# Electronic Supplementary Information

## pH-Responsive Hierarchical Transformation of Charged Lipid Assemblies within Polyelectrolyte Gel Layers with Applications for Controlled Drug Release and MR Imaging Contrast

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*Materials*

$FeCl_2 \cdot 4H_2O$ and $FeCl_3 \cdot 6H_2O$ were purchased from J. T. Baker. 1,2-Dioleoyl-3-trimethylammonium propane (DOTAP) was obtained from Avanti Polar Lipids. Poly(acrylic acid) (PAAc) (Mn = 8000 g $mol^{-1}$), 2-hydroxylethyl methacrylate (HEMA), cholesteryl choloformate, RPMI medium, *N*-hydroxysuccinimide (NHS) and *N,N'*-dicyclohexylcarbodiimide (DCC) were obtained from Sigma-Aldrich. Maleimide-PEG-$NH_2$ ($M_w$ = 2000 g $mol^{-1}$) was acquired from JenKem Technology. Folic acid and citric acid monohydrate were purchased from Fisher. DOX was obtained from Seedchem. Human cervical tumor cell line, HeLa cell, was purchased from Food Industry Research and Development Institute of Taiwan. Dulbecco's modified Eagle medium (DMEM) and Hoechst 33258 were purchased from Invitrogen. AlamarBlue was purchased from AbD Serotec. Deionized water was produced from Milli-Q Synthesis (18 MΩ, Millipore). All other chemicals were reagent grade and used as received.

*Synthesis of citric acid-coated SPION*

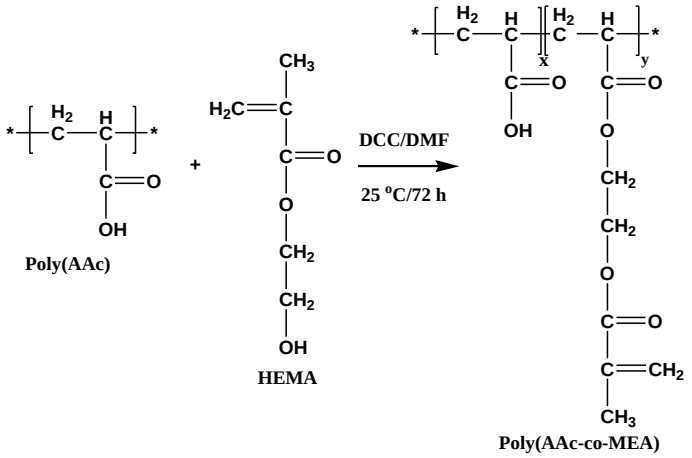
Citric acid-coated SPION were prepared by co-precipitation of $Fe_3O_4$ from a mixture of $FeCl_2$ and $FeCl_3$ (1:2 molar ratio) via addition of $NH_4OH$.[1] In brief, 0.43 g $FeCl_2$ and 1.17 g $FeCl_3$ were mixed in deionized water (20 mL) and heated to 80 $^oC$ under nitrogen atmosphere. $NH_4OH$ (35 %, 2.5 mL) was added by syringe under vigorous stirring with continuous heating for 30 min. This was followed by addition of citric acid (0.5 g) in 1.0 mL water and then an increase in temperature to 95 $^oC$. The reaction was carried out for 90 min. Finally, the resultant citric acid-covered SPION dispersion was dialyzed (Cellu Sep MWCO 12000-14000) against deionized water for 3 days to remove excessive unbound citric acid.

*Synthesis of poly(acrylic acid-co-methacryloylethyl acrylate) (poly(AAc-co-MEA))*

The copolymer was prepared by partial esterification reaction of PAAc in anhydrous DMF with purified HEMA (20 mol% with respect to the AAc residues) using DCC as the coupling agent. The reaction was performed at 25 $^oC$ for 72 h under stirring. The side product, *N,N'*-dicyclohexylurea, was fully removed by repeated filtration of concentrated polymer solution. Subsequently, the polymer solution was dialyzed (Cellu Sep MWCO 3500) against deionized water for over a period of 5 days to remove DMF and unreacted HEMA. The final product was collected by lyophilization. The composition of copolymer was determined by $^1$H-NMR, using

$D_2O$ as the solvent and DMF in a sealed capillary as an external standard. The calibration curve was established by the relative feature signal integrals of HEMA (with different concentrations in $D_2O$) at δ 1.9 ppm to the signal integral of DMF at δ 8.5 ppm. The composition was thus determined by a mass balance based on the characteristic signal integral of MEA at 1.9 ppm in combination with the calibration curve.

Scheme S1. Synthetic route of poly(AAc-co-MEA).

