Supplementary Material (ESI) for Chemical Communications

<Supplementary Information>

Spatially mineralized self-assembled polymeric nanocarriers with enhanced robustness and controlled drug-releasing property

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Experimental

Materials and Equipment. α-Methoxy-ω-amino-poly(ethylene glycol) (CH_3O-PEG-NH_2) of M_n of 2000 g/mol was purchased from SunBio Inc. (Seoul, Korea) and used as received. β-Benzyl L-aspartate (BAsp), L-phenylalanine (Phe), and doxorubicin hydrochloride (DOX· HCl) were purchased from Sigma Co. (St. Louis, MO) and used without further purification. Triphosgene, pyrene, and 1-dodecylpyridinium chloride (DPC) were purchased from Aldrich Co. (Milwaukee, WI) and used as received. Tetrahydrofuran (THF) was distilled from Na/benzophenone under N_2, prior to use. *N,N*-Dimethylformamide (DMF) was dried and distilled over calcium hydride. Calcium chloride ($CaCl_2$), disodium hydrogen phosphate (Na_2HPO_4), 35% hydrogen chloride (HCl), and sodium hydroxide (NaOH) were of the reagent grade. *β*-Benzyl L-aspartate *N*-carboxyanhydride (BAsp-NCA) and L-phenylalanine *N*-carboxyanhydride (Phe-NCA) of high purity were synthesized by the Fuchs-Farthing method using triphosgene [1]. Anal. Calcd for BAsp-NCA ($C_{12}H_{11}NO_5$): C, 57.83; H, 4.45; N, 5.62. Found: C, 57.95; H, 4.48; N, 5.63. Calcd for Phe-NCA ($C_{10}H_9NO_3$): C, 62.82; H, 4.74; N, 7.33. Found: C, 62.24; H, 4.91; N, 7.26.

^{1}H NMR spectra were recorded at 400 MHz on a Varian INOVA400 NMR spectrometer with a sample spinning rate of 5 kHz at 25 ^{o}C. 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 THF with a flow rate of 1 mL/min. The molecular weights were calibrated with polystyrene standards. Elemental analysis was performed on a PERKIN ELMER Series II CHNS/O Analyzer 2400. The zeta potential (ζ) was measured using a 90 PLUS (BrookHAVEN Instruments Cooperation, New York, USA) particle size analyzer. The TGA measurements were carried out using a SDT Q600 (TA Instruments, Delaware. USA) from room temperature to 1000℃ at a rate of 10℃/ min under a nitrogen atmosphere.

[1] W. H. Daly, D. Poché, *Tetrahedron Lett*. **1988**, 29, 5859-5862.

Synthesis of a PEG-*b*-PAsp-*b*-PPhe Triblock Copolymer. Poly(ethylene glycol)-*b*-poly(*β*-benzyl L-aspartate)-*b*-poly(L-phenylalanine) (PEG_{45}-$PBAsp_9$-$PPhe_{18}$) that have EG units of 45, BAsp units of 9, and Phe units of 18 was synthesized as follows: To a stirred solution of CH_3O-PEG-NH_2 (5 g, 0.0025 mol) in dry DMF (50 mL) was added BAsp-NCA (6.23 g, 0.025 mol) at 35 ^oC under nitrogen. After 24 h, Phe-NCA (9.56 g, 0.050 mol) and dry DMF (100 mL) were added to the reaction mixture, and the reaction was maintained for further 24 h. PEG_{45}-$PAsp_9$-$PPhe_{18}$ was isolated by repeated precipitation from DMF into diethyl ether. Yield 90 %. Finally, the deprotection of PEG_{45}-$PBAsp_9$-$PPhe_{18}$ was performed by treating the block copolymer (4 g) with 0.1 N NaOH (200 mL) to remove benzyl groups. The aqueous solution was then dialyzed using a membrane (Molecular weight cut-off (MWCO): 1000) for 24 h, followed by freeze-drying.

Mineralization of Core-Shell-Corona Polymer Micelles. Polymer micelles of

PEG_{45}-$PAsp_9$-$PPhe_{18}$ consisting of PPhe cores, PAsp shells, and PEG corona were prepared by dialyzing the polymer solution in DMF against doubly distilled water. To induce calcium phosphate mineralization on polymer micelles, an aqueous $CaCl_2$ solution (0.1 mL, 2.9 mM) was firstly added to a stirred solution of polymer micelles (0.9 mL, 1 g/L) at pH 7.0 and equilibrated for 5 h under stirring at 400 rpm. The molar concentration ratios of [Asp] to [Ca^{2+}] were 5:1 and 2:1. An aqueous solution of Na_2HPO_4 (0.2 mL, 2.9 mM) was slowly dropped to the reaction mixture, and the solution was stirred magnetically at 400 rpm at room temperature for 10 h. The solution was dialyzed to remove unreacted ionic species, and the dialyzate was lyophilized to obtain the mineralized hybrid nanoparticles.

Stability of Mineralized Micelles. Kinetic stability of mineralized micelles was investigated by interaction with the sodium dodecyl sulfate (SDS), which acts as a destabilizing agent in aqueous media. The effect of SDS on mineralized micelles in aqueous media was estimated by dynamic light scattering analysis and fluorescence spectroscopy. A SDS solution (1 mL, 7.5 g/L) was added to the mineralized micelle solution (2 mL, 0.75 g/L), and the solution was stirred at 400 rpm. At predetermined time intervals, scattered light intensity of the mineralized micelles was monitored by dynamic light scattering analysis. For time-dependent stability of DOX-loaded micelles, fluorescence spectroscopy was utilized to monitor the change of DOX fluorescence intensity in the presence of SDS. For comparison, the SDS treatment of the non-mineralized micelle solution was conducted under the same condition.

