

Supplementary Information

Nonidentical Intracellular Drug Release Rates in Raman and Fluorescence Spectroscopic Determination

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Experimental details:

NP Synthesis and Characterization. MTX dihydrochloride (>97%, molecular weight: 517.40) was purchased from Sigma Aldrich and used as received. The AuNP drug delivery platform was prepared by the citrate reduction method.¹ Absorption spectroscopy (Mecasys UV-3220 spectrophotometer) was used to check the surface plasmon band. Fluorescence measurements were performed by a Scinco FS-2 spectrometer. The hydrodynamic radius and the surface potential were measured using the quasi-elastic light scattering (QELS) measurements with an Otsuka ELSZ-2 analyzer. The atomic percentage of Au in NP solutions was measured using a Perkin-Elmer OPTIMA 4300DV ICP-AES.

Fluorescence Measurements. The fluorescence decay lifetime was measured using time-correlated single photon counting (TCSPC). A pulsed diode laser (635 nm, 50-ps pulsewidth) excited the MTX solution. Fluorescence signals from the sample were sent to a monochromator and then detected by a microchannel plate detector (R3809U, Hamamatsu). The instrument response function of the system was 132 ps (full-width at half maximum), as shown in **Fig. S1c**. Both 10- μ M MTX solution and 10- μ M MTX mixed with 5-nM AuNP solution (1:1 volume ratio) were prepared for fluorescence lifetime measurements. Cellular uptake of MTX-coated AuNPs was measured using an Olympus IX-71 inverted microscope. A single cell microscopic and multiple (1.5×10^3) cell measurements were performed by a Metamorph software and a Tecan F200 microreader, respectively.

DFM and SERRS Methods. Cellular uptake of NPs was also monitored using DFM with a Leica DL LM upright microscope and a high-resolution CytoViva 150 adapter. In most cases, the yellowish AuNPs can be discerned from the white color organelles in the DFM images. Raman spectra were obtained using a confocal system model 1000 spectrometer (Renishaw) equipped with an integral microscope (Leica DM LM). Spontaneous Raman scattering was detected with 180° geometry using a peltier cooled (-70°C) CCD camera (400 x 600 pixels). An appropriate holographic supernotch filter was set in the spectrometer for 633 nm from a 20 mW air-cooled HeNe laser (Melles Griots Model 25 LHP 928) with the plasma line filter. To test the z -dependence of the DFM image, we adjusted a micrometer to ensure that DFM images could be obtained inside the cellular parts. DFM images and corresponding SERS spectra at certain local points showed that the AuNPs are located inside a single HeLa cell upon uptake of MTX-modified AuNPs (Fig. S4). The maximum resolution of the DFM was ~ 90 nm ($\lambda/5$) with a narrower diffraction limit enforced by the annular aperture.² We were able to differentiate the nucleus from other parts of the cytoplasm owing to the color differences in the DFM image without the use of any fluorescent dyes. Raman mapping with a 1- μ m interval was performed for Cellular distributions of MTX-AuNPs using a Horiba Aramis dispersive Raman microspectrometer. Intracellular drug release of MTX from AuNPs was monitored using DFM and confocal Raman spectroscopy. Since the SERRS intensities of MTX were much larger than those of 6MP and 6TG, more accurate examination was possible to estimate the drug release rate for the first time in the present experiment.

Intracellular TEM Measurements: The size distributions of AuNPs inside the cell were checked using a JEOL JEM-1010 transmission electron microscope. Fig. S5 reveals solid evidence that AuNPs are mostly found in aggregated form inside vesicular structures (endosomes or lysosomes). Most MTX appeared to be released inside

cytosols regardless of the encapsulation, as suggested from the fluorescence microscopy image and cell viability test.

Cell Viability Test and In Vivo Measurements. HeLa cells (ATCC CCL-2) were grown on DMEM medium supplemented with 10% FBS and antibiotics at 37 °C in a 5% atmosphere Thermo Scientific Forma Steri-Cycle CO₂ incubator. DMEM and fetal bovine serum (FBS) were obtained from WelGene (Seoul, Korea). HeLa cells were plated at a concentration of 1×10^6 cells per dish on a 100 mm cell culture dish (SPL, KOREA) containing growth medium. For microreader measurement, 1.5×10^3 cells were seeded per well in a 96-well plate. AuNPs were added and the cells were incubated at 37°C with 5 % CO₂. After 24 h, the cells were harvested and washed with DPBS. Cell viability tests were performed using a highly water-soluble tetrazolium Donjindo CCK-8 kit. Considering the IC₅₀ values of MTX and the signal-to-noise ratio of the Raman intensities, our method could be applicable to physiological experiments.³ In vivo experiments were performed for six-week-old male nude mice (Orient Bio Inc, Gyeonggi, Korea). All animal experiments were performed in compliance with the guidelines of the Institute of Laboratory Animal Resources, Seoul National University (SNU-101221-1).