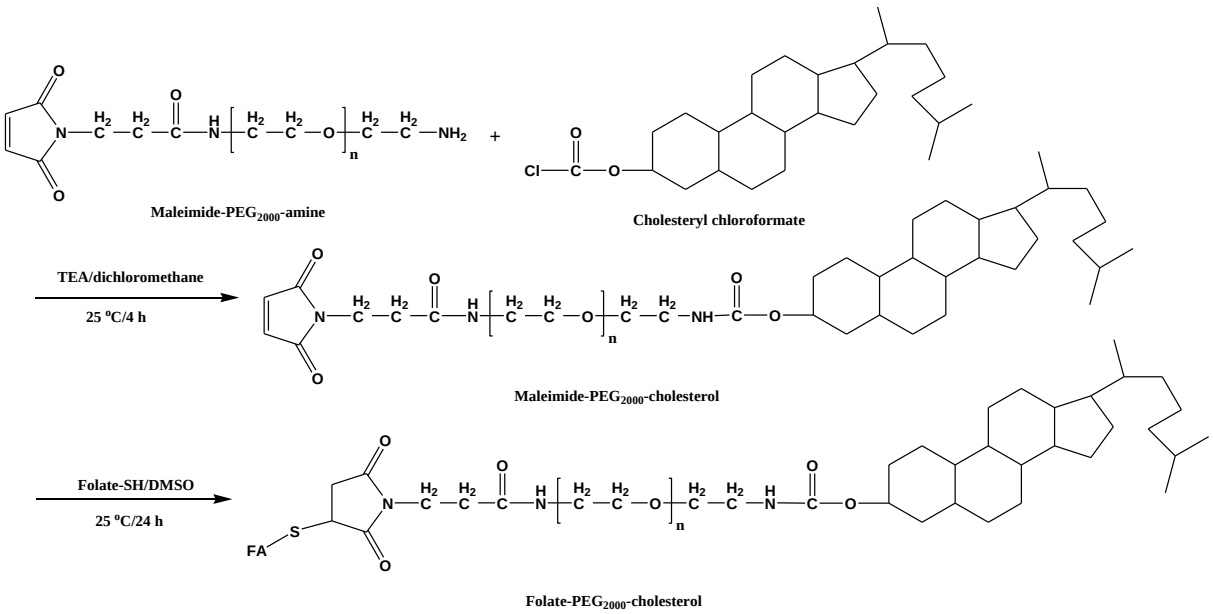



*Synthesis of ω-cholesterol-PEG-folate*

Maleimide-PEG-$NH_2$ (0.25 mmol) and cholesteryl choloformate (0.5 mmol) were dissolved in anhydrous dichloromethane (6.0 mL) in the presence of triethylamine (0.5 mmol) as the catalyst. The reaction was carried out at 25 °C for 4 h under stirring, followed by complete removal of anhydrous dichloromethane by evaporation under reduced pressure. Afterward, the resultant maleimide-PEG-cholesterol was re-dissolved in anhydrous DMSO solution (8.0 mL)

of thiol-functionalized folate (folate-SH, 0.5 mmol) and the reaction solution was stirred at room temperature for additional 24 h. This was followed by repeated precipitation in anhydrous diethyl ether to eliminate unreacted cholesterol choloformate and folate-SH and then filtration to collect the product. The yield of folate-PEG-cholesterol is ca. 85%. The cholesterol conjugation efficiency of folate-PEG-cholesterol was determined by $^{1}$H-NMR, using $CDCl_3$ as the solvent. According to the relative integral ratio of feature signals of methyl protons at δ 0.72 ppm from the end-methyl group of cholesterol and ethylene protons at δ 3.6 ppm from PEG, the efficiency was determined to be 92%. The folate conjugation efficiency was evaluated by UV spectroscopy to be ca. 90% based on the characteristic absorbance intensity at 285 nm using the pertinent calibration curve of folic acid with various concentrations in DMSO.

Scheme S2. Synthetic pathway of ω-cholesterol-PEG-folate.