Fluorescence Measurements. Pyrene emission spectra were recorded on a JASCO FP-6500 spectrofluorometer. For the measurement of pyrene emission spectra,

emission and excitation band widths were set at 1 nm and 5 nm, respectively. The excitation wavelength was 336 nm, and the pyrene emission was recorded in the wavelength range of 350-600 nm. The spectra were accumulated with an integration of 3 s/nm.

Steady-State Fluorescence Quenching. Fluorescence quenching experiments were performed by adding aqueous solutions of DPC (0.75 mL) with a various concentration (0.0016 mM - 0.5 mM) to a pyrene-loaded non-mineralized polymer micelles and mineralized polymer micelles (pyrene concentration = 1×10^{-6} M, polymer concentration = 0.75 g/L, 0.75 mL). In a Stern-Volmer equation ($I_0/I - 1 = k_q\tau_0[Q] = K_{SV}[Q]$), I_0 and I represent the integrated fluorescence intensity of pyrene in the absence and in the presence of quencher, respectively. [Q] is the molar concentration of quencher, and τ_0 is the fluorescence lifetime in the absence of the quencher. Time-resolved fluorescence measurement in our study showed that pyrene exhibited a single-exponential decay with a lifetime (τ_0) of 128 ns in aqueous phase. For the fluorescence lifetime of pyrene, the second harmonic output of a Nd:YAG laser (Spectra-Physics, GCR-150) was used to pump a dye laser (Lambda Physik, Scanmate II) to generate the laser pulse in 676 nm using LDS698 dyes. The output of the dye laser was frequency doubled via a KD*P crystal placed on a homemade autotracker to give the UV laser output in the 338 nm with a pulse duration of 7 ns and the power of ~1 mJ/pulse. Fluorescence decay data were recorded on a 300 MHz digitized oscilloscope (LeCroy 9361). The sample solution containing pyrene was excited at 338 nm. The pyrene fluorescence was detected at 393 nm.

Light Scattering Measurements. Dynamic light scattering measurements were performed using a 90 Plus particle size analyzer (Brookhaven Instruments Corporation). The sample solutions were purified by passing through a Millipore 0.45 μm filter. The scattered light of a vertically polarized He-Ne laser (632.8 nm) was measured at an angle of 90 ° and was collected on an autocorrelator. The hydrodynamic diameters (d) of micelles were calculated by using the Stokes-Einstein equation $d = k_B T/3\pi\eta D$ where k_B is the Boltzmann constant, T is the absolute temperature, η is the solvent viscosity, and D is the diffusion coefficient. The polydispersity factor of micelles, represented as μ_2/Γ^2, where μ_2 is the second cumulant of the decay function and Γ is the average characteristic line width, was calculated from the cumulant method [2].

[2] A. Harada, K. Kataoka, *Macromolecules* **1998**, *31*, 288-294.

Transmission Electron Microscopy. Transmission electron microscopy (TEM) was performed on a JEM-2000EX (JEOL Tokyo, Japan), operating at an acceleration voltage of 200 kV. For the observation of size and distribution of micellar particles, a drop of sample solution (concentration = 1 g/L) was placed onto a 200-mesh copper grid coated with carbon. About 2 min after deposition, the grid was tapped with a filter paper to remove surface water, followed by air-drying. Negative staining was performed for non-mineralized polymer micelles by using a droplet of a 5 wt % uranyl acetate solution. Mineralized polymer micelles were observed without a staining process.

TEM-associated Energy-dispersive X-ray Photoelectron Spectroscopy (EDS). EDS measurement was carried out using JEOL JEM-2100F equipped with an EDAX Genesis Series -TEM at 200 kV. The area of mineralized micelles was analyzed by EDS to reveal atomic components.

Drug Loading into Polymer Micelles. DOX was loaded into PEG_{45}-$PAsp_9$-$PPhe_{18}$ micelles by the dialysis method. Before loading DOX to the PEG_{45}-$PAsp_9$-$PPhe_{18}$ micelles, DOX ·HCl (1 mg, 0.0017 mmol) was stirred with TEA (0.31 $\mu\ell$, 0.0022 mmol) in DMF (0.3 mL) overnight in the dark. The triblock copolymer (10 mg) was dissolved in 1 mL of DMF for 3 h at 70 ℃, and then the DOX solution (0.3 mL) was subsequently added and stirred in the dark at room temperature. The solution was dialyzed using a membrane (Spectrapor, MWCO: 1000) for 10 h and followed by lyophilization in the dark. To determine the drug loading content and loading efficiency, DOX-loaded polymer micelles were dissolved in DMF, and then measured by fluorescence emission intensity at 588 nm (excitation at 480 nm). The drug loading content was calculated based on the standard curve obtained using DOX in DMF.

Preparation of DOX-loaded Mineralized Polymer Micelles. The DOX-loaded mineralized micelles were prepared based on an identical process used for mineralization of DOX-free polymer micelles, except that the DOX-loaded micelles were used as a nanotemplate for CaP deposition instead of unloaded micelles.

