Supplementary Information

pH-Responsive robust polymer micelles with metal-ligand coordinated core cross-links

Gyu Ha Hwang, Kyung Hyun Min, Hong Jae Lee, Hye Young Nam, Gi Hyun Choi, Byung Joo Kim, Seo Young Jeong, and Sang Cheon Lee*

1. Materials

α-Methoxy-ω-amino-poly(ethylene glycol) (CH_3O-PEG-NH_2) with number average molecular weight (M_n) of 5000 g/mol was purchased from IDBIOCHEM Inc. (Ulsan, Korea) and used as received. 3,4-Dihydroxy-L-phenylalanine (L-DOPA), docetaxel (DTX), acetic anhydride, triphosgene, piperidine, iron (III) chloride ($FeCl_3$), and Cremophor EL were purchased from Sigma Co. (St. Louis, MO) and used without further purification. Glacial acetic acid was purchased from Biosesang Co. (Korea) and used as received. Tetrahydrofuran (THF) was distilled from Na/benzophenone under N_2, prior to use. *N*,*N*-Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were dried and vacuum distilled over calcium hydride. Calcium chloride ($CaCl_2$), 37 % hydrogen chloride (HCl), and sodium hydroxide (NaOH) were of the reagent grade.

2. Instrumentation

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz, respectively, on a Varian INOVA400 NMR spectrometer with a sample spinning rate of 5 kHz at 25 ^{o}C. Samples for NMR measurement were prepared by dissolving 10 mg of samples in 1 mL of DMSO-d_6 or D_2O.

Gel Permeation Chromatography (GPC). Molecular weight distributions were determined using a GPC equipped with a Waters 2414 refractive index detector, 515 HPLC pump, and three consecutive Styragel columns (HR1, HR2, and HR4). The eluent was DMF with a flow rate of 1 mL/min. The molecular weights were calibrated with polystyrene standards.

3. Synthesis of poly(ethylene glycol)-b-poly(L-3,4-dihydroxy-L-phenylalanine) (PEG-PDOPA) copolymer

3.1. Synthesis of di-O,O'-acetyl-L-DOPA-N-carboxyanhydride ((AC_2)-DOPA-NCA)

(AC_2)-DOPA-NCA was synthesized by a two-step literature process [1]. First, di-O,O'-acetyl-L-DOPA hydrochloride ((AC_2)-DOPA) was synthesized. The stirred suspension of L-DOPA (10 g, 50.7 mmol) in glacial acetic acid (100 mL) was purged with dry HCl gas for 2 h at room temperature. Under continuous purging with HCl gas, acetic anhydride (10 mL, 105.8 mmol) was added to the suspension, and the reaction mixture was stirred at room temperature. After 1.5 h, additional acetic anhydride (10 mL, 105.8 mmol) was added, and the reaction temperature was raised to 54 ℃. After 1 h, the reaction mixture became clear. The reaction mixture was concentrated on a rotary evaporator to smaller volume, and ethanol (30 mL) was added to destroy any remaining acetic anhydride. (AC_2)-DOPA was isolated by repeated precipitation into diethyl ether. Yield: 71.2 % ^{1}H NMR (DMSO-d_6): δ 2.2 (s, 6H), 3.1 (m, 2H), 4.5 (m, 1H), 7.0-7.3 (m, 3H). Second, for (AC_2)-DOPA-NCA, to a stirred suspension of the reaction product (3 g, 9.4 mmol) in dry THF (15 mL) was added triphosgene (1.1 g, 3.7 mmol) in dry THF (5 mL) at room temperature under nitrogen. After the reaction temperature was raised to 60 ℃, and the reaction was maintained for 3 h. (AC_2)-DOPA-NCA was isolated by repeated precipitation from THF into *n*-hexane. Yield : 93.5 %

