Electronic Supplementary Information (ESI)

S-Nitrosothiols (SNO) as Light-Responsive Molecular Activators for Post-Synthesis Fluorescence Augmentation in Fluorophore-Loaded Nanospheres

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Materials and Methods

Materials

The following chemicals or reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.): rhodamine 6G, 3-mercaptopropyltrimethoxysilane (MPTMS), dimethyl sulfoxide (DMSO), 2,3-diaminonaphthalene (DAN), sodium fluorescein, ascorbic acid, and trehalose dehydrate. Doxorubicin was obtained from Zhejiang Hisun Pharmaceutical Co. Ltd. (China). Sodium nitrite ($NaNO_2$) and mercuric chloride were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Diethylenetriaminepentaacetic acid (DTPA) was obtained from TCI (Tokyo, Japan). All chemicals and solvents were of reagent grade and used as received. Deionized water (18.2 MΩ·cm at 25 °C) was prepared using a Millipore Milli-Q gradient A-10 water-purification system (Bedford, MA, U.S.A.).

Synthesis of Fluorophore-Loaded SNO-Silica NPs

Fluorophore-loaded S-nitroso silica NPs were synthesized according to our previous method with slight modification for individual fluorophores.[1] Briefly, a nanoprecipitation scheme was adopted, where the silane source (MPTMS, 200 mM) was S-nitrosated and polycondensed simultaneously in 10 mL of a DMSO-based organic phase (H_2O content = 15%) containing 400 mM sodium nitrite, 50 μM DTPA, and 0.5 M HCl. After reacting at room temperature and in the dark for 24 h, followed by rapidly injecting 1 mL of the organic phase into 10 mL of a water phase containing 80 μM of various fluorophores (DOX and R6G) with constant stirring (300 rpm) at room temperature. After aging (1 h) and multiple washing steps, the NP pellets were collected and lyophilized (with 5% trehalose). For SNO-R6G, ascorbic acid (80 mM) was added in the water phase to avoid nitrous acid-mediated degradation of R6G.

Basic Characterization of NPs

For each synthesis, the following properties of the as-prepared NPs were routinely characterized to ensure batch-to-batch consistency: hydrodynamic sizes (DLS) and the contents of NO and fluorophores. Hydrodynamic sizes were determined using dynamic light scattering (DLS) measurements (Horiba LB-500). The NO contents were determined using a fluorescence method.[1] The freeze-dried powder was redispersed in 1 mL of deionized water. After 20-fold dilution in water, an aliquot of 150 μL was taken and mixed with 20 μL of 2 mM 2,3-diaminonaphthalene (DAN) reagent (containing 0.62 M HCl) and 30 μL of 100 mM mercuric chloride, followed by incubation at room temperature for 60 min. Then, 10 μL of the reaction mixture was taken and mixed with 240 μL of 10 mM NaOH in a black 96-well microplate. The fluorescence intensity was measured at $\lambda_{Ex}/\lambda_{Em}$ of 375 nm/415 nm (Infinite M200, Tecan Austria GmbH). Then, the amount of NO loaded in NPs was determined by a standard curve of sodium nitrite. The concentrations of fluorophores were fluorescently measured at $\lambda_{Ex}/\lambda_{Em}$ of 479 nm/593 nm for DOX and of 525 nm/562 nm for R6G, respectively. After nanoprecipitation, the efficiency (%) of fluorophores entrapped in the NPs was estimated using the following equation: entrapment efficiency (%) = $(C_{initial} - C_{supernatant})/C_{inital} \times 100$, where $C_{initial}$ is the concentration of fluorophore initially added in the water phase, and $C_{supernatant}$ is the concentration of fluorophore in the supernatant of the resulting NP solution. The contents of DOX and R6G in the lyophilized powder were determined by first performing DMSO extraction (0.1 mL NP dispersion to 5 mL DMSO) under sonication (10 min), then by measuring the fluorescence intensity of the extracted solution with a standard curve constructed using the same solvent composition. The loading of the final lyophilized powder was estimated using the following equation: loading (%) = $(C_{fluorophore} \times M.W.)/(Q_{powder} - Q_{trehalose}) \times 100$, where $C_{fluorophore}$ is the concentration of fluorophore determined and M.W. is the molecular weight of the fluorophore; Q_{powder} is the total amount of the lyophilized powder and $Q_{trehalose}$ is the amount of trehalose added for lyophilization.

