Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Data

Title: Live-cell monitoring of the glutathione-triggered release of anticancer drug topotecan on gold nanoparticles in serum-containing media

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Experimental details

Materials:
The colloidal dispersions of AuNPs were prepared by the citrate reduction method. First, 30 mL of aqueous 14 mM $HAuCl_4$ (99.9 %, Sigma Aldrich) solution was boiled for the AuNP sample. Approximately 3 mL of 1 % sodium citrate solution was then added to the $HAuCl_4$ solution under vigorous stirring, and boiling was continued for ca. 1 h. Topotecan (>99%) was purchased from Selleck Chemical. TOPO dissolved in aqueous solutions was attached onto AuNPs by self-assembly and purified by centrifugation at 15000 rpm for 1 h. The Ellman reagent DTNB was purchased from Aldrich (Catalogue # 218200). The supernatant solution was decanted. GSH-OET and Rhodamine B dye-tagged GSH-OME were synthesized from Peptron (Daejeon, Korea). The endosomal marker (CellLight® Early Endosomes-GFP Catalogue # C10586) was purchased from Invitrogen (Grand Island, USA). A cell Counting Kit-8 (CCK-8) was purchased from Dojindo.

Physical characterization:
UV-Vis absorbance spectrum of the AuNP solution was taken using a Mecasys 3220 spectrophotometer. The dynamic light scattering (DLS) measurements were used to estimate the hydrodynamic radius of the AuNPs particles with an Otsuka ELSZ-2 analyzer. High-resolution TEM image was obtained with a Tecnai F20 Philips transmission electron microscope. The fabrication of TOPO on AuNPs via self-assembly was checked by attenuated total reflectance infrared spectroscopic tools using a thermoelectron 6700 Fourier-transform infrared spectrometer with a nominal resolution of 4 cm^{-1} and 256 scanning times. Surface-enhanced Raman spectra were obtained using a Raman confocal system model 1000 spectrometer (Renishaw) equipped with an integral microscope (Leica DM LM) and a 20 mW air-cooled HeNe laser (Melles Griots Model 25 LHP 928). The percentage of Au in the NP solutions was measured using the Perkin-Elmer Optima 4300DV ICP-AES.

Cell culture:
A549 (human lung carcinoma) cells were grown on RPMI 1640 in a steri-cycle CO_2 incubator (Thermo Fisher Scientific). All cells were supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin antibiotics (Gibco)/0.2 ppm plasmocin and maintained in 5 % CO_2 / 95 % humidified air at 37 ℃. Approximately 10^5~10^6 cells were washed with phosphate buffer saline and 0.25 % trypsin-EDTA was added for 3 min to detach the cells and counted by a hematocytometer. Dulbecco's phosphate buffered saline (DPBS) was used to wash seeded cells. After adding 1 mL of trypsin solution, the cells were incubated at 37 ℃ for 3 min and then 5 mL of the medium solution was added subsequently. Detached cells were transferred into a new culture dish and incubated at 37 ℃ with a 5 % CO_2 incubator.

Cytotoxicity and GSH assay:
We applied methylthiazol tetrazolium (MTT) and lactate dehydrogenase (LDH) leakage cytotoxicity assays of AuNPs. Intracellular GSH levels were measured using a fluorescence method. We used a fluorescence assay kit (Sigma Catalogue #CS1020)

with a Tecan F200 96 well plate reader at an excitation/emission of 360/485 nm using a fluorescent plate reader. Estimation of protein was done using a bicinchoninic acid (BCA) protein assay kit (Intron Biotechnology Catalogue # 21071) for the colorimetric detection and quantification of total protein using a Tecan F50 microplate reader.

Live-cell imaging:
A cover glass (Fisher) was embedded into a 100 mm × 20 mm size Petri dish. A 0.2 % gelatin was coated onto a cover glass and remained for 30 min at room temperature. After 30 min, a number of 3 x 10^5 cells were seeded onto a medium of a 10 % FBS solution and a 1 % antibacterial reagent at 37 ℃, 5 % CO_2 in an incubator for overnight. A cover glass seeded the cells was placed in a live cell chamber at a maintained temperature of 37 ℃. A live cell chamber (Model: Chamlide TC) was purchased from LCI instruments and used with a supply of 5% CO_2 gas.

Fluorescence images:
Cellular uptake of TOPO-coated AuNPs was also monitored using a conventional fluorescence microscopy. An Olympus IX-71 inverted microscope was employed with a mercury 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 NPs consists of 330-385 nm excitation, 400 nm dichroic, and 520 nm long pass emission (Olympus (U-MWB2). 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. A549 cells were seeded to a 35 mm confocal dish. On the next day, a volume of 2 uL Rhodamine B-GSH-OME was treated overnight. Then a volume of 2 uL endosome marker was incubated for 16h for the colocalization measurements.

