Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Data

Title: Hydrophobic end-gated silica nanotubes for intracellular glutathione-stimulated drug delivery in drug-resistant cancer cells

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Experimental details

Materials:

L-Glutathione reduced (GSH, Sigma Aldrich, 98%), 1-octadecanethiol (Aldrich, 98%), chloroauric acid (Aldrich, 98%), tetrakis(hydroxymethyl)phosphonium chloride (THPC, Aldrich, 80% aq.), sodium citrate tribasic dehydrate (Sigma, ＞98%), silicon tetrachloride ($SiCl_4$, Acros Organics, 99.8%), (3-trimethoxysilypropyl)diethyenetriamine (DETA-silane, Geleat, 95%), and aluminum foils (Alfa Aesar, 99.99%) were purchased and were used without further purifications. Since GSH is known to be not able to internalize cell membranes due to its anionic nature, glutathione ethyl ester (GSH-OEt) (Sigma Aldrich, 90%) was used as an external stimulus to trigger DOX release (1). Control tripeptide (95%) and rhodamine B dye-tagged glutathione methyl ester (GSH-OMe, 90%) were synthesized from Peptron (Daejeon, Korea). DOX was purchased from Selleck Chemicals and used as received. The lysosomal marker (LysoTracker Blue Catalogue # L-7525) and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (Carlsbad, USA).

Preparation of the AAO templates

Alumina template was prepared according to the literature (2). Briefly, preannealed aluminum foils (0.25 mm thick) were degreased in acetone for 1 h, and then electropolished at 5 °C and 15 V for 6 min in the mixed solution of perchloric acid and ethanol (volume ratio 1:5). The first anodization was performed in a 0.3 M oxalic acid solution for 10~15 h at 10 °C and 40 V, then the resulting aluminum oxide layer was removed in a solution of phosphoric acid (6 wt%) and chromic acid (1.5 wt%) at 60 °C. To obtain AAO templates with 500-nm pore length, the etched aluminum foil was anodized again for 7 min and then pore-widened in phosphoric acid solution (5 wt%) for 30 min at 30 °C.

2.3. Preparation of SNTs with an amine moiety inside

SNTs were prepared using modified surface sol-gel method described previously (3). Briefly, AAO template was immersed in $SiCl_4$ solution for 5 min, and quickly washed with hexane three times to remove the unreacted $SiCl_4$ remaining on the surface. The template was then immersed in a mixed solution of hexane and methanol and in ethanol for 2 min, respectively. After dried under nitrogen gas for 5 min, the template was immersed in D.I. water to hydrolyze $SiCl_4$. To prepare 500-nm SNTs, the whole process mentioned above was repeated twice. The inner surfaces of nanotubes were then functionalized with amine group, by dipping the template in a solution of 5% (v/v) DETA-silane in ethanol/water (95% v/v) for 10 min at RT. After washed with ethanol, the template was cured in a vacuum oven for 20 min at 120°C.

2.4. Preparation of Au nanorings at the open ends of SNTs

Au nanoring structures were prepared specifically at the open ends of SNTs using modified diffusion-controlled partial functionalization method, described in the author's previous report (4). First, THPC gold solution composed of 2 nm AuNPs colloid was prepared according to the literature (5). To a solution of 1.5 mL of 2 nm AuNPs, a small piece of AAO template containing SNTs with amine moiety inside was added and incubated on a rocking platform for 24 h at RT. After the template was washed with D.I. water three times, SNTs were partially functionalized with Au NPs at the open ends. To prepare solid Au layer, seed-mediated gold growth reaction was performed according to

the literature (6). Briefly, to a 30-mL solution of previously boiled 0.01% (wt/v) $HAuCl_4$, the template containing SNTs with AuNPs inside was added. Gold growth reaction was then initiated by adding 50 μL of 1% (wt/v) sodium citrate solution. After 1 h later, the template was taken out and washed with DI water three times. To obtain free-standing SNTs with Au nanoring at the open ends, the AAO template was mechanically polished for discarding silica layer on the top of the template and was dissolved in 25% (wt/v) phosphoric acid solution. Free-standing SNTs liberated from AAO template were then collected by filtration and were kept in D.I. water for the further use. The morphologies of SNTs were checked using a scanning electron microscope (SEM, JEOL JEM-3010) and a transmission electron microscope (TEM, FEI TECNAI G2).

Our experimental approach to the hydrophobicity-controlled gatekeeper is more advantageous over MCM-41 type materials in terms of the range of much larger pore size to which it is applicable. In addition, it is inevitable for the whole inner surface of MCM-41 to be modified with the thiol group when 3-mercaptopropyltrimethoxysilane is treated to form the thiol group as anchoring moiety necessary for the introduction of gatekeeper via a disulfide bond. The unnecessary thiol groups inside pores are supposed to remain even after drug loading and gatekeeper preparation steps, which may lead to unwanted reactions between the thiol group and thiol susceptible drug molecules inside the pores. In contrast, the hydrophobic end-gated SNTs employ Au-thiol interaction for the introduction of the gatekeeper, C18-SH, so that the inner surfaces of SNTs remain intact after the whole synthetic processes end, which can offer inert environment to the loaded drug molecules.