Kinetic Fluorometric Measurements

The effect of visible-light irradiation on NP fluorescence was performed by placing NP samples (redispersed in water) under a fluorescent desk lamp (13 W, PL-S, Philips) at a distance of 14 cm. The fluorescence change upon light irradiation or in the dark was then monitored over time using a fluorometer (Infinite M200, Tecan Austria GmbH). The instrument parameters are: excitation bandwidth = 9 nm; emission bandwidth = 20 nm; gain = 70 or 100; number of flashes = 10; integration time = 20 μs. Briefly, for continuous measurements, NP dispersions were divided into light and dark groups (6 mL each). The light group was under continuous light irradiation in a transparent glass vessel, whereas the dark group was kept in an amber glass bottle wrapped with aluminum foil. At a pre-determined time (up to 150 min), an aliquot of 200 μL of the sample was taken and measured (for both the whole NP dispersion and the separated supernatant) at $\lambda_{Ex}/\lambda_{Em}$ of 479 nm/593 nm for DOX and of 525 nm/560 nm for R6G, respectively. For the ON/OFF experiments, an aqueous NP dispersion of (200 μL) was placed in the sample chamber of a fluorometer and its fluorescence continuously monitored every minute for 5 min (the OFF period).

Then, the sample was removed from the chamber and irradiated with light for 5 min (the ON period). After that, the sample was sent back to the instrument for another 5-min fluorescence measurement. The ON/OFF cycle was repeated several times. At the end, the sample solution was centrifuged and its supernatant removed for another fluorescence measurement.

Fluorophore Release

The content of a bottle of lyophilized NPs were redispersed in 2 mL of deionized water containing 160 mM NaCl to remove surface-bound fluorophores. After centrifugation (17211g, 15 min), the NP pellet was dispersed in 2 mL of deionized water, which was divided into two 1-mL portions: one was light irradiated for 4 h, while the other was kept in the dark during the time (as control). Then, the light-treated and control NP dispersions were separately transferred to dialysis bags (MW cutoff: 12-14 kDa). The sealed dialysis bag was immersed in 225 mL of PBS-based release medium (pH 7.4, 37 °C) with constant stirring at 300 rpm for 24 h (Note that the release setup was placed in the dark and protected from light). At each sampling time (0, 0.5, 1, 2, 4, 8, 12, 24 h), 25 mL of the medium was removed and then replenished with 25 mL of fresh medium. The amount of fluorophore released was fluorescently measured at $\lambda_{Ex}/\lambda_{Em}$ of 479 nm/593 nm for DOX and of 525 nm/560 nm for R6G, respectively. Cumulative fluorophore release was estimated according to the following equation: % release $= 100 \times \left(C_n \times V_t + \sum_{i=1}^{n-1} C_i \times V_s\right)/A_0$, where C_n is the fluorophore concentrations measured at the nth sampling and C_i is concentration measured before the nth sampling; V_t and V_s are the total volume of the release medium (i.e. 225 mL) and the sampling volume (i.e. 25 mL), respectively; A_0 is the initial amount of fluorophore contained in the sample.

Fluorescence Spectroscopy

Lyophilized NPs were redispersed in water containing 160 mM NaCl to remove surface-bound fluorophores. Then, the NPs were redispersed and diluted in pure water, and divided into the light and dark groups: the light group was placed under a 13-watt light source (at a distance of 14 cm) for 3 h, whereas the dark group was wrapped with aluminum foils and placed in dark. For free DOX and SNO-DOX (light and dark), the emission spectra were obtained at emission wavelengths in the range of 520 nm to 800 nm (gain = 100, excited at 479 nm). For free R6G and SNO-R6G (light and dark), the emission scan was performed at wavelengths of 520 nm to 650 nm (gain = 70, excited at 480 nm). Finally, the NPs were extracted using DMSO (200 µL NPs in 800 µL DMSO), and after centrifugation (17211g, 15 min), the emission scans for the supernatant were obtained under the same spectral conditions.