Cellular utake of AuNPs:
The internalization of AuNPs was examined using a JEOL JEM-1010 transmission electron microscope. A549 cells were plated on a 100 mm culture dish (SPL, Korea) at a concentration of 1x10^4 cells per dish containing growth medium before the cells were exposed to the NPs. NPs were added, and the cells were incubated at 37^{o}C with 5% CO_2. After 24 h, the cells were washed twice with DPBS, fixed in Karnovsky's fixative for 24 h, and post-fixed in 0.5 M of osmium tetroxide. After fixation, specimens were rinsed with and dehydrated in a graded series of 30, 50, 70, 80, 90% ethanol and three times in 100% ethanol, for 15 min each. Samples were embedded in a mixture of resin in propylene oxide polymerized at 80 ^{o}C. Ultrathin sections for TEM measurements were prepared with a diamond knife. The relative uptake amounts for each AuNPs were also checked by a Varian 820 ICP-MS spectrometer.

***In vitro* fluorescence images:**
Cellular uptake of AuNPs was monitored using a conventional optical microscopy. An Olympus IX-71 inverted microscope was employed with a high sensitive CCD camera (CoolSnap HQ2, Roper Scientific). An objective lens (×40 or ×60) with a corrected thickness for the cover glass was used to obtain the image. The quantitative fluorescence measurements of TOPO were measured using a BD Biosciences LSR Fortessa cell analyzer at the excitation 355 nm and the emission at 530 nm.

***In vivo* fluorescence images:**

The fluorescence emission profiles of TOPO in living nude mice were obtained using a fully automated CRi Maestro^{TM} 2 in vivo imaging system at the detection wavelength between 500-800 nm using a Xenon illuminator excitation. A volume of 50 μL of TOPO (20 ng)-coated AuNPs (5.5 ug) was subcutaneously injected to mice. A volume of 16 μL of GSH (9.8 ug) was subsequently applied to the same spot. Considering the initial volume of the AuNPs and the TOPO mixture (66 uL) became diluted at least to 300 uL and the final AuNP and GSH concentrations could be estimated to be as low as 0.66 nM and 1 mM, respectively. The images were obtained with 30 min after the initial injection. We found that the fluorescence changes were not affected by the injected sites by switching the positions.

Figure captions

Fig. S1. Chemical structure of TOPO, GSH, tripeptide, and GSH-OET.

Fig. S2. Chemical structure and synthetic procedure of rhodamine B-GSH-OME.

Fig. S3. High resolution-transmission electron microscope image of AuNPs.

Fig. S4. UV-Vis absorbance spectra of initial and supernatant TOPO after being centrifuged and separated from adsorbates on AuNPs.

Fig. S5. UV-Vis spectra of pristine (AuNPs), TOPO-modified AuNPs (Au+TOPO), and 2 mM GSH-applied TOPO-coated AuNPs (2mM GSH).

Fig. S6. Release of TOPO in PBS buffer and RPMI (10 % FBS) for 30min-24 hr. 5 mM GSH is also compared.

Fig. S7. Ellman assay of thiol groups using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). We could find the strong absorption band at 400 nm not by serum proteins but by GSH.

Fig. S8. Time-dependent intensity plot of tripeptide and GSH.

Fig. S9. Au-N stretching band in the SERS spectrum of TOPO.

Fig. S10. TEM images of AuNPs in A549 cells. The arrows indicate the internalized AuNPs

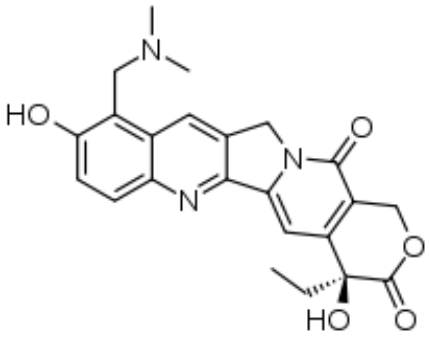
Fig. S11. Autofluorescence from (a) a single cell and (b) mice with those of TOPO.

Fig. S12. Co-localization experiments of endosome (GFP) and GSH-OME (rhodamine B).

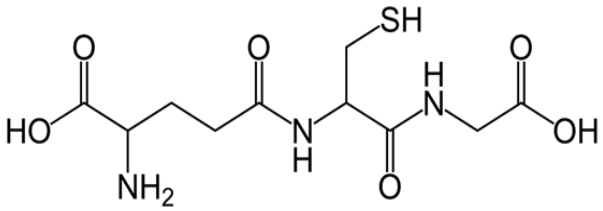
Fig. S13. Cell viability assay of A549 cells after treatment with free TOPO and TOPO-coated AuNPs (60 pM) for incubation for 48 h.

Fig. S14. Endosomal rupture of drug-conjugated AuNPs in A549 cells after treating with GSH-OET.

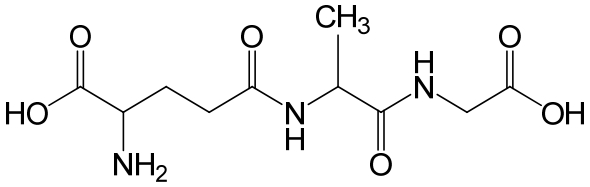
Fig. S1. Chemical structure of TOPO, GSH, Tripeptide, and GSH-OET.