Controlled Drug Release from DOX-loaded Mineralized Polymer Micelles. In vitro release profiles of DOX from DOX-loaded non-mineralized micelles and mineralized micelles were investigated in the aqueous buffer solutions of which pH and the concentrations of calcium and phosphate ions are similar to an intracellular endosomal fluid ($CaCl_2$ 100 nM, Na_2HPO_4 40 mM, pH 4.5, NaCl 140 mM) or an extracellular fluid ($CaCl_2$ 2 mM, Na_2HPO_4 1 mM, Tris 25 mM, pH 7.4, NaCl 140 mM). Various DOX-loaded micelles were dispersed in aqueous buffer solutions (1 mL, 1 g/L), and transferred to a dialysis membrane bag (MWCO: 1000). 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 160 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 released DOX in the samples was determined by measurement of fluorescence emission intensity at 588 nm (excitation at 480 nm) based on the standard curve obtained using DOX in DMF.

Calcium Dissolution of Mineralized Polymer Micelles. To support the drug release behavior, in vitro calcium dissolution experiments of mineralized micelles were performed in simulated endosomal/lysosomal fluids or extracellular fluid. Mineralized polymer micelles solution (1 g/L) was sealed in a dialysis membrane bag (MWCO: 1000), was shaken at 160 rpm at 37 ^{o}C. The release medium (10 mL) was withdrawn at predetermined time intervals and replaced with an equal volume of the fresh medium. The release rate of calcium ions was monitored by taking a 100 $\mu\ell$ of sample and diluting it in the Arsenazo Ⅲ solution (2 mL, 0.2 mM) in HBS (HEPES-buffered saline where [HEPES] = 20 mM and [NaCl] = 150 mM at pH 7.4). The absorbance of the solution at 656 nm was then measured, and the concentration of calcium ions was calculated based on the standard curve.

Cell culture. Human breast cancer MCF-7 cells were obtained from the Korean Cell Line Bank (KCLB, Seoul). Cells were propagated 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 three days.

Intracellular distribution of DOX-loaded mineralized micelles. MCF-7 cells (1 × 10^5 cells/mL per well) were seeded onto a Lab-Tek® II chamber slide (Nalgen Nunc International, Napevillem, IL) in 1 mL of Dulbecco's modified eagle's medium (DMEM) medium supplemented with 10 % fetal bovine serum (FBS), 1 % antibiotics (penicillin 100 U/mM, streptomycin 0.1 mg/mL). After the cells reached 70-80% confluence, endosomes of MCF-7 cells were transiently transfected with green fluorescence protein (GFP) along with the indicated plasmids after overnight incubation. The total amount of DNA was held constant with pcDNA 3.1 for each transfection with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). After 24 h of transfection (37°C, 5% CO_2), the medium was carefully aspirated and replaced with 1 mL of medium containing 5 μg/mL DOX equivalent of DOX-loaded mineralized micelles. The cells were incubated for 20 min, and then washed three times with PBS. Formaldehyde (1 mL, 3.7 %) was added, and the cells were fixed for 5 min. Finally, the solution was aspirated, and the cells were rinsed three times with PBS, and then the coverslips were transferred to glass slides. The confocal laser scanning microscopy (CLSM) images of GFP-transfected MCF-7 cells treated with DOX-loaded mineralized micelles were obtained using a confocal laser scanning microscope (C1si, Nicon, Japan) by green-fluorescing (λ_{ex} = 470-490 nm) and red-fluorescing (λ_{ex} = 520-550 nm).

Cellular uptake of free DOX and DOX-loaded mineralized micelles. To observe the cellular uptake, MCF-7 cells were seeded in six-well plate at a density of 2 x 10^5 cells/well in 2 mL of DMEM medium supplemented with 10 % FBS, 1 % penicillin-streptomycin. After 24 h incubation, the medium was removed and replaced with 2 mL of medium containing an equivalent DOX concentration (300 μg/mL) of free DOX and

DOX-loaded mineralized micelles. The MCF-7 cells treated with free DOX and DOX-loaded mineralized micelles were incubated at various incubation times (10, 30, 60 min). At the designated time, the cells were washed three times with PBS and then harvested by trypsin treatment. The cell suspensions were centrifugated at 1000 rpm for 5 min. The supernatants were discarded, and cells were dispersed and fixed with 400 μL of 3.7% formaldehyde. The cell suspension was pipetted onto a glass cover slide before imaging. Cellular uptake images were examined under a confocal laser scanning microscopy (C1si, Nicon, Japan). The excitation and emission wavelengths were 488 nm and 510 nm, respectively.

Biocompatibility of Mineralized Polymer Micelles. MCF-7 cells were seeded into 96-well flat-bottomed tissue-culture plate at 1000 cells/well, and incubated for 24 h in a humidified atmosphere of 5 % CO_2 at 37 ^{o}C. The micelles solution was diluted with culture medium to obtain a concentration range from 1 to 300 μg/mL. After the incubation for 24 h, 50 μL of a 10 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution in PBS was added to each well, and the plate was incubated for 4 h at 37 ^{o}C, allowing viable cells to reduce the MTT into purple formazan crystal. The formazan crystal was dissolved by adding 200 μL of dimethyl sulfoxide (DMSO) and 25 μL of Sörensen's glycine buffer. The absorbance of individual wells was measured at 570 nm by a microplate reader (SOFTmax® PRO, Molecular Devices Corporation, CA).

