Electronic Supplementary Information (ESI)

An efficient S-NO-polysilsesquioxane nano-platform for the co-delivery of nitric oxide and an anticancer drug

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1. Materials and Methods

1.1 Materials

3-Mercaptopropyltrimethoxysilane (MPTMS), dimethyl sulfoxide (DMSO), 2,3-diaminonaphthalene (DAN), sodium fluorescein and trehalose dihydrate were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Sodium nitrite ($NaNO_2$) and mercuric chloride were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Hydrochloric acid (HCl) was purchased from Merck (Darmstadt, Germany). Diethylenetriaminepentaacetic acid (DTPA) was obtained from TCI (Tokyo, Japan). Doxorubicin was obtained from Zhejiang Hisun Pharmaceutical Co. Ltd. (China). 4,5-diaminofluorescein diacetate (DAF-2DA) was purchased from Calbiochem (San Diego, CA, USA). All chemicals and solvents were ACS 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.).

1.2 Preparation of DOX-Loaded S-Nitroso (SNO) Polysilsesquioxane Nanoparticles (SNODOX)

The preparation method is based on a nanoprecipitation procedure, in which 1 mL of an organic phase was injected into 10 mL of a water phase with constant stirring at room temperature. The organic phase was prepared by mixing 0.375 mL of MPTMS (5.384 M), 8.125 mL of DMSO, 0.5 mL of 8 M $NaNO_2$ solution (containing 1 mM DTPA), and 1 mL of 5 M HCl in a 10-mL tightly capped glass bottle; the reaction mixture was initially left standing in an ice bath for 10 min. Then, the reaction bottle was removed from the ice bath and kept at room temperature for 24 h (protected from light). At the end of the reaction, the reacted organic phase was centrifuged at 3000 rpm (4141*g*) for 10 min, and then 1 mL of which was taken with a 1-mL 27G syringe and rapidly injected into 10 mL of deionized water (the water phase), which contains 80 μM doxorubicin (DOX). After 30 sec of constant stirring (300 rpm), followed by 1-h aging at room temperature (protected from light), a total of 8 injections were combined and centrifuged at 5500 rpm (7591*g*) for 30 min at 4 °C to separate the pellet from the supernatant. The supernatant was further centrifuged to collect remaining particles (repeating the process 4 times). The pellets were redispersed and combined, and then washed three times using 20 mL of ice-cold water. Finally, the washed particles were redispersed in 4 mL of deionized water, followed by mixing with 4 mL of 10% trehalose solution. The resulting colloidal solution was divided into 500-μL aliquots in glass vials. After freezing at −80 °C, the samples were placed into the drying chamber of a lyophilizer (FD-5030, Panchum Scientific Corp., Kaohsiung, Taiwan), precooled to −40 °C. Drying was performed at a pressure of 100 mTorr for 12 h.

1.3 Optimization of the Preparation Method and Determination of Encapsulation Efficiency

The effect of varying various nanoprecipitation parameters on particle formation and DOX encapsulation was investigated. The tested parameters include: DOX concentrations in the water phase, organic phase reaction time, solvent type, and the presence of ethanol in the water phase. After nanoprecipitation, the resulting colloid solutions were subjected to particle size analysis (DLS) and determination of DOX entrapment. The encapsulation efficiecy (E.E.) of doxorubicin was determined using a fluorometic method. An aliquot of 1 mL of colloid dispersion was centrifuged at 14000 rpm (15997g, 4 °C, 10 min) to collect the supernantant. Then, the amount of DOX remained in the supernatant was determined at excitation and emission wavelengths of 479 nm/593 nm. The encapsulation efficiecy was calculated according to the following equation: E.E.

(%) = 100*(total DOX amount added–supernatant DOX amount measured)/total DOX amount added.

1.4 Determinations of Hydrodynamic Particle Sizes and Zeta Potential

Particle sizes were determined by a dynamic-light-scattering (DLS) instrument (LB-500, Horiba instruments Inc., Irvine, CA, U.S.A.). Three size readings were taken and the mean value was used for each sample (1 mL). The surface charges of particles were measured by a zeta-potential analyzer (ZetaPlus, Brookhaven Instruments Co., Holtsville, NY, U.S.A.). For each sample, the average of 10 zeta-potential readings were recorded.

