SUPPORTING INFORMATION

Multi-Stimuli Responsive Cu_2S Nanocrystals as Trimodal Imaging and Synergistic Chemo-Photothermal Therapy Agents

Aby Cheruvathoor Poulose[1◊], Srivani Veeranarayanan[1◊], M. Sheikh Mohamed[1], Yutaka Nagaoka[1], Rebeca Romero Aburto[2], Trevor Mitcham[3], Pulickel M. Ajayan[2], Richard R. Bouchard[3], Yasushi Sakamoto[4], Yasuhiko Yoshida[1], Toru Maekawa[1], D. Sakthi Kumar[1]*

[1]*Bio Nano Electronics Research Centre, Graduate School of Interdisciplinary New Science, Toyo University, Kawagoe, Japan- 350-8585*

[2]*Department of Material Science and NanoEngineering, Rice University, 6100 Main Street, Houston, TX 77005, USA*

[3]*Department of Imaging Physics, University of Texas MD Anderson Cancer Center, Houston, TX 77054, USA*

[4] *Biomedical Research Centre, Division of Analytical Science, Saitama Medical University, Saitama 350-0495, Japan*

◊ **Both Authors Contributed Equally**

Corresponding author*

Prof. D. Sakthi Kumar,

Ph: 81-492-39-1636

Fax: 81-492-34-2502

E-mail: sakthi@toyo.jp

METHODOLOGY

Materials:

Cu (I) Cl, chloroform, ethanol, dimethylsulfoxide were purchased from Kanto chemicals, Japan. Oleylamine and octadecene were purchased from Tokyo chemical industry, Japan. Sulfur powder, doxorubicin hydrochloride and live/dead cell double staining kit were purchased from Aldrich. All chemicals and reagents were of analytical grade. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG-Fol) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG-Amine) were purchased from Avanti Polar Lipids, Alabama. Alamar blue, Lysotracker and DAPI were from Invitrogen.

Synthesis of Cu_2S NCs:

In a typical procedure, Cu_2S NCs were prepared by hot injection method using organic solvents. 0.2 mmol of Cu (I) Cl was dissolved in 10 mL of octadecene with 1.2 mL of oleylamine by stirring at 100 ^oC under vacuum for 30 min. This solution served as copper precursor. The sulfur precursor was prepared using 0.5 mmol of sulfur dissolved in 5 mL of octadecene under vigorous stirring and heating which yielded a transparent sulfur precursor solution. 1 mL of sulfur precursor solution was quickly injected into copper precursor solution and the reaction was carried out at 180 ^oC for 1 h under Ar atmosphere. The reaction was quenched in a cold-bath and the NCs were precipitated with ethanol and centrifuged thrice and obtained precipitate was re-dispersed in chloroform.

Lipid-PEGylation of Cu_2S NCs:

The lipid-PEGylation of Cu_2S NCs was performed using thin-film hydration method. The schematic representation of the same is provided in **Scheme S1**. Typically, DSPE-PEG-Amine dissolved in chloroform (1 mg/mL) was added to 1 mg/mL of the NCs suspended in chloroform. The solution was gently evaporated to obtain a thin film of NCs and lipid-PEG. This thin film was then hydrated with PBS buffer and the suspension was sonicated for 15 min. The PEGylated Cu_2S NCs were

separated by repeated centrifugation and washing. A clear aqueous suspension of NCs functionalized with PEG were obtained. To obtain folate targeted Cu_2S NCs, same procedure of thin-film hydration was followed with DSPE-PEG-Fol substituting DSPE-PEG-Amine.

Characterization and Instrumentation:

The morphology of as prepared Cu_2S NCs was analyzed with the help of field emission transmission electron microscope (TEM), (JEOL JEM-2100) and scanning electron microscopy (JEOL, JSM-7400F). The elemental composition of the NCs was analyzed using EDS (JEOL JED-2300T). The zeta potential and average hydrodynamic size of the NCs before and after PEGylation was determined by Nano-ZS 168 Zetasizer (Malvern Instruments Ltd). To investigate the surface chemical bonding and characteristics of bare and PEGylated NCs Fourier transform infrared spectroscopy was performed with the help of Spectrum 100 FT-IR Spectrometer (Perkin Elmer) connected with Universal ATR Sampling Accessory. The absorption spectrum of the Cu_2S NCs was analyzed using a UV-Vis-NIR spectroscope (JASCO V-570 UV/Vis/NIR spectrophotometer). Photoluminescence spectra of Cu_2S NCs were recorded with excitation wavelength 365 nm using JASCO FP 750 spectrofluorometer. X-ray photoelectron spectroscopy (XPS) measurement was carried out using Kratos, Shimadzu with anode mono Al, pass energy 40, Current 10 mA and Voltage 12 kV. The powder X-ray diffraction (XRD) pattern was carried out on a Rigaku (RINT) diffractometer equipped with a rotating anode. The 2θ angle for the XRD spectra was recorded at a scanning rate of 5° /min. UV–Vis spectrophotometry (DU 730 Life Science, Beckman Coulter UV/Vis spectrometer) was carried out to analyze the DOX loading and release. Cell viability assessment was done with a microplate spectroflurimeter (Multidetection microplate scanner, Dainippon Sumitomo Pharma). High-speed confocal laser-scanning microscope (CLSM, Olympus IX 81 under DU897 mode) was used to analyze particle uptake, NC-drug trafficking, visible light photoexcitation and DOX release study and to analyze live/dead stained cell population. A highly monochromatic, collimated beam of NIR range (800 nm) [Chameleon Ultra diode-Pumped Mode Locked-Sub Femtosecond Laser (Coherent 80 MHz repetition rate)] with power 2027 mW (Laser power meter: VEGA, OPHIR, Japan) was utilized for all PTT experiments.

PEGylated Cu_2S NCs at a concentration of 1, 10, 30, 50 and 100 ppm was utilized to analyze the efficiency of the NCs to convert photo energy into thermal energy whereas the heat induced drug release was studied using PEGylated-Cu_2S-DOX NCs (1 mg/mL in pH 4 solution). The temperature variations were measured with an infrared (IR) thermometer [Thermal imager test 881-2 (Testo AG, Germany)]. Photoacoustic imaging was performed using VisualSonics Vevo LAZR- 2100 high-frequency photoacoustic system (VisualSonics Inc., Toronto, Canada). In the photoacoustic imaging study, PEGylated Cu_2S was injected into vinyl tubing that did not generate a significant PA signal. Once the tubing was filled, the tubes were sealed at the ends with epoxy and suspended in a container filled with a mixture of water with milk (2% fat by volume), added for optical scattering. The sample was imaged spectroscopically with a 21 MHz linear array from 680 to 970 nm with a step size of 2 mm and a PA gain of 25 dB to determine the peak signal produced by the NCs. The microCT imaging of PEGylated Cu_2S was analyzed at 45 KeV tube voltage using microCT syatem (ScanCo, Switzerland). The PEG- Cu_2S NCs which was partially dried and the slightly moistened NCs were loaded into phantom tube and were analyzed.