Loading DOX into SNTs and preparation GSH-sensitive labile cap:

To a 1 mL of methanol/DMSO (80% v/v) solution containing 30 mM DOX and ca 1.2 x 10^{12} SNTs with Au nanoring at the open end, 400μL of 1-octadecanethiol solution (4 mM, dissolved in ethanol) was added and reacted for 24 h under darkness. After the reaction finished, SNTs was filtered and washed with methanol and D.I. water four times, respectively. The final concentration of DOX-loaded SNTs stock solution was adjusted to 1.2 x 10^{12} SNTs/mL. The amount of DOX loaded inside SNT was determined by measuring the fluorescence intensity after releasing DOXs from the SNTs in GSH solution as follows: to a 360 μL of 4 mM GSH solution in D.I. water, 40 μL of DOX-loaded SNT stock solution was added and reacted for 12 h under darkness. The fluorescence intensity of the solution was measured at 592 nm with excitation at 500 nm. The standard curve was obtained by plotting fluorescence intensity against DOX concentrations ranging from 0 to 30 μM. The concentration of DOX in the sample solution was measured to be 7.3 μM and in turn the concentration of DOX contained in 1 mL of SNT stock solution can be calculated to be 73 μM. Considering the total loaded concentration of DOX inside $1.2x10^{12}$ SNTs in a 1 mL of stock solution is 73 μM, we can calculate the amount of DOX in a single SNT as 1.2 x 10^{-20} mol per SNT (ca. 37000 DOX molecules per SNT). Assuming that the volume of a 500-nm long SNT with 80-nm pore diameter is $2.5x10^{-18}$ L, the effective concentration of DOX inside the SNT can be estimated to be ca. 24 mM, which is slightly bigger than a theoretical value (21.4 mM) obtained from the initial DOX concentration used in the loading experiment. We reason that the adsorption of DOX on SNT contributes to a slight increase in DOX concentration loaded to SNT.

Cell culture and cytotoxicity assay:
Doxorubicin resistant MCF-7 cell line (MCF-7/ADR) (7) was kindly provided by Prof. H. J. Lee from the Ewha Women's University in Korea. Human lung carcinoma A549 cells (ATCC CCL-185) were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a 5% CO_2 atmosphere incubator.

For cell viability test, MTT cytotoxicity assays were applied for SNTs. The number of 5×10^4 MCF-7/ADR cells was cultivated at a 35 mm cell culture dish and incubated overnight at 37℃, 5% CO_2. We eliminated the cell culture medium at a seeded dish, washed with a 1 mL of PBS, and then added a fresh medium solution of 2 mL. We then applied SNTs by varying the concentrations and stored inside the incubator for 24 h. We then removed the medium at a seeded dish, washed with DPBS (1 mL), added 2 mL of MTT solution that was diluted from the original product by a factor of 10, and incubated for additional 4 h. After 4 h, we removed the MTT solution, put DMSO (1 mL) and stayed at room temperature for 10 min, and poured a 200 μL of each solution to each 96 well plate, and read the absorbance values at 570 nm.

TEM images of SNTs' uptake and fluorescence measurement:
The internalization of SNTs was examined using a JEOL JEM-1010 transmission electron microscope. Fluorescence spectroscopy (Scinco FS-2 spectrometer) was used to examine the GSH-triggered fluorescence recovery effect at the excitation wavelength of 525 nm with the silt width of 2.5 nm. An Olympus IX-71 inverted microscope was employed with a Hg lamp (U-LH100HG) and a high sensitive CCD camera (CoolSnap HQ, Roper Scientific). An objective lens (×40 or ×60) with a corrected thickness for the cover glass was used to obtain the image using the CCD camera. The filter set for DOX consists of 460-490 nm excitation, 506 nm dichroic, and 575 nm emission (Semrock). A shutter (Ludl Electronic Products Ltd.) with a MAC6000 shutter controller was employed to give a 300 ms exposure time by a MetaMorph ID34072 software. Cells were seeded to a 35 mm confocal dish. Cells were loaded with LysoTracker Blue at 50 nM in growth medium for 1 h for the colocalization experiment.

Xenograft model and SNTs treatment:
In order to investigate the ability of SNTs to target drug resistant cells both *in vitro* and *in vivo*, we performed *in vivo* experiment using a xenograft model. A 40 μL SNT solution was premixed with a 30 μL of matrigel (BD Sciences, Catalogue #356231) (8). The 50 μL mixed solution was injected to a tumor site. A 50 μL of 5 mM GSH was subsequently injected into the same spot of the tumor site, where the SNT solution was injected. After orthotopically implanting MCF7/ADR cells into nude mice, mice were checked for tumor growth and randomized thereafter before the initiation of the treatment. Five-week-old male CAnN.Cg-Foxn1^{nu}/CrljOriBALB/c nude mice (Orient Bio Inc, Gyeonggi, Korea) were housed in sterile cages and supplied with sterile food and water. MCF7/ADR cells (5×10^5 cells suspension in 10% RPMI medium) were injected subcutaneously after the mice were anesthetized with 25 mg/kg Zoletil 50 (Virbac Laboratory, Carros, France) and 2% Rompun (BAYER, Leverkusen, Germany) (2:1 ratio, 1 mL/kg). Injection of SNTs (50 μL) was started when the tumor volume was reached a size of approximately 500 mm^3. SNTs were injected every other day into the