1.5 Transmission Electron Microscopy (TEM).

The TEM images were acquired from a Hitachi HT7700 120 kV high-contrast/high resolution digital TEM instrument operated at 75.0 kV, 8.0 μA. The freeze-dried SNODOX powder was redispersed in deionized water, followed by centrifugation at 6600 rpm (7591*g*) to remove the supernatant. The particle pellet was redispersed in deionized water and one drop of the sample was placed on a carbon-Formvar-coated copper grid (300 mesh, type A; Electron Microscopy Sciences, Hatfield, PA, U.S.A.). After air-drying for 3 h, the TEM pictures were taken.

1.6 Solid-State ^{29}Si NMR

Solid-state magic angle spinning (MAS) ^{29}Si NMR spectroscopy was performed on a Bruker Avance III spectrometer. The instrument was equipped with a wide-bore 14.1 T magnet and a 4 mm double-resonance MAS probehead. Settings: sample spinning rate, 10 kHz; ^{29}Si Larmor frequency, 119.24 MHz; spectra acquisition parameters, 2.5 μs pulse (π /4), 1H TPPM decoupling scheme (70 kHz rf); recycle delay, 120 s.

1.7 Assay of NO and DOX contents in SNODOX

The amounts of SNO groups in SNODOX were determined using a fluorescence method. SNODOX freeze-dried powder was redispersed in 500 μL of deionized water. Then, a total of 150 μL of diluted particle solution (100-fold dilution) was 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. A total of 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 excitation/emission wavelengths of 375 nm/415 nm and the equivalent SNO concentration was determined by a standard curve of sodium nitrite. For the determination of the DOX content, a total of 100 μL of redispersed SNODOX was added to 5 mL of an extraction solution (DMSO/deionized water = 4:1), followed by sonication extraction for 10 min. The solution was then centrifuged at 11590 rpm (15997g) for 10 min, and 200 μL of the supernatant was transferred to a black microplate for fluorescence measurement (479 nm/593 nm). DOX standard curves were constructed for the estimation of DOX concentrations.

1.8 Kinetics of Nitric Oxide Release.

Cumulative NO release was kinetically monitored using a nitric oxide-specific fluorescent probe, 2,3-diaminonaphthalene (DAN). A total of 100 μL of redispersed SNODOX was added to 9.9 mL of a PBS-based, release medium (containing 0.4 mM DAN, pH at 7.4 or adjusted to 5.7). The release solution was incubated at 37 °C and protected from light. At predetermined time intervals, 10 μL of sample was taken and added to 240 μL of 10 mM NaOH solution, followed by

fluorometric measurements (375 nm/415 nm; Infinite M200, Tecan Austria GmbH). The concentration of the fluorescent product (2,3-napthotriazole, NAT) was determined using a synthetic NAT standard.

1.9 Kinetics of DOX Release

A total of 1 mL of redispersed SNODOX (in deionized water) was added into a dialysis bag (MW cutoff: 12-14 kDa). The sealed dialysis bag was immersed in 225 mL of PBS-based release medium (pH 7.4 or 5.7, 37 °C) with constant stirring at 300 rpm (protected from light). At each sampling time, 25 mL of the medium was removed and then replenished with 25 mL of fresh medium. A total of 200 μL release medium was transferred to a black microplate for the fluorescence measurement of DOX (479 nm/593 nm). Cumulative DOX release at each sampling time 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 DOX 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 DOX added.

1.10 Cellular Uptake

Thirty thousand MDA-MB-231 cells were plated on glass cover slides and grown in RPMI 1640 culture medium (containing 10% FBS) for 48 h at 37°C. Cells were washed with PBS twice, and incubated with FBS-free culture medium containing 10 μM DAF-2DA for 30 min at 37°C. After removing the medium, cells were again washed with PBS twice, and incubated with cell culture medium containing free DOX, SNO silica NPs, or SNODOX for 24 h at 37°C. After incubation, cells were washed with PBS twice, and fixed in a 4% formaldehyde/PBS solution for 15 min. Then, the fixative was removed and cells washed again with PBS twice. The slides were incubated with DAPI-containing solution for cellular nuclei staining. Cell images were obtained using a deconvolution fluorescence microscope (DeltaVision, Applied Precision) equipped with a 40× 1.30 NA oil objective and standard DAP/FITC/TRITC filter settings.

1.11 Cytotoxicity

MDA-MB-231 or H9c2 cells were seeded in triplicates at 10000 cells (for 72 h study) and 40000 cells (for 24 h study) per well in 96-well plates and incubated in RPMI 1640 medium containing 10% FBS (culture medium). Following the overnight incubation, cells were incubated with culture medium containing various concentrations of free DOX, SNO NPs, SNODOX, or free DOX combined with SNO NPs for 72 h or 24 h. After incubation, the medium was removed and replaced with 100 μL of 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 540 nm using a microplate ELISA reader. The data were presented as mean ± s.e. of three independent experiments.