DOX loading:

The loading of anti-cancerous drug, DOX was carried out based on the principle of hydrophobic interactions that can happen between hydrophobic DOX and lipid moieties (of Lipid-PEG conjugate) on NCs. To the suspension of PEG-Cu_2S-Fol (10 mg/mL), 1 mg of DOX dispersed in 0.1 mL of DMSO was added and the suspension was allowed to mix overnight in a rotator. Post loading, the conjugate was repeatedly washed to remove unbound drug and the pelleted PEG-Cu_2S-DOX-Fol was dried and stored at 4° C until use. The drug-loading efficacy was calculated using the following formula:

$$\text{DOX loading efficacy (\%)} = \frac{\text{Absorbance of DOX bound to NCs}}{\text{Absorbance of total DOX used}} \times 100$$

DOX release study:

The standard absorption of the drug, DOX at various concentrations was plotted and the drug release profile at varied time intervals was analyzed using the

standard curve of the drug's absorbance. pH 7, depicting the physiological pH, pH 4, 5 and 6, depicting the acidic pH of cancer cells were utilized for drug release studies. 1 mL of freshly prepared PEG-Cu_2S-DOX-Fol NCs in respective pH solutions was dispersed and the supernatant thus obtained after centrifugation of the conjugate at different point of time (0 - 120 h) were analyzed using UV–Vis absorption spectroscopy. For photosensitization mediated DOX release studies, 1 mL of freshly prepared PEG-Cu_2S-DOX-Fol NCs in pH 4 solution was irradiated with 488 nm light for 10 min. For NIR-mediated DOX release, the pH 4 suspension of PEG-Cu_2S-DOX-Fol NCs was irradiated with 800 nm light for 10 min. The non-irradiated sample, pH 4 suspension of PEG-Cu_2S-DOX-Fol NCs, was also used to quantify the pH-mediated DOX release for 10 min. After the experiment (photosensitization, NIR and pH-mediated release studies), the suspension was centrifuged and the supernatant is read to quantify released DOX. The percentage of released DOX after 10 min by pH, photosensitization and NIR exposure was plotted (**Figure S12**).

Cell studies:

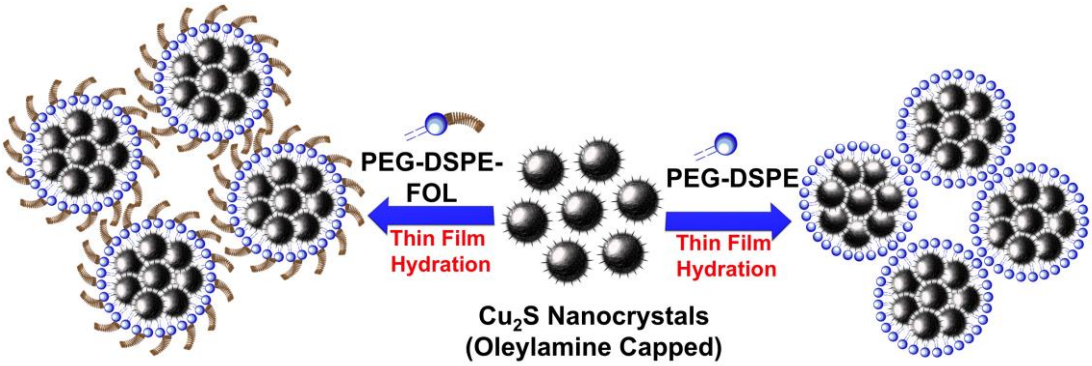
HCN-1A human cortical neurons were purchased from ATCC and Gl-1 human glioma cell line was purchased from Riken Bioresources. Cells were grown under ambient conditions using DMEM medium supplemented with 10% FCS. For toxicity analysis, approximately 10,000 cells were plated onto each well of 96 well plates and allowed to grow until visual confluency. Post visual confluency, cells were treated with NCs (PEG-Cu_2S-NCs) of varying concentration (0.01 – 1 mg) for 7 d. Relative cell viabilities were determined by the standard Alamar blue assay. For analyzing the drug-induced toxicity, cells were treated with PEG-Cu_2S-Fol-DOX NCs/free DOX at varying concentration (Free DOX: 2 – 10 μg/mL and PEG-Cu_2S-Fol-DOX NCs: 0.2 - 1.2 mg/mL of NCs carrying 2 – 10 μg/mL DOX). The drug mediated cytotoxicity was read post 24 h and the corresponding IC_{50} values of PEG-Cu_2S-Fol-DOX NCs/free DOX against Gl-1 cells were quantified based on the 50 % cell killing concentration of DOX. For NC entry imaging assay, cells were incubated with PEG-Cu_2S-NCs/PEG-Cu_2S-Fol-DOX NCs (0.1 mg/mL) for 2 h. For PEG-Cu_2S entry, post-incubation the cells were stained for nucleus with DAPI and lysosomes with lysotracker, rinsed with PBS and then imaged using confocal microscope with appropriate excitation and emission filters for NCs, DAPI and lysotracker. To confirm

the entry of PEG-Cu_2S-Fol-DOX NCs, 0.1 mg/mL of NCs were incubated with cells for 2 h, post that the cells were washed and imaged using appropriate excitation and emission filters for NCs and DOX. For NC-DOX intracellular trafficking assay, NC-DOX conjugate (0.05 mg/mL) were added to Gl-1 cells grown on glass base dish and immediately were visualized for intracellular trafficking of NCs at a stationary cell position. Images of NC's luminescence and DOX fluorescence along with the respective bright field images were recorded every 15 min until 120 min. For photoexcitation mediated drug release assay, Gl-1 cells grown on glass base dish were exposed to PEG-Cu_2S-Fol-DOX NCs conjugate (0.05 mg/mL) for 30 min, post which the NCs were sensitized using appropriate excitation wavelength for 10 min. Post irradiation, cells were imaged for DOX fluorescence to understand the release of the same. For in vitro PTT, Gl-1 cancer cells were incubated with or without PEG-Cu_2S-NCs/PEG-Cu_2S-Fol-DOX NCs (0.05 mg/mL) for 2 h and then irradiated by an 800-nm laser at power of 2.027 W for 3 min. Post intracellular tracking, photosensitization and PTT experiments, cells were stained for live/dead cell population using Calcein/PPi, rinsed and then viewed using appropriate excitation and emission filters. The excitation and emission wavelength used for the experiments are tabulated in **Table S1**.

Mechanism of PEG-lipid coating onto NCs:

In a typical micelle synthesis procedure, a concentrated suspension of surfactant NCs in chloroform is added to chloroform/methanol solution of phospholipids-PEG conjugate. The chloroform is evaporated during desiccation, following which hydration in aqueous solvent (here, PBS, pH 7.4) is carried out. The hydration technique helps in transferring the NCs to aqueous phase by an interfacial process propelled by the hydrophobic van der Waals interfaces amongst the primary alkane of the phospholipids-PEG and the secondary alkane of the oleylamine, resulting in micelle coating over NCs.

SUPPLEMENTARY IMAGES:



Scheme S1: Preparation of PEG-Cu_2S and PEG-Cu_2S-Fol NCs by thin-film hydration method.