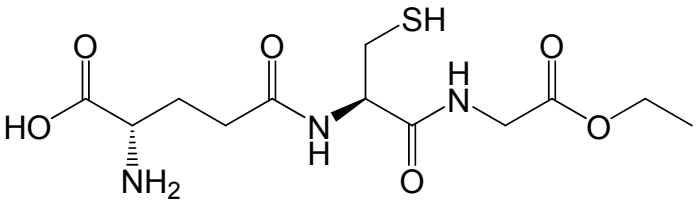
TOPO



GSH



Tripeptide



GSH-OET

Fig. S2. Chemical structure and synthetic procedure of rhodamine B-GSH-OME.

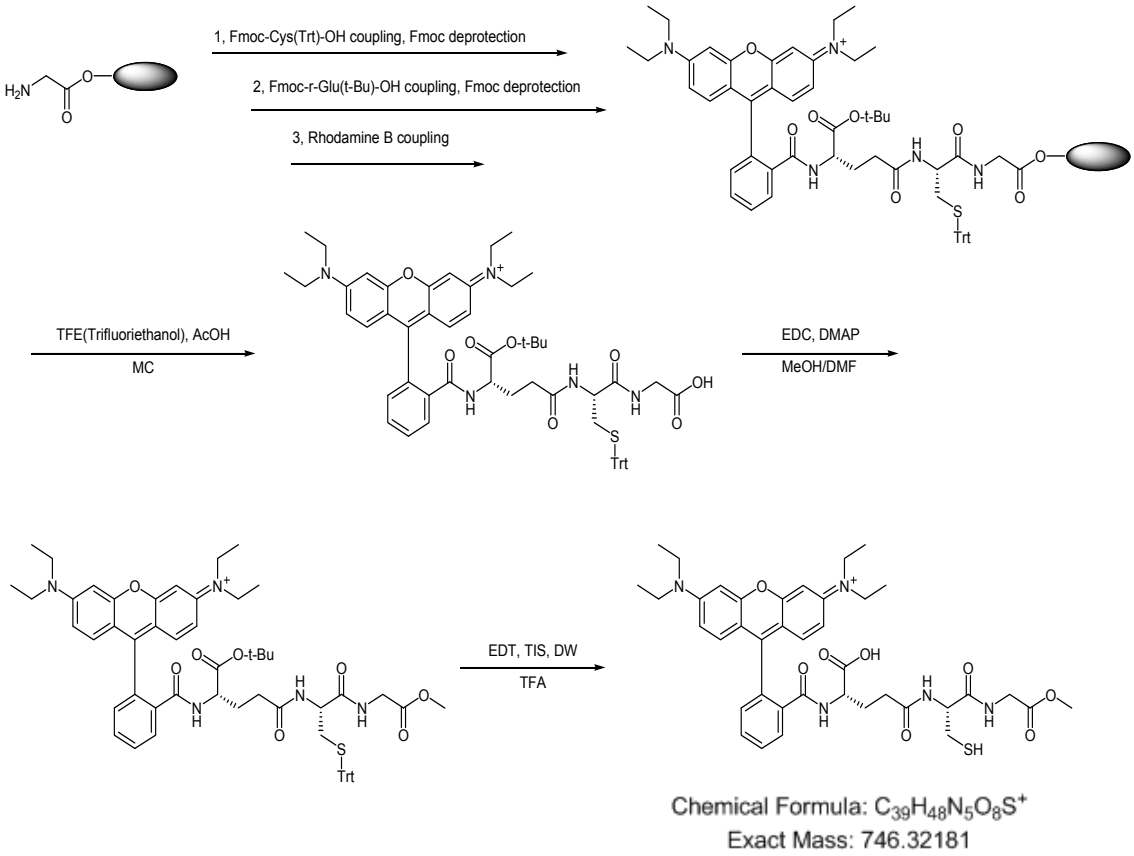
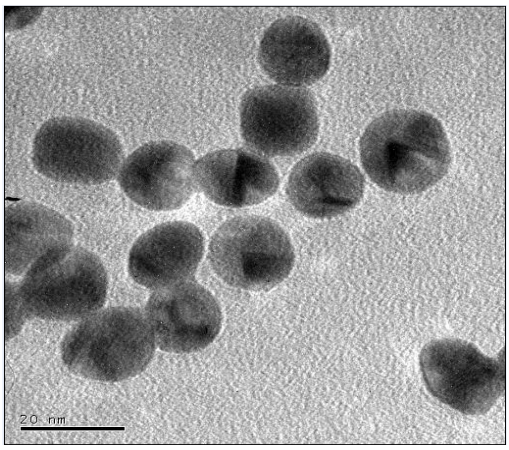
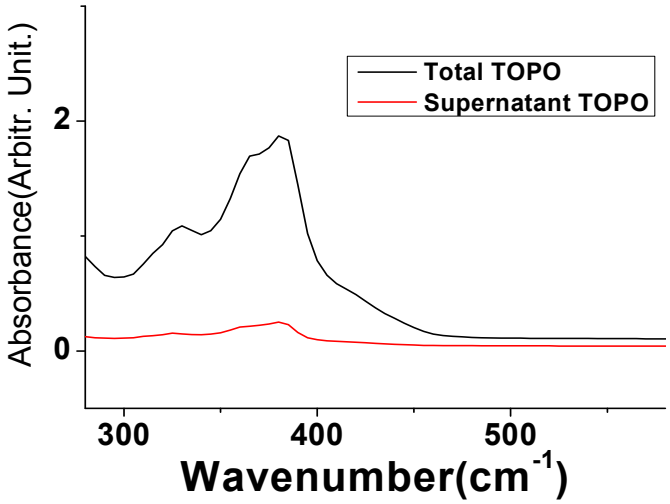


Fig. S3. High resolution-transmission electron microscope (HR-TEM) image of AuNPs.