Fluorescence Response in the Context of Light-triggered NO Release vs. Slow NO Release

Purified NP dispersions were divided in two black microplates; one was placed under a fluorescent desk lamp (13 W, PL-S, Philips) at a distance of 14 cm (the light group), the other was kept in the dark and protected from light (the dark group). In this experiment, the fluorescence of both the

fluorophores (DOX or R6G) and the NO-adduct of an NO probe (DAN)[2] were separately measured (i.e. in two separate wells, with or without 1 mM DAN). To ensure extensive release of NO in the dark, the kinetic measurements were extended over 9 days. Fluorescence intensities were determined at $\lambda_{Ex}/\lambda_{Em}$ of 479 nm/593 nm for DOX, 525 nm/560 nm for R6G, and 375 nm/415 nm for the fluorescent NO probe, respectively.

Effect of Mercuric Ions on the Fluorescence of NPs

Purified NPs were redispersed and diluted in aqueous solutions containing various concentrations of mercuric chloride, followed by loading a portion of the resulting solution (200 µL) into a 96-well plate. The whole procedure was protected from light. At a pre-determined interval (up to 150 min), the fluorescence intensity was continuously measured at $\lambda_{Ex}/\lambda_{Em}$ of 479 nm/593 nm for DOX and of 525 nm/560 nm for R6G, respectively. To examine whether mercuric ions would affect fluorophore release, another portion of the NP solution was taken and centrifuged (17211g, 15 min), and the supernatant was measured for fluorescence intensity. Later at 180 min, the continuous fluorescence measurement in the microplate was resumed, only that the plate was thereafter placed under a 13-watt light source.

Transmission Electron Microscopy

The TEM images of light-treated and control NPs were acquired from a Hitachi HT7700 120 kV high-contrast/high resolution digital TEM instrument operated at 75.0 kV, 6.0 µA.

Solid-State MAS ^{29}Si NMR spectroscopy

A purified NP dispersion was irradiated with visible light for 24 h. Then the light-irradiated sample was centrifuged to obtain particles, which was then vacuum dried. Control particles were prepared, purified and dried in the dark and protected from light. Solid-state MAS ^{29}Si NMR spectroscopy was performed on a Bruker Avance III spectrometer.

Solvent Extraction Study

Lyophilized SNO-DOX was redispersed in water containing 160 mM NaCl to remove surface-bound DOX. Then, the NPs were redispersed and diluted in pure water, and divided in two groups (light vs. dark); each containing five 50-µL aliquots of the particle dispersion in an Eppendorf tube. The light group was placed under a 13-watt light source (at a distance of 14 cm), whereas the dark group was wrapped with aluminum foils and placed in dark. At a predetermined interval (15, 30, 60, 120, and 240 min), one tube from each group was removed and an aliquot of 10 µL dispersion was taken and added to 190 µL of 60% DMSO. After waiting for 30 min, the extraction mixture was centrifuged (17211g, 15 min) to obtain the supernatant. The concentration of the extraction solvent (i.e. 60% DMSO) and extraction time (i.e. 30 min) was optimized and chosen in a

preliminary test. The amount of DOX in the supernatant was measured at $\lambda_{excitation}/\lambda_{emission}$ of 479 nm/593 nm, based on a standard curve of DOX dissolved in the same solvent.