3.2. Synthesis of PEG-b-PDOPA copolymer (PEG-PDOPA)

PEG-PDOPA that has EG units of 113 and DOPA units of 6 was synthesized by ring-opening polymerization of (AC_2)-DOPA-NCA in the presence of a CH_3O-PEG-NH_2 macroinitiator and a subsequent deprotection process. First, to a stirred solution of CH_3O-PEG-NH_2 (2 g, 0.4 mmol) in dry DMF (10 mL) was added (AC_2)-DOPA-NCA (1.23 g, 4 mmol) at room temperature under nitrogen. The reaction was maintained for 24 h. PEG-P((AC_2)-DOPA) was isolated by repeated precipitation from DMF into diethyl ether. Second, in order to remove AC groups, a stirred solution of PEG-P((AC_2)-DOPA) (2 g, 0.3 mmol) in dry DMF (20 mL) was bubbled with nitrogen. After 5 min, piperidine (0.27

mL) was added, and the reaction was stirred for 15 min. The reaction mixture was isolated by repeated precipitation from DMF into diethyl ether. A solution of the obtained precipitate in 0.1 N HCl was dialyzed against an acidic water (HCl, pH 4.5) using a membrane (molecular weight cut-off (MWCO): 1000), followed by lyophilization. Yield : 52.5 %.

4. Preparation of DTX-loaded polymer micelles with catechol-Fe^{3+} cross-linked cores

DTX-loaded PEG-PDOPA micelles with core-specific catechol-Fe^{3+} coordinated cross-linking were performed by the dialysis method and a subsequent core-cross-linking process. DTX (10 mg) in DMF (1 mL) was added to a stirred solution of PEG-PDOPA (100 mg) in DMF (20 mL). The solution was dialyzed using a membrane (Spectrapor, MWCO: 1,000 g/mol) against doubly distilled de-ionized water. After 10 h, the solution of DTX-loaded micelles was collected. To form catechol-Fe^{3+} cross-linked cores on the polymer micelles, an aqueous solution of $FeCl_3$ (40 mM, pH 1.73) was firstly added to a stirred solution of polymer micelles (pH 6.0) at the feed molar ratio of [DOPA] : [Fe^{3+}] = 2 : 1. The pH of the solution mixture was 1.87. For Fe^{3+}-catechol bis-complex formation, the solution pH was adjusted to pH 7.4. After 1 h, the solution was dialyzed to remove unreacted ionic species. Unloaded DTX was eliminated by centrifugation (3000 rpm) of the dialyzed solution, and the supernatant solution was freeze-dried for isolation of DTX-CLPMs. The loading contents and loading efficiency of DTX within the polymer micelles were measured by high-performance liquid chromatography (HPLC). The concentration of DTX was determined by HPLC after solubilization of DTX-loaded micelles in DMF/acetonitrile (50:50). The HPLC system consisted of a reverse-phase silica column (ZORBAX Eclipse XDB-C18, 4.6 × 250mm, 5-Micron, Agilent, USA), a mobile phase of acetonitrile, and water (60:40 v/v) pumped (LC-20AT, Shimadzu) at a flow rate of 1.0 mL/min at 25 ^{o}C. A 50 μL aliquot of sample was injected, and detected at 230 nm with a UV detector (CBM-20A, Shimadzu). The DTX loading amount and efficiency was determined using a calibration curve of various DTX concentrations (0.5-250 μg/mL).

5. Characterization of DTX-loaded core-cross-linked polymer micelles (DTX-CLPMs)

Dynamic light scattering (DLS) measurements were carried out using a 90 Plus particle size analyzer (Brookhaven Instruments Corporation). As a light source, a vertically polarized He-Ne laser (632.8 nm) was used. The scattered light was measured at an angle of 90° and was collected on an autocorrelator. The hydrodynamic diameters of DTX-loaded non-cross-linked polymer micelles (DTX-NPMs) and DTX-loaded core-cross-linked polymer micelles (DTX-CLPMs) were calculated by using the Stokes-Einstein equation [2]. The polydispersity factor of various polymer micelles, represented as μ_2/Γ^2, was calculated from the cumulant method [2]. The morphology of DTX-loaded core-cross-linked polymer micelles was examined by transmission electron microscopy (TEM) (CM30, Philips, CA, USA). For visualization of polymer micelles, negative staining was performed using a uranyl acetate solution (3 wt %).