Cell cytotoxicity. In vitro cytotoxicity of DOX-loaded mineralized micelles were evaluated by measuring the IC_{50} using the MTT colorimetric assay. Briefly, MCF-7 cells were seeded onto a 96-well plate at a density of 5×10^3 cells per well in 200 μl of

medium and incubated at 37^{o}C and 5 % CO_2. After 24 h, the medium of each well was then replaced by 200 μL of new medium containing free DOX, DOX-loaded non-mineralized micelles, DOX-loaded mineralized micelles at various drug concentrations from 0.01 to 100 μg/mL, and then the plates were incubated (37 ^{o}C, 5 % CO_2). After 24 h, the medium was removed, and cells were washed with the fresh medium, followed by the MTT assay. The inhibitory concentration of 50 % (IC_{50}) was calculated as the concentration of DOX yielding 50 % of MTT reduction compared to the untreated control.

Table S1 Characteristics of the PEG-*b*-PAsp-*b*-PPhe copolymer

copolymer	feed ratio ([EG]:[BAsp]:[Phe])	composition ratio [a] ([EG]:[Asp]:[Phe])	conversion of BAsp [a] (%)	conversion of Phe [a] (%)	M_n [a]	M_w/M_n [b]	cmc [c] (mg/L)	d [d] (μ_2/Γ^2)
PEG_{45}-$PAsp_9$-$PPhe_{18}$	45 : 10 : 20	45 : 9 : 18	90	90	5700	1.06	18	31.2 nm (0.18)

*The copolymers were synthesized using the macroinitiator (CH_3O-PEG-NH_2) with M_n of 2,000 and polydispersity of 1.09, respectively.

[a] Calculated by ^1H NMR spectra [b] Estimated by GPC. [c] critical micelle concentration at 25 °C. [d] mean diameter of polymer micelles at pH 7.4.

Fig. S1 Synthetic route for the PEG-*b*-PAsp-*b*-PPhe copolymer.

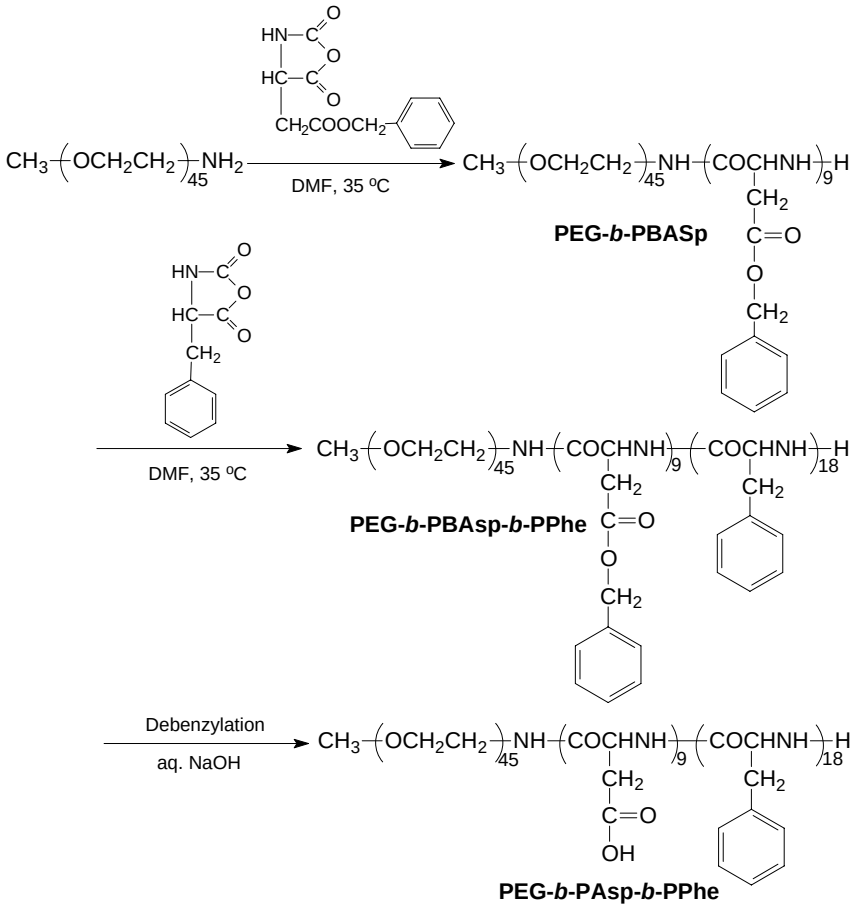

Fig. S2 ^{1}H NMR spectra of CH_3O-PEG-NH_2 (a), PEG_{45}-$PBAsp_9$ (b), PEG_{45}-$PBAsp_9$-$PPhe_{18}$ (c), and PEG_{45}-$PAsp_9$-$PPhe_{18}$ (d) in DMSO-d_6. As shown in Fig. S2(d), the resonance for the methylene protons (-***CH*****$_2$**Ph) of PBAsp blocks at 5.12 ppm completely disappeared after the hydrolysis.