tumors for 15 days. The volume of tumor was evaluated every other day using the formula; Volume = (width × length × depth) × (π/6). All animal experiments were performed in compliance with the guidelines of the Institute of Laboratory Animal Resources, Seoul National University (SNU-101221-1).

Reference

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Figure captions

Fig. S1. (a) GSH (0-20 mM)-mediated release of a portion of DOX drug molecules loaded in C18-SH gated SNTs by observing an increase in fluorescent intensity in real time. (b) Fluorescence spectra of DOX released from the C18-SH gated SNTs after treating with GSH (2 mM) for 12 h. As a control, the tripeptide (2mM) analogue of GSH, containing a methyl group instead of a thiol group, did not efficiently uncap the end at the given conditions. Serum containing media (RPMI FBS (10%) was compared along with SNTs.

Fig. S2. (a) TEM images of SNTs in a single mammalian cancer cell after incubation for 24 h. The arrows indicate the internalized SNTs. (b) TEM images at a magnification of 100k. The scale bars are 200 nm. Gold capping was seen at the end of SNTs.

Fig. S3. Colocalization images of lysosome-stained mammalian cancer cells after treating with SNTs-DOX. (a) Phase-contrast image, (b) lysosome, (c) SNTs-DOX, and (d) their overlaid images. Scale bars are 10 μm.

Fig. S4. Colocalization images of lysosome-stained mammalian cancer cells after treating with rhodamine B-tagged GSH-OMe. Scale bars are 10 μm.

Fig. S5. (a) Another example of the GSH-OEt-stimulated release of DOX from SNTs was examined by fluorescence live-cell imaging. Scale bars are 10 μm. (b) Plot of measured fluorescence intensities.

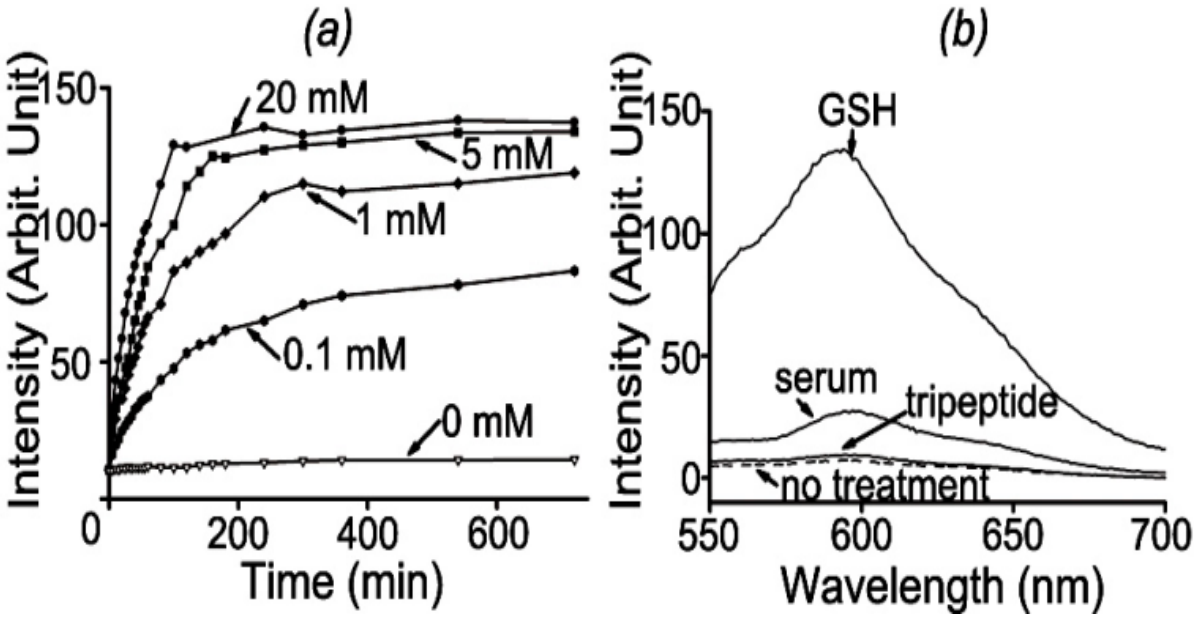
Fig. S6. (a) The release of DOX from SNTs was examined by fluorescence live cell imaging in a single A549 cell. Scale bars are 10 μm. b) Plot of measured fluorescent intensities.

Fig. S7. Colocalization images of (a) SNTs-DOX, (b) DAPI-stained mammalian cells, and (c) their overlaid images. Scale bars are 10 μm.

