Supplementary Material (ESI) for Chemical Communications

<Supplementary Information>

Disulfide-Cross-linked PEG-Poly(amino acid)s Copolymer Micelles for Glutathione-Mediated Intracellular Drug Delivery

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Experimental

Materials and Equipment. α-Methoxy-ω-amino-poly(ethylene glycol) (CH_3O-PEG-NH_2) of M_n of 5000 g/mol was purchased from SunBio Inc. (Seoul, Korea) and used as received. N^6-Carbobenzyloxy-L-lysine (H-Lys(Z)-OH)), L-phenylalanine (Pha), and methotrexate were purchased from Sigma Co. (St. Louis, MO) and used without further purification. Triphosgene and pyrene were purchased from Aldrich Co. (Milwaukee, WI) and used as received. 3,3'-Dithiobis(sulfosuccinimidylpropionate) (DTSSP) was purchased from Pierce (Perbio Science Deutschland GmbH, Bonn, Germany). Tetrahydrofuran (THF) was distilled from Na/benzophenone under N_2, prior to use. *N,N*-Dimethylformamide (DMF) was dried and distilled over calcium hydride.

The ^{1}H NMR spectra were recorded at 400 MHz on a Varian INOVA400 NMR spectrometer with a sample spinning rate of 5 kHz at 25 ^{o}C. Molecular weight distributions were determined using a GPC equipped with a Waters 2414 refractive index detector, 515 HPLC pump, and three consecutive Styragel® columns (HR1, HR2, and HR4). The eluent was THF with a flow rate of 1 mL/min. The molecular weights were calibrated with polystyrene standards. The zeta potential (ζ) was measured using a 90 PLUS (BrookHAVEN Instruments Cooperation, New York, USA) particle size analyzer.

***N*^{6}-Carbobenzyloxy-L-lysine *N*-carboxyanhydride (Lys(Z)-NCA) and L-phenylalanine N-carboxyanhydride (Pha-NCA).** Synthesis of *N*-carboxyanhydride of L-lysine and L-phenylalanine (Lys(Z)-NCA and Pha-NCA) was carried out by the Fuchs-Farthing method using triphosgene [1]. In brief, for Lys(Z)-NCA, Lys(Z) (30g, 107 mmol) was suspended in THF (300 mL) at 50 ^{o}C and triphosgene (10.58 g, 35.68mmol) was then added into the Lys(Z)-suspended solution. After 3 h, the crude product was

precipitated into *n*-hexane, and Lys(Z)-NCA was recrystallized from THF/*n*-hexane. Yield 92%. For Pha-NCA, Pha (5 g, 30.3 mmol) was suspended in THF (50 mL) and heated to 50 ^{o}C in a nitrogen atmosphere. A solution of triphosgene (3 g, 12.1 mmol) in THF was then added dropwise to the stirred reaction mixture. After 3 h, the reaction mixture was filtered to remove insoluble materials, and the filtrate was poured into *n*-hexane (300 mL). The resulting suspension stored at -20 ^{o}C overnight to assure complete crystallization. Pha-NCA was recrystallized at -20 ^{o}C from a mixture of THF/n-hexane. Yield 84 %. Anal. Calcd for Lys(Z)-NCA ($C_{15}H_{18}N_2O_5$): C, 58.82; H, 5.92; N, 9.15. Found: C, 58.69; H, 5.99; N, 9.02. Calcd for Pha-NCA ($C_{10}H_9NO_3$): C, 62.82; H, 4.74; N, 7.33. Found: C, 62.24; H, 4.91; N, 7.26.

[1] W. H. Daly, D. Poche´, *Tetrahedron Lett*., 1988, **29**, 5859.

