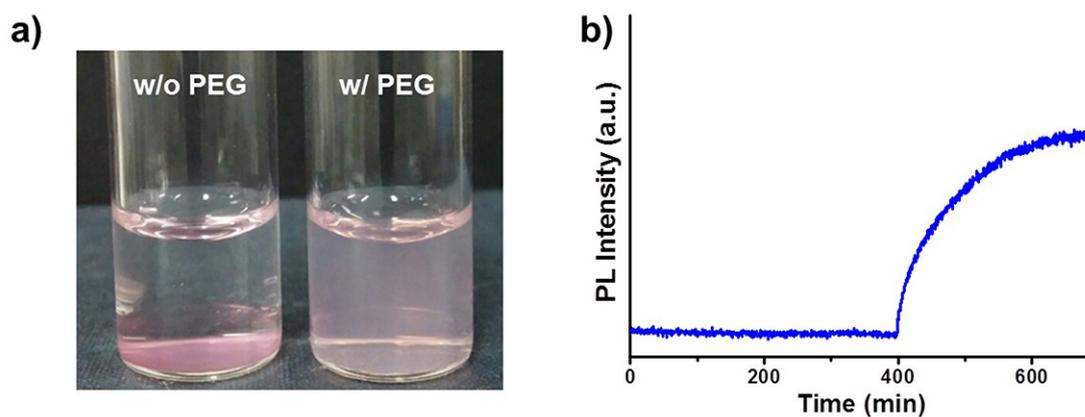


Fig. S5 a) Images of WCGKC-SS-Si and PEG-WCGKC-SS-Si dispersed in PBS buffer after 1 week. b) Release profile of PEG-WCGKC-SS-Si with GSH.

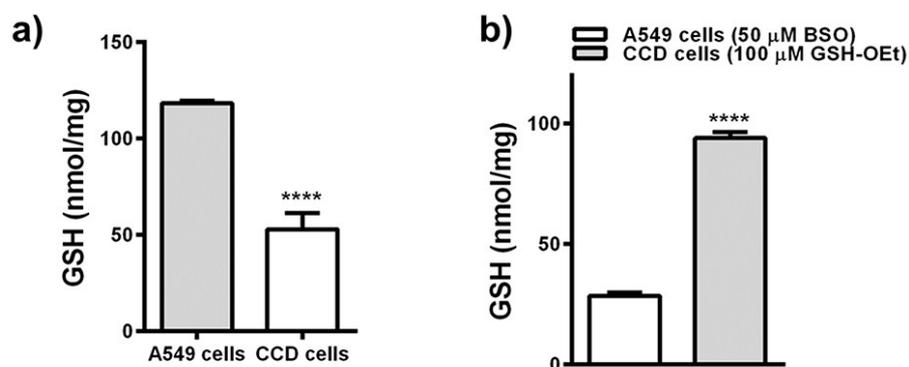


Fig. S6 a) Glutathione (GSH) concentration in A549 and CCD cells. Columns represent compiled data derived from five independent experiments \pm standard deviation. ****: $P < 0.0001$. b) Depletion or increase of GSH concentration in A549 or CCD cells. Columns represent compiled data derived from five independent experiments \pm standard deviation. ****: $P < 0.0001$.