Fig. S8. Pore diameter distribution of (A) native SNTs before incubation with 2-nm AuNPs and (B) SNTs with 2-nm AuNP seeds; Histogram for maximum (C) and minimum (D) pore diameters of SNTs after Au growth reaction.

Table. S1. Pore diameter of SNTs.

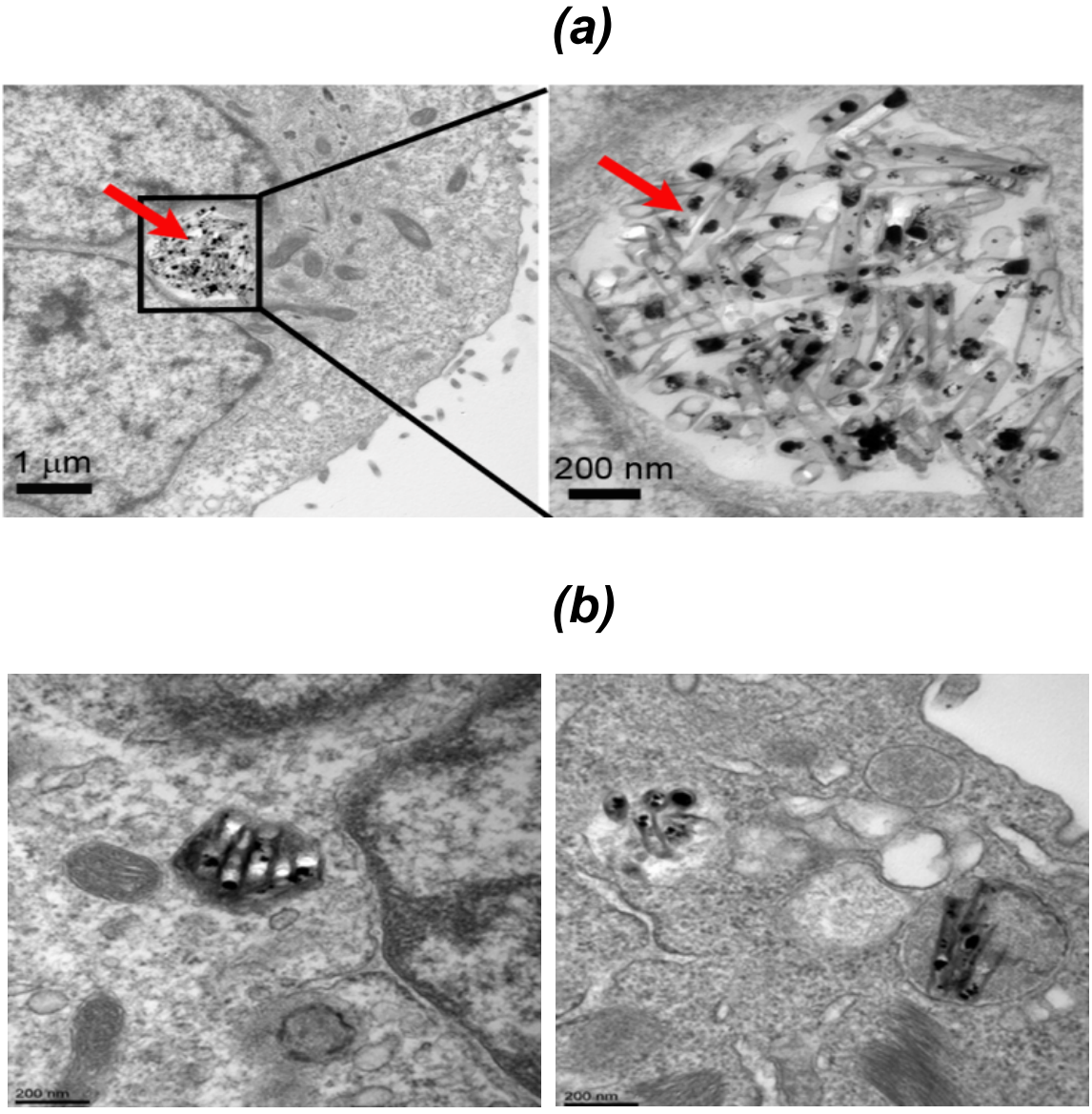
Fig. S1. (a) GSH (0-20 mM)-mediated release of a portion of DOX drug molecules loaded in C18-SH gated SNTs by observing an increase in fluorescent intensity in real time. (b) Fluorescence spectra of DOX released from the C18-SH gated SNTs after treating with GSH (2 mM) for 12 h. As a control, the tripeptide (2mM) analogue of GSH, containing a methyl group instead of a thiol group, did not efficiently uncap the end at the given conditions. Serum containing media (RPMI FBS (10%) was compared along with SNTs.



Since the fluorescence signal of DOX molecules confined in the SNT at relatively high concentration (21.4 mM in this experiment) is suppressed through a self-quenching effect, an increase in fluorescence intensity is ascribed to the release of DOX from the SNT. After treating with GSH (1-20 mM), a significant intensity increase was observed, which reached a plateau above 5 mM as illustrated in Fig. S1(a).