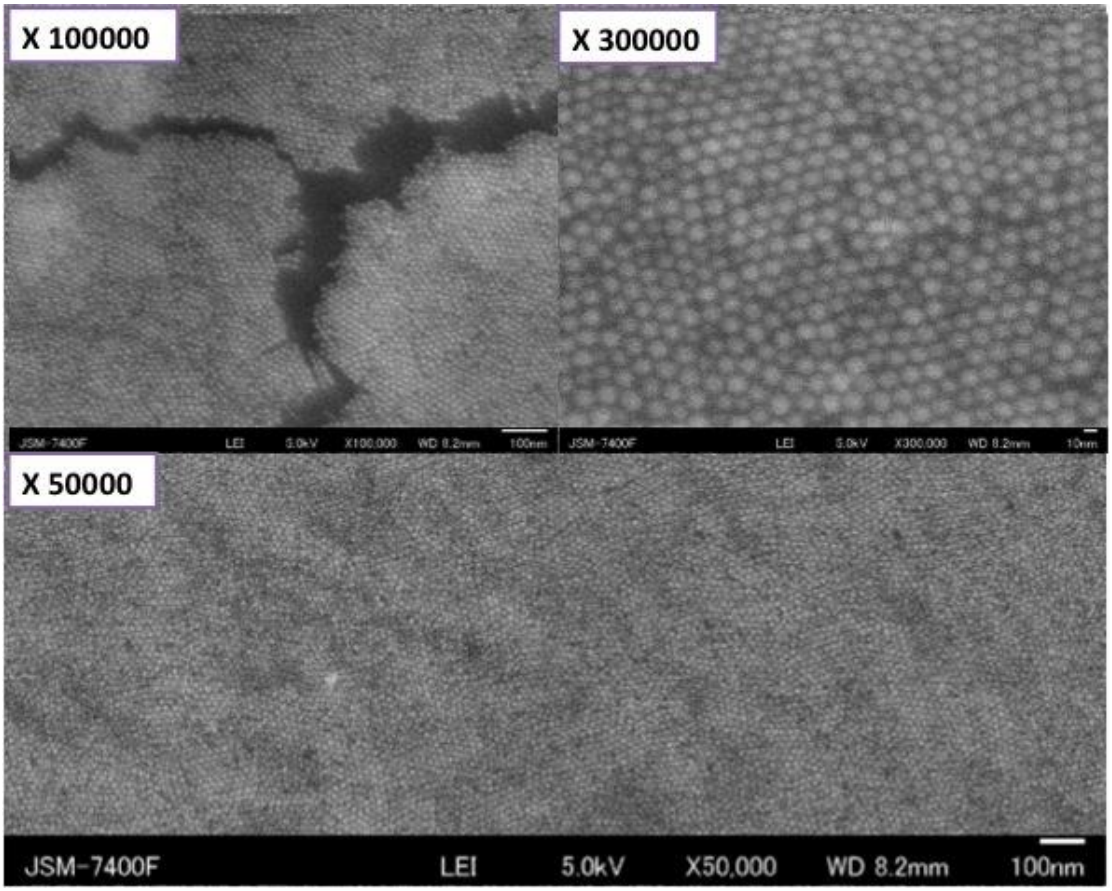


Figure S1: SEM images of Cu_2S NCs recorded at various magnifications.

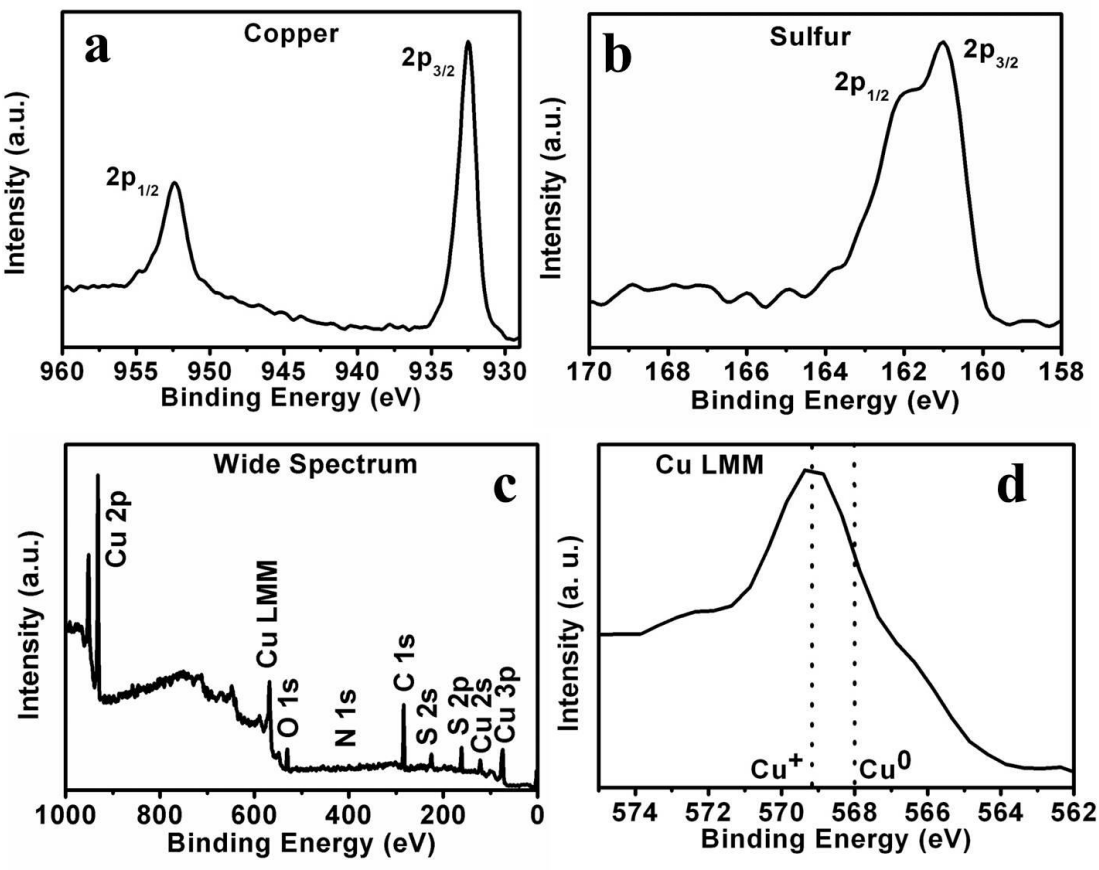


Figure S2: XPS analysis of Cu_2S NCs showing spectrum of Cu 2p (a), S 2p (b), wide spectrum (c) and Cu LMM peaks (d).