Synthesis of PEG-*b*-PLys-*b*-PPha Triblock Copolymer. PEG_{113}-*b*-$PLys_{11}$-*b*-$PPha_{24}$ that have EG units of 113, Lys of 11, and Phe units of 24 was synthesized as follows: To a stirred solution of CH_3O-PEG-NH_2 (3 g, 0.6 mmol) in dry DMF (30 mL) was added Lys(Z)-NCA (2.02 g, 6.6 mmol) at 35 ^{o}C under nitrogen. After 24 h, Pha-NCA (3.44 g, 18 mmol) and dry DMF (100 mL) were added to the reaction mixture, and the reaction was maintained for further 24 h. PEG_{45}-*b*-$PLys(Z)_{11}$-*b*-$PPha_{24}$ was isolated by repeated precipitation from DMF into diethyl ether. Yield 92 %. Finally, the deprotection of PEG_{113}-*b*-$PLys(Z)_{11}$-*b*-$PPha_{24}$ was performed by treating the block copolymer (0.5 g) with trifluoroacetic acid (TFA) (5 mL) and HBr/HOAc (0.2 mL) to remove Z groups. The product, PEG_{113}-*b*-$PLys_{11}$-*b*-$PPha_{24}$, was isolated by dialysis using a membrane (molecular weight cut-off: 1000) for 24 h, followed by freeze-drying.

Shell Cross-Linking of Core-Shell-Corona Micelles. Polymer micelles of PEG_{113}-*b*-$PLys_{11}$-*b*-$PPha_{24}$, consisting of PEG coronas, PLys shells, and PPha cores, were prepared by dialyzing the polymer solution in DMF against doubly distilled water. Shell cross-linking was carried out by adding an aqueous solution of DTSSP to a micellar solution of PEG_{113}-*b*-$PLys_{11}$-*b*-$PPha_{24}$ (1 g/L) at pH 9.0. The reaction mixture was allowed to stir for 8 h at room temperature and then dialyzed against doubly distilled water for 3 h to remove unreacted DTSSP, and the dialyzate was lyophilized to obtain the shell cross-linked polymer micelles. To control the degree of cross-linking, the feed molar ratio of DTSSP to Lys repeating units of PLys middle shells was varied from 1:1 to 2:1. Consumption of primary amines of Lys units during the cross-linking reaction was quantified using a fluorescamine assay.

Stability of Shell Cross-Linked Polymer Micelles.

Kinetic stability of non-cross-linked micelles and shell cross-linked micelles was investigated by interaction with the sodium dodecyl sulfate (SDS), which acts as a destabilizing agent in aqueous media. The effect of SDS on micelles in aqueous media was estimated by dynamic light scattering analysis. A SDS solution (1 ml, 7.5 g/L) was added to aqueous solutions of non-cross-linked micelles or shell cross-linked polymer micelles (2 ml, 0.75 g/L) and the solution was stirred at 400 rpm. The final SDS concentration was 2.5 g/L. At predetermined time intervals, scattered light intensity of the micellar solutions containing SDS was monitored.

Fluorescence Measurements. Pyrene excitation spectra were recorded on a JASCO FP-6500 spectrofluorometer. Excitation and emission band widths were set at 1

nm and 5 nm, respectively. The emission wavelength was 390 nm, and the pyrene excitation spectra were recorded in the wavelength range of 280-360 nm.

Light Scattering Measurements. Dynamic light scattering measurements were performed using a 90 Plus particle size analyzer (Brookhaven Instruments Corporation). All the measurements were carried out at 25 ^{o}C. The sample solutions were purified by passing through a Millipore 0.45 μm filter. The scattered light of a vertically polarized He-Ne laser (632.8 nm) was measured at an angle of 90 ° and was collected on an autocorrelator. The hydrodynamic diameters (d) of micelles were calculated by using the Stokes-Einstein equation $d = k_B T/3\pi\eta D$ where k_B is the Boltzmann constant, T is the absolute temperature, η is the solvent viscosity, and D is the diffusion coefficient. The polydispersity factor, represented as μ_2/Γ^2, where μ_2 is the second cumulant of the decay function and Γ is the average characteristic line width, was calculated from the cumulant method [2].

[2] A. Harada and K. Kataoka, *Macromolecules,* 1998, **31**, 288.

Transmission Electron Microscopy. Transmission electron microscopy (TEM) was performed on a JEM-2000EX (JEOL Tokyo, Japan), operating at an acceleration voltage of 200 kV. To observe the size and distribution of micellar particles, a drop of sample solution (concentration = 2 g/L) was placed onto a 200-mesh copper grid coated with carbon. About 1 min after deposition, surface water was removed using a filter paper, followed by air-drying. A drop of uranyl acetate solution (5 wt%) was used for negative staining.