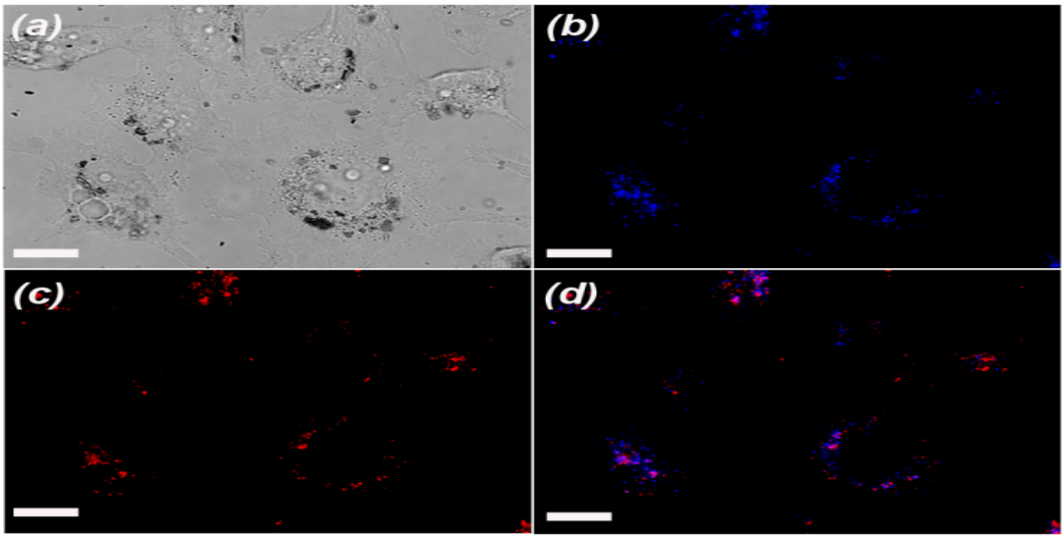
As shown in Fig. S1(b), serum presumably with thiol-containing species appeared to detach the C18-SH cap slightly. Fluorescence spectra were found to increase in the cell culture media containing serum proteins, indicating the partial release of DOX from SNTs. After treatment with GSH (2 mM), the fluorescence became intensified further, indicating the substantial release of DOX by uncapping the end.

Fig. S2. (a) TEM images of SNTs in a single mammalian cancer cell after incubation for 24 h. The arrows indicate the internalized SNTs. (b) TEM images at a magnification of 100k. The scale bars are 200 nm. Gold capping was seen at the end of SNTs.



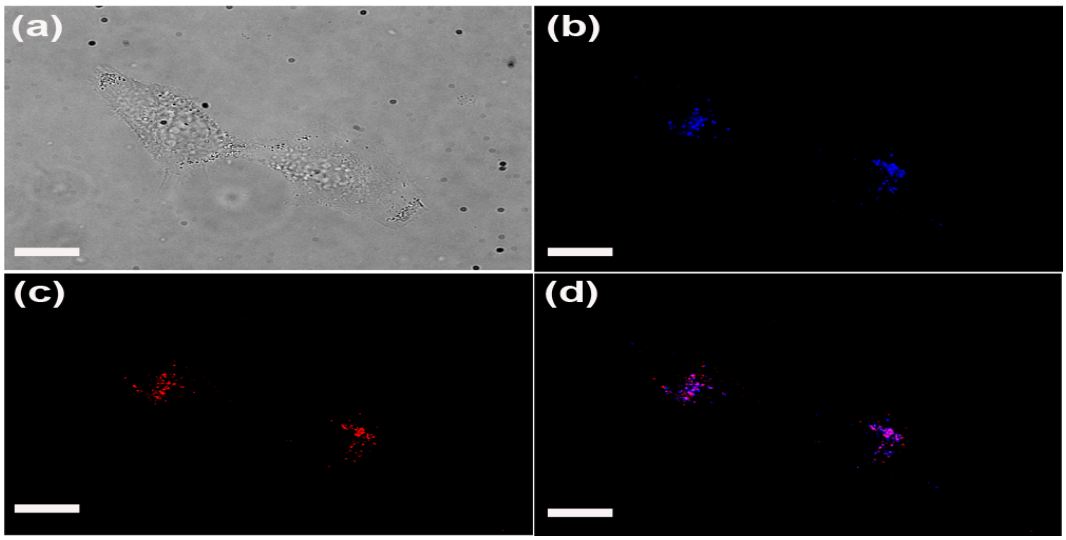
TEM images indicated a lysosomal entrapment in an aggregated state for the uptake of C18-SH-modified Au-capped SNTs in mammalian cells

Fig. S3. Colocalization images of lysosome-stained mammalian cancer cells after treating with SNTs-DOX. (a) Phase-contrast image, (b) lysosome, (c) SNTs-DOX, and (d) their overlaid images. Scale bars are 10 μm.