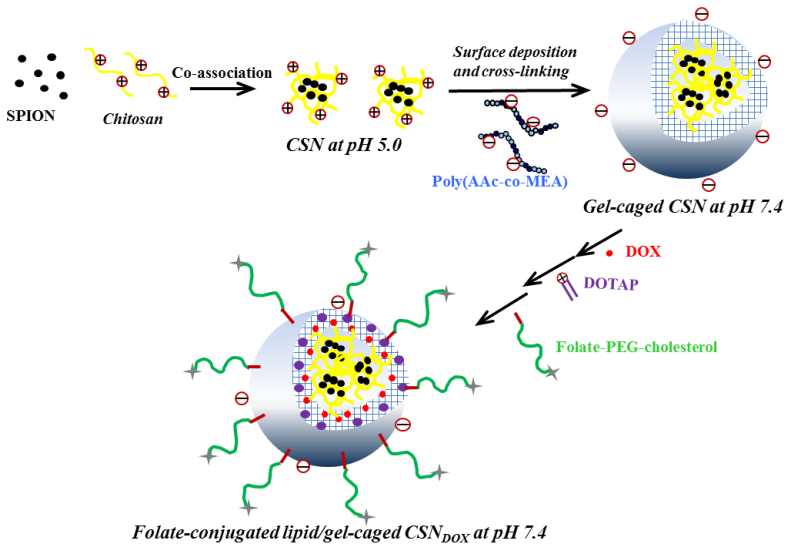



*Preparation of the folate-conjugated lipid/gel-caged $CSN_{DOX}$*

To attain folate-decorated lipid/gel-caged $CSN_{DOX}$, the hybrid chitosan/citric acid-covered SPION nanoassemblies (CSN) were first prepared by co-association of citric acid-covered SPION and chitosan via charge attraction between citric acid and primary amine residues. In brief, the citric acid-coated SPION suspension (1.0 mg $mL^{-1}$, 1.0 mL) was added into aqueous solution of chitosan (2.0 mg $mL^{-1}$, 1.0 mL) at pH 5.0 under vigorous stirring. This was followed by centrifugation (3000 rpm, 15 min) to remove unloaded SPION. Afterward, the CSN dispersion (1.5 mg $mL^{-1}$, 2.0 mL) was added dropwise into aqueous solution of poly(AAc-co-MEA) (2.4 mg $mL^{-1}$, 2.0 mL, pH 6.0) and then the pH of the mixture solution was adjusted to 6.0 by 0.1 M NaOH. After stirring over a period of 12 h under $N_2$ atmosphere, the polymer-caged

CSN suspension in the presence of diethoxyacetophenone (10 wt%) as the photo-initiator was placed into ultraviolet crosslinkers (UVP CL-1000) equipped with five 8-watt 254 nm UV tubes. Covalent cross-linking of poly(AAc-co-MEA)-caged CSN was achieved by radical polymerization of MEA residues under UV light of 80 $mW\,cm^{-2}$ for 40 min. Subsequently, the aqueous DOX solution ($3.7\times10^{-4}$ M, 1.0 mL, pH 7.4) was added directly into the aqueous gel-caged CSN dispersion (2.0 mL, pH 7.4) and the mixture was stirred at 25 $^{o}$C for 2 h. To remove unloaded DOX, the gel-caged $CSN_{DOX}$ dispersion was dialyzed (Cellu Sep MWCO 12000-14000) against the phosphate buffer of pH 7.4 for 3 days. Then, DOTAP in methanol (0.143 M) was added dropwise into the gel-caged $CSN_{DOX}$ dispersion (pH 7.4) at a preset weight ratio of DOTAP to poly(AAc-co-MEA) and the dispersion was stirred under reduced pressure over a period of 12 h to eliminate methanol. Finally, cholesterol-PEG-folate dissolved in methanol was directly added into the above dispersion and the solution was stirred for additional 12 h. For comparison, the folate-free lipid/gel-caged $CSN_{DOX}$ was acquired by the aforementioned approach using the maleimide-PEG-cholesterol instead of folate-PEG-cholesterol.

Scheme S3. Development of the folate-conjugated lipid/gel-caged $CSN_{DOX}$.




*Characterization of the hybrid nanoassemblies*

The particle size, size distribution and zeta potential were determined by a Malvern ZetaSizer Nano Series Instrument (He-Ne laser 4 mW, $\lambda = 633$ nm). The experimental results presented herein represent an average of at least triplicate measurements. The morphology of the citric acid-covered SPION, gel-caged CSN and lipid/gel-caged CSN was examined by transmission electron microscopy (TEM) (JEOL JEM-1200 CXII microscope, Japan) at an accelerated voltage of 120 kV. The sample was prepared by placing a few drops of the hybrid nanoparticle dispersion on a 300-mesh copper grid covered with carbon and then negatively stained with uranyl acetate solution (5.0 wt%) for 20 s. The sample was dried at 25 $^{o}$C for 2 days prior to TEM examination. For small angle X-ray scattering (SAXS) analysis, samples were run at beamline 23A1 at the National Synchrotron Radiation Research Center

(NSRRC), Hsinchu, Taiwan. For SAXS experiments, the energy of X-ray source was 14 keV. The scattering signals were collected by MarCCD detector of 512×512 pixel resolution. The scattering intensity profile was depicted from the scattering intensity (I) vs. scattering vector, $q = (4\pi/\lambda)\sin(\theta/2)$ (where $\theta$ is the scattering angle), after corrections for solvent background, sample transmission, empty cell transmission, empty cell scattering and the detector sensitivity.[2] For static experiments, the FA-decorated lipid/nanogel-caged $CSN_{DOX}$ dispersion at the preset pH was placed in the center of a steel washer and closely sealed with Kapton tape and run for 5 min. All data were normalized and the background corrected before analysis.