Cytotoxicity

Cytotoxicity were evaluated in MDA-MB-231 (breast cancer; ATCC® HTB-26™) and A2058 cells (melanoma; ATCC® CRL11147™) using the MTT assay. MDA-MB-231 or A2058 cells were seeded at 2×10^4 cells and at 1×10^4 cells, respectively, per well in 96-well plates and incubated in RPMI 1640 medium containing 10% FBS. After overnight incubation, cells were incubated with SNO-R6G- or R6G-containing culture medium for 6 h (R6G concentrations: 0.05, 0.25, 0.5, 2.5, 5, 25, and 50 μM), followed by light irradiation for 1 h (the control group was protected from light). Then, cells were re-incubated for another 48 h (MDA-MB-231) and 24 h (A2058), respectively. Finally, the medium was removed and replaced with 100 μL of 0.5 mg/mL MTT solution (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 3 h at 37 °C. Then, the MTT solution was removed, and 100 μL of DMSO was added. Cell viability was determined by the absorbance measurement at 600 nm using a microplate ELISA reader.

Flow Cytometry

MDA-MB-231 cells (2×10^5) were plated in a 6-well plate and then incubated in a culture medium (RPMI 1640 with 10% FBS) for 48 h. Then, the culture medium was removed and replaced with SNO-R6G dispersed in the culture medium. The SNO-R6G dosing solution contained SNO-R6G with or without light pretreatment (4 h). The R6G concentration in the dosing solution was varied from 0.05 to 10 μM. After adding SNO-R6G and further incubation for 6 h, the dosing solution was removed and the cells were washed (with PBS), and then collected using 0.05% trypsin/EDTA. After centrifugation (1000 rpm, 20 °C), the cell pellets were redispersed in PBS. Fluorescence histograms were recorded with a flow cytometer (FACSCalibur, BD Bioscience) and analyzed using CellQuest Pro software supplied by the manufacturer. A minimum of 10,000 events were analyzed to generate each histogram. The gate was arbitrary set for the detection of orange fluorescence (FL2-H, 565-606nm). For quantitative comparison, geometric mean fluorescence intensity (gMFI) was estimated from the cellular fluorescence data.

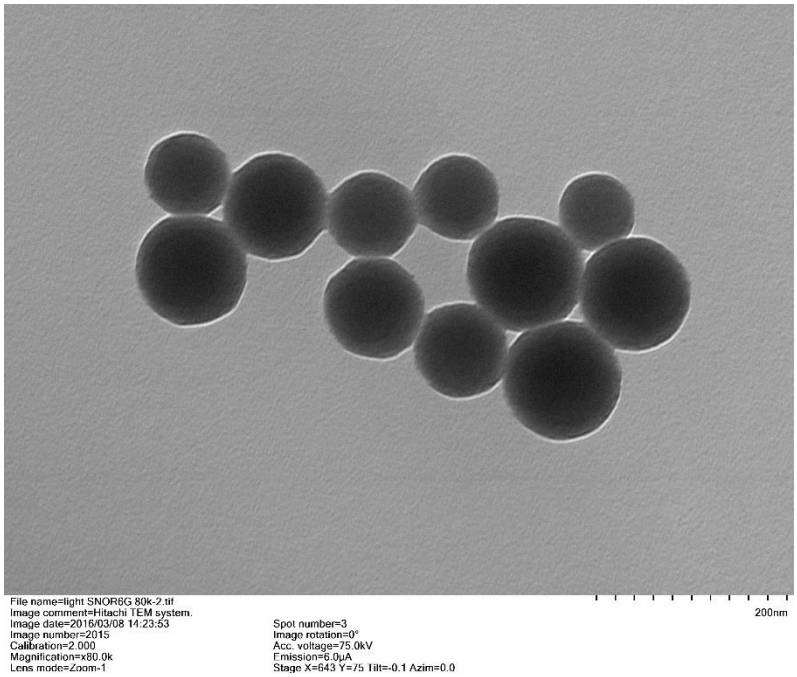


Figure S1. TEM image of SNO-R6G. The TEM image was acquired from a Hitachi HT7700 120 kV high-contrast/high resolution digital TEM instrument operated at 75.0 kV, 6.0 μA. Magnification: 80 K.