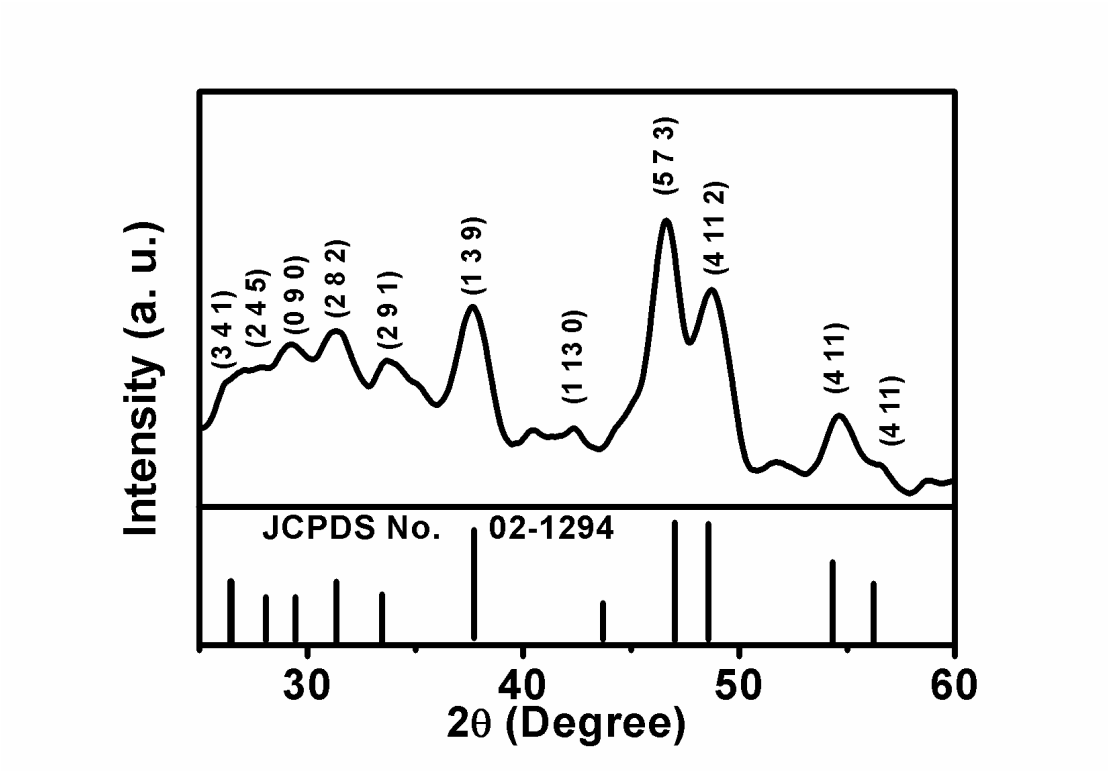


Figure S3: XRD analysis of Cu_2S NCs confirming that the synthesized Cu_2S is chalcocite Cu_2S (Orthorhombic, JCPDS 02-1294).

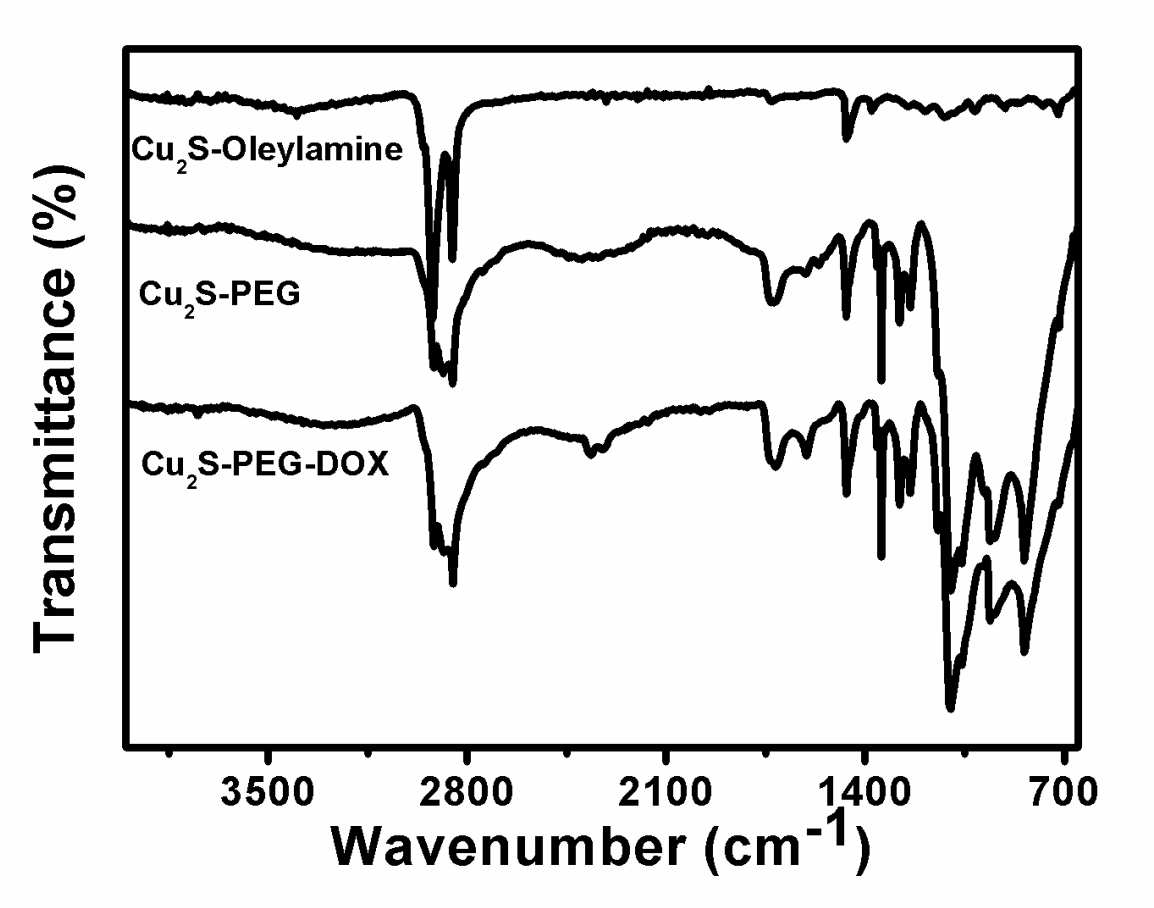


Figure S4: Surface characteristics of the Cu_2S NCs, PEG-Cu_2S NCs and PEG-Cu_2S-DOX NCs analyzed by FT-IR.

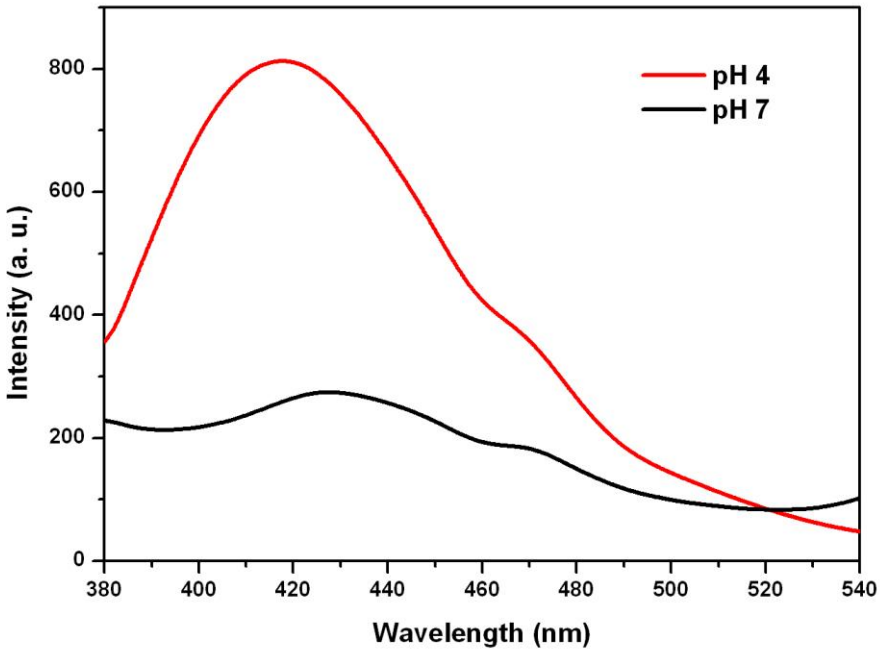


Figure S5: pH dependent luminescence enhancement of Cu_2S NCs.