Fluorescence co-localization experiments of lysosome (lysotracker) with DOX were performed in order to check DOX to release at the intracellular compartment, showing significant overlap.

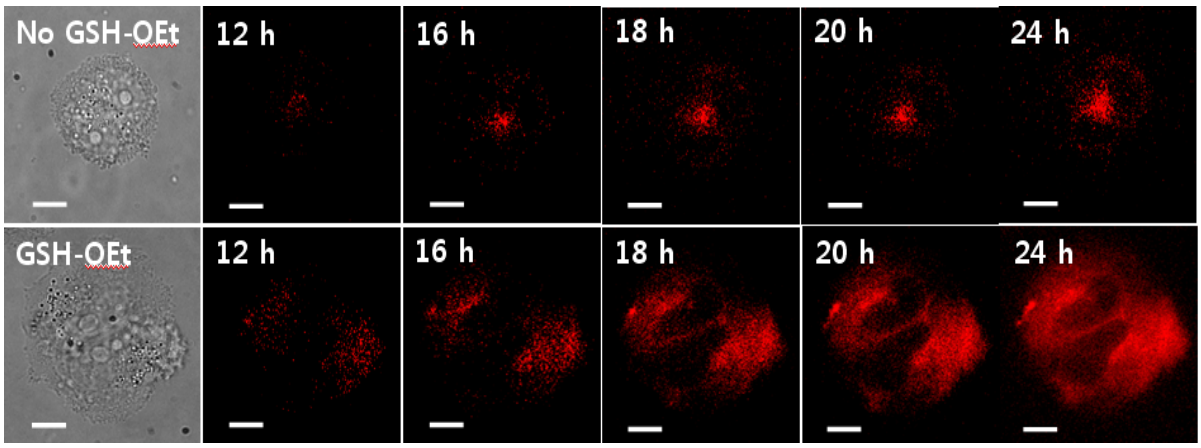
Fig. S4. Colocalization images of lysosome-stained mammalian cancer cells after treating with rhodamine B-tagged GSH-OMe. Scale bars are 10 μm.



In order to confirm that GSH reached the lysosome, we performed the colocalization experiments of rhodamine B-tagged GSH-OMe (red) with lysosome (blue). The red and blue emission patterns showed significant overlap. Colocalization images of SNTs and lysosome in mammalican cancer cells, (a) phase-contrast image, (b) lysosome, (c), rhodamine B-tagged GSH-OMe, and (d) their overlaid images.

Fig. S5. (a) Another example of the GSH-OEt-stimulated release of DOX from SNTs was examined by fluorescence live-cell imaging. Scale bars are 10 μm. (b) Plot of measured fluorescence intensities.

(a)



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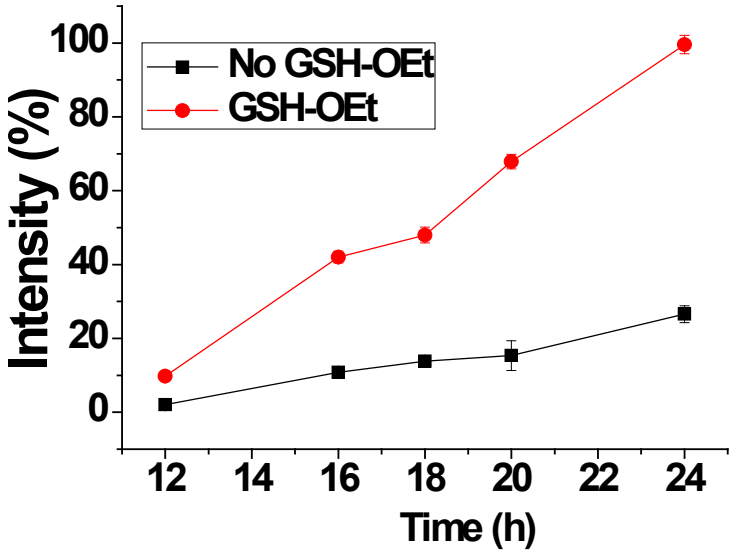
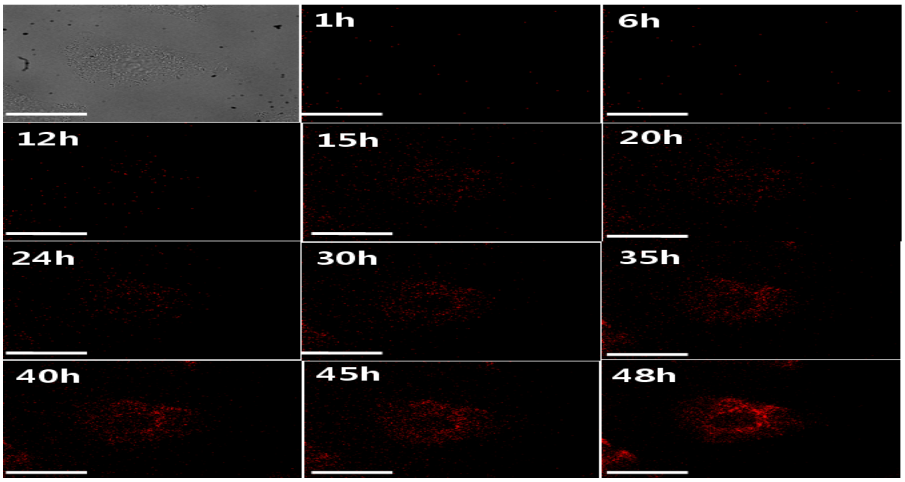


Time-lapse fluorescence live cell images in a single MCF-7/ADR cell indicating the *in situ* release of DOX from SNTs for 24 h after the treatment of the DOX-embedded SNTs.