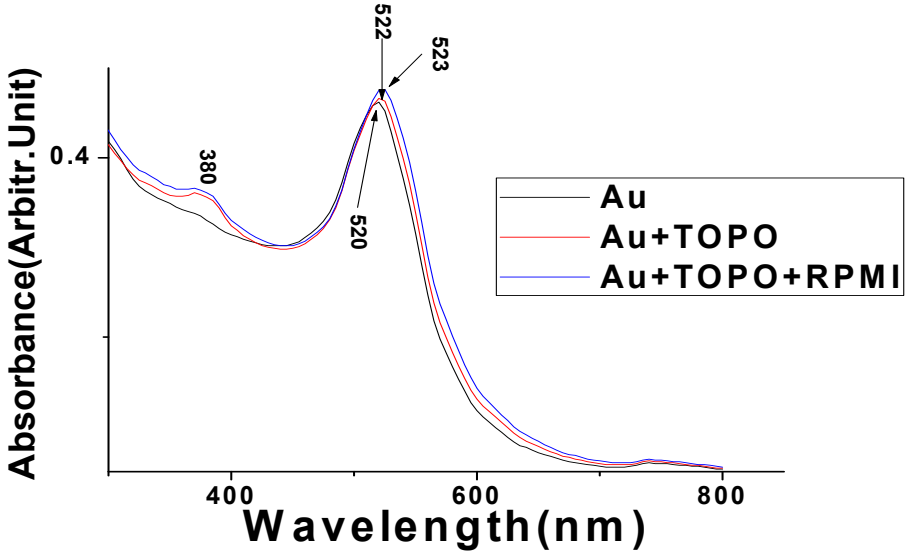
The average diameter is 15.0 (±1.1) nm. Not much difference was noted in the TEM images of either the TOPO-coated or serum protein-coated AuNPs. A single particle with an average diameter of 15 nm is composed of ~1.4 x 10^5 Au atoms and the concentration of the AuNPs is estimated to be approximately 4.0 x 10^{-9} M from the ICP-AES value of 111.4 ppm (ug/mL).

Fig. S4. UV-Vis absorbance spectra of initial and supernatant TOPO after being centrifuged from adsorption on AuNPs.

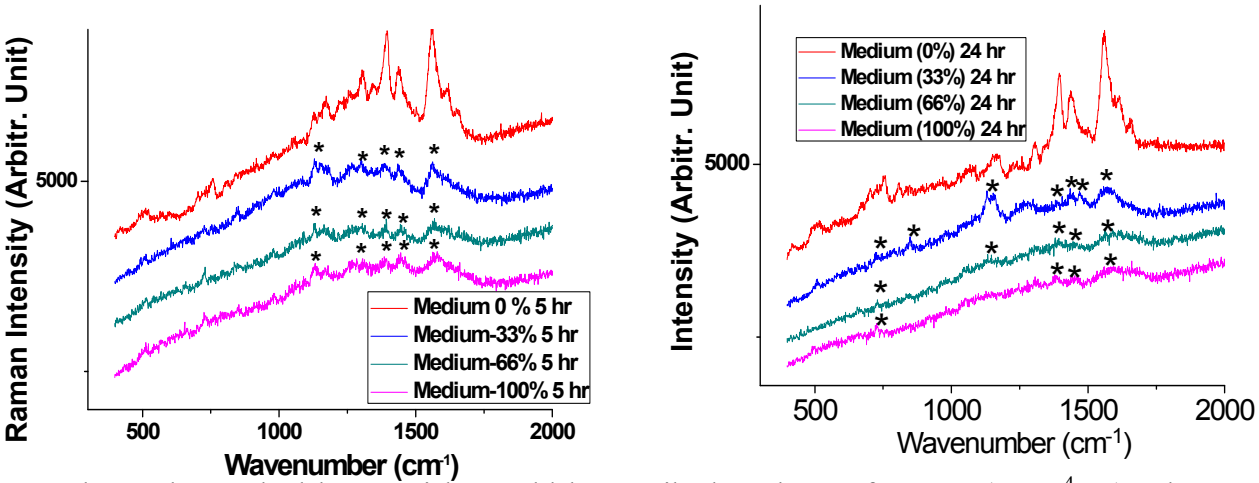


Loading efficiency (%) = [(total amount of TOPO added - amount of TOPO in supernatant)/total amount of TOPO added] x 100 = 86.6%

Fig. S5. UV-Vis spectra of pristine (AuNPs), TOPO-modified AuNPs (Au+TOPO), and TOPO-coated AuNPs in RPMI (Au+TOPO+RPMI).



The UV-Vis spectra were obtained from the AuNP samples assembled by TOPO and diluted in RPMI medium. The absorption band at 380 nm can be ascribed to TOPO. The absorption bands at 380 nm are ascribed to TOPO. A slight redshift of surface Plasmon bands was observed for AuNPs (1 nM) in 2 mL from 520 nm to 522 and 523 nm, after coating with TOPO (5.0×10^{-6}M) and RPMI medium (10 % FBS) solution (0.5 mL), respectively.