2. Supplementary data

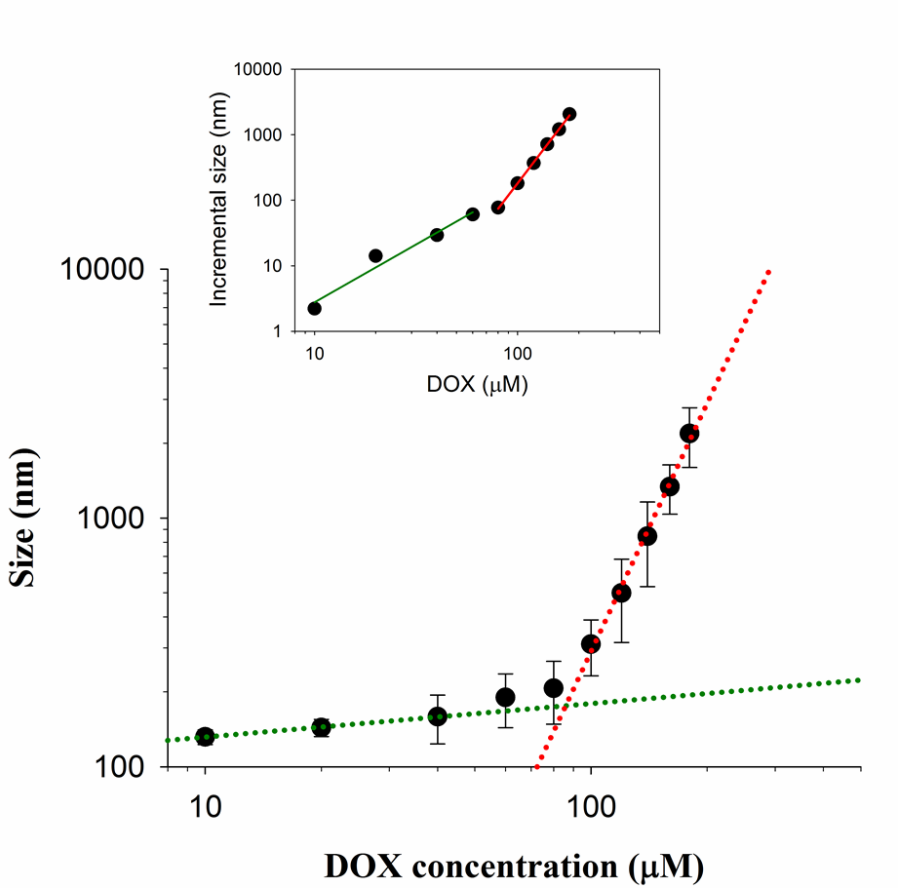


Figure S1. Hydrodynamic sizes as a function of DOX concentration. To prepare nanoparticles, 1 mL of organic phase was injected into 10 mL of water phase containing various concentrations of DOX (10 – 180 μM). DLS measurements were conducted after 1-h aging. The data reported are mean ± s.d. of three independent preparations. For each preparation, triplicate DLS readings were recorded. Dotted lines were drawn visually through the log-log linear portions of the graph. The two log-log linear portions cross at a critical concentration at about 80 μM. The incremental size due to the presence of DOX was calculated by subtracting the control size (i.e. without DOX), and the corresponding plot was inserted. For the low concentration region, the fitted line (solid green line) is: Log Y = 1.77 Log X – 1.32 , r = 0.9811; for the high concentration region, the fitted line (red) is: Log Y = 4.04 Log X – 5.81, r = 0.9997.