*Determination of DOX and SPION loading efficiency*

In order to determine the drug loading level, a small aliquot of gel-caged $CSN_{DOX}$ (0.1 mL) was withdrawn and diluted with DMF (0.9 mL) to a volume ratio of DMF/$H_2O$ = 9/1. The amount of DOX encapsulated was quantitatively determined by a fluorescence spectrometer (Hitachi F-7500, Japan). The excitation was performed at 480 nm and the emission spectrum was recorded in the range 500-700 nm. The calibration curve used for drug loading characterization was established by the fluorescence intensity of DOX with different concentrations in DMF/$H_2O$ (9/1 (v/v)) solutions. The drug loading efficiency was defined as the ratio of the amount of

DOX encapsulated in the gel-caged $CSN_{DOX}$ to that added in feed. As to the iron content of the purified gel-caged CSN, it was determined by inductively coupled plasma mass spectroscopy (ICP-MS) (Agilent 7500ce, Japan). In brief, the lyophilized gel-caged CSN dispersion was weighed precisely, followed by addition of 1.0 M HCl solution to achieve complete dissolution of SPION crystals. Iron concentration was determined at the specific Fe absorption wavelength (248.3 nm) based on a previously established calibration curve. The SPION loading efficiency was calculated as the ratio of the weight of embedded SPION to that added in feed.

*In vitro pH-triggered drug release*

The in vitro drug release profile of the gel-caged $CSN_{DOX}$ (or folate-conjugated lipid/gel-caged $CSN_{DOX}$ of varying DOTAP contents) was determined by the dialysis technique. The dispersion of drug-loaded hybrid nanoassemblies (1.0 mL) was put in a dialysis tube (Cellu Sep MWCO 12000-14000) and dialyzed against succinic acid buffers of pH 5.4 and 6.0, and phosphate buffer saline of pH 7.4 (50 mL, ionic strength 0.15 M), respectively, at 37 $^{o}$C. At selected time intervals, 1.0 mL of the external solution was withdrawn for fluorescence analysis, and the lost part replaced with equal volume of fresh medium. This was followed by determination of the drug concentration by fluorescence measurements using the appropriate calibration curve

of DOX with various concentrations in aqueous solution of pH 5.4, 6.0 or 7.4.

*Cellular uptake*

HeLa cells ($2\times10^5$ cells per well) were seeded in 6-well plates in DMEM containing 10% FBS and 1% penicillin and then incubated in a humidified incubator (5% $CO_2$) at 37 $^oC$ over a period of 24 h. The medium was then replaced by fresh DMEM containing either free DOX or folate-conjugated (or folate-free) lipid/gel-caged $CSN_{DOX}$ at a DOX concentration of 10 μM. Subsequently, HeLa cells were cultured at 37 $^oC$ for 2 h. After washing twice with PBS, HeLa cells were detached by the trypsin-EDTA solution and then dispersed in PBS (1.0 mL). Cellular drug uptake was then analyzed on a FACSCalibur flow cytometer (BD Biosciences, USA). For confocal laser scanning microscopy (CLSM) examination, HeLa cells were treated by the aforementioned approach, washed twice with PBS and then fixed with 2.5% formaldehyde. Finally, HeLa cells were stained with Hoechst 33258 for 10 min, and the slides rinsed with PBS three times. Coverslips were placed onto the microscope slides, and cellular uptake of DOX visualized by a ZESS LSM 510 META at the excitation and emission wavelength of 488 and 590 nm, respectively.

*Cytotoxicity evaluation*

HeLa cells were seeded in a 96-well plate at a density of $1\times10^4$ cells per well in DMEM containing 10% FBS and 1% penicillin and incubated at 37 ºC over a period of 24 h. The medium was then replaced with 100 μL of DMEM containing either free DOX or folate-conjugated (or folate-free) lipid/gel-caged $CSN_{DOX}$ at various DOX doses or drug-free lipid/gel-caged CSN of different concentrations. This was followed by incubation of HeLa cells for additional 24 h. Metabolic activity of the treated HeLa cells as an indirect measure of the number of living cells was then determined by AlamarBlue in RPMI (10% v/v, 100 μL). After incubation for 2 h, the absorbance at both 570 and 600 nm for the medium/AlamarBlue solution was recorded using a SpectraMax M5 microplate reader, and the cell viability (%) estimated according to the absorbance of the sample relative to the blank control.[3]