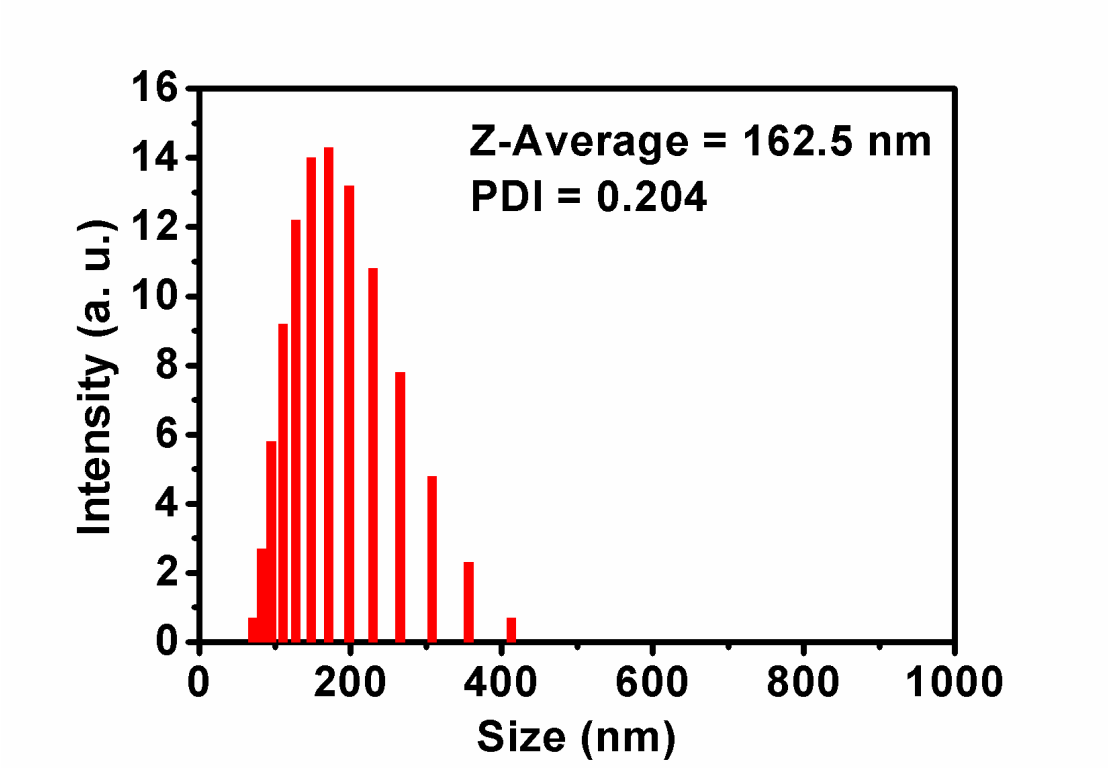


Figure S6: Hydrodynamic diameter of PEG-Cu_2S NCs measured by DLS.

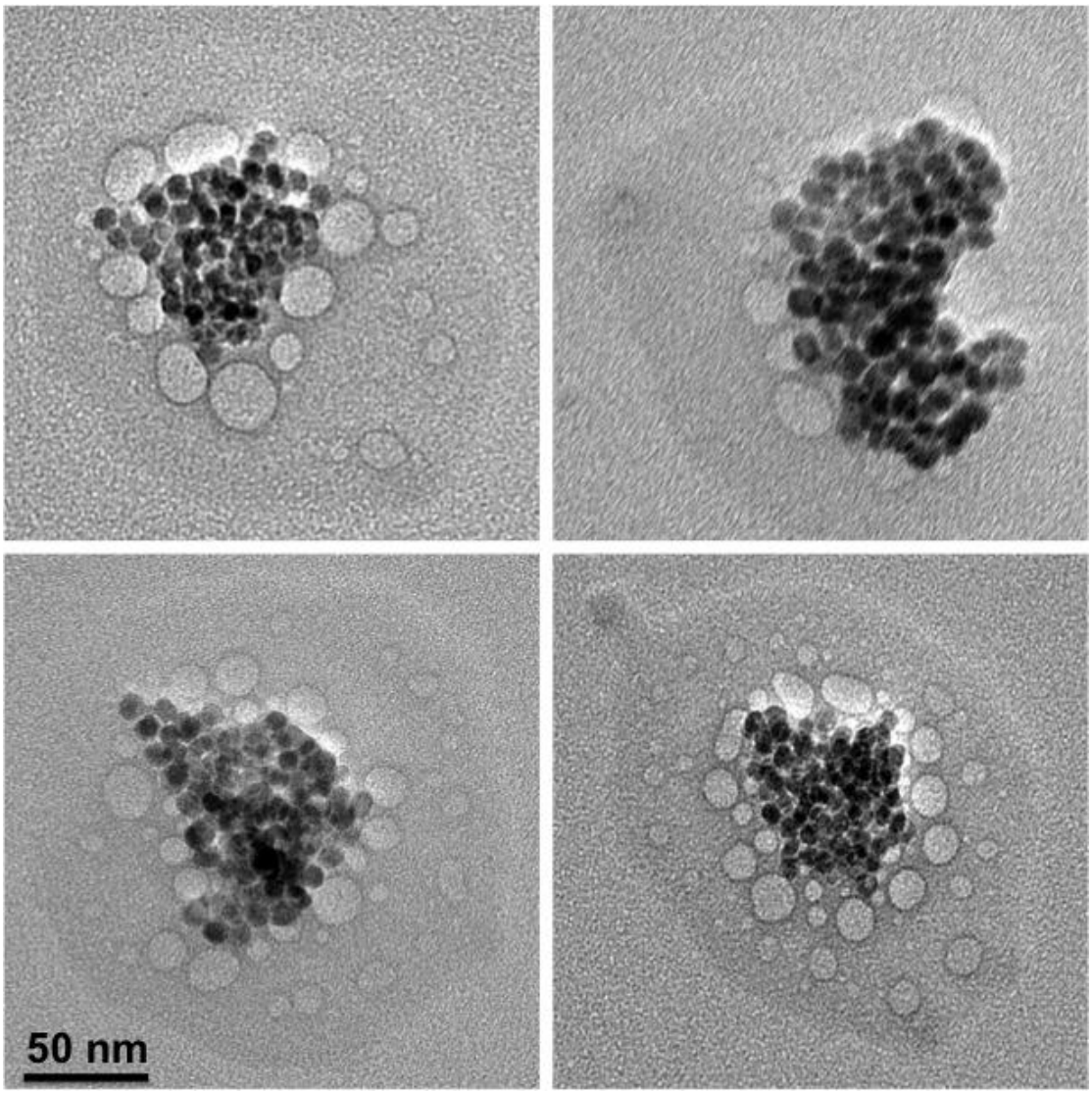


Figure S7: TEM images of PEG-Cu_2S NCs.

