Supporting Information

Experimental section

Materials

All glassware was stored in a drying oven for several hours at 120 ^{o}C. Doxorubicin hydrochloride (DOX. HCl) was purchased from Biotang Inc (Waltham, MA, USA). The 2,2'-azo-bis(isobutyronitrile) (AIBN), chloroauric acid ($HAuCl_4.3H_2O$), lithium borohydride ($LiBH_4$), cholesterol (96%), n-butylamine, and pyrene were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Triethylamine (TEA) and dimethyl formamide (DMF) were purchased from Fisher Scientific (Boston, MA, USA). Polyethylene oxide methyl ether (MW= 2000), 1,4-dioxane (99.8%, extra dry), dichloromethane (DCM) (99.9%, extra dry), methacryloyl chloride (>97%) were purchased from Acros Organics USA. Liquid crystalline monomer, cholesteryl 6-methacryloyloxyhexaneoate (C5MA), was prepared according to published work.[1] The RAFT agent S-1-dodecyl-S'-(α,α'-dimethyl-α"-acetic acid) tricarbonate (CTA) was synthesized according to a published procedure.[2] Penicillin-streptomycin, 0.25% (w/v) trypsine-0.03% (w/v) EDTA solution, and DMEM medium were purchased from American Type Culture Collection (Rockville, MD, USA). Human cervical cancer cells (Hela) was purchased from National Cancer Institute (Frederick, MD, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA, USA). *In vitro* toxicology assay kit (MTT based) was obtained from Invitrogen (Carlsbad, CA, USA). Spectra/Pro membranes were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA). All chemicals were analytical grade and used without purification.

Synthesis of methacrylate graft polyethylene oxide (MA-*g*-PEO)

To synthesize macromonomer, MA-*g*-PEO, polyethylene oxide methyl ether (MW: 2000 Da, 4 g, 2 mmol) was dissolved in DCM, followed by addition of methacryloyl chloride and triethylamine (TEA) and stirred overnight under nitrogen. The molar ratio of PEO, methacryloyl chloride and TEA was 1:2:2. After 12 h, the insoluble salt (TEA.HCl) was filtered out, and the solution was collected. The macromonomer product was precipitated in excess cold diethyl ether and collected as light yellow solid.

^{1}H NMR ($CDCl_3$, δ ppm): 6.15, 5.54 (m, 2H, CH_2-CH=COO-), 4.27 (t, 2H, -COOCH_2-, in PEO end group), 3.79-3.44 (m, -CH_2CH_2O-, repeating units of PEO), 3.36 (s, -OCH_3).^{13}C NMR ($CDCl_3$, δ ppm): 170.9 (-*C*OO), 133, 126.6 (-*C*H_2, *C*H in vinyl group), 70.3 and 64.8 (-*C*H_2 repeat unit in PEO).

Synthesis of polymethacrylates bearing PEO (PMA-*g*-PEO-thioester)

The PMA-*g*-PEO-thioester macro chain transfer agent was synthesized using RAFT polymerization. In a Schlenk tube equipped with a stir bar, macromonomer (MA-*g*-PEO, 0.5 g, 0.93 mmol), was dissolved in 1,4-dioxane (3 mL), followed by the addition of the RAFT agent (CTA, 18.3 mg, 0.05 mmol), and the initiator (AIBN, 0.82 mg, 0.005 mmol). The Schlenk tube was then degassed by three freeze-evacuate-thaw cycles and then placed in an oil bath maintained at 80 ^{o}C. The reaction was allowed to proceed for 24 h. The reaction mixture was concentrated and precipitated in diethyl ether. The product was collected and dried in vacuum overnight.

^{1}H NMR ($CDCl_3$, δ ppm): 4.27 (t, 2H, -COOCH_2-, in PEO end group), 3.79-3.44 (m, -CH_2CH_2O-, repeating units of PEO), 3.36 (s, -OCH_3), 3.2 (t, 2H, $CH_3C_{10}H_{20}CH_2$-S-), 1.60-1.75 (m, 6H, -S-C(CH_3)$_2$COO-), 1.25 (m, 20H, $CH_3C_{10}H_{20}CH_2$S-), 0.87 (t, 3H, $CH_3C_{10}H_{20}CH_2$S-).

^{13}C NMR ($CDCl_3$, δ ppm): 170.9 (-*C*OO), 133, 126.6 (-*C*H_2, *C*H in vinyl group), 74.5 (-COO*C*H-), 70.3 and 64.8 (-*C*H_2 repeat unit in PEO), 51.3-11.2 (-*C*H_2-*C*(*C*H3)COO-).

GPC (40 ^{o}C, THF mobile phase, polystyrene standards): M_n = 8950 g/mol, PDI = 1.27.

Synthesis of (PMA-*g*-PEO)-*b*-PC5MA-thioester (brush-chol-BCP-thioester)

In a representative procedure, mixture of the PMA-*g*-PEO macro chain transfer agent (1.2 g, 0.2 mmol), C5MA (3.8 g, 28.0 mmol), and AIBN (6 mg, 0.04 mmol) were dissolved in 1,4-dioxane (3 mL) and degassed by performing three freeze-evacuate-thaw cycles. The reaction mixture was sealed and then placed in an oil bath maintained at 90 ^{o}C for 20 h. The resulting mixture was concentrated and precipitated in a large excess of methanol. The crude product was collected, Soxhlet extracted overnight using methanol to remove unreacted monomer, then extracted with THF and reprecipitated into methanol. The product, brush-chol-BCP-thioester, was collected and dried under vacuum. The thioester peak was appeared at 310 nm, as measured by UV-visible spectroscopy.