Preparation of MTX-Loaded Shell Cross-Linked Micelles. MTX-loaded PEG_{113}-*b*-$PLys_{11}$-*b*-$PPha_{24}$ micelles were prepared by the dialysis method and subsequent shell cross-linking reaction. The block copolymer (20 mg) was dissolved in 0.5 mL of DMF at 70 ^{o}C, and MTX (2 mg) was subsequently added. The solution was stirred for 6 h at room temperature and then dialyzed against doubly distilled water using a membrane (Spectrapor, MWCO: 1000). After 12 h dialysis, the solution of MTX-loaded micelles was collected. DTSSP as a shell cross-linking agent was then added to this solution by varying the feed molar ratio of [DTSSP]:[Lys] (1:2 or 1:1). The reaction was maintained for 8 h at pH 9.0, and the solution was dialyzed for 3 h to remove residual DTSSP, unloaded MTX, and reaction by-products. The dialyzate was lyophilized to obtain MTX-loaded shell cross-linked polymer micelles. For preparation of non-cross-linked MTX-loaded micelles, an identical process and time period were employed, except for the DTSSP addition. To determine the drug loading content and loading efficiency, MTX-loaded polymer micelles were dissolved in DMF, and the absorbance of MTX was then measured using UV-VIS spectrophotometer at 303 nm.

Glutathione-Mediated Controlled Drug Release. MTX-loaded micelles were precisely weighed, redispersed in the phosphate-buffered saline (1 mL PBS, pH 7.4, 1 g/L) solution, and introduced into a dialysis membrane bag (MWCO=1,000). The release experiment was initiated by placing the dialysis bag in release media of various GSH concentrations (0, 2 μM, 0.1 mM, 1 mM, and 10 mM). The release medium was shaken at a speed of 100 rpm at 37 ^{o}C. At predetermined time intervals, samples (10 mL) were withdrawn and replaced with an equal volume of the fresh medium. The concentration of MTX in the samples was measured by a UV-VIS spectrophotometer at 303 nm. The

assay for MTX was based on a linear standard curve obtained using the concentration range of 0.00125-0.02 mg/mL.

Cell Culture. A549 human lung carcinoma cells was obtained from the Korean Cell Line Bank (KCLB, Seoul). Cells were propagated in RPMI-1640 medium (Gibco BRL) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco BRL), and 1% (v/v) penicillin–streptomycin (Gibco BRL). Cells were cultured in a humidified incubator at 37 ℃ with 5% CO_2.

Cellular Uptake of SCM. To monitor time–dependent cellular uptake of SCM, fluorescence isothiocyanate (FITC) was conjugated to the PLys middle layers of SCM. A549 cells (5×10^3 cells/well) incubated with FITC-labeled SCM (100 μg/mL) were imaged by a IX71 fluorescence microscope (Olympus, Japan).

Cytotoxicity of Polymer Micelles and SCMs. A549 cells were maintained in RPMI-1640 medium containing 10% FBS and 1% antibiotic-antimycotic. The cells were seeded into 96-well flat-bottomed tissue-culture plate at 1000 cells/well, and incubated for 24 h in a humidified atmosphere of 5 % CO_2 at 37 ^oC. The concentration of SCM was diluted with culture medium to obtain a concentration range from 50 to 300 μg/mL. After the incubation for 24 h, 50 μL of a 10 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution in PBS was added to each well, and the plate was incubated for 4 h at 37 ^oC, allowing viable cells to reduce the MTT into purple formazan crystal. The formazan crystal was dissolved by adding 200 μL of dimethyl sulfoxide (DMSO) and 25 μL of Sörensen's glycine buffer. The absorbance of individual wells was measured at 570 nm by a microplate reader (SOFTmax® PRO, Molecular Devices Corporation, CA).

GSH-Controlled Intracellular MTX Delivery. To estimate the dependency of the intracellular MTX release on GSH concentrations, the intracellular level of GSH was manipulated by adding glutathione ethyl ester (GSH-OEt) into the cell culture media. A549 cells were seeded evenly into 96-well flat-bottomed tissue-culture plate (Corning Glass Works, Corning, NY) at a 5000 cells/well concentration, and incubated for 24 h in a humidified atmosphere of 5 % CO_2 at 37 ^{o}C. The cells were firstly treated with varied concentrations of GSH-OEt (0, 5, and 10 mM) for 2 h and washed. MTX-loaded SCM olutions (100 μL, concentration= 50 μg/mL) were then added and, the plates were incubated. The MTX concentration was fixed at 4.3 μg/mL. After the incubation for 5 and 10 h, the cytotoxicity of SCMs was evaluated by an MTT assay.