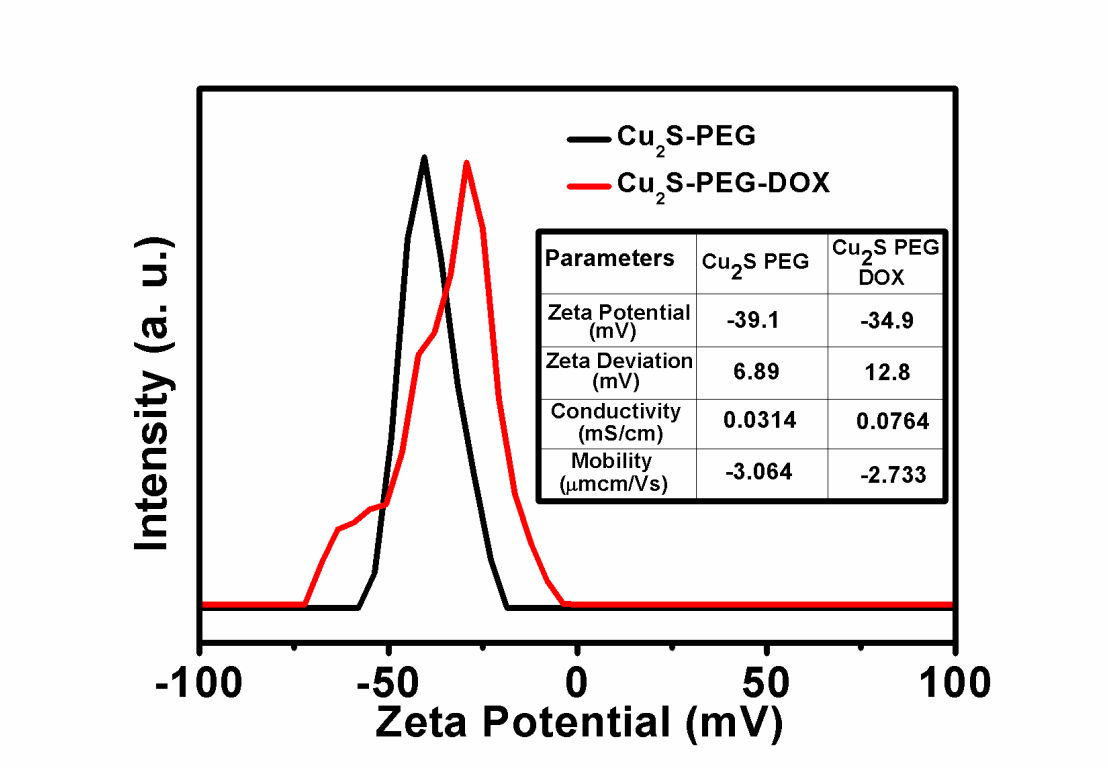


Figure S8: Zeta Potential of PEG-Cu_2S and PEG-Fol-Cu_2S-DOX NCs.

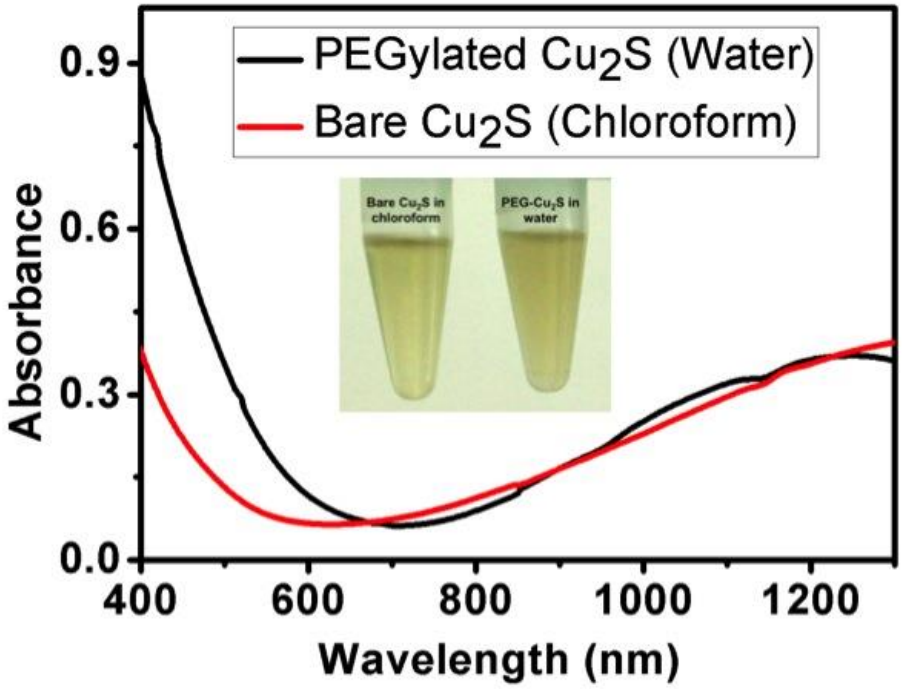


Figure S9: UV-Vis-NIR absorption spectra of bare and PEG-Cu_2S NCs.