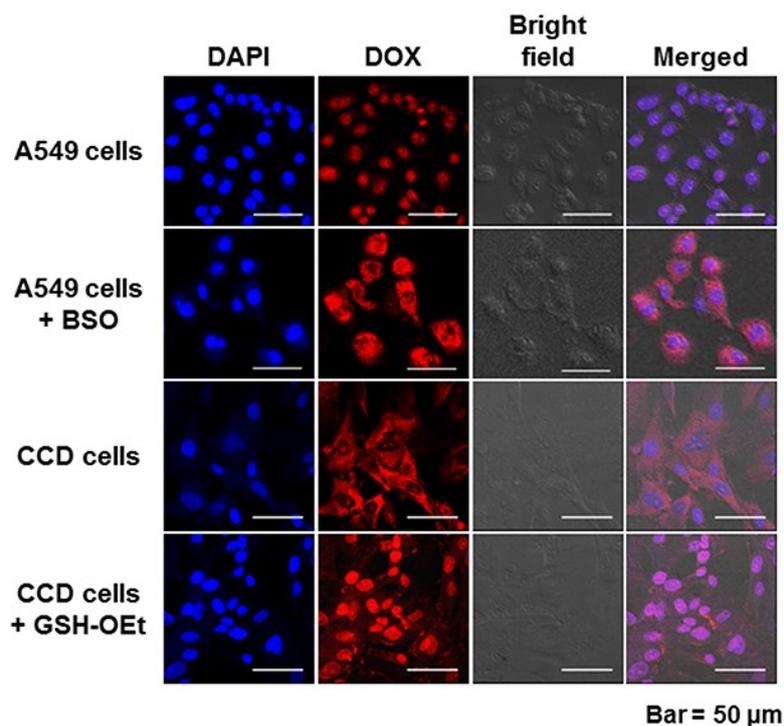


Fig. S7 The GSH-induced release of DOX from PEG-WCGKC-SS-Si and the accumulation of DOX in the nuclei. Cells were treated with PEG-WCGKC-SS-Si loaded with 5 μ M DOX for 24 hr. The cells were fixed with 3.7% PFA, washed three times with PBS and stained with DAPI. The fluorescence intensity of DOX and DAPI-stained nucleus were examined by laser scanning TE2000E confocal microscope (Nikon, tokyo, Japan).

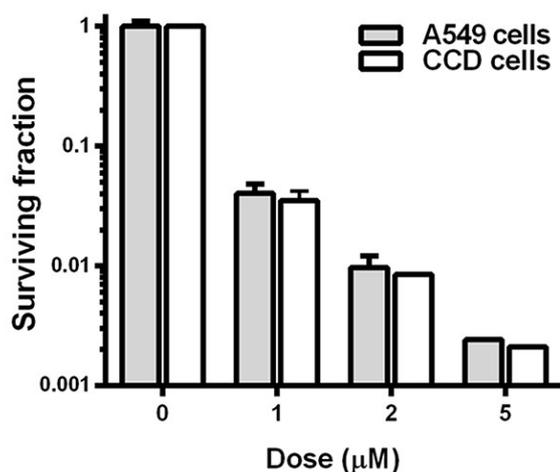


Fig. S8 The clonogenic survival of A549 and CCD cells treated with 0, 1, 2, and 5 μ M of DOX for 6 hr. After the incubation, cells were washed three times with PBS and cultured for an additional 14 days, and survival proportions were calculated.