References

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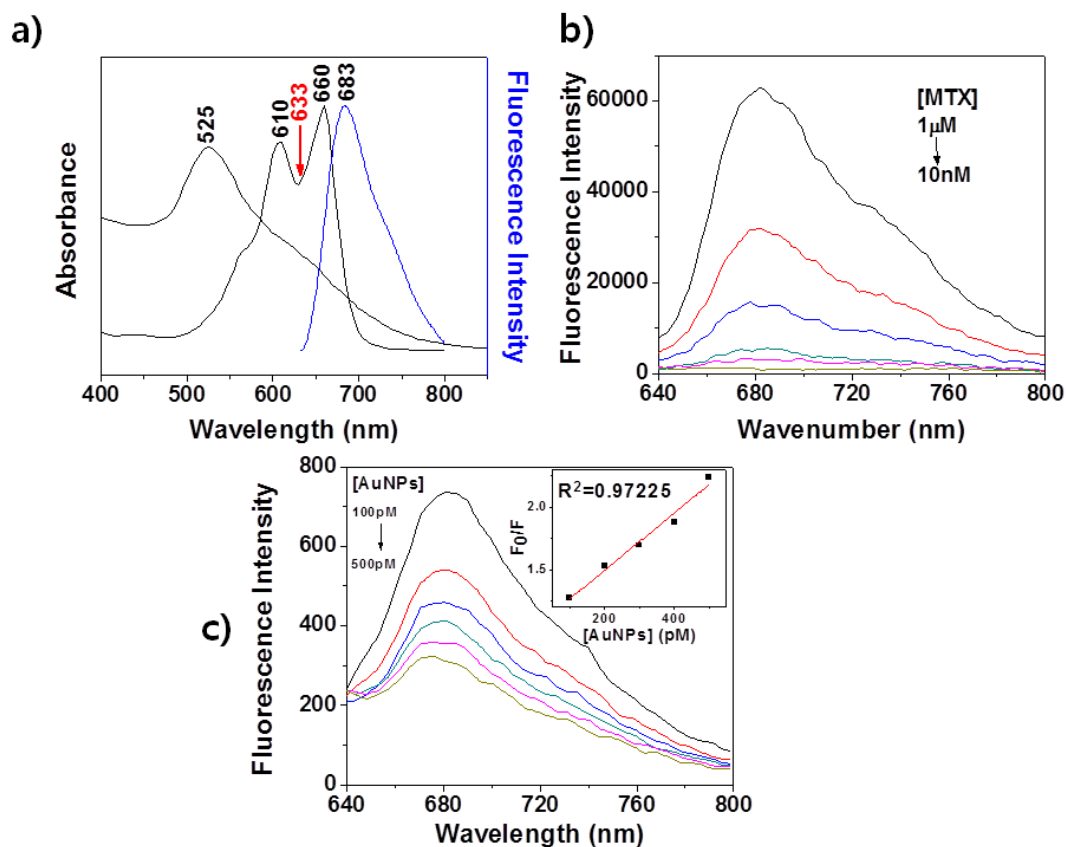
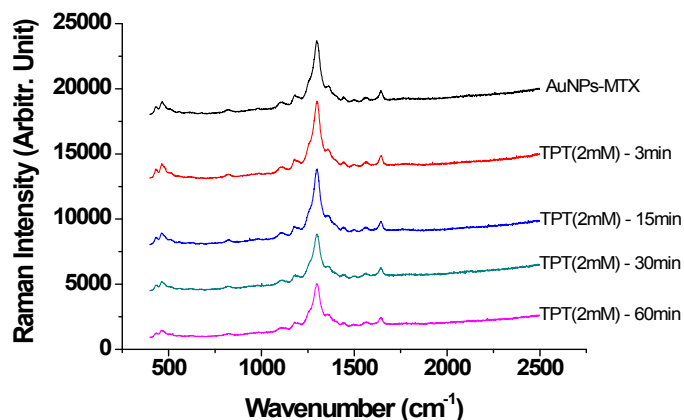