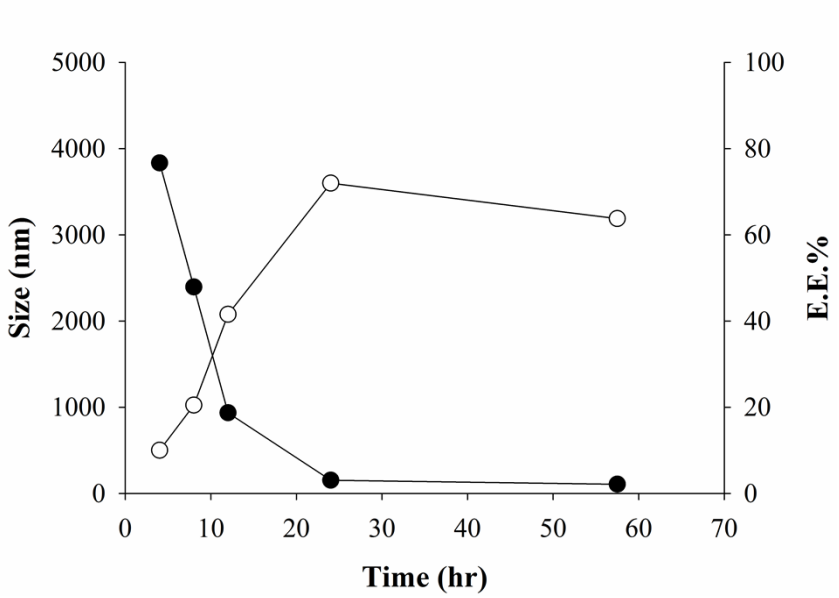


Figure S2. Effect of polycondensation reaction time on particle formation and DOX encapsulation. The organic phase (containing 200 mM MPTMS, 400 mM $NaNO_2$, 0.5 M HCl, in DMSO) was left standing at room temperature for 4, 8, 12, 24 and 57 h. At each reaction time, 1 mL of the organic phase was injected into 10 mL of the DOX-containing water phase. Sizes were measured by DLS. Encapsulation efficiency (E.E.) was determined according to the method described in the Materials and Methods.

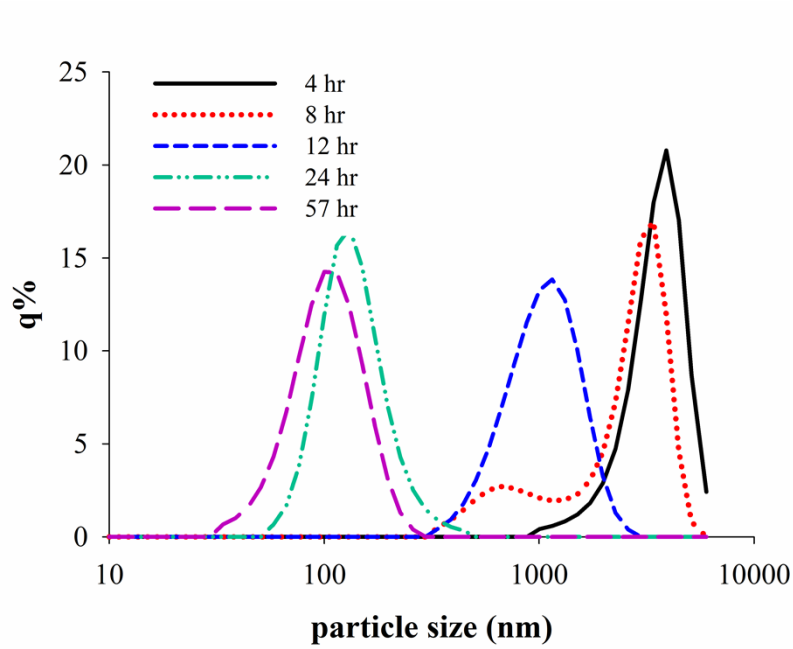


Figure S3. The corresponding particle size distributions for colloidal solutions generated at different reaction times (as in Figure S2).