6. Spectrophotometric analysis of pH-dependent coordination between catechols and Fe^{3+} ions in PEG-PDOPA micelle cores

UV-Visible absorption spectra were obtained using a UV-1650PC (Shimadzu, Japan). The aqueous mixtures (1 g/L) of DTX-loaded PEG-PDOPA micelles and $FeCl_3$ (the molar ratio of [DOPA] : [Fe^{3+}] = 2 : 1) at various pH conditions (pH 5.0, 7.4, 12.0) were prepared by increasing the pH (1.73) of initial acidic solution mixture of $FeCl_3$ and DTX-loaded micelles. pH-Dependent variation in the absorption spectra was monitored at 400 ~ 650 nm. Photo images of the aqueous mixture at various pH were obtained in 4 clear window cuvettes.

7. Stability of DTX-CLPMs in Serums

For the stability evaluation in serum conditions, scattered light intensity (SLI) of the DTX-CLPMs (1.25 g/L) in serum-containing solution (pH. 7.4, 50 % fetal bovine serum (FBS)) was measured by dynamic light scattering. At predetermined time intervals, SLI was monitored and compared to the initial scattered light intensity (SLI_0). The ratio of scattered light intensity (SLI/SLI_0) was monitored. As a control experiment, the stability of the DTX-NPMs was examined under the identical conditions.

8. pH-Controlled DTX release from DTX-CLPMs

In vitro release profiles of DTX from DTX-CLPMs were investigated in the aqueous buffer solutions (pH 7.4 and 5.0 phosphate buffer). DTX-CLPM solutions (1 mL, 1 g/L) in a dialysis membrane bag (MWCO: 1,000 g/mol) were prepared. The release experiment was initiated by placing the dialysis bag in 10 mL of release media. The release medium was shaken at a speed of 90 rpm at 37 ^{o}C. At predetermined time intervals, samples (10 mL) were withdrawn and replaced with an equal volume of the fresh medium. The concentration of DTX in the samples was measured by HPLC at 230 nm. The assay for DTX was based on a calibration curve of various DTX concentrations (0.5-250 μg/mL).

9. Visualization of cellular uptake of DTX-CLPMs

For visualization of cellular uptake of DTX-CLPMs, fluorescein isothiocyanate (FITC)-labeled DTX-CLPMs were prepared as follows: First, for synthesis of FITC-labeled PEG-b-PDOPA, FITC (0.014 g, 0.03 mmol) was added to a stirred solution (2 mL) of PEG-b-PDOPA (0.2 g, 0.03 mmol) in DMF (2 mL). After 24 h, the reaction mixture was dialyzed against distilled de-ionized water using a membrane (molecular weight cut-off (MWCO): 3,500), followed by lyophilization. The labeling efficiency of FITC to PEG-b-PDOPA was calculated to be 90.2%, which was determined using a calibration curve of various FITC concentrations in DMF. Second, FITC-labeled DTX-CLPMs were prepared based on an identical procedure formerly established for DTX-CLPMs, except the use of FITC-labeled PEG-b-PDOPA instead of PEG-b-PDOPA.

To observe the cellular uptake, MCF-7 cells were seeded in six-well plate at a density of 1×10^5 cells/well in 2 mL of RPMI 1640 medium supplemented with 10 % (v/v) FBS, 1 %(v/v) penicillin-streptomycin. After 24 h incubation at 37 ^{o}C with 5 % CO_2, the medium was removed and replaced with 2 mL of medium containing FITC-labeled DTX-CLPMs (100 μg/mL). The MCF-7 cells treated with FITC-labeled DTX-CLPMs were incubated at various incubation times (10 min, 30 min, 1 h, 2 h). At the designated time, the cells were washed three times with PBS and then fixed with 3.7% formaldehyde.

Cellular uptake images were confirmed by IX71 fluorescence microscope (Olympus Optical Co., Tokyo, Japan).

10. Biocompatibility of NPMs, CLPMs and Cremophor EL

MCF-7 human breast cancer cells were obtained from the Korean Cell Line Bank (KCLB, Seoul) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Gaithersburg, MD) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS, Gibco BRL), and 1 % (v/v) penicillin-streptomycin (Gibco BRL). Cells were cultured in a humidified incubator at 37 ^{o}C with 5 % CO_2. The culture medium was replaced every two days. Cells were seeded at a number of 5×10^3 cells per well in 96-well flat-bottomed plates for 1 day. The cells were washed with PBS and incubated with 200 μL of a fresh medium containing NPMs, CLPMs and Cremophor EL at 37 ^{o}C with 5 % CO_2. The concentration of NPMs, CLPMs and Cremophor EL was diluted with culture medium to obtain a concentration range from 10 to 500 μg/mL. After 24 h, the cells were washed with PBS, followed by the cell counting kit (CCK) assay. In brief, the medium was replaced with CCK-8 solutions (Dojindo Laboratories, Kumamoto). The absorbance of each wells was measured at 450 nm by a microplate reader (Biorad Elizer, PA). Cell viability was evaluated by relative to the untreated control group.