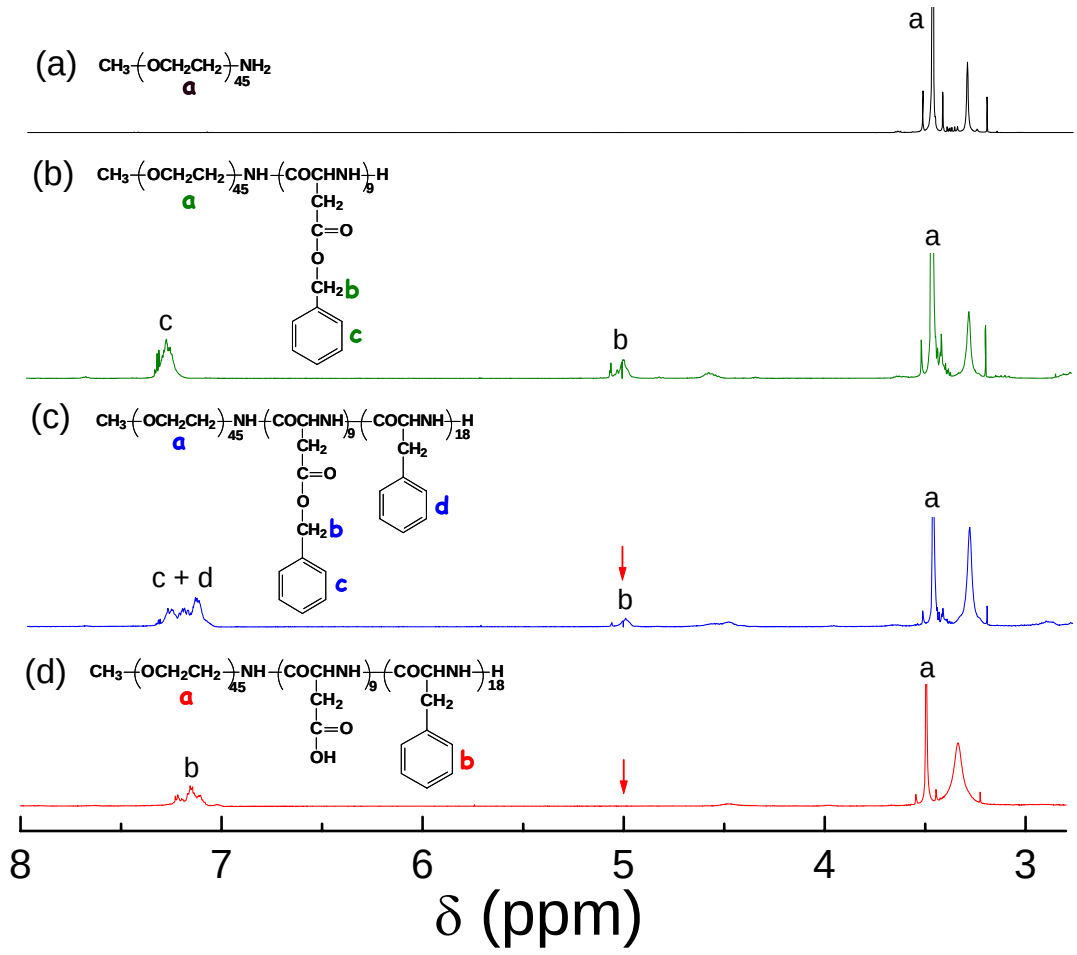

Fig. S3 Gel permeation chromatograms of CH_3O-PEG-NH_2 (a), PEG_{45}-$PBAsp_9$ (b), PEG_{45}-$PBAsp_9$-$PPhe_{18}$ (c)

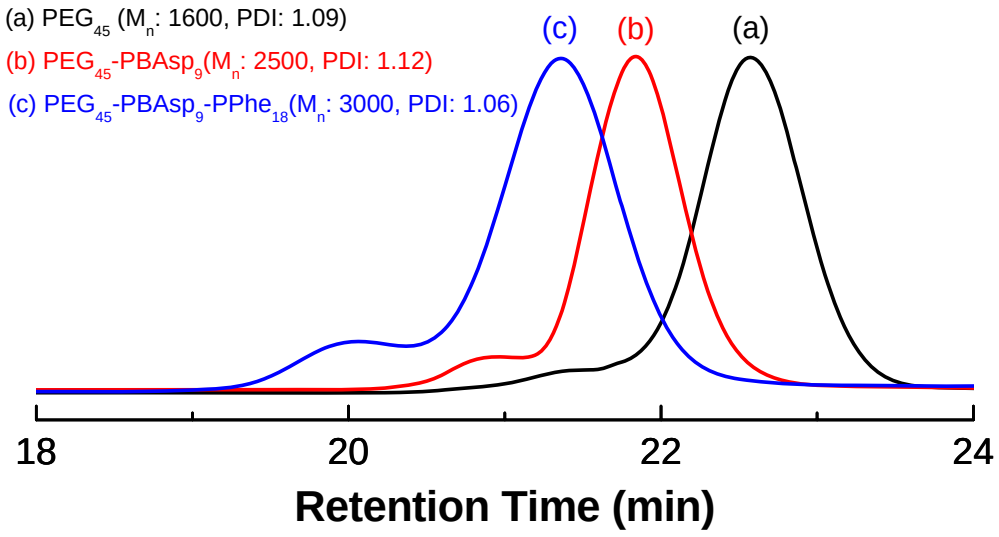

Fig. S4 (a) Excitation spectra of pyrene as a function of the PEG_{45}-$PAsp_9$-$PPhe_{18}$ concentration in water. (b) Plot of I_{336}/I_{332} (from pyrene excitation spectra) vs. Log C for PEG_{45}-$PAsp_9$-$PPhe_{18}$.