Imaging of Cell Viability. To obtain cellular vital/dead images, the stock solutions of fluorescein diacetate (5 mg/mL in acetone) and ethidium bromide (1.25 mg/mL in PBS) were prepared. Shortly before use, 5 μL of the fluorescein diacetate solution and 5 μL of the ethidium bromide solution were mixed in 1 mL of PBS and stored at 5 ^{o}C. The cells were stained with the solution mixture (50 μL) and incubated for 10 min at 37 ^{o}C, before being subjected to fluorescence microscopic observation.

Table S1. Characteristics of the PEG_{113}-$PLys_{11}$-$PPha_{24}$ copolymer

copolymer	feed ratio ([EG]:[Lys(Z)]:[Pha])	composition ratio [a] ([EG]:[Lys]:[Pha])	conversion of Lys(Z) [a] (%)	conversion of Pha [a] (%)	M_n [a]	M_w/M_n [b]	cmc [c] (mg/L)
PEG_{113}-$PLys_{11}$-$PPha_{24}$	113 : 12 : 30	113 : 11 : 24	92	80	11,400	1.08	39

*The copolymers were synthesized using the macroinitiator (CH_3O-PEG-NH_2) with M_n of 5,000 and polydispersity of 1.06. [a] Calculated by ^1H NMR spectra. [b] M_w/M_n of PEG_{113}-$Plys(Z)_{11}$-$PPha_{24}$ estimated by GPC. [c] Critical micelle concentration at 25 °C.