The peaks marked by asterisks could be ascribed to those of TOPO (2×10^{-4} M). The percentages of serum-containing media 0, 33, 66, and 100 % are the ratios with respect to AuNP (3.3×10^{-9} M) solutions.

Fig. S6. Release of TOPO (2 x 10^{-4} M) from AuNPs (300 pM) in RPMI (10 % FBS) for 30min-24 hrs. (a) UV absorption spectra and (b) stick diagram of the increased absorbance of TOPO at ~380 nm in PBS buffer and RPMI (10 % FBS) for 30min-24 hrs. The released amounts of TOPO could be estimated from the supernatant solution from AuNPs.

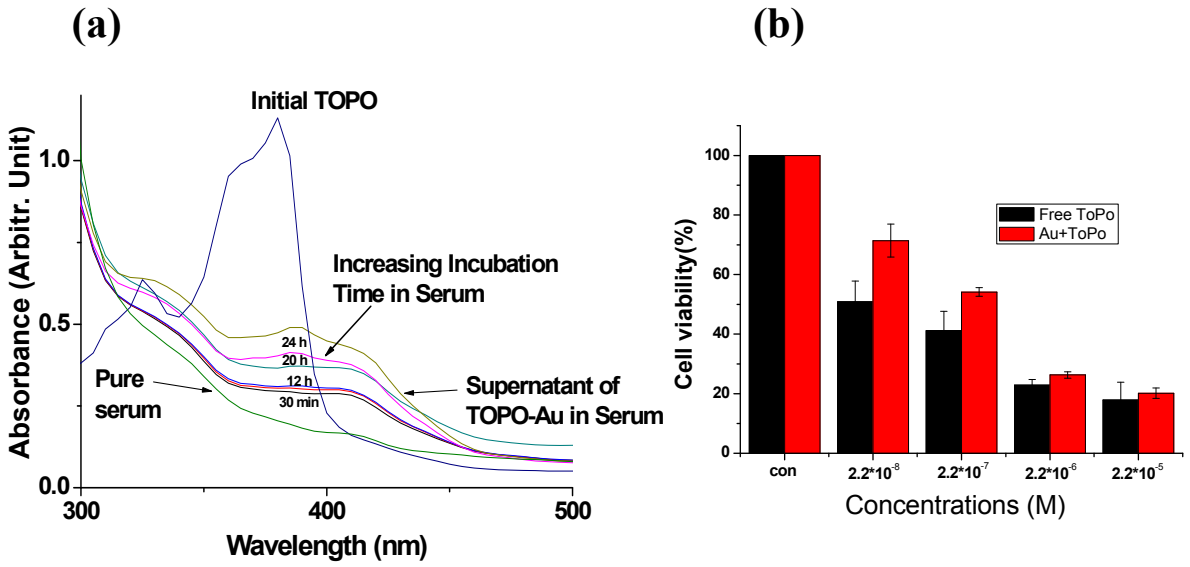
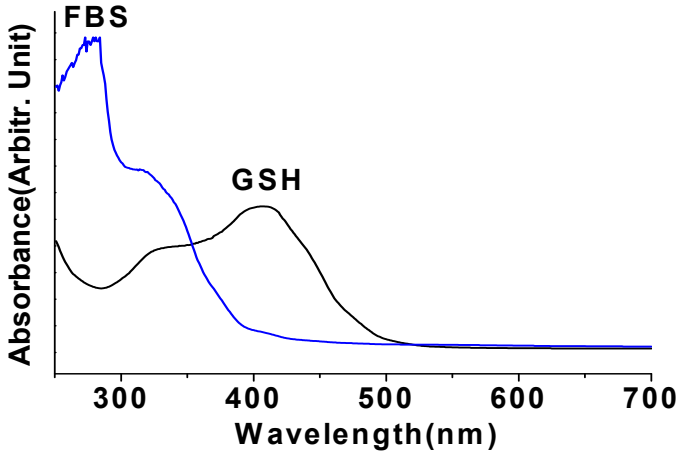


Fig. S7. Ellman assay of thiol groups by using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). We found the strong absorption band at 412 nm by GSH, not by serum proteins (FBS).



The Ellman reagent with a volume of 2mM DTNB (50 uL) and 1M Tris buffer (100 uL) was used to compare the thiol group amounts of GSH (30 ug) and fetal bovine serum (FBS) (50 ug).

Fig. S8. Time-dependent intensity plot of tripeptide and GSH. The error bars indicate the standard deviations of the three independent measurements. The excessive amount of GSH with respect to TOPO was used to simulate milimolar intracellular concentration conditions.