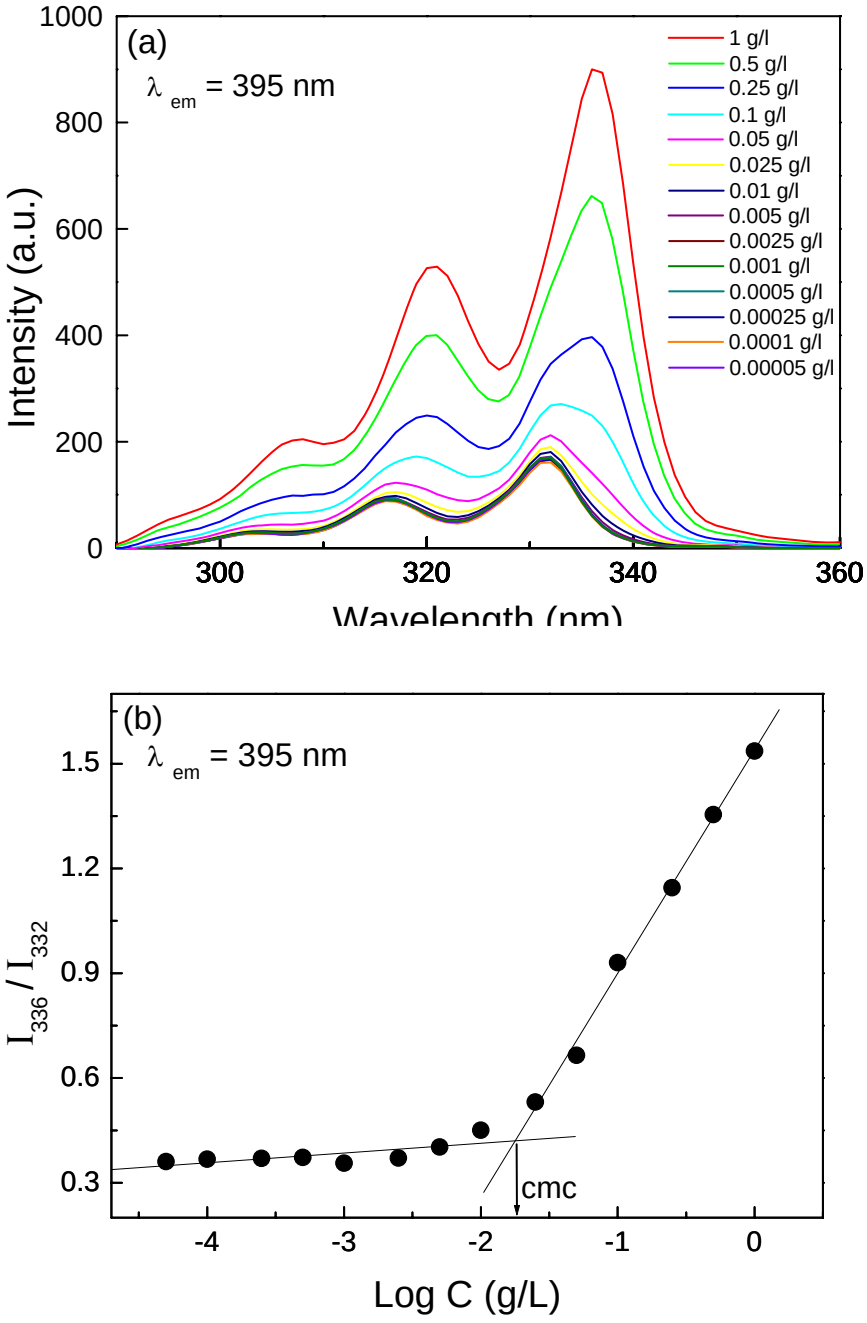

Fig. S5 TEM images for estimation of CaP-deposited shell thickness of mineralized polymer micelles.

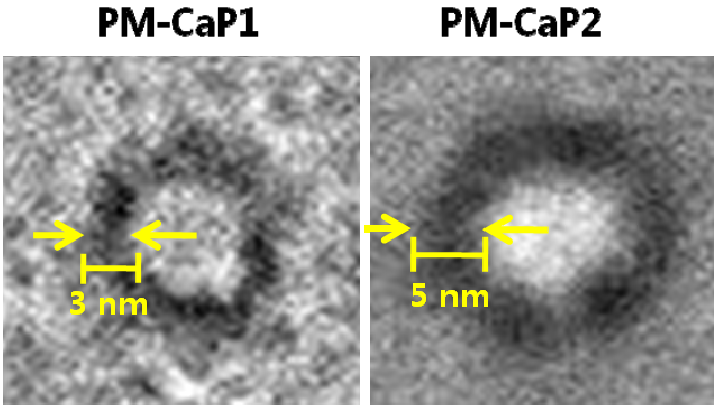

Fig. S6 (a) Mineral masses on polymer micelle nanotemplates estimated by TGA .