Fig. S1 Synthetic route to the PEG_{113}-$PLys_{11}$-$PPha_{24}$ copolymer

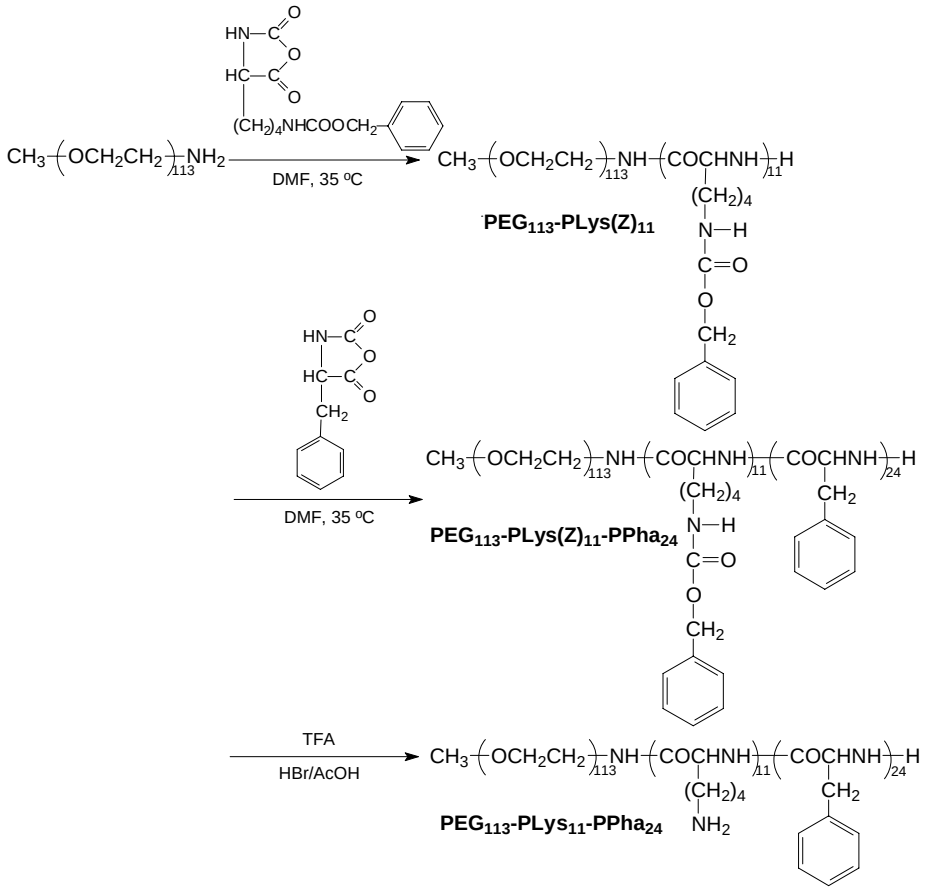


Fig. S2 ^{1}H NMR spectra of CH_3O-PEG-NH_2 (a), PEG_{113}-$PLys(Z)_{11}$ (b), PEG_{113}-$PLys(Z)_{11}$-$PPha_{24}$ (c), and PEG_{113}-$PLys_{11}$-$PPha_{24}$ (d) in DMSO-d_6.

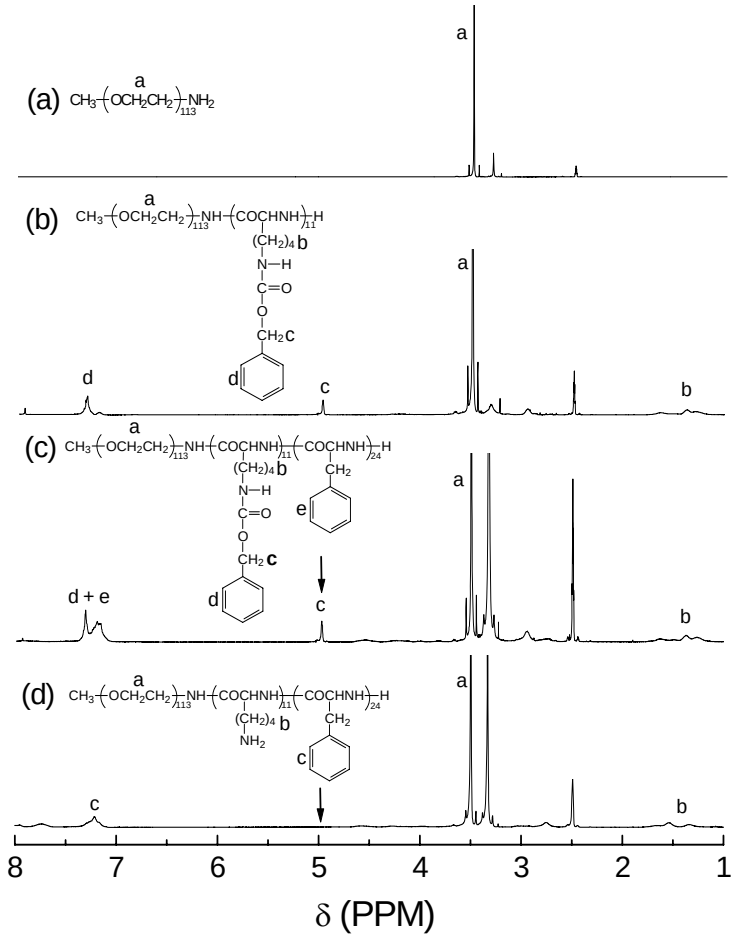


Fig. S3 Gel permeation chromatograms of CH_3O-PEG-NH_2 (a), PEG_{113}-$PLys(Z)_{11}$ (b), and PEG_{113}-$PLys(Z)_{11}$-$PPha_{24}$ (c).