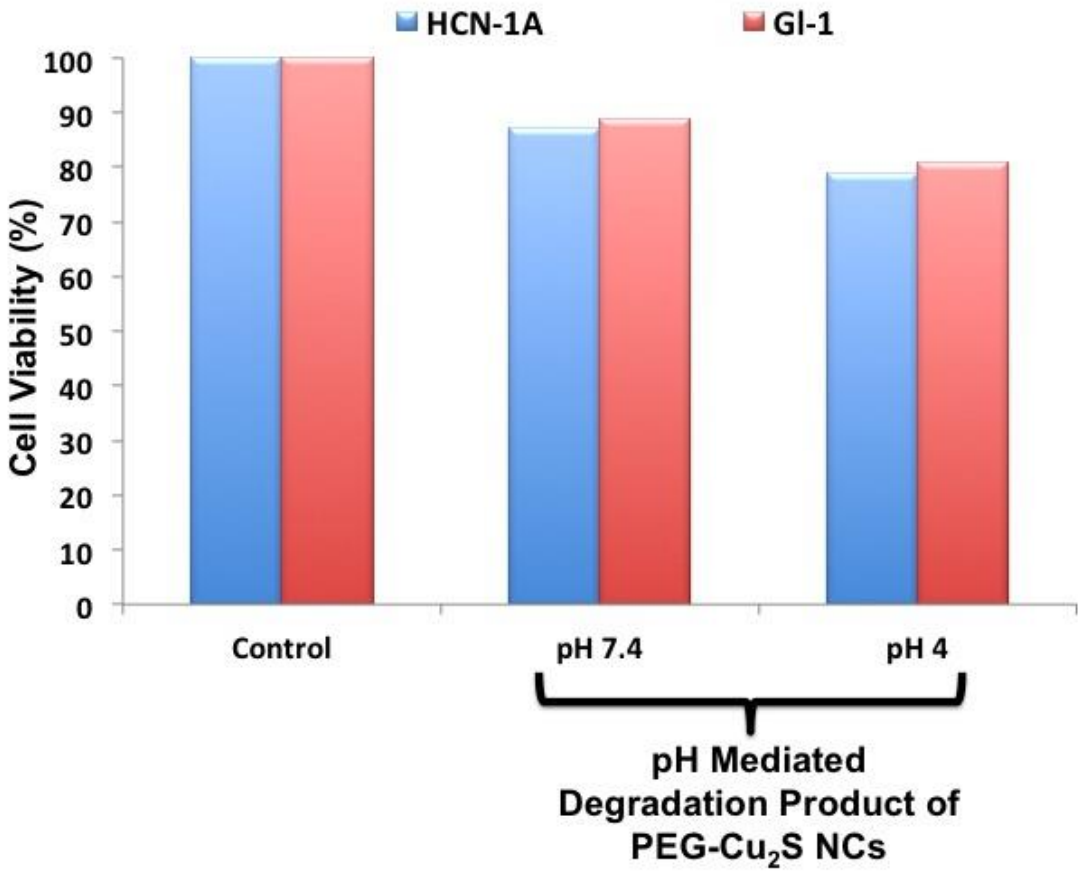


Figure S10: Cytotoxicity of degradation product of PEG-Cu_2S post 7 days of co-incubation with HCN-1A and Gl-1 cells. The NCs are pre-incubated at pH 7.4 or 4 for 24 h respectively and then centrifuged to collect the degradation product. The collected product is incubated for 7 days with the cells, post which the cell viability is analyzed.

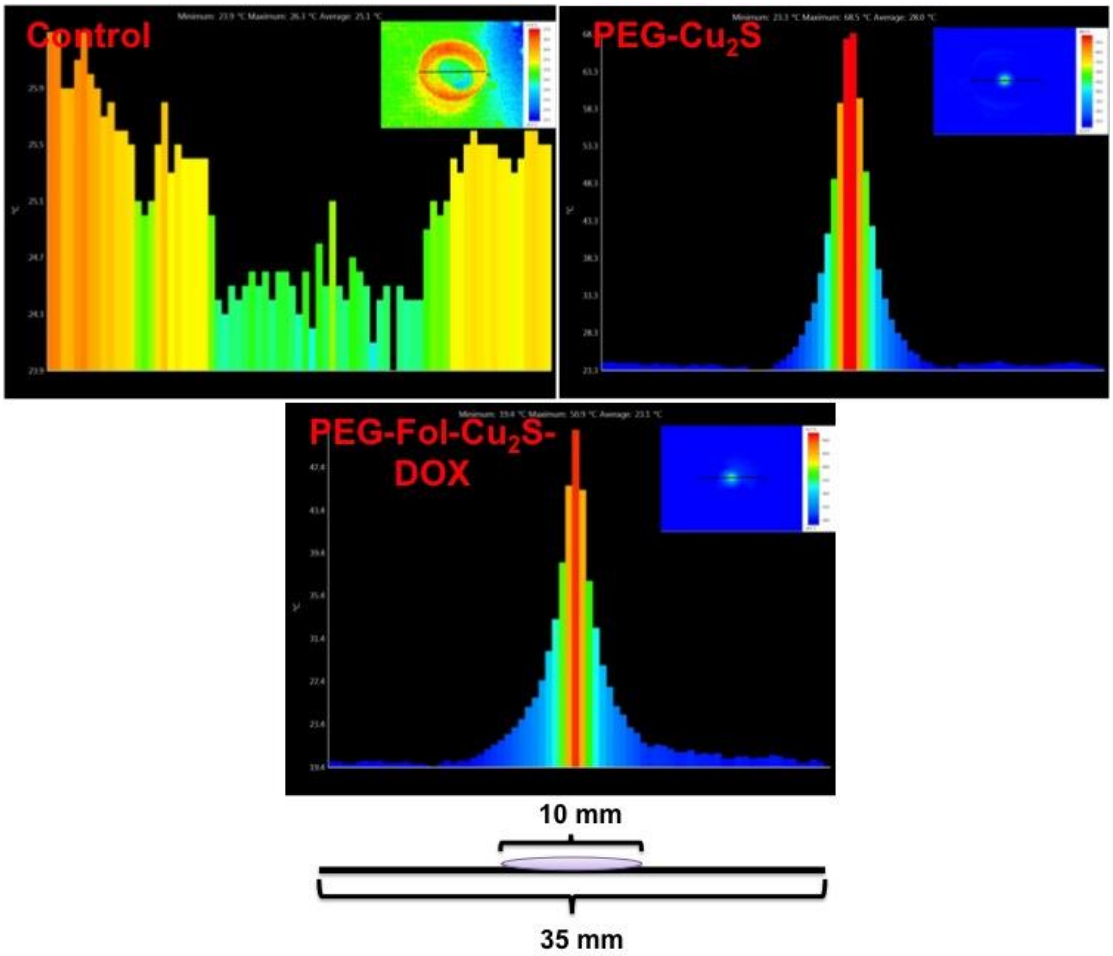


Figure S11: Spatiotemporal temperature distribution of cells grown in confocal dish exposed to PEG-Cu_2S and PEG-Fol-Cu_2S-DOX in comparison with control cell. The length of the line across the center of confocal plate (shown in inset) and the length of X-axis of the histogram corresponds to the diameter of the confocal plate (35 mm). The center 10 mm of the plate is the culture area. The heat distribution/dissipation from point of irradiation to surrounding cells were mapped and shown as histogram. It could be clearly visualized the heat dissipation happens exactly at the cell culture area (center 10 mm area) whereas the surrounding area shows no temperature rise.

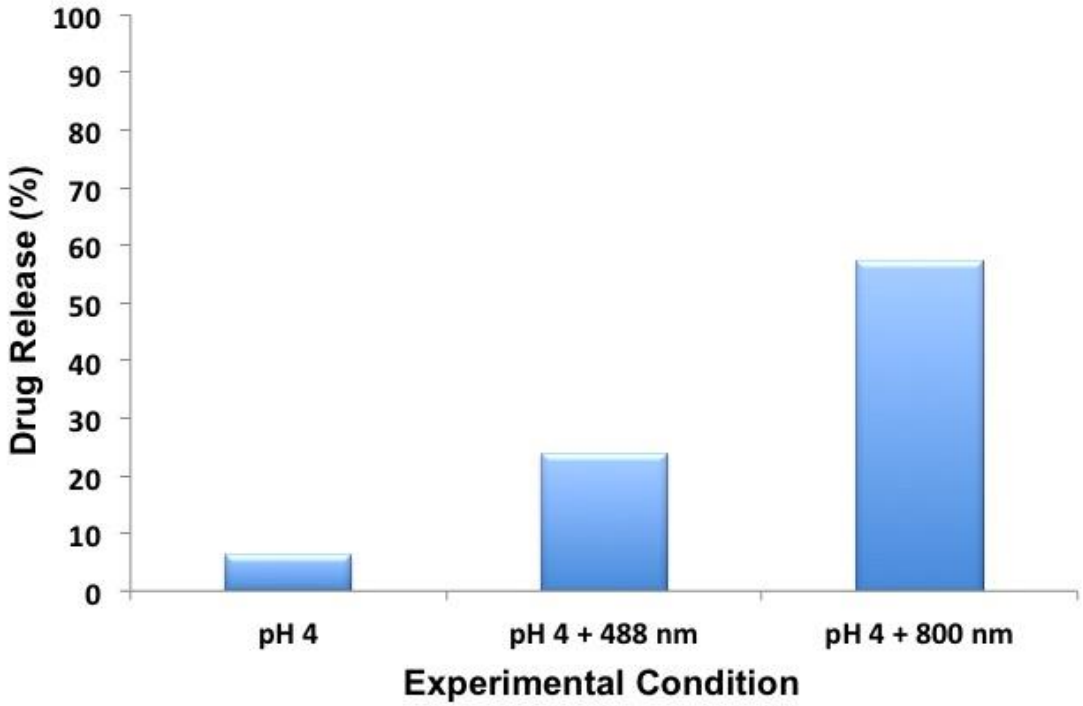


Figure S12: Comparison of drug release after 10 min by pH, photosensitization and NIR exposure.