Fig. S6. (a) The release of DOX from SNTs was examined by fluorescence live cell imaging in a single A549 cell. Scale bars are 10 μm. b) Plot of measured fluorescent intensities.

(a)



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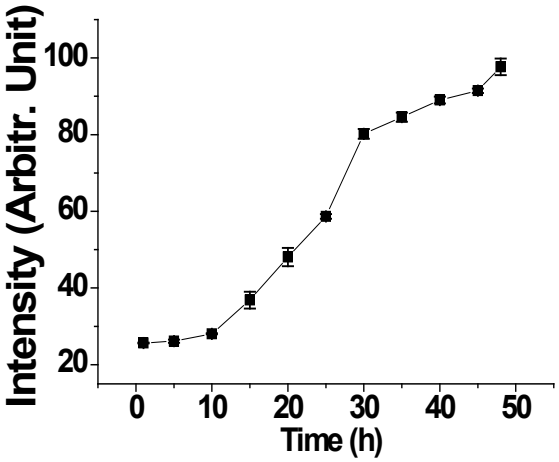
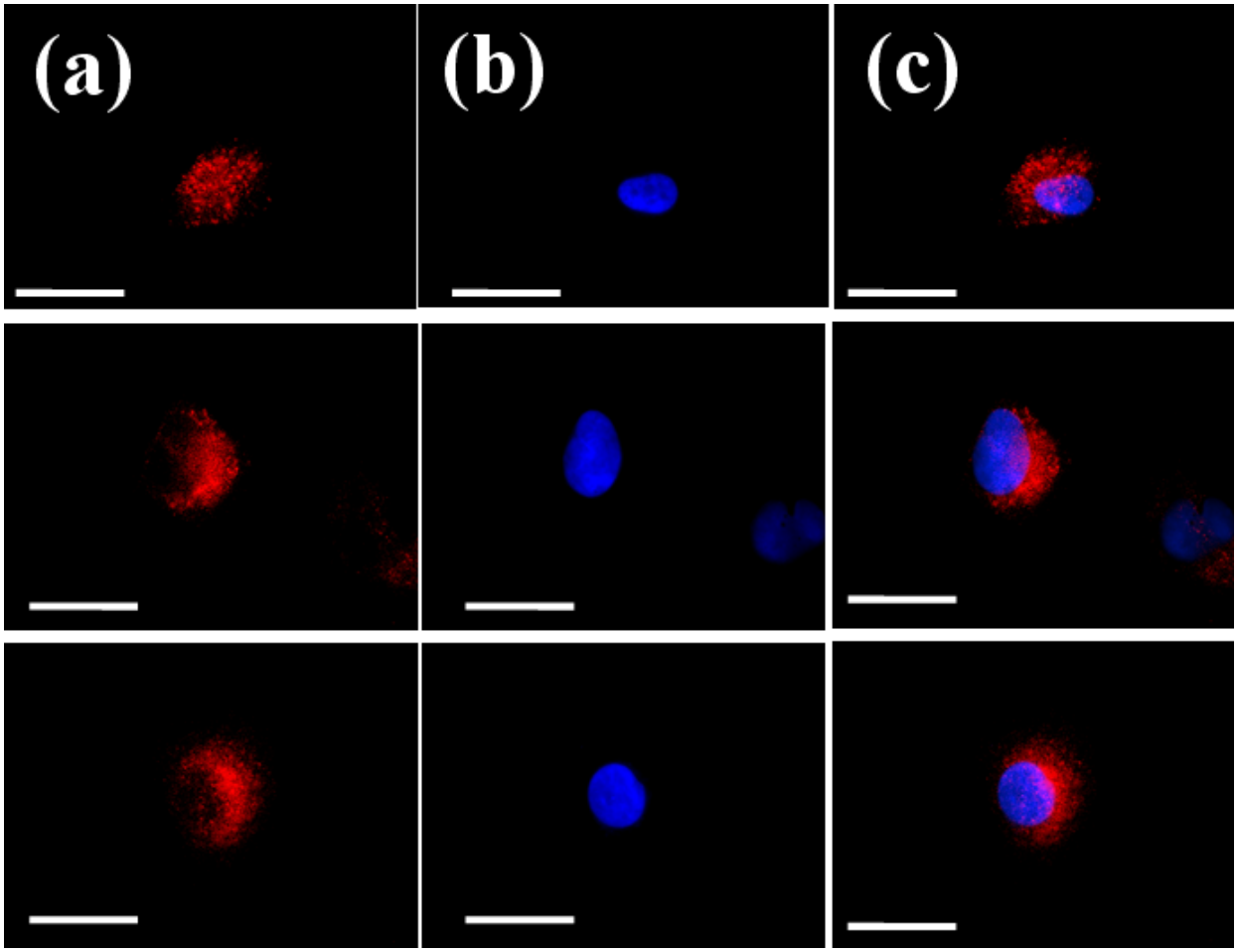


Time-lapse fluorescence live cell images in a single A549 cell indicating the *in situ* release of DOX from SNTs. At some certain local points inside a single A549 cell for 1-48 h after treatment of the DOX-embedded SNTs.