Fig. S1. (a) Absorption bands of MTX (red) and MTX-AuNPs (black). Emission spectrum of MTX (blue). The Raman excitation wavelength at 633 nm is marked by an arrow. The emission band of MTX overlaps with the absorption band of MTX-AuNPs. The estimated loading efficiency and amount of MTX released from the AuNPs were both $\sim 90\%$ based on UV-Vis absorbance measurements. (b) Concentration-dependent fluorescence spectra in MTX between 10 nM and 1 μ M. (c) Concentration-dependent fluorescence intensities of MTX (AuNP concentration: 100–500 pM). The inset shows the dependence of F_0/F on the AuNP concentration, where F_0 and F are the fluorescence emission intensities of MTX in the absence and presence of the AuNPs, respectively.



a)

b)

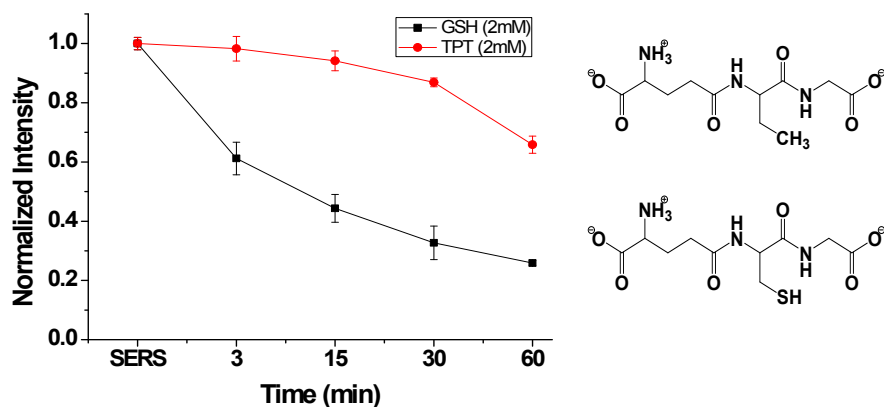


Fig. S2. (a) Control tripeptide (TPT) concentration-dependent SERS intensities of MTX ($\sim 10^{-5}$ M) on AuNP surfaces in an aqueous solution. (b) Plots of decrease in the band intensities depending on the GSH and TPT concentrations. The error bars indicate the standard deviation of the three measurements. The peaks at 1291 cm^{-1} were used to compare the relative intensities from MTX, respectively. TPT with the same concentration of GSH (2 mM) were used as a control. Raman spectra were obtained

after a few minutes from the injection time of peptides. The release mechanism of MTX from the MTX-AuNPs presumably originates from intracellular substitution by GSH, which is known to exist at a high concentration (1–10 mM) in the cytosol of cancer cells. Therefore, the MTX is rapidly released from the AuNP surfaces by GSH substitution and results in enhancement of the MTX fluorescence. The influence of the other peptide without the -SH group on the drug release was shown to be far less important.