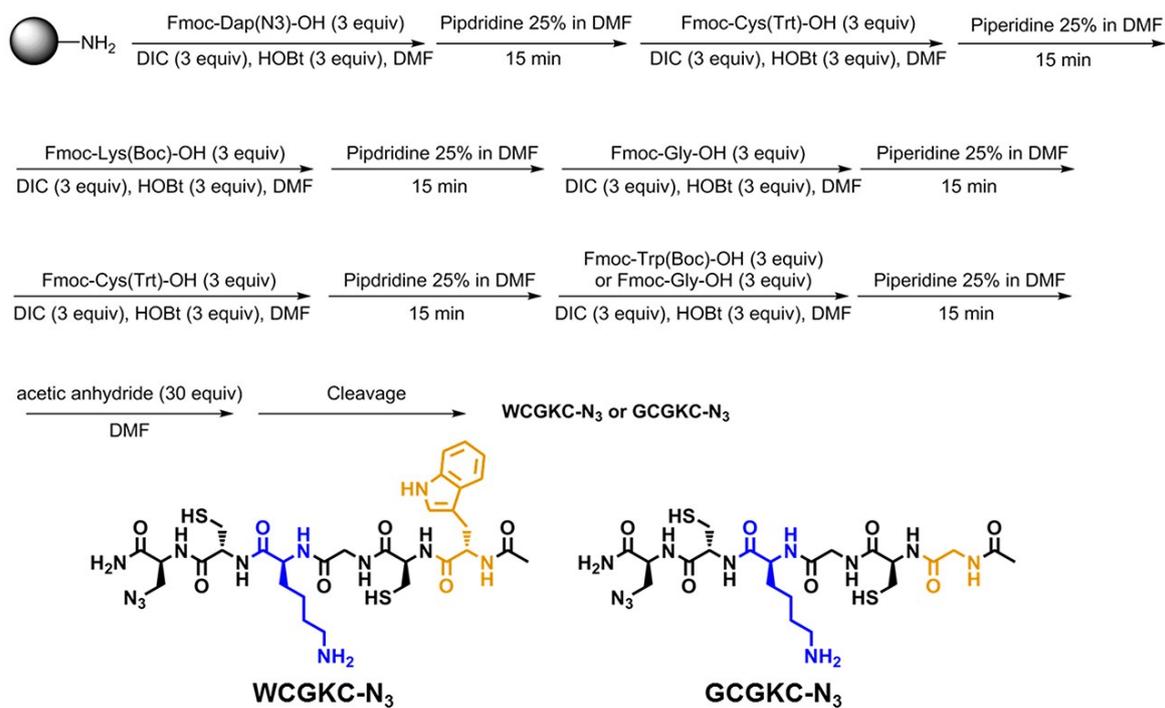


Fig. S9 Synthetic routes to WCGKC-N₃ and GCGKC-N₃.

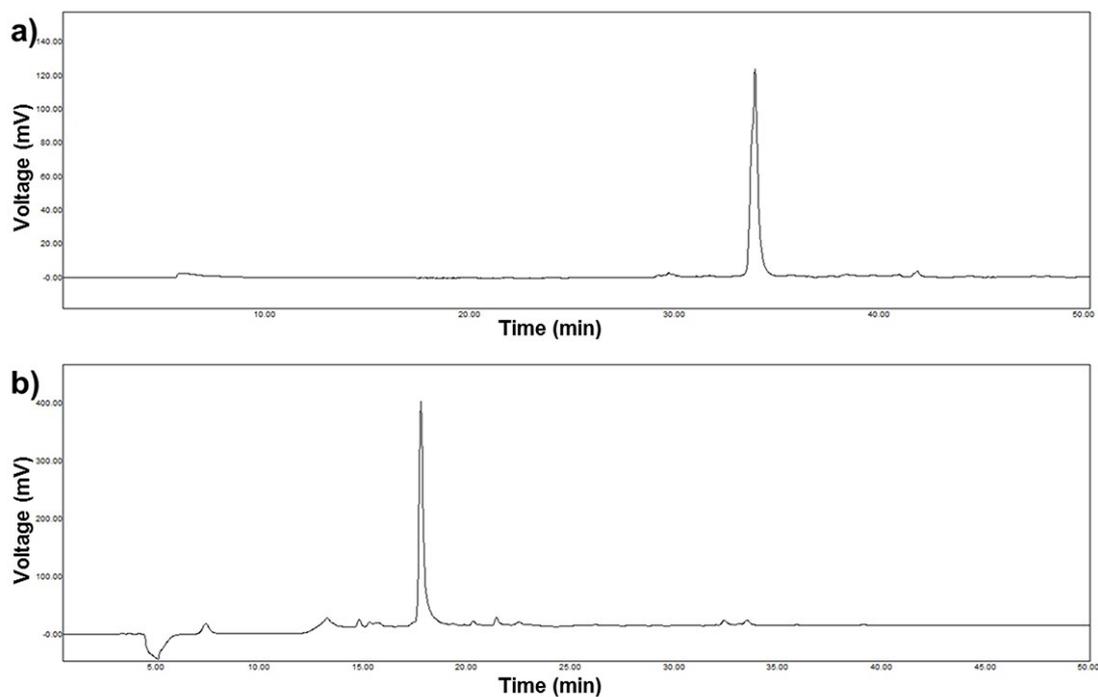


Fig. S10 HPLC chromatogram of WCGKC-N₃ (a) and GCGKC-N₃ (b).