*Relaxivity measurement*

$T_2$ relaxation times of the citric acid-covered SPION, CSN and folate-conjugated lipid/gel-caged $CSN_{DOX}$ in aqueous solution at different pH were determined by a Bruker S300 Biospec/Medspec MRI at 7 T (Bruker Biospec, USA). The $T_2$-weighted MR images of folate-conjugated lipid/gel-caged $CSN_{DOX}$ at different pH and of HeLa cells after being incubated with the citric acid-coated SPION or folate-conjugated (or

folate-free) hybrid $CSN_{DOX}$ for 2 h were also attained accordingly. The treated HeLa cells were washed twice with fresh PBS, and harvested by trypsin-EDTA solution. The cell pellets were collected by centrifugation (200 g) and then washed twice with fresh PBS. Afterward, the cell pellets were re-suspended in PBS (0.1 mL) and then embedded in agar. $T_2$-weighted MR images were acquired using the following parameters: repetition time (TR) = 1500 ms for folate-conjugated hybrid $CSN_{DOX}$ and 3000 ms for HeLa cells exposed to the citric acid-coated SPION or folate-conjugated (or folate-free) hybrid $CSN_{DOX}$, echo time (TE) = 15, 30, 45, 60, 75, 90, 105, 120, 135, 150 ms, number of experiment (NEX) = 3, field of view (FOV) = 25×25 $mm^2$, matrix size (MTX) = 192×192, number of axial slices = 3 and slice thickness (SI) = 1.0 mm.

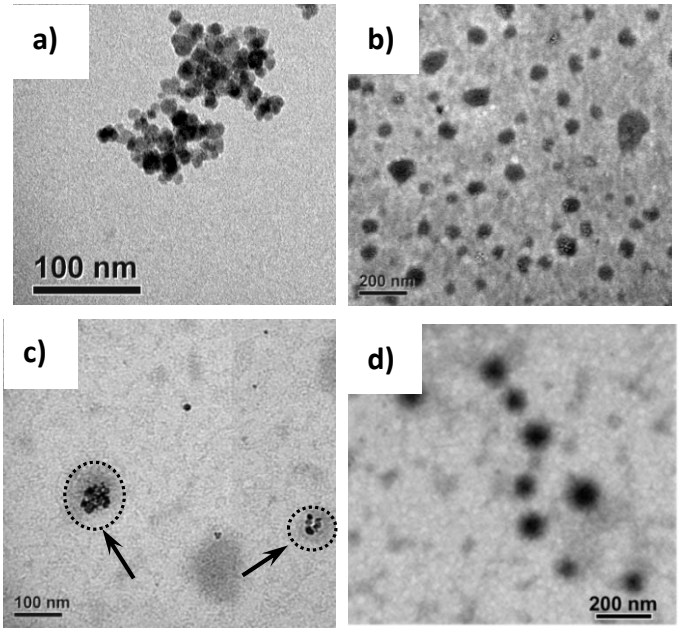



Figure S1. a) TEM images of the citric acid-coated SPION without negative staining, b) gel-caged CSN with negative staining, c) gel-caged CSN without negative staining and d) lipid/gel-caged CSN with negative staining using uranyl acetate.

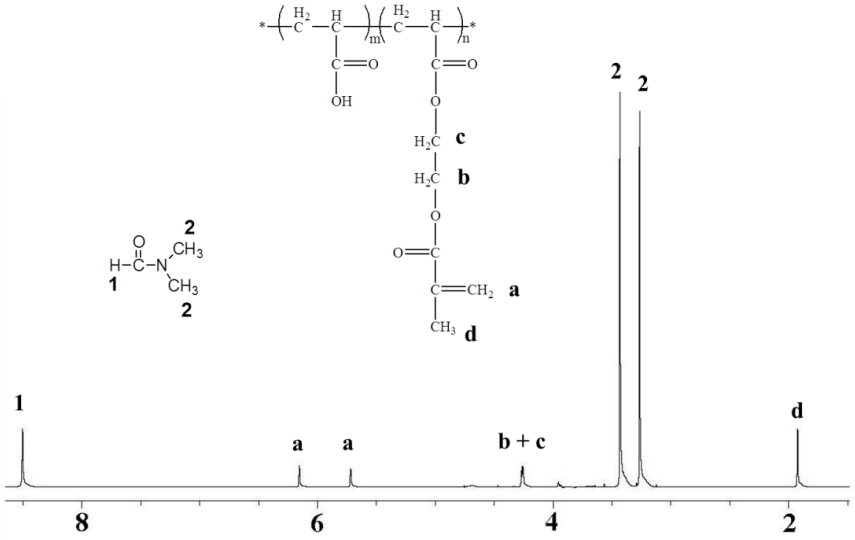



Figure S2. $^{1}$H-NMR spectrum of poly(AAc-co-MEA) in $D_2O$ (pD 9.0) using DMF in a sealed capillary placed coaxially in the NMR sample tube as an external standard.