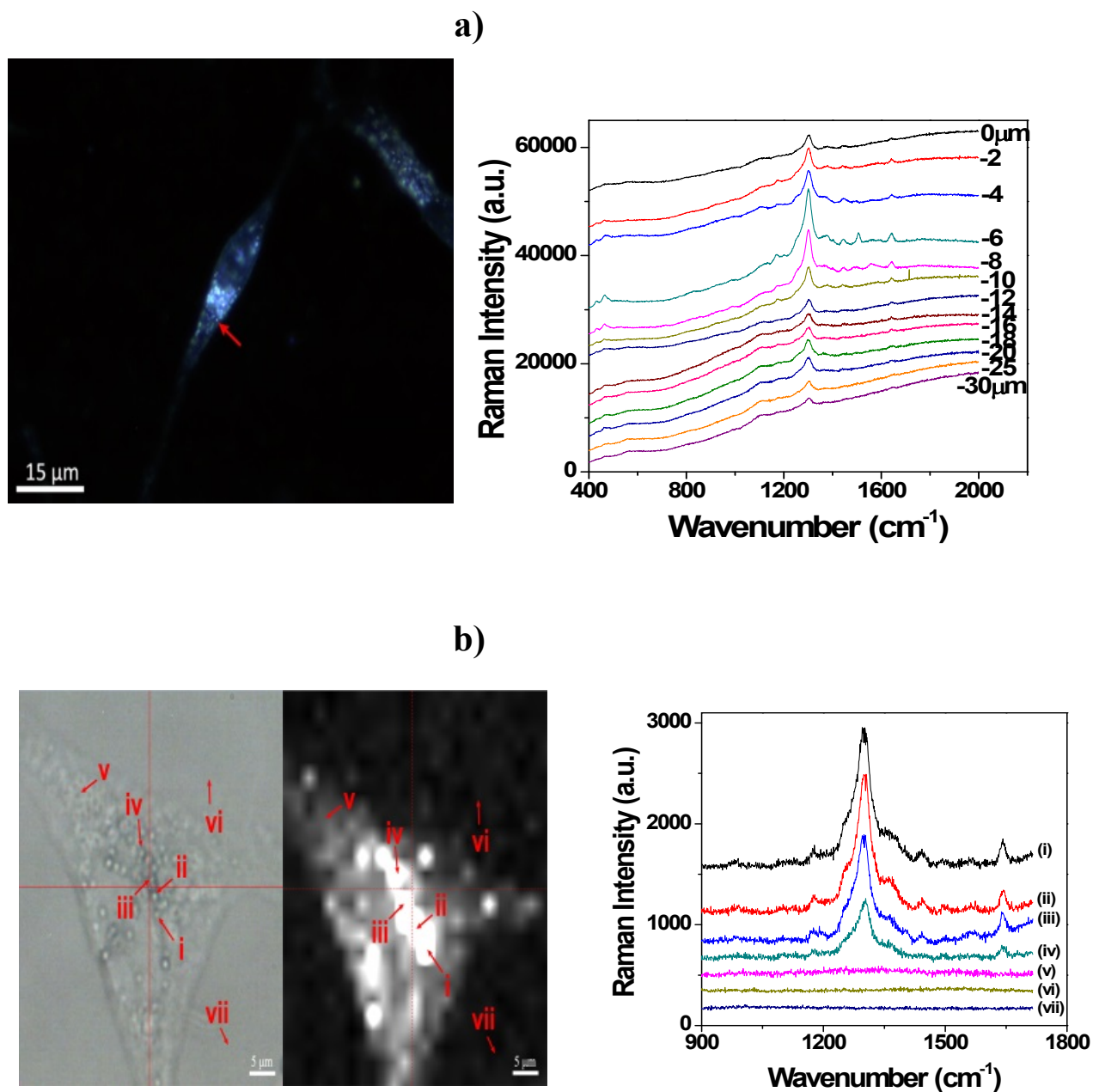
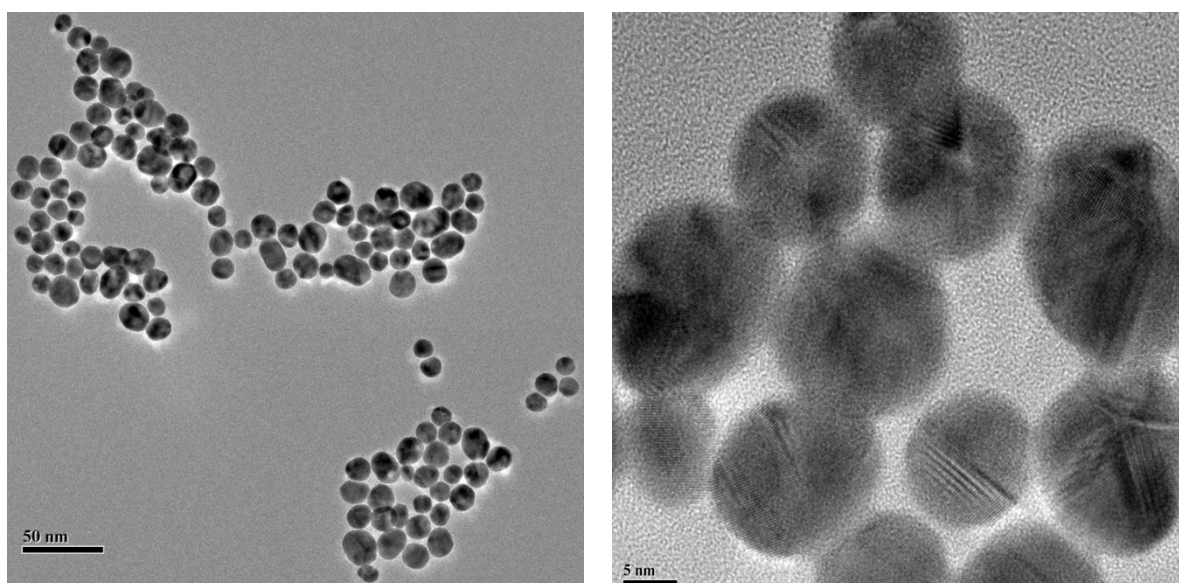