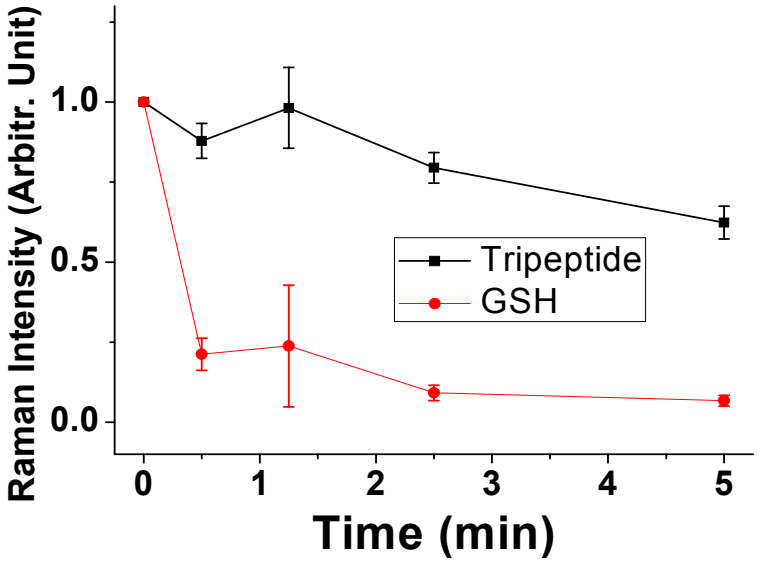
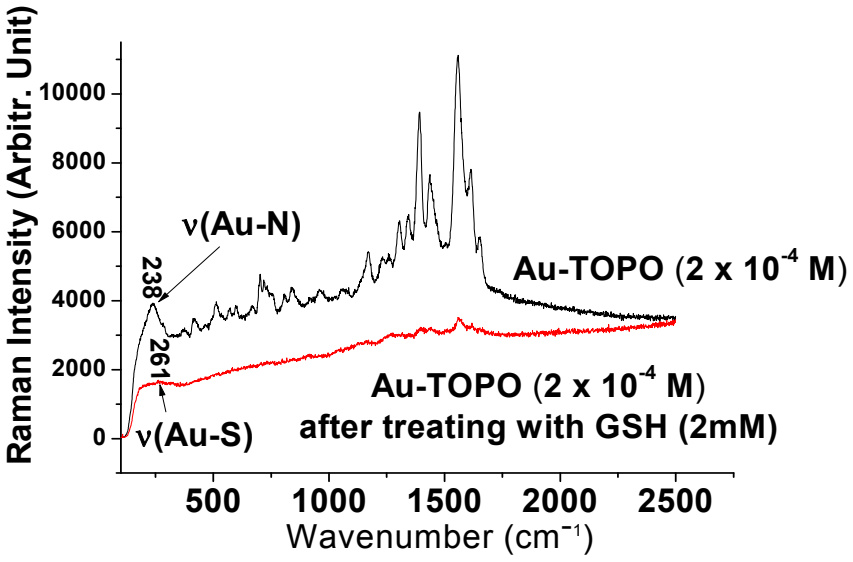


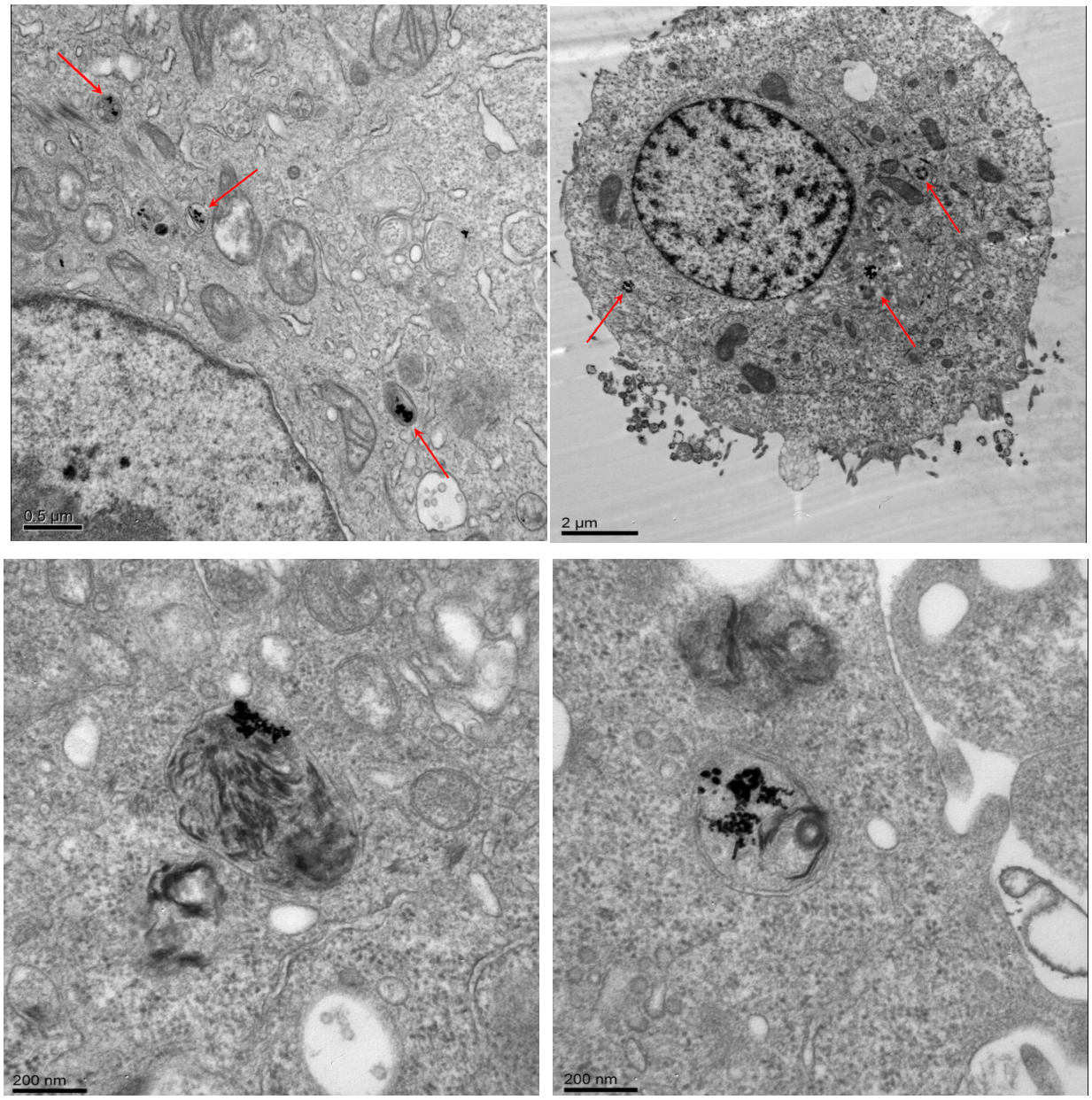
Fig. S9. Au-N stretching band in the SERS spectrum of TOPO.