11. Cytotoxicity of DTX-CLPMs

MCF-7 cells were seeded at a number of 5×10^3 cells per well in 96-well plate and incubated for 24 h at 37 ^{o}C with 5 % CO_2. The cells were washed with PBS and incubated with 200 μL of a fresh medium containing various concentration of DTX-NPMs, DTX-CLPMs and DTX-Cremophor EL at 37 ^{o}C with 5 % CO_2. For preparation of the DTX-Cremophor EL solution, DTX (5 mg) dissolved in Cremophor EL (1 mL), afterwards mixed with a fresh cell culture media. After 24 h incubation, the cells were washed with PBS, followed by the CCK assay. The absorbance of individual wells was measured at 450 nm by a microplate reader (Biorad Elizer, PA). The data are expressed as the percentages of viable cells compared to the survival of a control group. The IC_{50} value presents the concentration of DTX yielding 50 % inhibition of cell proliferation, compared to the untreated control.

References

[1] W. D. Fuller, M. S. Verlander, M. Goodman, *Biopolymers*, **1978**, *17*, 2939.

[2] A. Harada and K. Kataoka, *Macromolecules*, **1998**, *31*, 288.

Table S1 Characteristics of the PEG-b-PDOPA copolymer.

copolymer	composition ratio [a] ([EG] : [DOPA])	M_n [a]	M_w/M_n [b]
PEG-PDOPA	113 : 6	6,600	1.10

*The copolymers were synthesized using the macroinitiator (CH_3O-PEG-NH_2) with M_n of 5,000 and polydispersity index (M_w/M_n) of 1.13.
[a] Calculated by ^{1}H NMR spectra [b] Estimated by GPC.

Table S2 The micelle sizes, zeta potentials, and polydispersity factors of various micelles

micelles	d [a] (nm)	μ_2/Γ^{-2} [b]
NPMs	40.5	0.23
CLPMs	45.0	0.27
DTX-NPMs	43.5	0.21
DTX-CLPMs	43.6	0.30

[a] Mean hydrodynamic diameters at pH 7.4, 25 ^{o}C. [b] Polydispersity factor estimated by dynamic light scattering.

Fig. S1 Synthetic route to PEG-PDOPA.

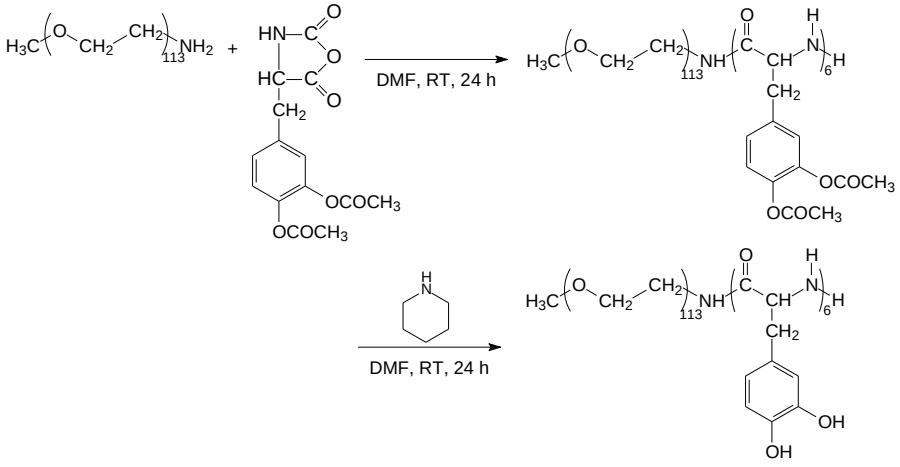

Fig. S2 ^{1}H NMR spectra of (a) PEG, (b) PEG-P((AC$_2$)-DOPA), and (c) PEG-PDOPA in DMSO-d_6.