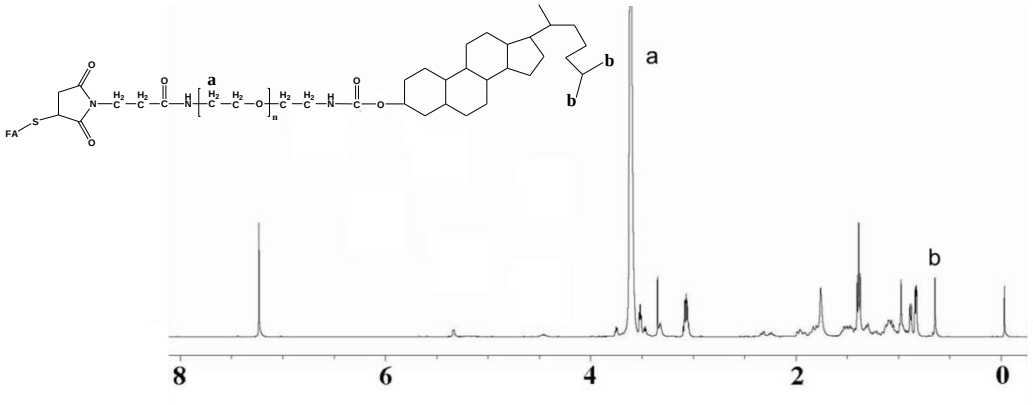



Figure S3. $^{1}$H-NMR spectrum of ω-cholesterol-PEG-folate in $CDCl_3$ at 20 $^{o}$C.

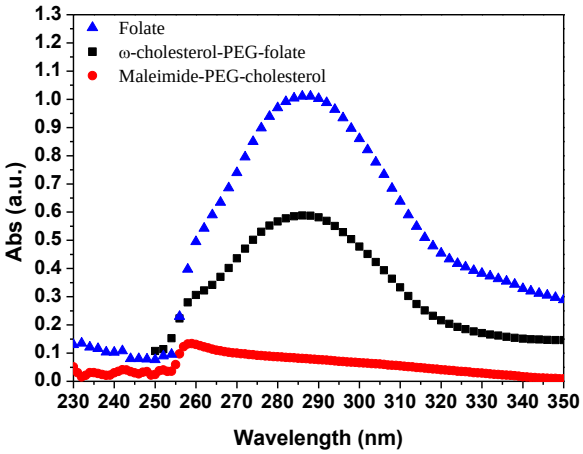



Figure S4. UV/Vis spectra of folate, ω-cholesterol-PEG-folate and maleimide-PEG-cholesterol in DMSO at room temperature.

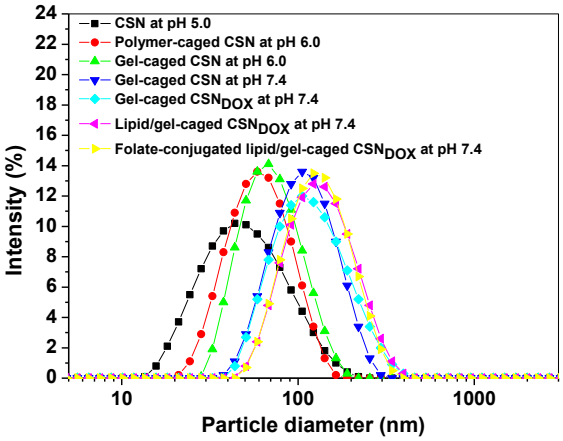



Figure S5. DLS particle size distribution profiles of various hybrid nanoassemblies in aqueous solution at 25 $^{o}$C.

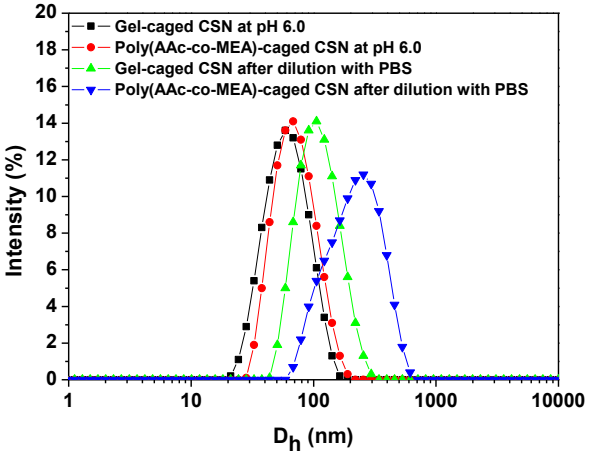



Figure S6. DLS particle size distribution profiles of gel-caged CSN and poly(AAc-co-MEA)-coated CSN (without photo-initiated cross-linking) before and after being subjected to 100-fold dilution with PBS.