WCGKC-2#45-69 RT: 1.40-1.92 AV: 6 NL: 1.99E8
T: + p ESI Full ms [200.00-1000.00]

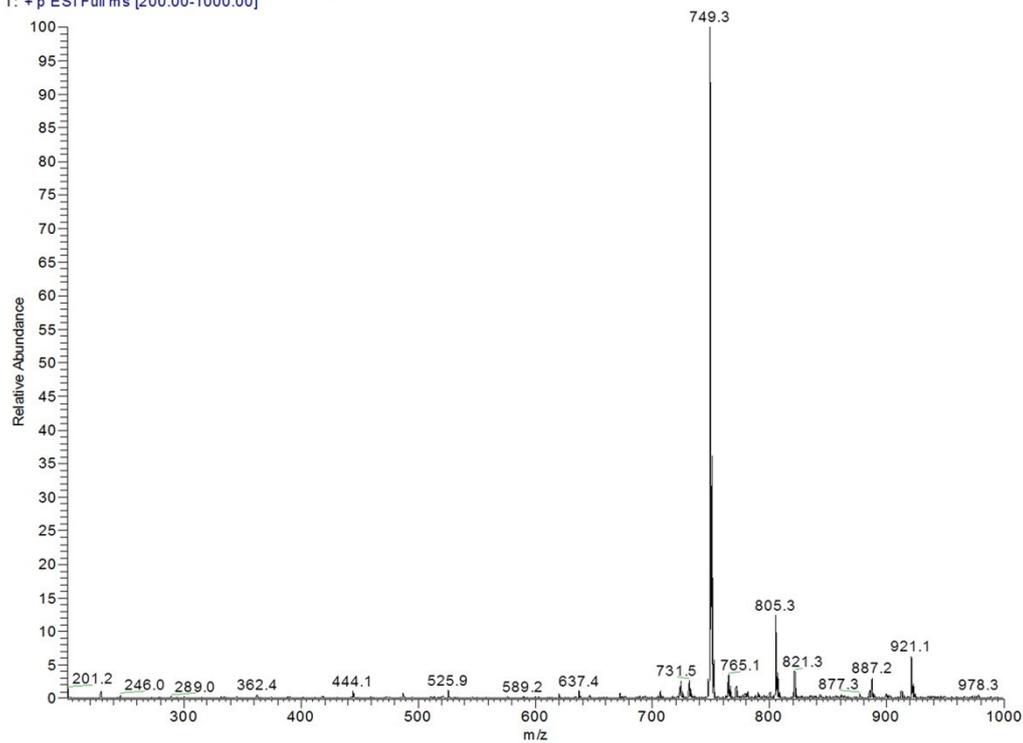


Fig. S11 ESI-Mass spectrum of WCGKC-N₃.

GCGKC #47-70 RT: 1.36-1.88 AV: 6 NL: 9.24E7
T: + p ESI Full ms [200.00-1000.00]