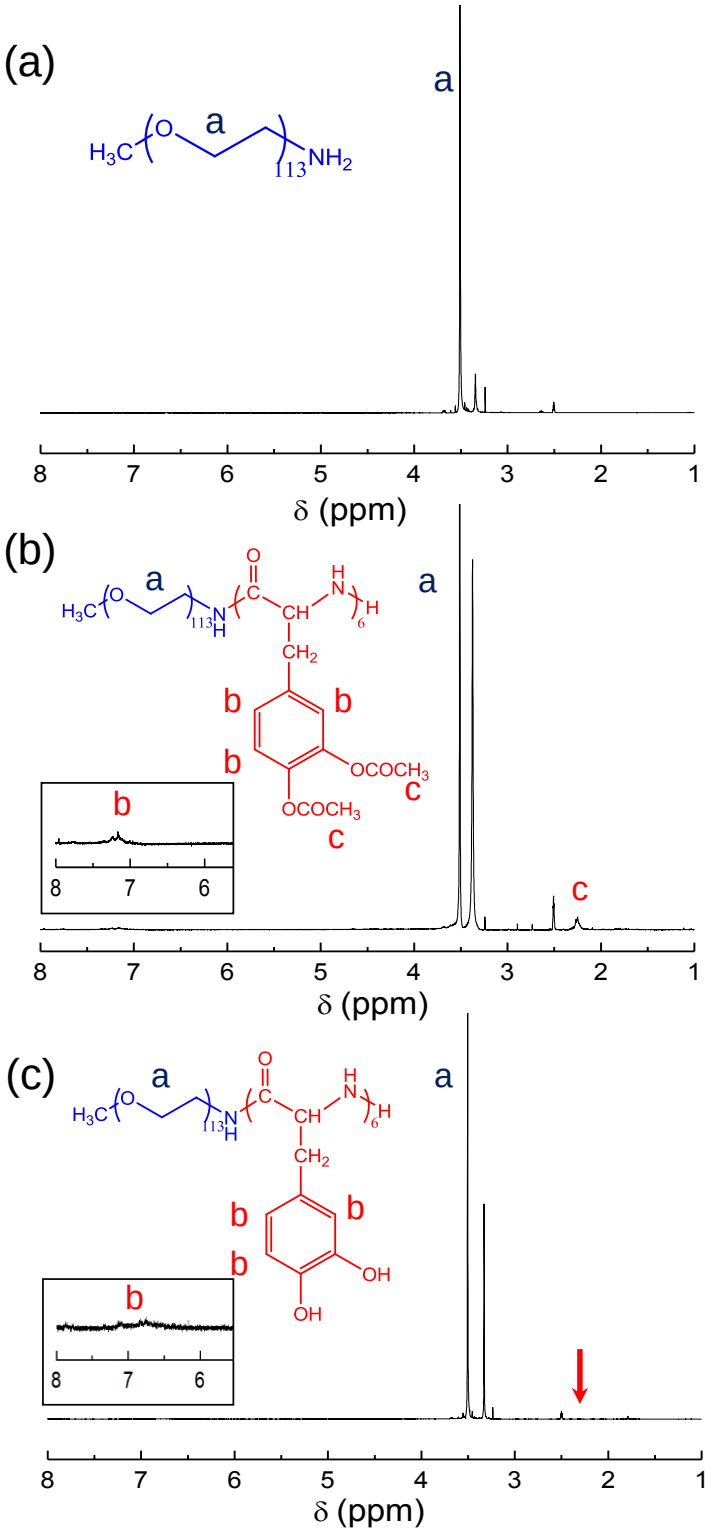

Fig. S3 ^{13}C NMR spectra of (a) PEG, (b) PEG-P((AC$_2$)-DOPA), and (c) PEG-PDOPA in DMSO-d_6.