Fig. S3. (a) DFM (left) and z-depth dependent SERS (right) images of MTX (40 nM)-AuNPs in HeLa cells. (b) Raman mapping of MTX (40 nM)-AuNPs in HeLa cells (left). The brighter site indicates more MTX based on the ratio of the peak intensities at $\sim 1291 \text{ cm}^{-1}$. Actual SERS spectra at the points marked with the arrows of (i)-(vii). Please note that no MTX peaks were observed outside of the cell at (vi) and (vii).

i)



ii)

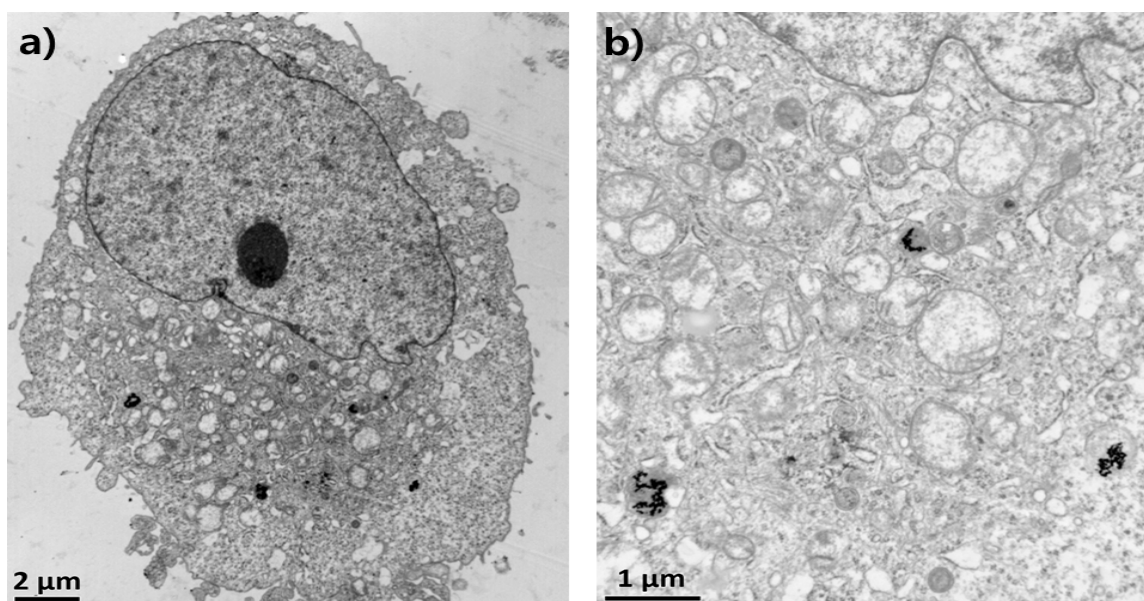


Fig. S4. (i) TEM images of AuNPs (a) in aqueous solution and (ii) inside either endosome or lysosome in cancer cells. The average diameter of ~ 15 nm, determined from TEM images, gives an AuNP concentration (~ 140 ppm from an inductively coupled plasma-atomic emission spectrometer measurement) of 5×10^{-9} M. AuNPs in a vesicular structure may form an aggregated structure with hot spots to exhibit extraordinary SERS intensities. (ii) (a) TEM image of MTX-coated AuNPs encapsulated in either an endosome or a lysosome. (b) Expanded view of the encapsulated structures.