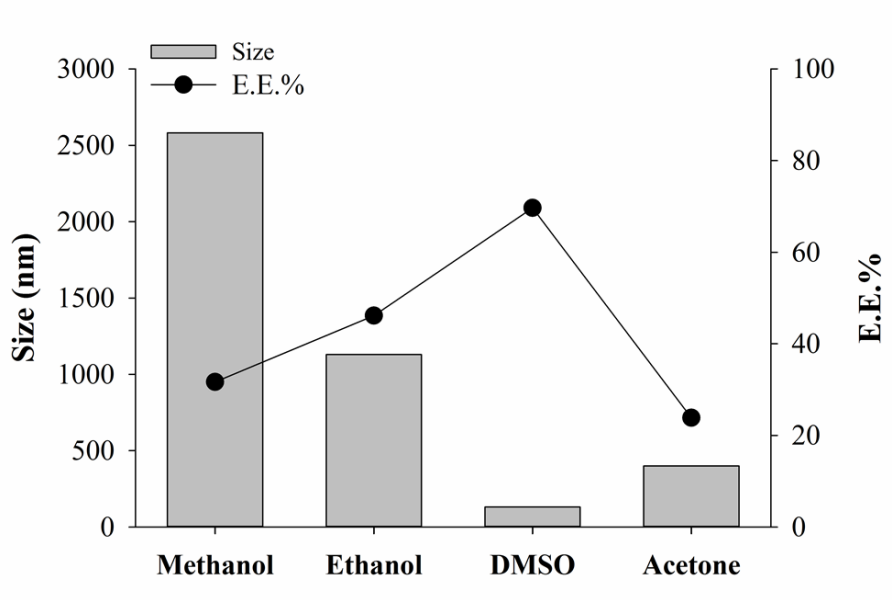


Figure S4. Effect of solvents used in the organic phase on particle formation and DOX encapsulation. Nanoprecipitation conditions: Organic phase reaction time, 24 h; injection volume, 1 mL; DOX conc., 80 μM.

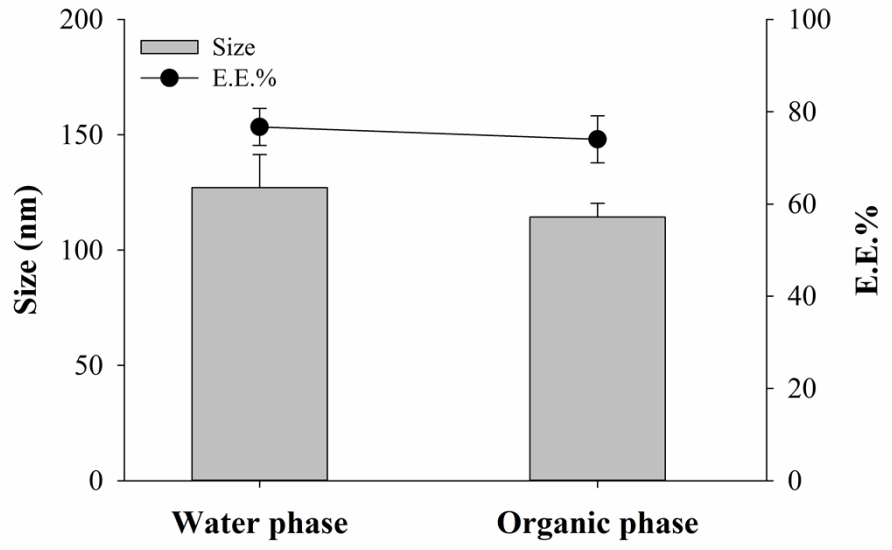


Figure S5. The effect of placing DOX in the water phase vs. in the organic phase on particle formation and DOX encapsulation. Instead of adding DOX in the water phase, DOX was added in the organic phase (800 μM) before nanoprecipitation, followed by injecting 1 mL of the DOX-containing organic phase to 10 mL water. The concentration of DOX in the final organic-water phase mixture was the same as in the control experiment, in which DOX (80 μM) was initially added in the water phase before nanoprecipitation.

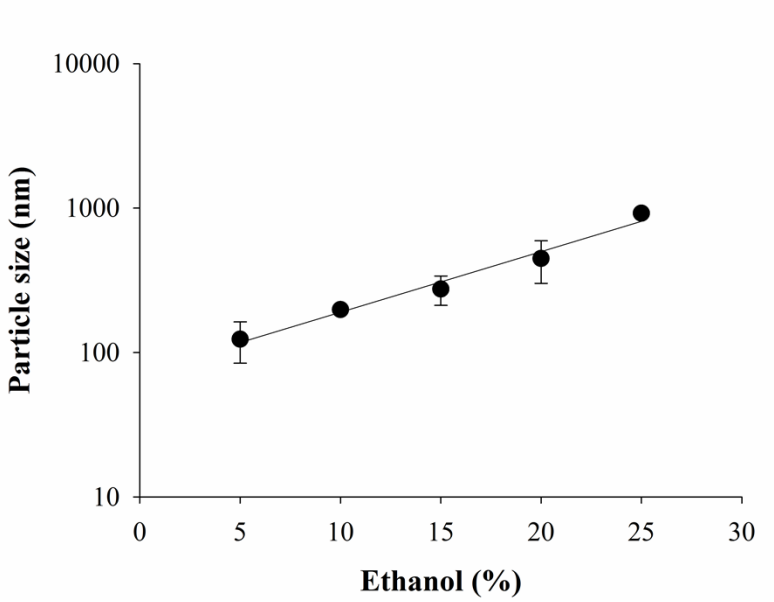


Figure S6. The particle size can be fine-tuned by adding ethanol in the water phase. Nanoprecipitation was conducted such that 1 mL of the organic phase was added to the DOX-containing water phase in the presence of 5 – 25% of ethanol. The DLS size data are mean ± s.d. of three independent experiments. A log-linear relationship was observed (the fitted line): Log size = 0.042*[ethanol] + 1.86, r = 0.990.