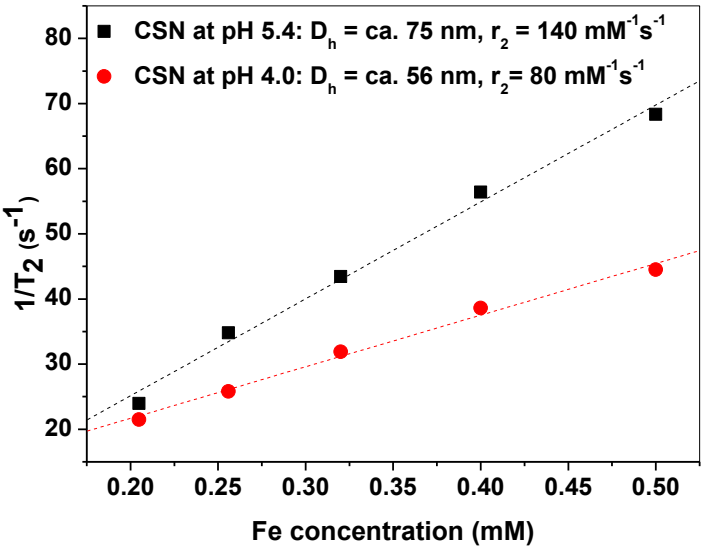



Figure S7. Reciprocal of $T_2$ (relaxation rate) of CSN alone in aqueous solutions at pH 5.4 and 4.0. At pH 6.0 and above, CSN undergo aggregation.