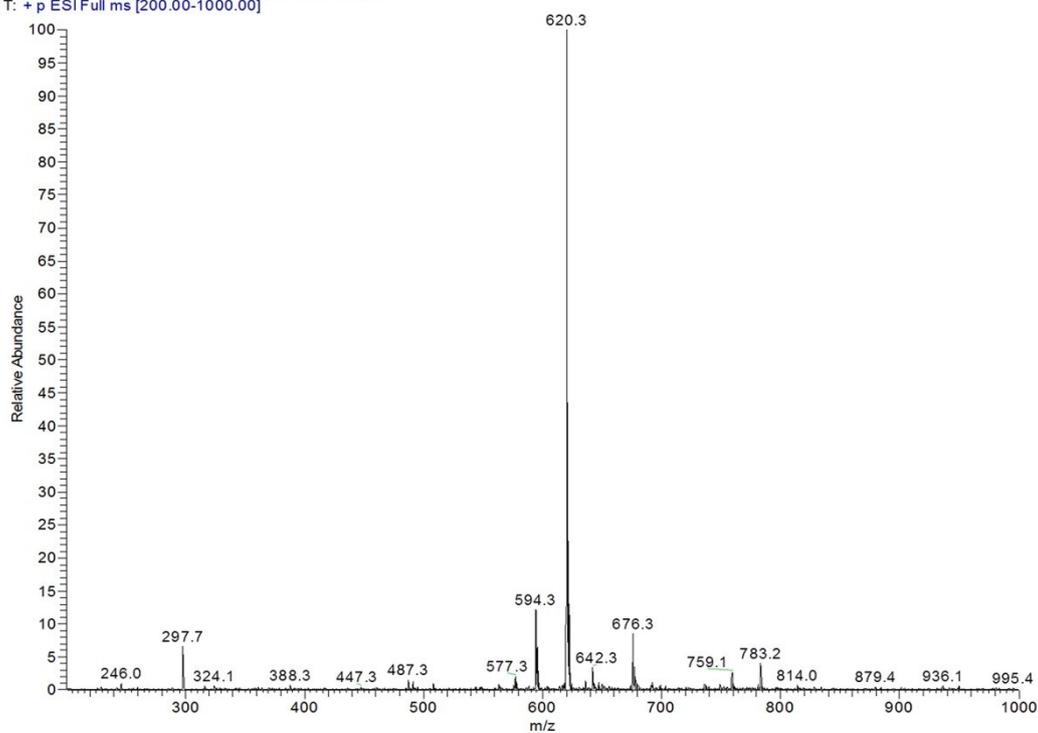


Fig. S12 ESI-Mass spectrum of GCGKC-N₃.

WCGKC-1#41-64 RT: 1.43-1.97 AV: 6 NL: 1.76E8
T: + p ESI Full ms [200.00-1000.00]