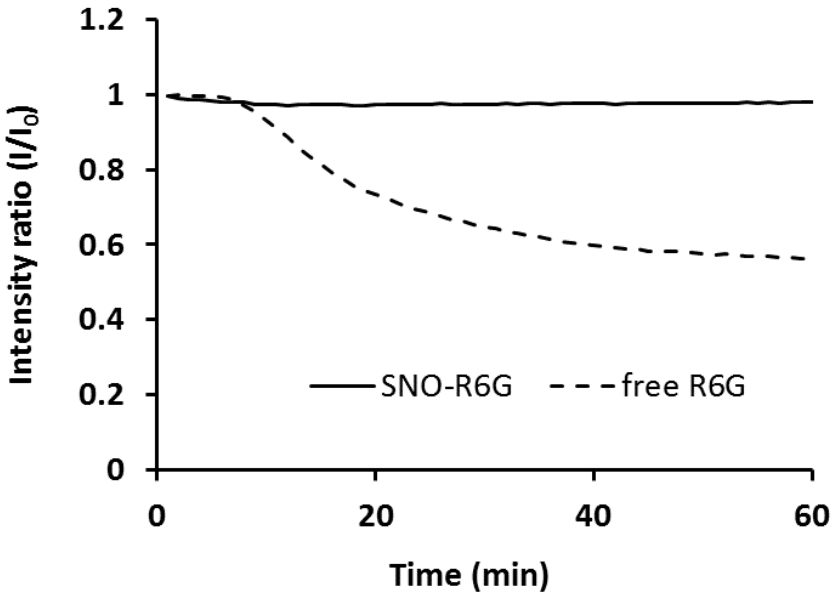


Figure S2. Photobleaching of R6G. Fluorescence intensities of an aqueous dispersion of SNO-R6G and of a solution of R6G (0.5 μM) were measured at $\lambda_{Ex}/\lambda_{Em}$ of 525 nm/560 nm (Tecan Infinite M200; gain = 70) over 60 min (at a 1-min interval). SNO-R6G was pre-treated with visible-light irradiation (13 W) for 2 h before the measurement. Data plotted were the measured intensity (I) at each time point normalized to the initial intensity (I_0).

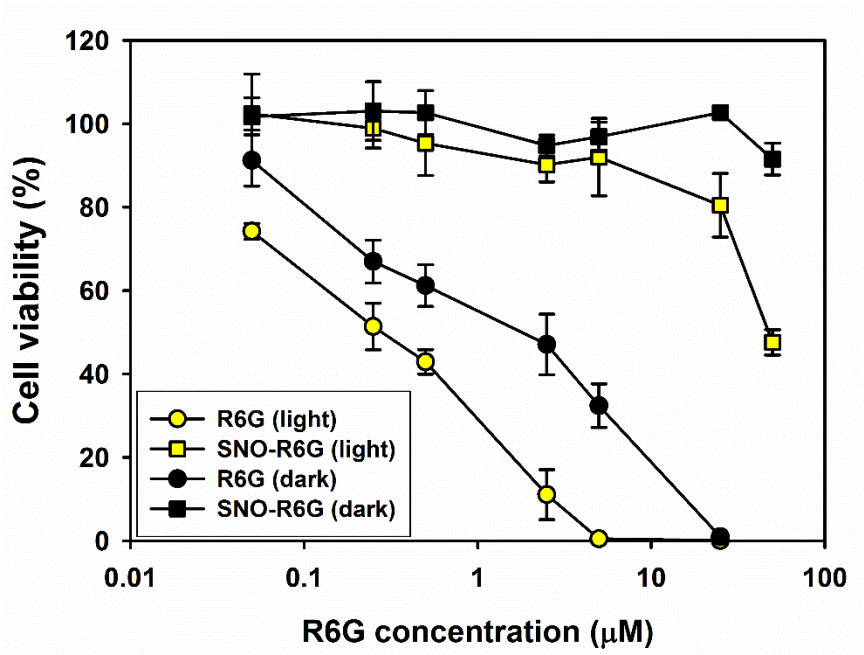


Figure S3. Cytotoxicity of SNO-R6G and R6G in A2058 melanoma cells with or without light exposure during incubation. A2058 cells were seeded at 1×10^4 cells per well in 96-well plates and incubated in a culture medium (RPMI 1640 with 10% FBS). After overnight incubation, cells were incubated with SNO-R6G- or R6G-containing culture medium for 6 h (R6G concentrations: 0.05, 0.25, 0.5, 2.5, 5, 25, and 50 μM), followed by light irradiation for 1 h (the control group was protected from light). Then, cells were re-incubated for another 24 h. Cell viability was determined by the absorbance measurement at 600 nm using a microplate ELISA reader.