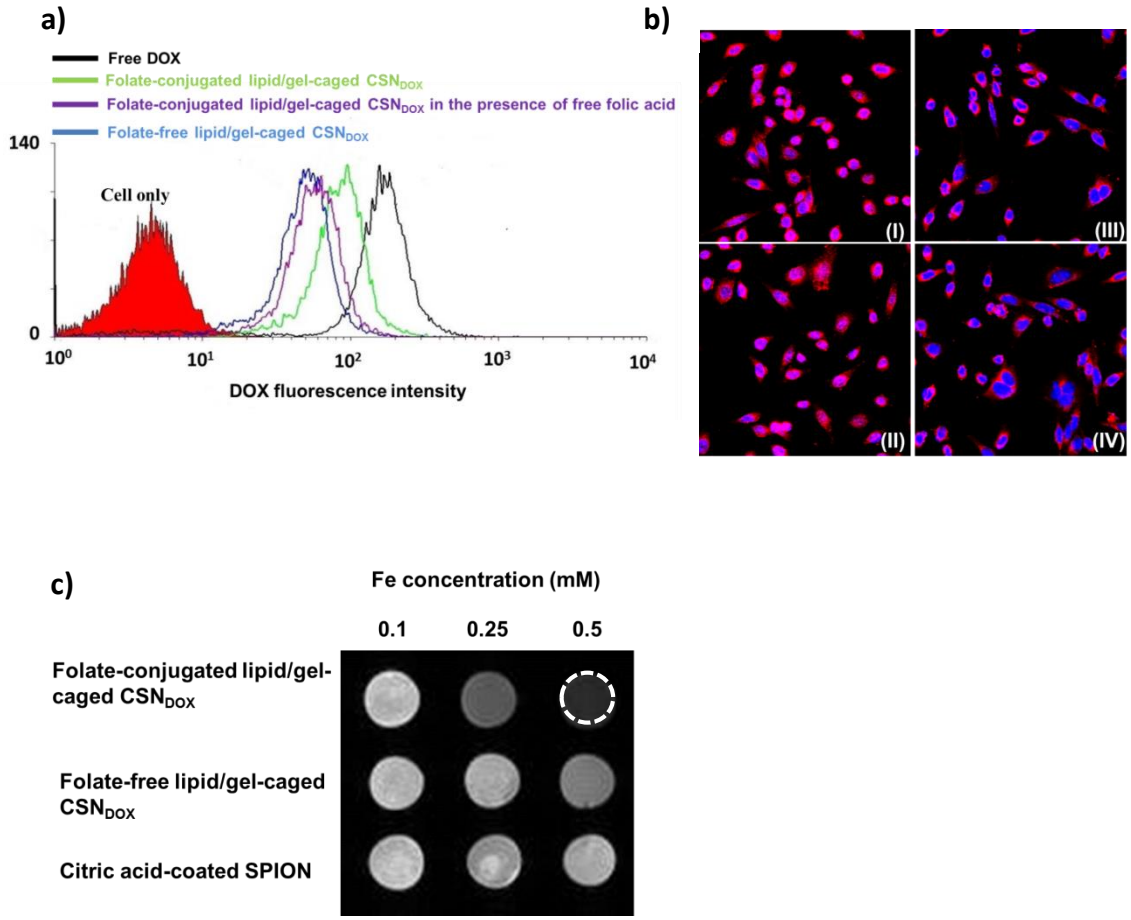



Figure S8. a) Flow cytometric histogram proflies of HeLa cells incubated with free DOX, folate-conjugated (or folate-free) lipid/gel-caged $CSN_{DOX}$ in the presence or absence of free folic acid (2.0 mM) at 37 ºC for 2 h (DOX concentration= 10 μM). b) CLSM images of HeLa cells incubated with free DOX (I), folate-conjugated lipid/gel-caged $CSN_{DOX}$ in the absence of free folic acid (II) or in the presence of free folic acid (III), and folate-free lipid/gel-caged $CSN_{DOX}$ (IV) for 2 h at 37 ºC. Cell nuclei were stained with Hoechst 33258. c) $T_2$-weighted MR images of HeLa cells incubated with the citric acid-coated SPION, folate-free (or folate-conjugated) lipid/gel-caged $CSN_{DOX}$ for 2 h at 37 ºC. As shown in CLSM images (Figure S8b), DOX species delivered by the lipid/gel-coated $CSN_{DOX}$ accumulate largely in the cytoplasmic region of HeLa cells. In contrast to the appreciable deposition of free DOX in cell nuclei, this observation demonstrates the distinct endocytotic uptake mechanism for DOX-loaded nanoparticles from the simple passive diffusion mechanism for free DOX. This also verifies effective drug release of the lipid/gel-coated $CSN_{DOX}$ from acidic endosomes/lysosomes, which is most likely achieved by the aid of the structural switch of lipid assemblies. Furthermore,

incorporation of folate-PEG-cholesterol into the gel layer greatly promotes cellular uptake via the folate receptor-mediated endocytosis for the lipid/gel-caged $CSN_{DOX}$. In agreement with CLSM images, the folate-decorated lipid/gel-caged $CSN_{DOX}$ show remarkably enhanced intracellular fluorescence intensity and $T_2$-weighted MR image contrasts compared to the folate-free counterpart.

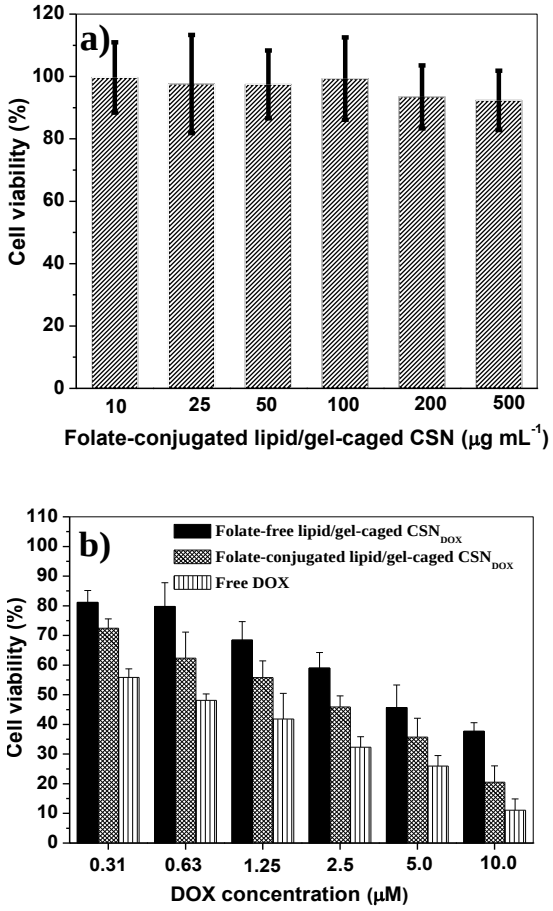



Figure S9. Cell viability of HeLa cells incubated with a) drug-free folate-conjugated lipid/gel-caged CSN, and b) free DOX, folate-conjugated (or folate-free) lipid/gel-caged $CSN_{DOX}$ with various DOX doses at 37 ºC for 24 h. In Figure S9a, the concentration of drug-free folate-conjugated lipid/gel-caged CSN corresponds to that of the counterpart carrying DOX used in the cytotoxicity evaluation. While the drug-free nanoparticles are virtually nontoxic to HeLa cells, the FA-conjugated lipid/gel-caged $CSN_{DOX}$ show superior capability to inhibit cancer cell proliferation with respect to the FA-free counterparts.

## References

[1] Y. Sahoo; A. Goodarzi; M. T. Swihart; T. Y. Ohulchanskyy; N. Kaur; E. P. Furlani;

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[3] D. S. Benoit; S. M. Henry; A. D. Shubin; A. S. Hoffman; P. S. Stayton, *Mol. Pharm*. **2010**, *7*, 442.