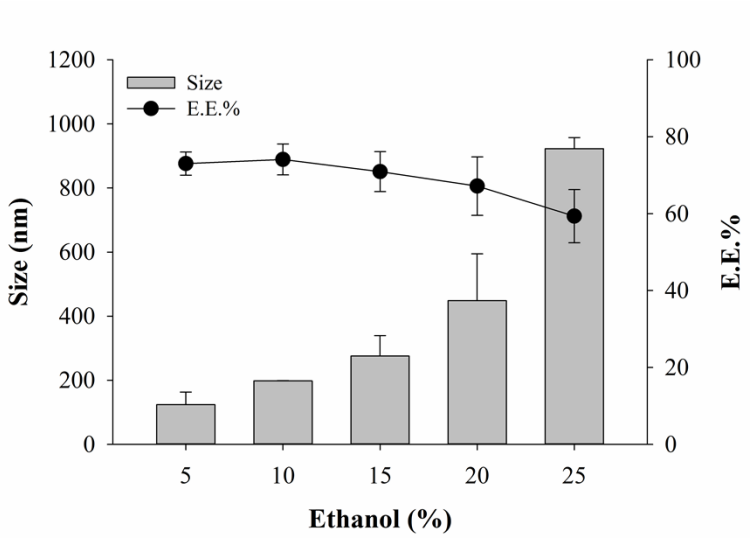


Figure S7. Effect of adding ethanol in the water phase on drug encapsulation. Note that the E.E% was only slightly changed, compared with size changes.

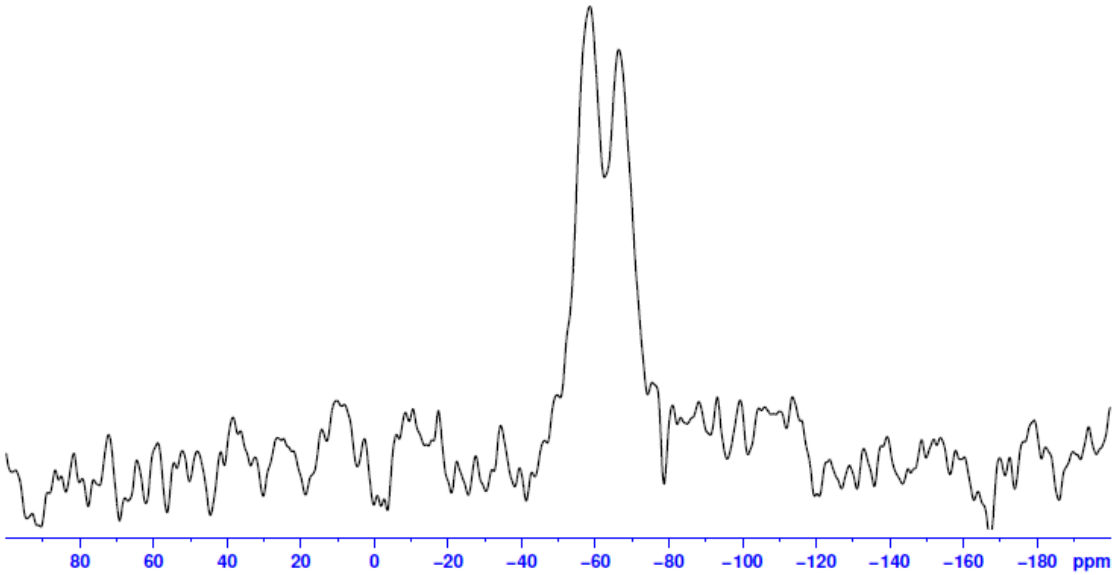


Figure S8. Solid-state ^{29}Si NMR spectrum for SNODOX.