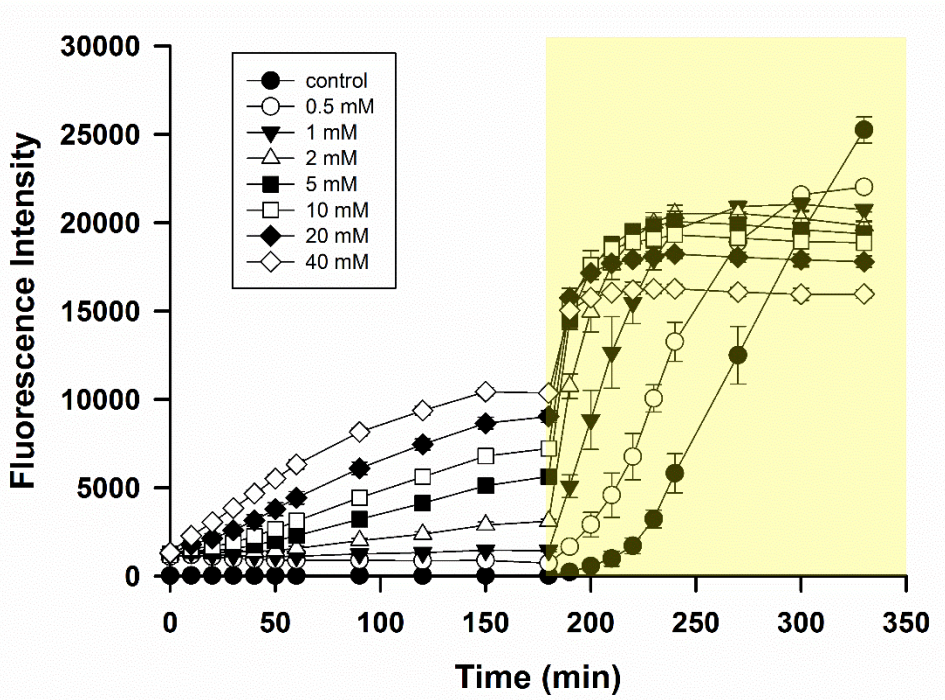
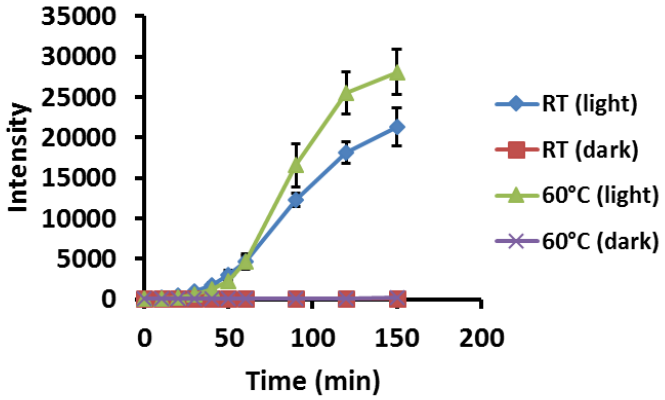
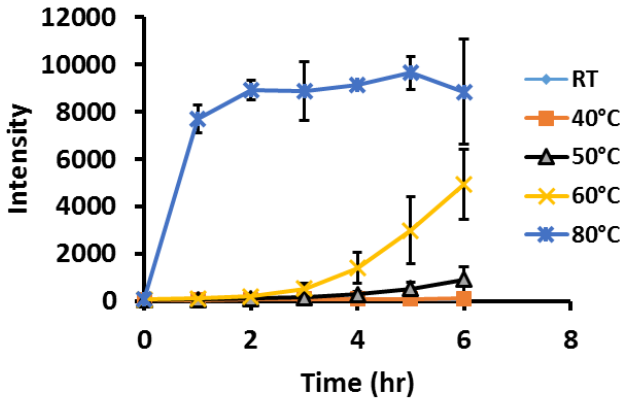