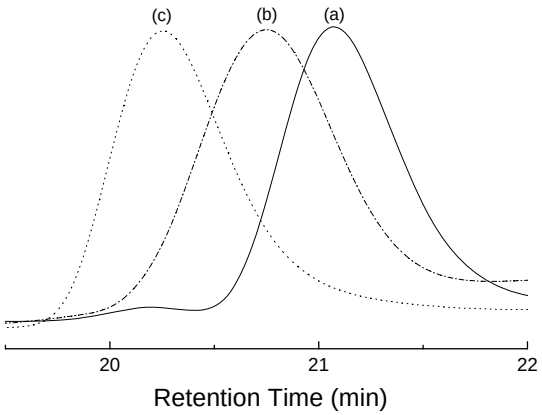
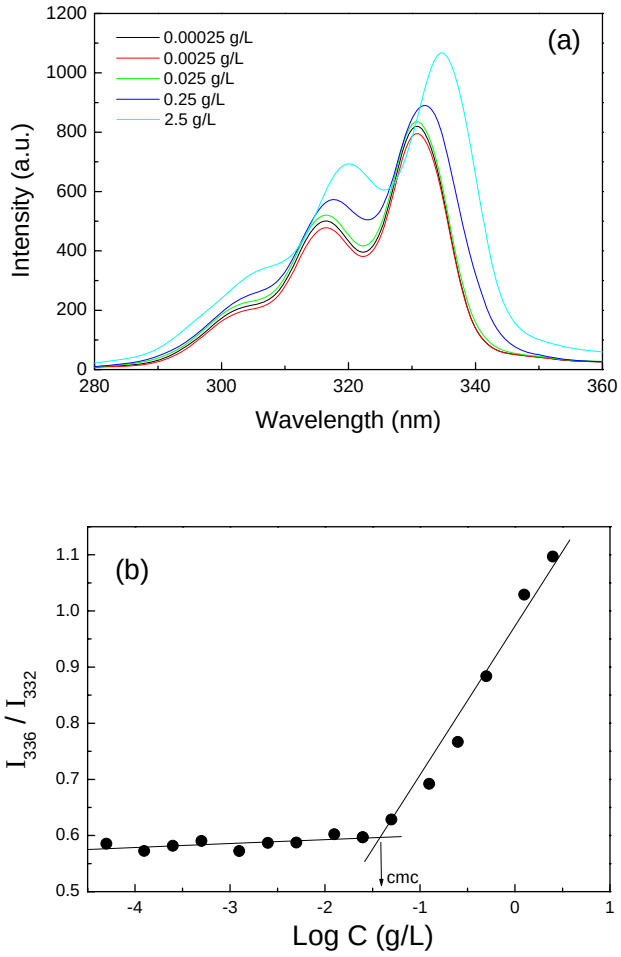


Fig. S4 (a) Excitation spectra of pyrene as a function of the PEG_{113}-$PLys_{11}$-$PPha_{24}$ concentration in water. (b) Plot of I_{336}/I_{332} (from pyrene excitation spectra) vs. Log C for PEG_{113}-$PLys_{11}$-$PPha_{24}$.



Fig, S5 TEM images of micelle nanoparticles before and after the shell cross-linking reaction (a: NCM, b: SCM1, c: SCM2)

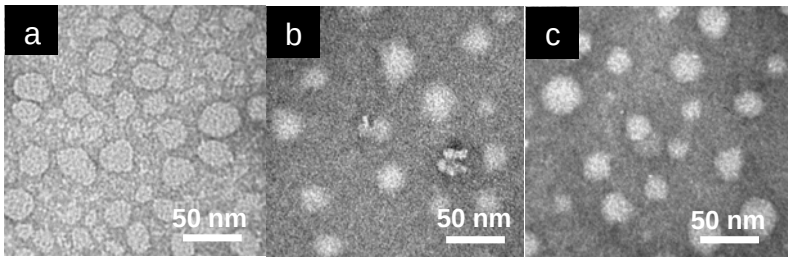


Fig. S6 (a) Time-dependent changes in light scattering intensities of aqueous NCM, SCM1, and SCM2 in the presence of SDS (2.5 g/L). (b) Time-dependent changes of micelle size distribution (polydispersity factor, μ_2/Γ^2) in the presence of SDS (2.5 g/L).