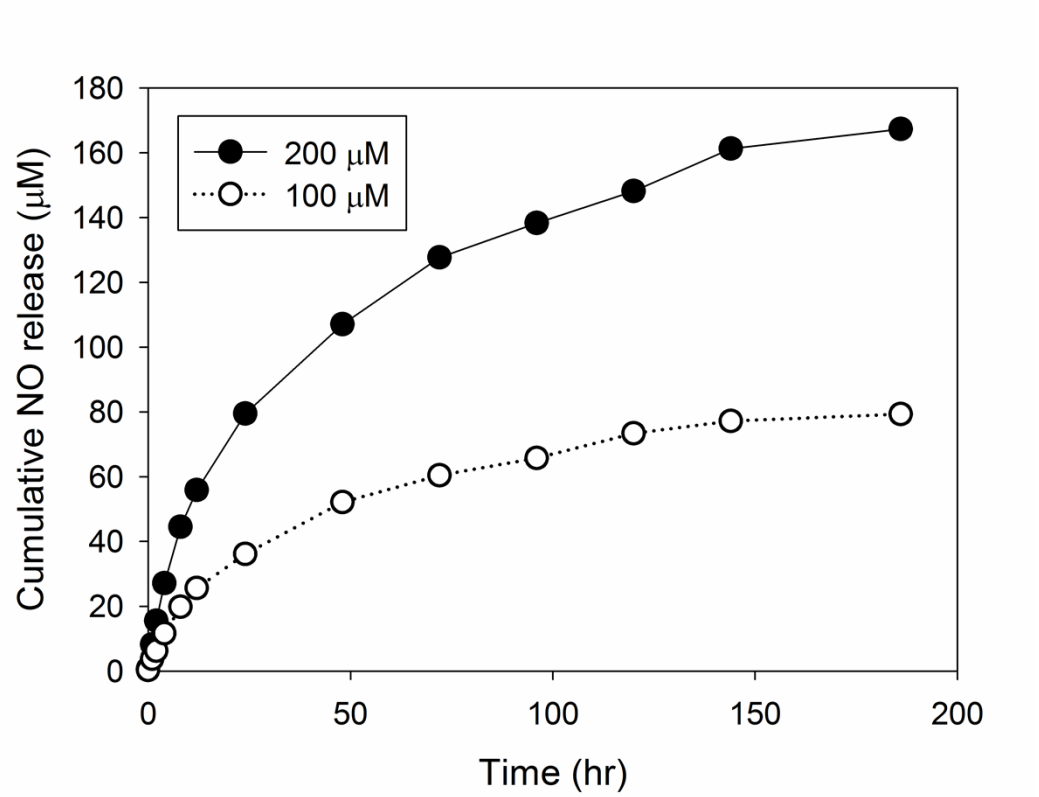


Figure S9. Cumulative NO release over a long incubation time. Lyophilized SNODOX powder was re-dispersed in water and diluted in the PBS-based release medium PBS (containing 0.4 mM DAN, pH 5.7). The initial SNO concentrations in particle dispersions were 200 and 100 μM, respectively. The cumulative NO release was determined using NAT standards (Materials and Methods), where 1 mol of NAT corresponds to 2 mol of NO (Miles et al. Fluorometric determination of nitric oxide. Methods, 1995, 7, 40-47).

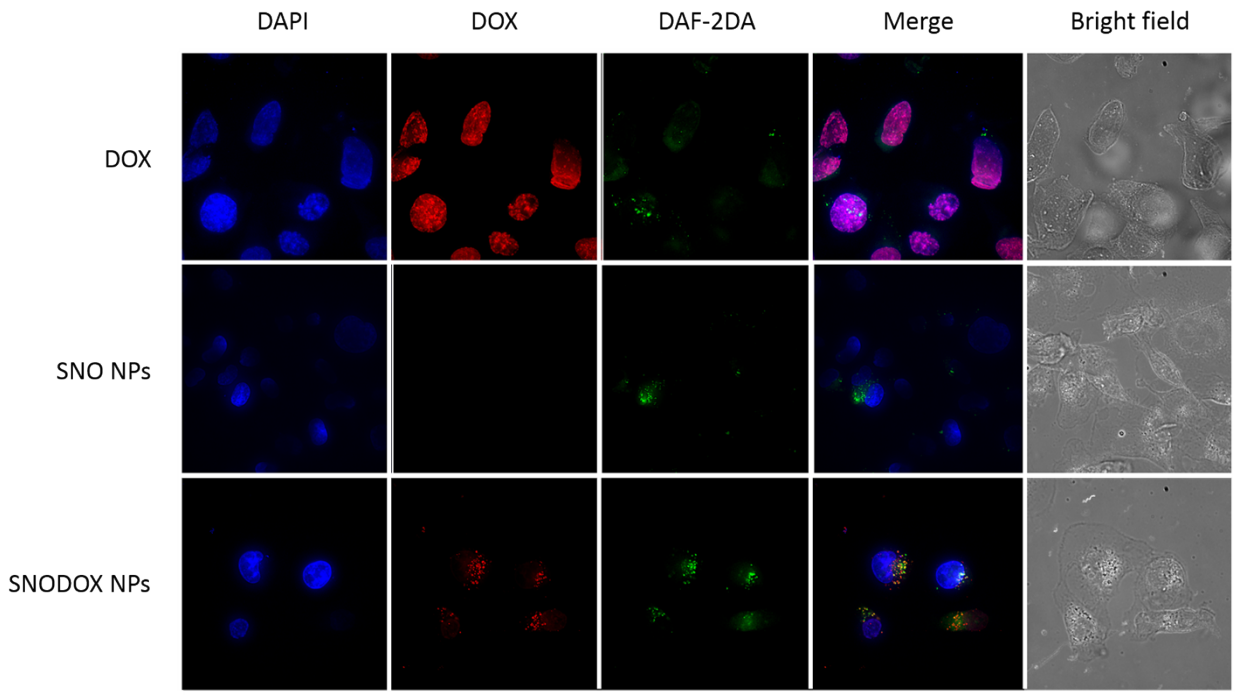
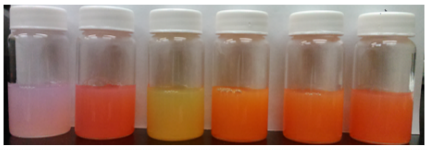