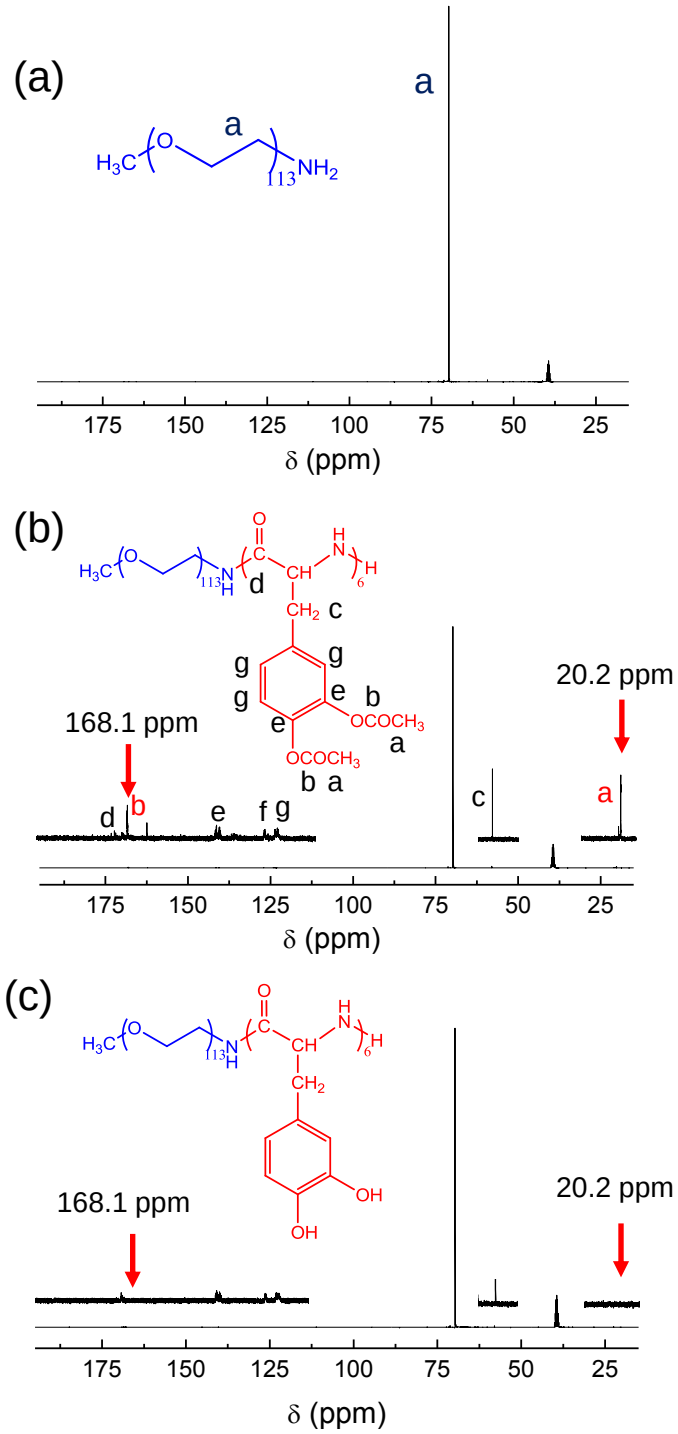

Fig. S4 Gel permeation chromatograms of (a) PEG and (b) PEG-P((AC_2)-DOPA).

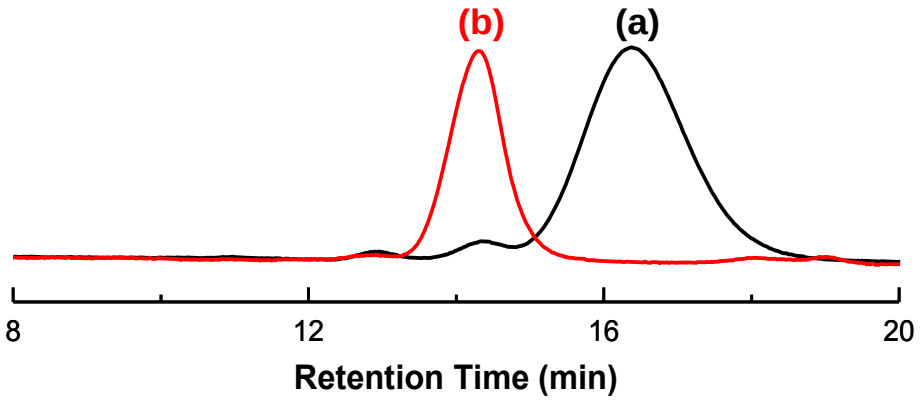

Fig. S5 Size distribution and morphology of DTX-NPMs (a and c) and DTX-CLPMs (b and d) estimated by dynamic light scattering and TEM.