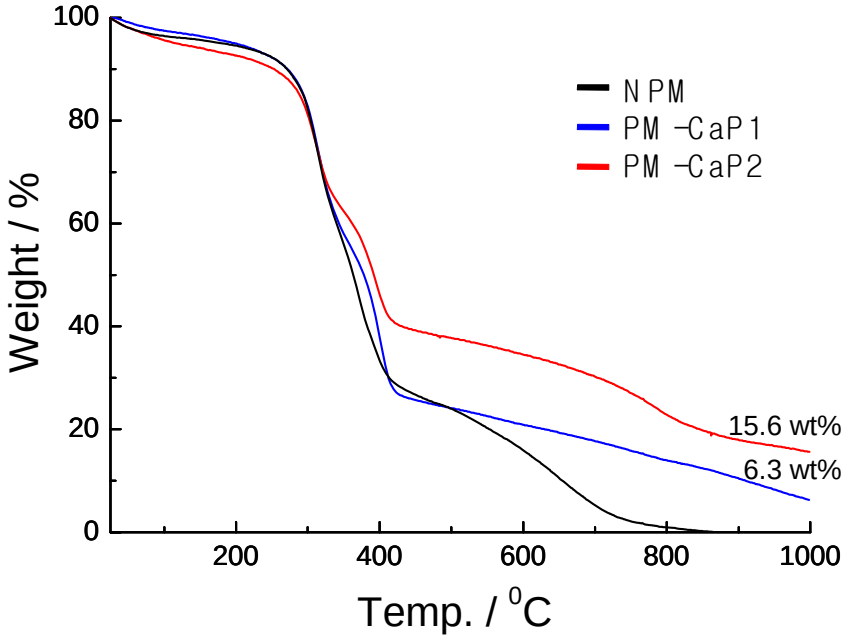

Fig. S7 Stern-Volmer plots for micelle-entrapped pyrene (6.0×10^{-6} M) quenched by DPC.

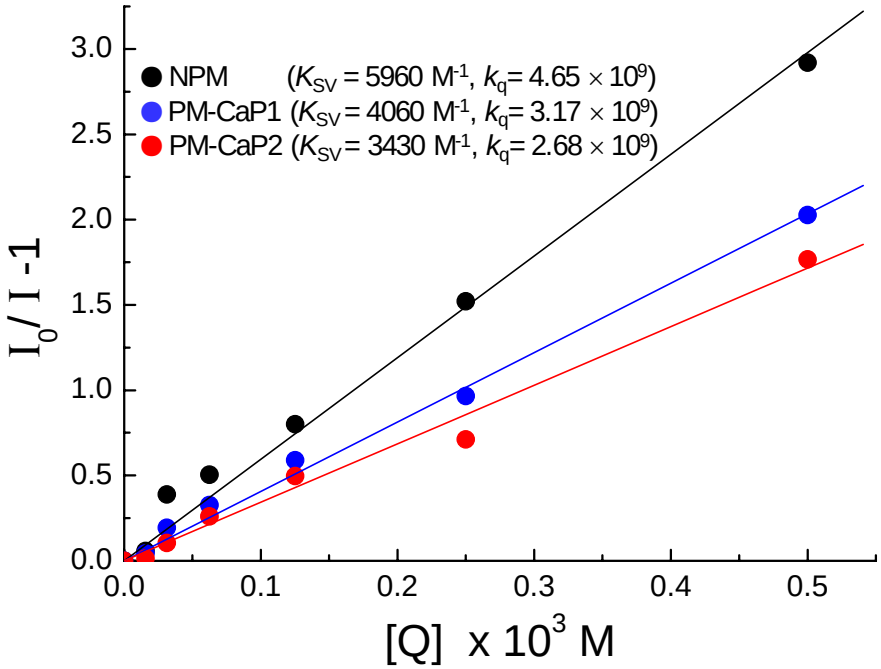

Fig. S8 (a) Kinetic changes in scattered light intensity (SLI/SLI_0) for NPM and PM-CaPs, and (b) the relative fluorescence intensity (FI/FI_0) of DOX-NPM and DOX-PM-CaPs in the presence of SDS (2.5 g/L).

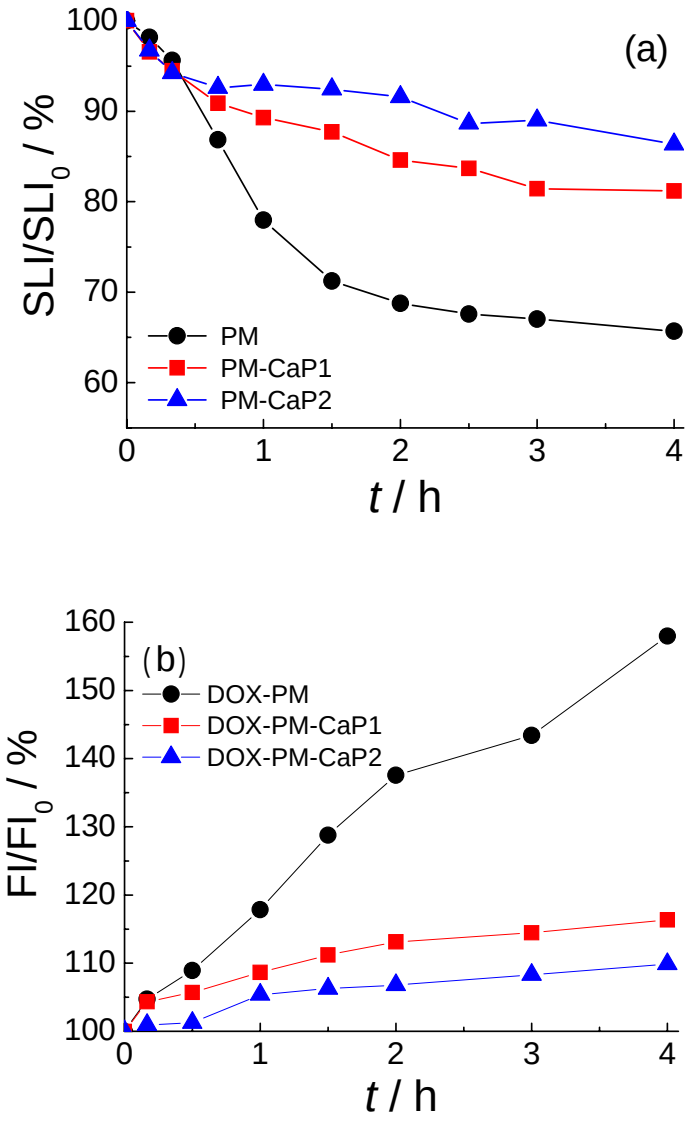

Fig. S9 Viability of MCF-7 cells at various concentrations of NPM (a) and PM-CaP2 (b).