Figure S10. Fluorescence deconvolution microscopy images for cellular uptake of free DOX, S-nitroso silica NPs (SNO NPs), and SNODOX NPs in MDA-MB-231 cells. The nuclei were stained with DAPI. Cells were pre-treated with a cell-permeable, intracellular fluorescent probe for NO (DAF-2DA), followed by 24 h incubation with DOX, SNO NPs, and SNODOX, respectively.

DOX (μM)	0	80	0	80	80	80
Flu (μM)	0	0	80	80	40	16



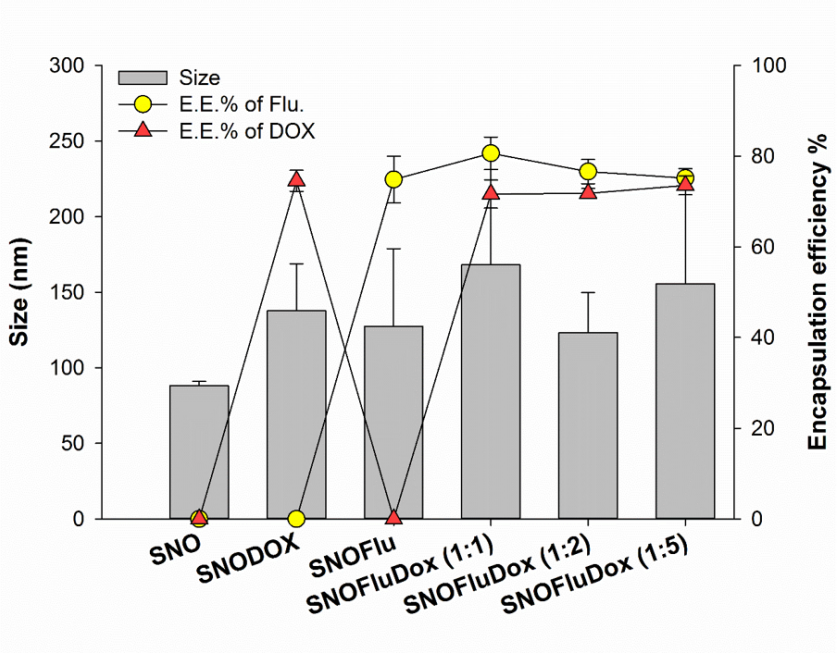


Figure S11. Preparation of fluorescein-loaded SNO NPs. Nanoprecipitation was conducted using the typical condition, except the compound added in the water phase was varied by including fluorescein. The concentration information for each experiment and the corresponding colloidal solutions are inserted. Abbreviations: SNO, SNO silica NPs; SNODOX, DOX-loaded SNO NPs; SNOFlu, fluorescein-loaded SNO NPs; SNOFluDox (1:1), fluorescein/DOX (1:1)-loaded SNO NPs; SNOFluDox (1:2), fluorescein/DOX (1:2)-loaded SNO NPs; SNOFluDox (1:5), fluorescein/DOX (1:5)-loaded SNO NPs. The encapsulation efficiecy (E.E.) of flourescein was determined similarly to that for DOX (see Materials and Methods), except that the excitation/emission wavelengths were 460 nm/520 nm, at which possible interference from DOX has been ruled out.