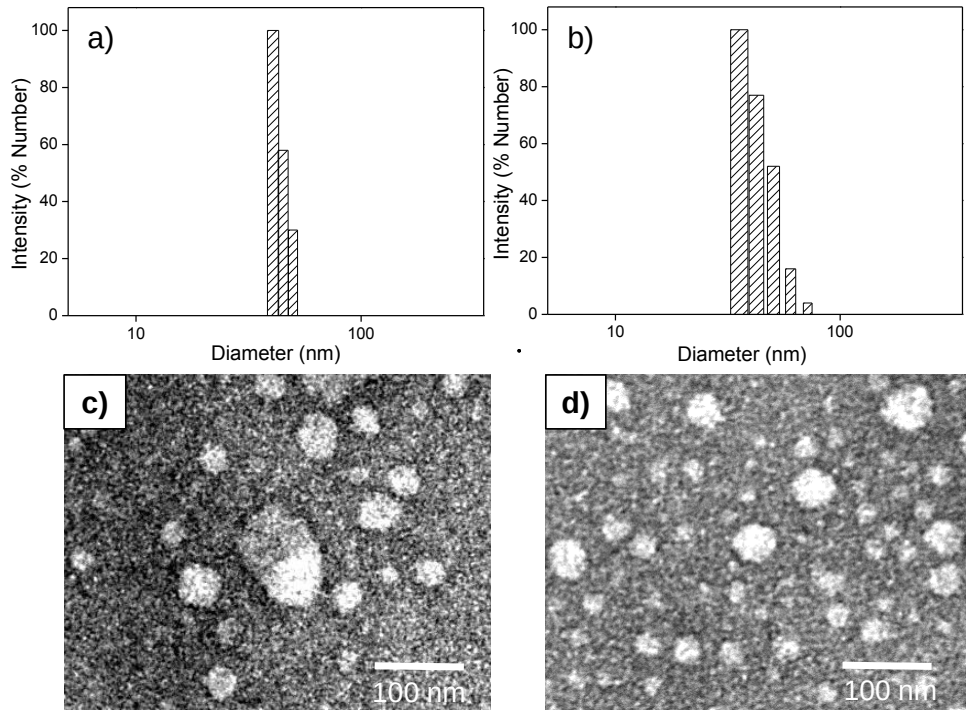

Fig. S6 Time-dependent change of the ratio of scattered light intensities (SLI/SLI_0 (%)) of DTX-CLPMs and DTX-NPMs in the FBS-containing solution (pH 7.4). Each point represents the mean value of *n* experiments ± S.D. (*n* = 3).

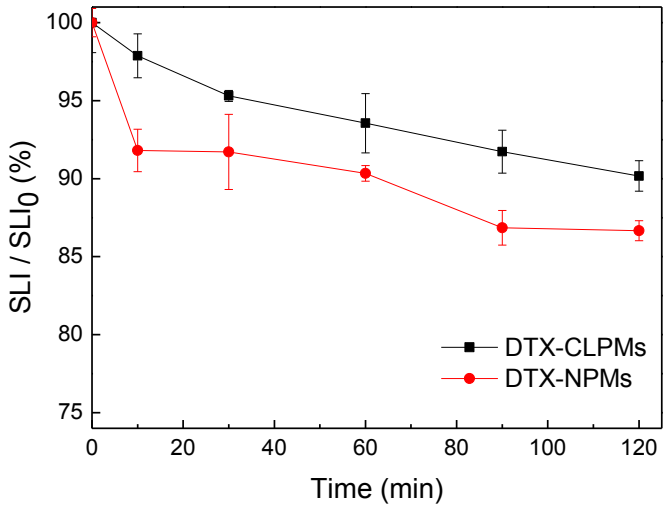

Fig. S7 Time-dependent visualization of cellular uptake of FITC-labeled DTX-CLPMs as a function of incubation time.