Fig. S7. Colocalization images of (a) SNTs-DOX, (b) DAPI-stained mammalian cells, and (c) their overlaid images. Scale bars are 10 μm.



This result indicates that DOX molecules released from SNTs in intracellular compartments could reach and damage nuclei.

Fig. S8. Pore diameter distribution of (A) native SNTs before incubation with 2-nm AuNPs and (B) SNTs with 2-nm AuNP seeds; Histogram for maximum (C) and minimum (D) pore diameters of SNTs after Au growth reaction.

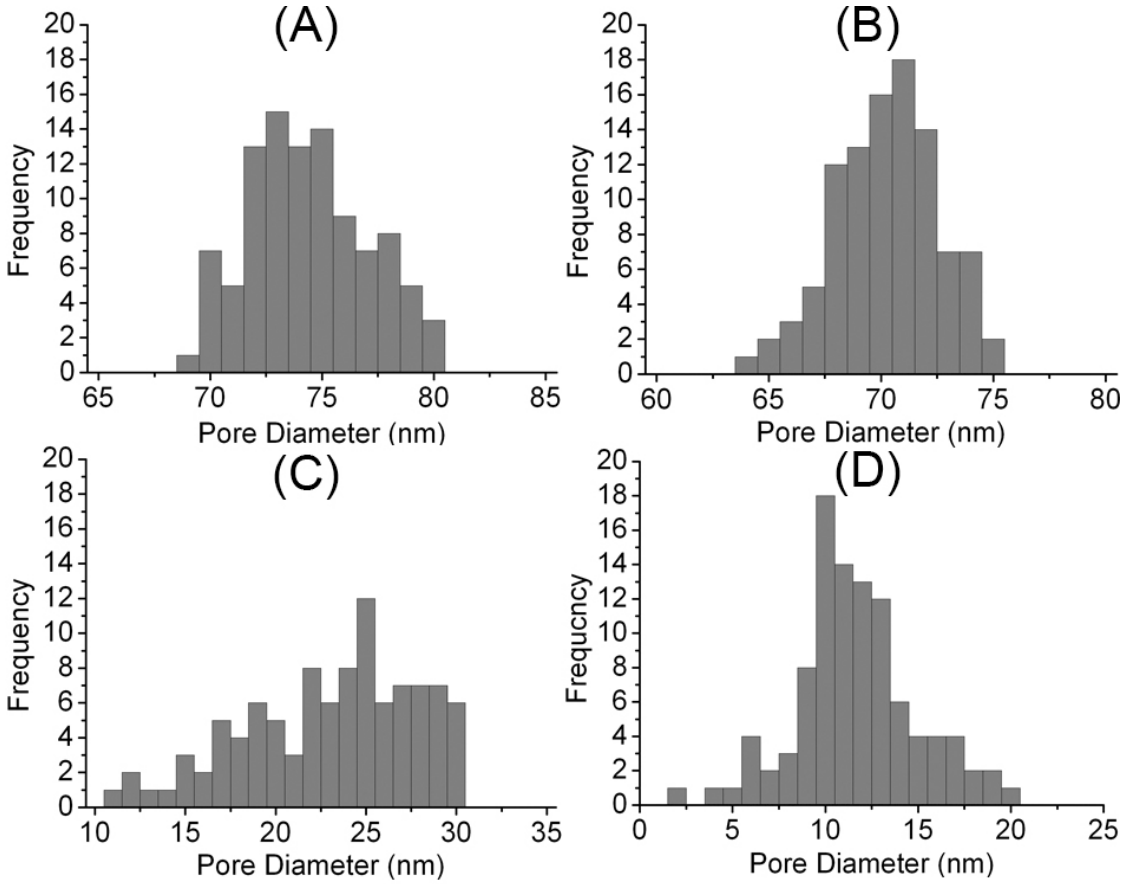


Table. S1. Pore diameter of SNTs.

Type	N total	Mean	SD	Minimum	Maximum
Native SNT before AuNP seeds	100	74.43	2.64	69.05	80.32
SNT with 2-nm AuNP seeds	100	70.24	2.30	64.13	75.17
SNT after Au growth reaction (Max. Values)	100	23.15	4.72	12.78	30.48
SNT after Au growth reaction (Min. Values)	100	11.61	3.27	1.58	20.51