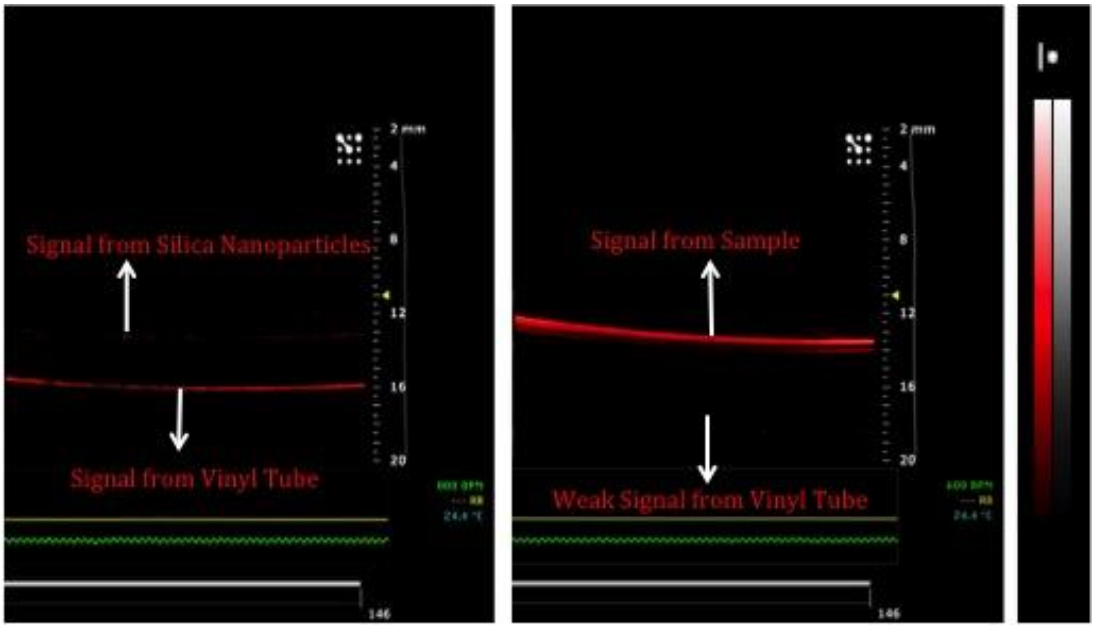


Figure S13: Photoacoustic imaging ability of PEG-Cu2S NCs (right) in comparison to Silica nanoparticles (negative control, left).

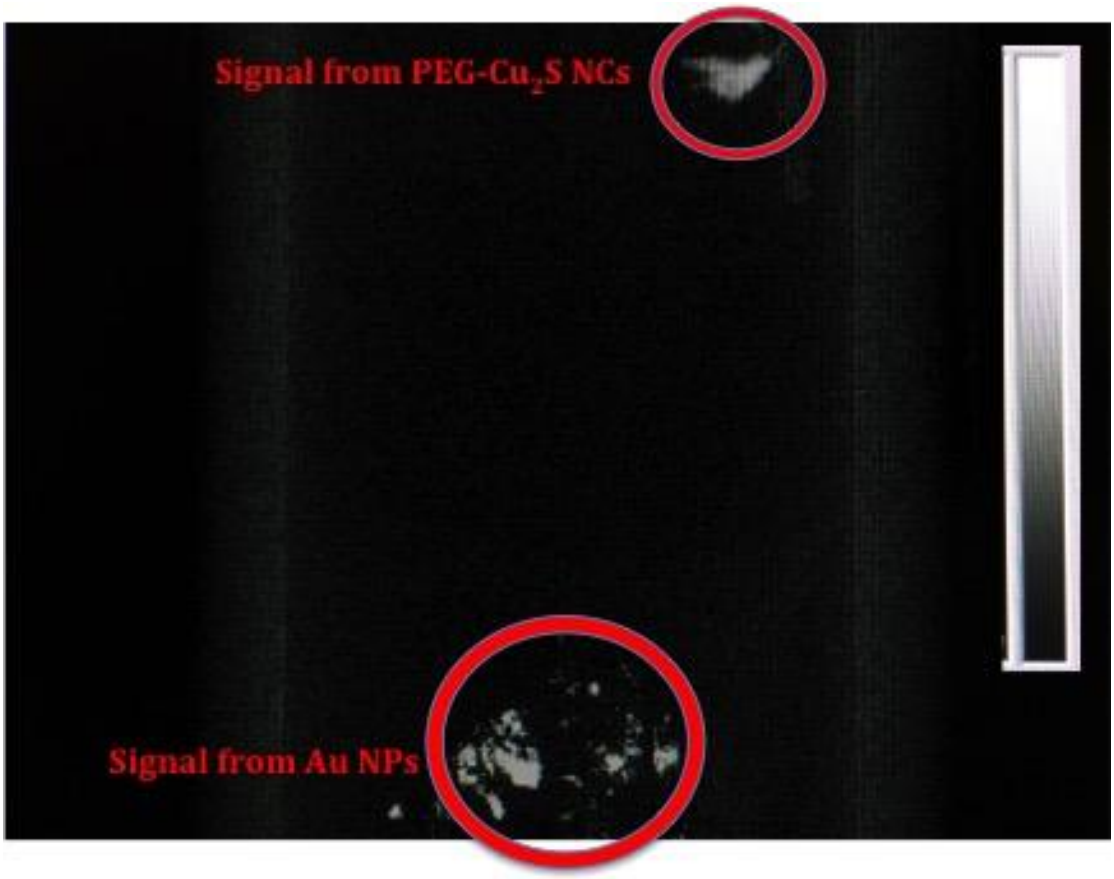


Figure S14: X-ray contrast imaging ability of PEG-Cu2S NCs in comparison to positive control, Gold nanoparticles.

Agent	Excitation Filter	Emission Filter (Band Pass)
Nanocrystal: **PEG-Cu_2S/PEG-Cu_2S-Fol-DOX NCs**	488 nm	520/35
DOX	561 nm	617/73
DAPI	405 nm	460/80
Lysotracker	561 nm	617/73
Calcein	488 nm	520/35
Propidium Iodide (PPi)	561 nm	617/73

Table S1: Excitation and Emission filters used for experiment.