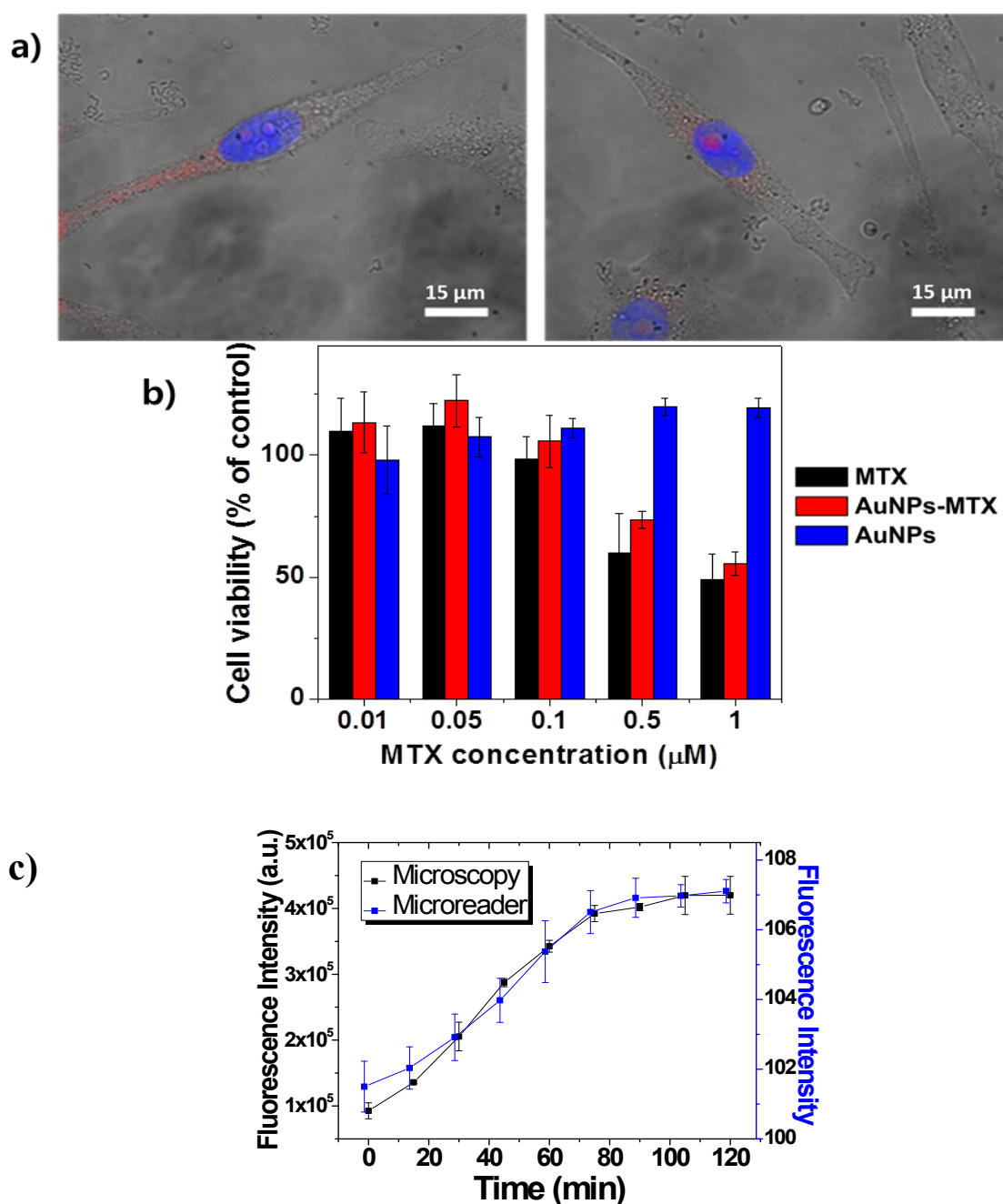


Fig. S5. (a) Overlaid fluorescence microscopy images of MTX (red) and 4',6-diamidino-2-phenylindole (DAPI) (blue) in HeLa cells on TEM images of the AuNPs. We also noticed from the overlaid fluorescent image that the MTX drug was safely released from the MTX-AuNPs to the cellular nuclei. (b) Cytotoxicity of MTX (40 nM)-AuNPs in HeLa cells. The used AuNPs were practically nontoxic in the HeLa cells according to the CCK-8 data. The 40-nM MTX concentration did not damage the cell under our experimental conditions. The comparable cytotoxicity data of the MTX-AuNPs and MTX itself implied that the drug efficiently detached from the AuNPs to induce cell apoptosis. (c) Comparative plot of fluorescence intensities by a microscope (single cell) and a microplate reader (1.5×10^3 cell).