Reference

(1) T. Yajima, Y. Yu, M. Futamata, *Phys. Chem. Chem. Phys*. 2011, **13**, 12454–12462
(2) S. Jang, J. Park, S. Shin, C. Yoon, B. K. Choi, M-s.Gong, S-W.Joo, *Langmuir* 2004, **20**, 1922-1927.

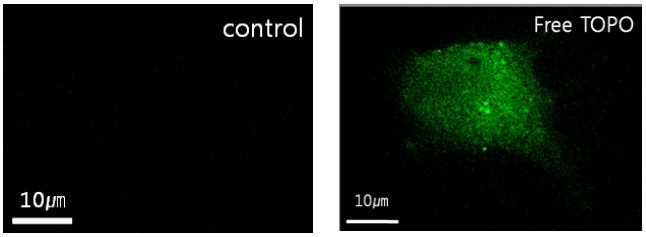
Fig. S10. TEM images of AuNPs in A549 cells. The arrows indicate the internalized AuNPs.



TEM images of TOPO-coated AuNPs encapsulated in either an endosome or a lysosome.

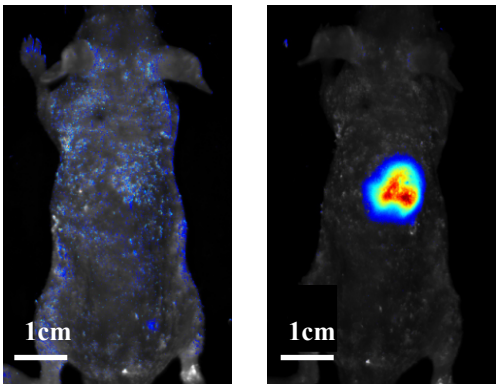
Fig. S11. Autofluorescence from (a) a single cell and (b) mice with those of TOPO.

(a)



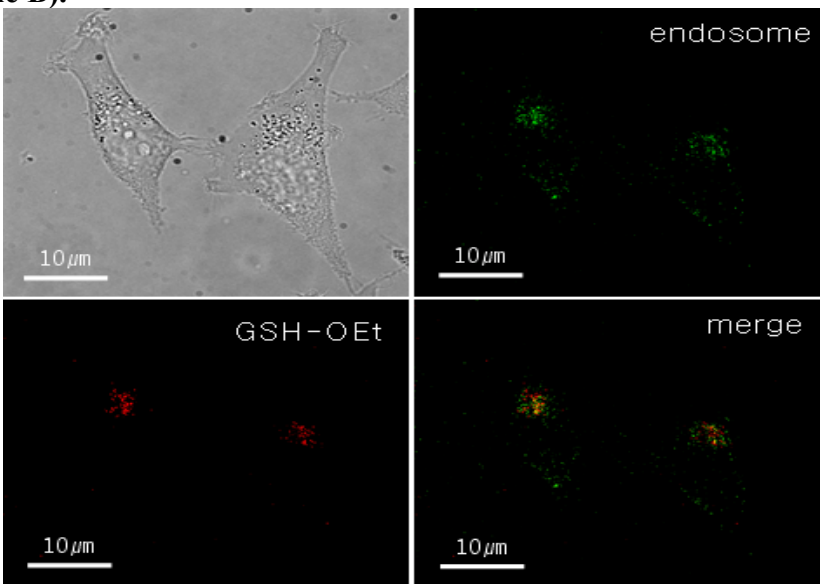
Autofluorescence Free TOPO (87 nM)

(b)