Figure S4. Mercuric ion-triggered fluorescent response of SNO-R6G before and after (yellow shaded area) light exposure. Purified SNO-R6G NPs were redispersed and diluted in aqueous solutions containing various concentrations (0.5 – 40 mM) of mercuric chloride, followed by loading a portion of the resulting solution (200 µL) into a 96-well plate. The whole procedure was protected from light. At a pre-determined interval (up to 150 min), the fluorescence intensity was continuously measured at $\lambda_{Ex}/\lambda_{Em}$ of 525 nm/560 nm (Tecan Infinite M200). At 180 min and thereafter, the samples were placed under a 13-watt light source and fluorescence intensities were measured at predetermined intervals.

(a)



(b)



(c)

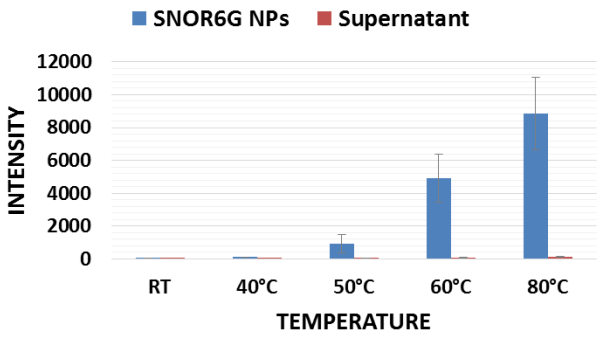


Figure S5. Effect of temperature on the fluorescence intensity of SNO-R6G NPs. (a) Kinetic fluorescence measurement for NP samples incubated at 60 °C and at room temperature, in the dark and under light irradiation over time. (b) Long-term fluorescence measurements up to 6 h for NP samples incubated at various temperatures and in the dark. (c) Comparison of the fluorescence intensities for NP dispersion and the supernatant of the NP dispersion (after separating NP from supernatant by centrifugation). The fluorescence measurement was performed in a new fluorometer (Tecan, Spark 10 M; excitation λ_{Ex} = 520 nm (bandwidth 20 nm); emission λ_{Em} =560 nm (bandwidth = 20 nm); gain = 60; number of flashes = 10).

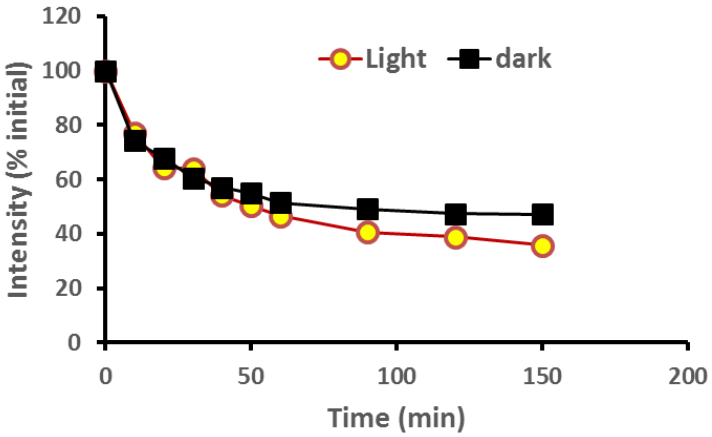


Figure S6. Kinetic fluorescence measurements for control R6G silica NP in the dark vs. under light irradiation for 150 min. The control NPs were prepared using a modified nanoprecipitation procedure[3] without the presence of a nitrosating agent in the DMSO-based organic phase containing MPTMS (100 mM), DTPA and HCl (similar to the preparation of SNO NPs, except that sodium nitrite was not added in the organic phase). After 24 h reaction in the organic phase, 1 mL of the organic phase was taken and injected into 10 mL of water containing 2 mM R6G. After aging at 60 °C for 2 h, the NP was collected and purified using repeat centrifugation and re-dispersing with pure water. The hydrodynamic size of the finished NPs was about 200 nm. The fluorescence intensity was measured using Tecan, Spark 10 M.