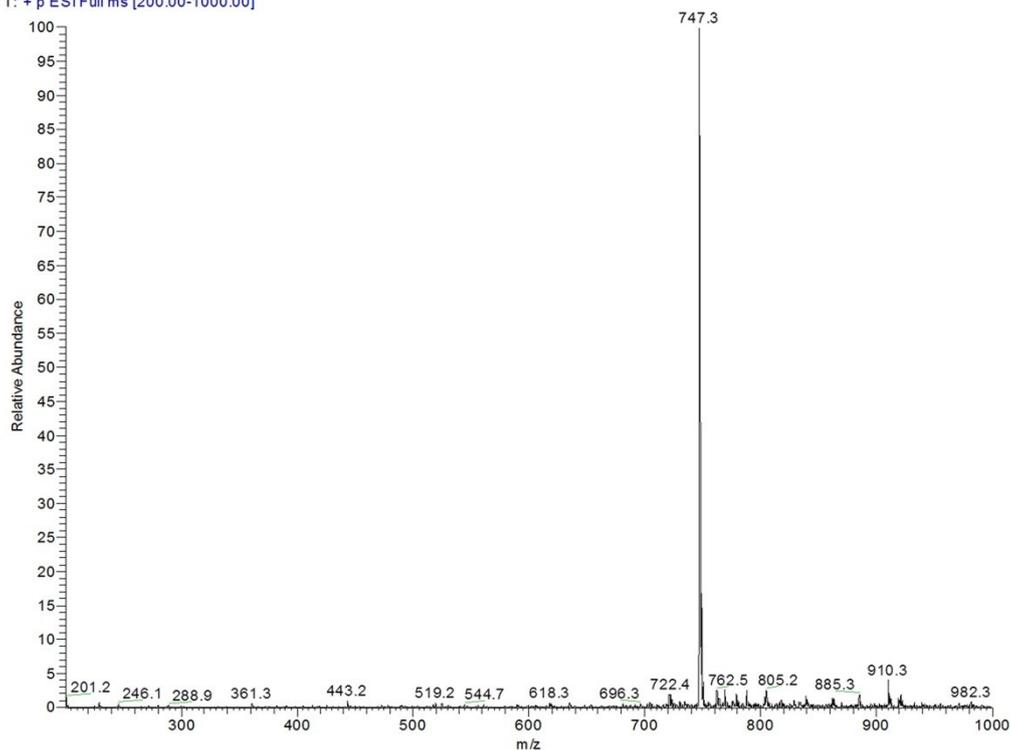


Fig. S13 ESI-Mass spectrum of WCGKC-SS-N₃.

3_Cyclic_GCGKC#38-57 RT: 1.37-1.83 AV: 5 NL: 8.22E6
T: + p ESI Full ms [50.00-1000.00]

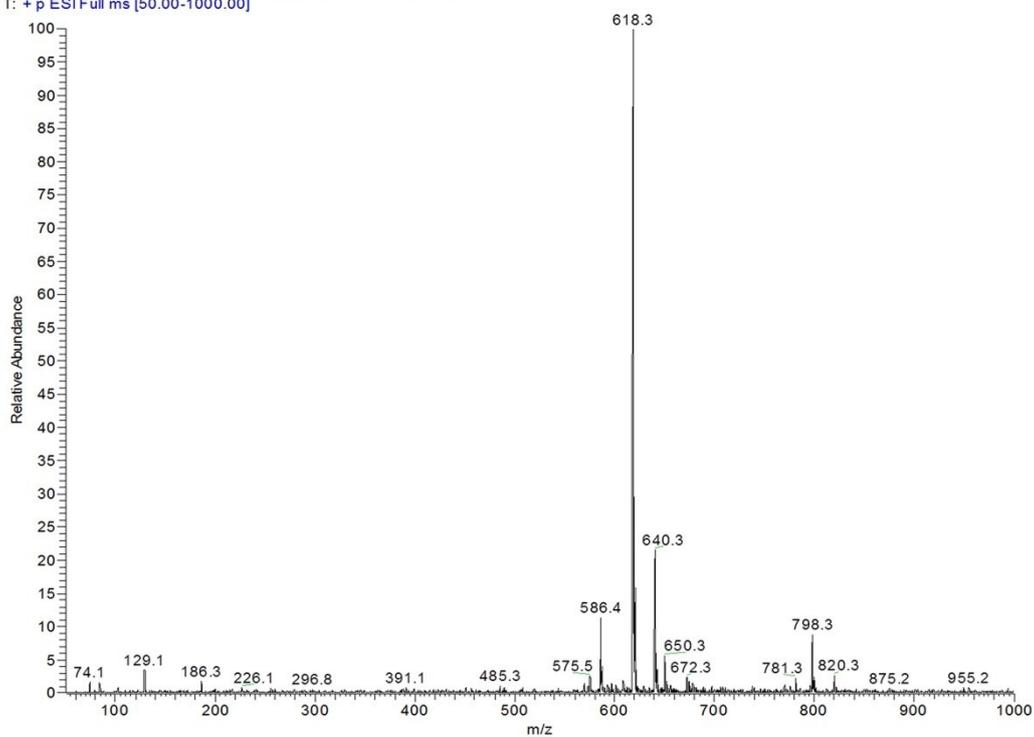


Fig. S14 ESI-Mass spectrum of GCGKC-SS-N₃.

References

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