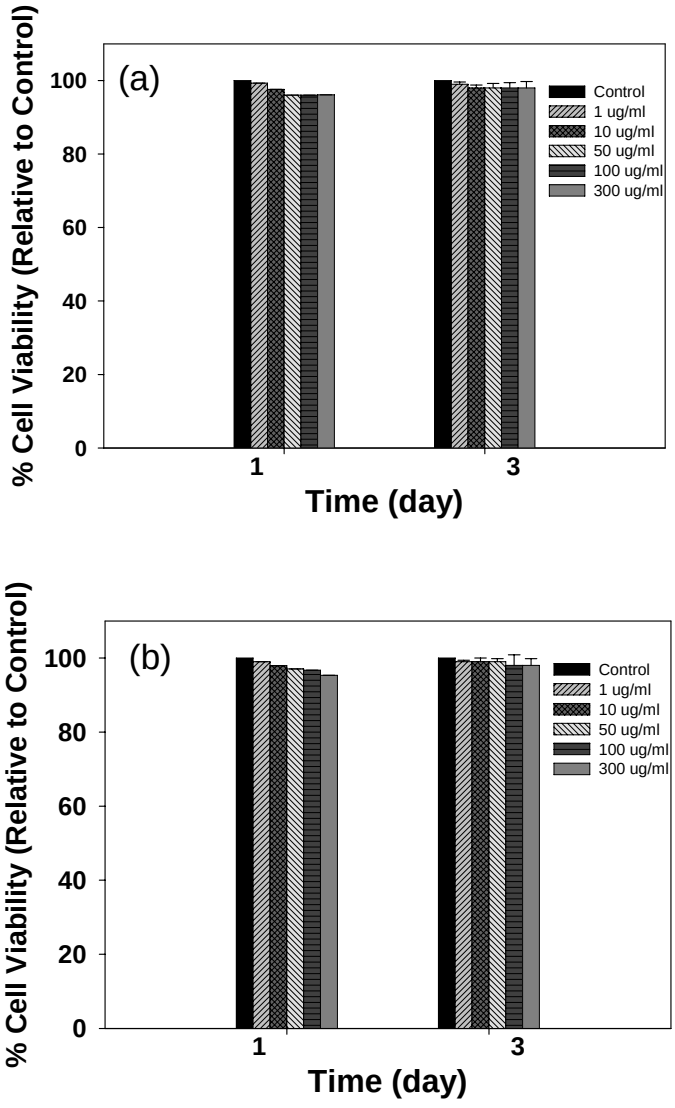

Fig. S10 In vitro cytotoxicity of DOX, DOX-NPM, DOX-PM-CaP2 with MCF-7 cells after 24 h (n=3)**.**

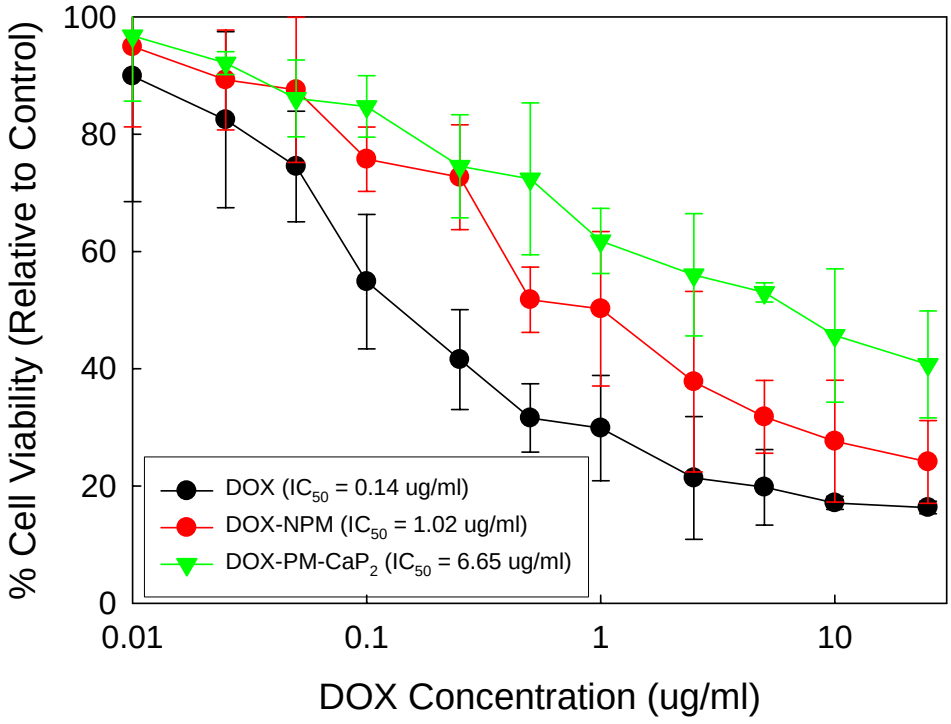

Fig. S11 Cellular uptake of free DOX and DOX-PM-CaP2. Differential interference contrast microscopy (DICM) image (left) and confocal laser scanning microscopy (CLSM) image (right) of MCF-7 cells treated with free DOX for (a) 10 min and (b) 60 min or DOX-PM-CaP2 for (c) 10 min and (d) 60 min, respectively.