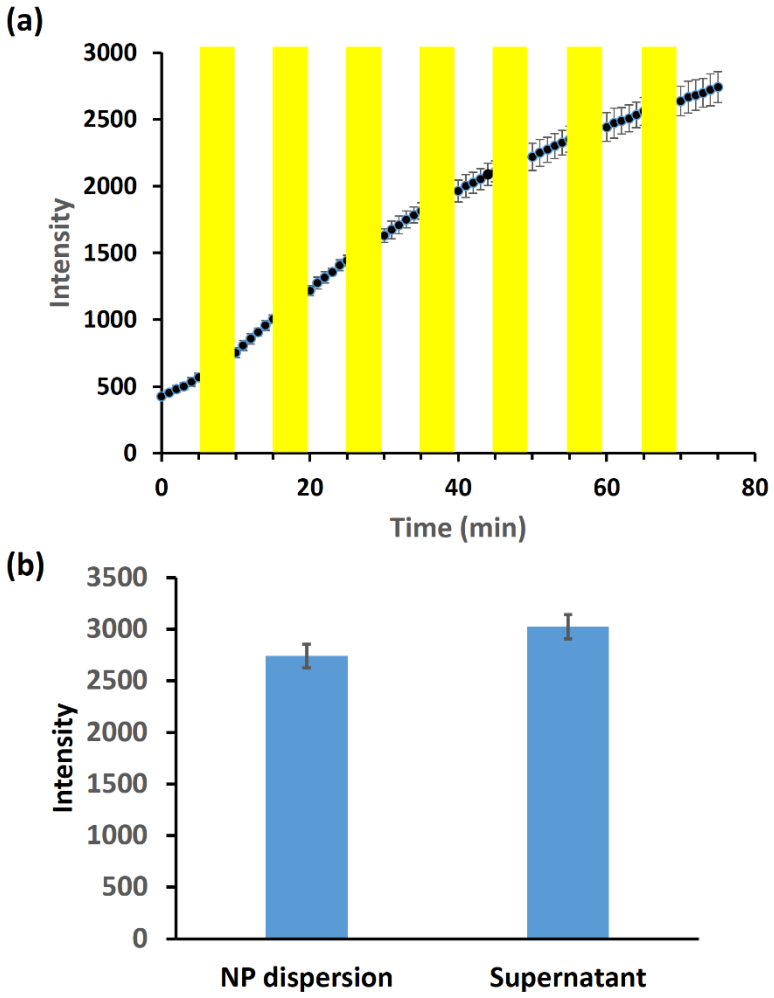


Figure S7. Kinetic fluorescence measurements for an aqueous dispersion of SNO-FLU under intermittent light irradiation. (a) An aqueous dispersion of SNO-FLU (200 µL) was placed in the sample chamber of a fluorometer, and its fluorescence ($\lambda_{Ex}/\lambda_{Em}$ of 460 nm/520 nm; gain = 70; (Tecan Infinite M200) was continuously monitored every minute for 5 min (OFF period). Then, the sample was removed from the chamber and irradiated with light for 5 min (ON period, yellow shaded). The ON/OFF cycle was repeated several times up to 75 min. (b) Comparison of the fluorescence intensity between the NP dispersion and its supernatant at 75 min.

References:

1. M. R. Wang, S. J. Chiu, H. C. Chou and T. M. Hu, *Chem Commun (Camb)*, 2015, **51**, 15649-15652.
2. T.-M. Hu, S.-J. Chiu and Y.-M. Hsu, *Biochem Biophys Res Commun*, 2014, **451**, 196-201.
3. S. J. Chiu, C. Y. Lin, H. C. Chou and T. M. Hu, *Langmuir*, 2016, **32**, 211-220.