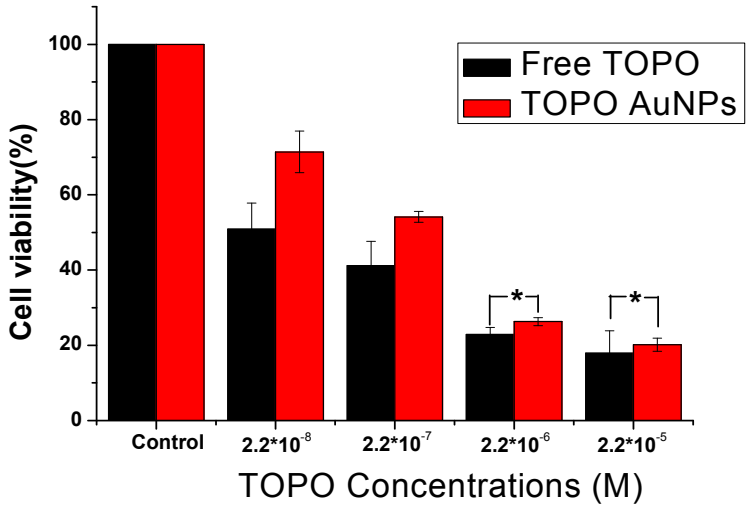
Autofluorescence Free TOPO (20 ng)

Fig. S12. Co-localization experiments of endosome (GFP) and GSH-OME (rhodamine B).



In order to check GSH to reach the endosome, we performed the co-localization experiments of rhodamine B-tagged GSH-OME (red) with endosomes (green). Red and green emission patterns show significant overlap.

Fig. S13. Cell viability assay of A549 cells after treatment with free TOPO and TOPO-coated AuNPs (60 pM) for incubation for 48 h. The control sample was unloaded sample without any AuNPs or TOPO. All data presented as means ± S.D. (*, $p < 0.05$).

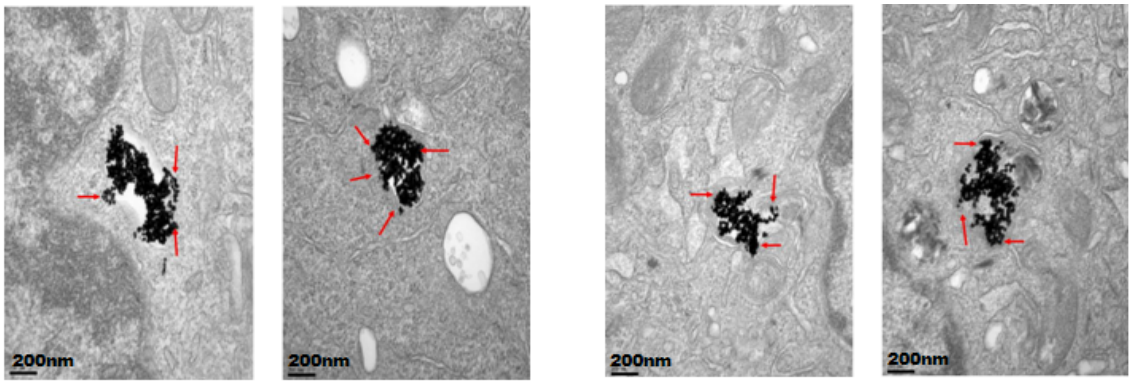


We also performed the cell viability test that TOPO-AuNP conjugates could reduce the cell viability of A549 cancer cells. IC_{50} values of AuNPs for 24 h were measured to be approximately 1.7 nM via our MTT and LDH assay (Reference (3) at the bottom of this page). To better discriminate the effect of cancer cell destruction, we chose to use a low concentration of AuNPs (60 pM) with a longer incubation time (48 h). The IC_{50} value of TOPO-AuNPs became increased from 2.2 x 10^{-8} M to ~2 x 10^{-7} M. The less reduction of cell viability of TOPO-AuNPs than free TOPO indicated that a part of TOPO remained onto AuNPs without much damaging the cells. The fluorescence images of nucleus-stained A549 cells indicated that TOPO-AuNP conjugates could damage DNA as in the case of TOPO. It was found that Au-TOPO system could reduce the cancer cell viability demonstrating that TOPO was still present through cell culture media to intracellular compartments."

Reference

(3) S. Y. Choi, et al. *In vitro* toxicity of serum protein-adsorbed citrate-reduced gold nanoparticles in human lung adenocarcinoma cells, *Toxicology in vitro*. 2012, **26**, 229-237.

Fig. S14. Endosomal Rupture of Drug-Conjugated AuNPs in A549 cells after treating with GSH-OET.



AuNPs outside the membrane suggesting the endosome rupture are marked as arrows.

Table S1: Diameters, size change after adsorption and stabilization of serum proteins of TOPO on AuNPs. DLS, of pristine AuNPs (20 pM), TOPO (4.1×10^{-7} M)-modified AuNPs (Au+TOPO), 2 mM GSH-applied TOPO-coated AuNPs (2mM GSH). Three independent measurements were performed to obtain the standard deviation.

Hydrodynamic Diameter of AuNPs (nm) [a]	Diameter after Adsorption of TOPO in RPMI with 10 % FBS (nm) [a]	Diameter after Treatment of 2 mM GSH
21.0±0.6	42.0±0.7	91.6±4.3

[a]Sizes are based on the DLS measurements. The transmission electron microscopy (TEM) images of TOPO-coated AuNPs exhibited an average diameter of ~15 nm. It was difficult to discern the aggregation difference from the TEM image due to the drying sample procedures.