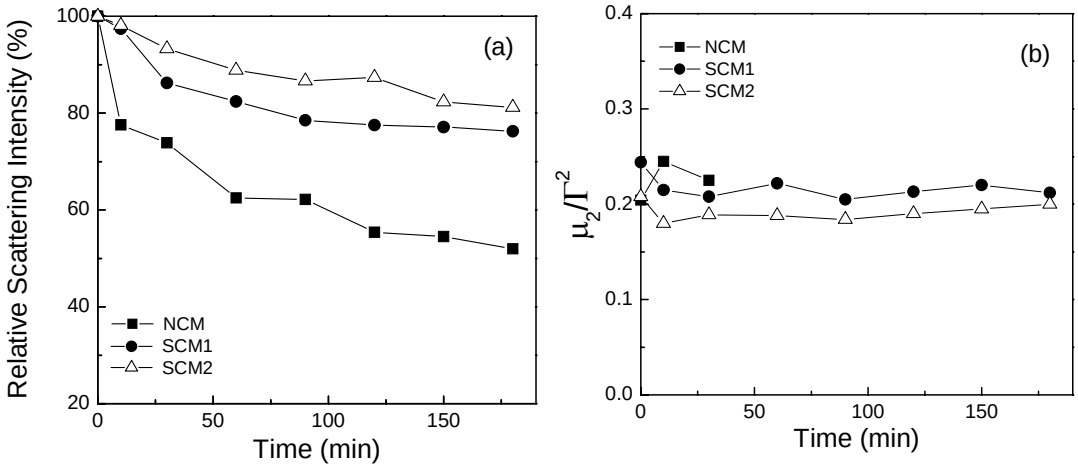


Fig. S7 (a) MTX release profiles from NCM, SCM1, and SCM2 at PBS (pH 7.4, 37 ^{o}C). Each point represents the mean value±S.D. (n=3).

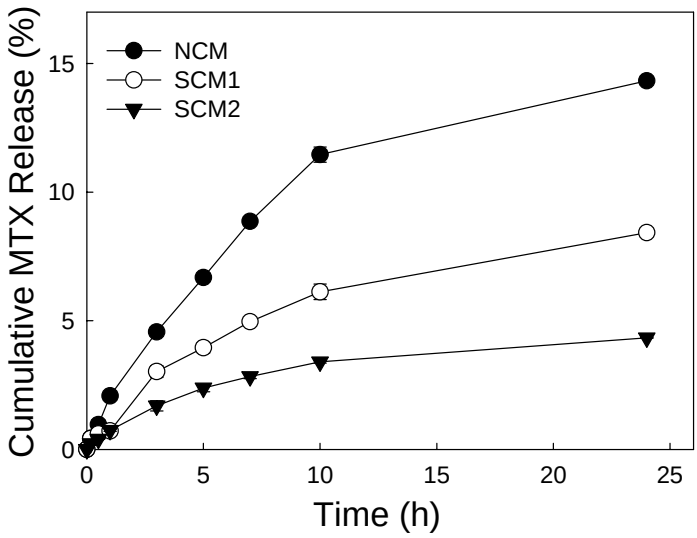


Fig. S8. Cellular uptake of FITC-labeled SCM2 as a function of incubation time.

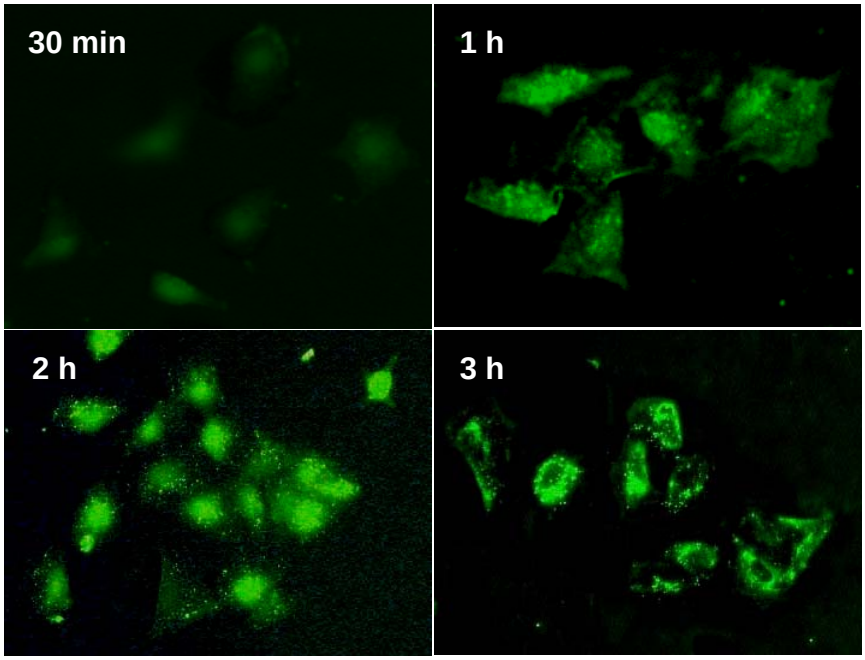


Fig. S9 Viability of A549 cells at various concentrations of NCM and SCMs