^{1}H NMR ($CDCl_3$, δ ppm): 5.33 (d, 1H, -C=C*H*-, olefin group in cholesteryl moiety), 4.5 (m, 1H, -CH_2-COO-C*H*), 3.9 (m, 2H, -COOCH_2CH_2), 3.64 (m, -CH_2CH_2O- repeating units of PEO), 3.45 (m, 2H, -CH_2OCH-), 3.36 (s, -OCH_3), 3.2 (t, 2H, $CH_3C_{10}H_{20}CH_2$-S-), 2.50-0.55 (m, 56H, -CH_3, -CH_2-, -CH-, -CH-(CH_3)- in cholesteryl moiety, -CH_2-C(CH_3)COO-, -CH_2CH_2-$CH_2CH_2CH_2$- in spacer).

^{13}C NMR ($CDCl_3$, δ ppm): 170.9 (-*C*OO), 140.9 (-*C*=CH-, olefin group in cholesterol), 121.9 (-C=*C*H-, olefin group in cholesterol), 133, 126.6 (-*C*H_2, *C*H in vinyl group), 74.5 (-COO*C*H-), 70.3 and 64.8 (-*C*H_2 repeat unit in PEO), 51.3-11.2 (-*C*H_2-*C*(*C*H3)COO- , -cholesterol).

GPC (40 ^{o}C, THF mobile phase, polystyrene standards): M_n = 18 320 g/mol, PDI = 1.16.

Preparation of (PMA-*g*-PEO)-*b*-PC5MA-thiol (brush-chol-BCP-thiol)

To obtain the brush-chol-BCP-thiol, the brush-chol-BCP-thioester was reduced by *n*-butylamine in THF. In a representative procedure, brush-chol-BCP-thioester (0.35 g, 0.02 mmol) and *n*-butylamine (80 mg, 1.1 mmol) were dissolved in THF under a blanket of nitrogen and stirred for 2 h until the color of solution changed from light yellow to colorless. The polymer was then precipitated in excess methanol. The crude product was collected; Soxhlet extracted overnight with methanol to remove unreacted monomer, and then extracted with THF, and finally reprecipitated into methanol. The product was collected and dried in vacuum. To establish the reaction kinetics, a solution of brush-chol-BCP-thioester in THF (1 mg/mL) was placed in a quartz cuvette fitted in the sample compartment of a UV-visible spectrometer. The appropriate amount of n-butylamine solution in THF was added and the absorbance of the solution at 310 nm was measured as a function of time.

Characterization of the brush-chol-BCPs

The ^{1}H NMR spectra (Bruker DMX 400 MHz NMR spectrometer) of macromonomer and polymers were recorded in $CDCl_3$ and the 7.24 ppm peak was used as an internal standard. Molecular weight and polydispersity indices (PDI) of the polymers were determined by gel permeation chromatography (GPC) by using a Waters 150-C ALC/GPC equipped with Evaporative Light Scattering Detector. THF was used as the eluent with a flow rate of 2.0 mL/min at 40 ^{o}C with polystyrene as the standard. Fourier transform infrared (FT-IR) data were obtained using a Niclotet Magna 560 FTIR spectrometer with transmission mode.

Preparation and characterization of self-assembled nanoparticles

Preparation of brush-chol-BCP-thiol self-assembled nanoparticles (blank NPs)

The self-assembled nanoparticles based on brush-chol-BCP-thiol were prepared by a nanoprecipitation method. Briefly, brush-chol-BCP-thiol (10 mg) was dissolved in a mixture of DMF and THF (3 mL, DMF: THF=2:1), followed by a dropwise injection into distilled water (10 mL). The solution was then transferred to a dialysis bag (MWCO: 6,000-8,000 Da) and dialyzed against distilled water for 48 h.

Preparation of AuNPs-templated brush-chol-BCP-thiol in an organic solvent

To prepare AuNPs-templated brush-chol-BCP-thiol, brush-chol-BCPs-thiol (0.15 g, 0.025 mmol) and $HAuCl_4.3H_2O$ (0.01 g, 0.05 mmol) were dissolved in DMF (10 mL) and stirred in the dark under nitrogen at room temperature for 24 h. Freshly prepared 0.25 M $LiBH_4$ (1.2 mL, 0.25 mmol) was then added quickly to the solution with vigorous stirring. The reaction mixture immediately turned from yellow to dark purple, violent gas evolution was observed. The solution was stirred for 4 h at room temperature. The reaction mixture was then transferred into dialysis bag (MWCO: 6,000-8,000 Da), followed by dialysis against DMF for 48 h to remove byproducts.

Preparation of AuNPs-encapsulated brush-chol-BCP-thiol nanoparticles in water (AuNPs-encapsulated NPs)

To prepare AuNPs-encapsulated NPs, THF was mixed with of an aliquot of AuNPs-templated brush-chol-BCP-thiol solution in DMF (10 mg copolymer equivalence) (DMF: THF=2:1). The resulting solution was injected dropwise into distilled water (10 mL), followed by the dialysis against distilled water for 48 h (MWCO: 6,000-8,000 Da). The resulting solutions

were centrifuged at 11,000 rpm for 10 min, followed by the filtration through 0.45 μm syringe to remove any precipitate.

Preparation of DOX-encapsulated brush-chol-BCP-thiol nanoparticles in water (DOX-encapsulated NPs)

To prepare DOX-encapsulated NPs, DOX.HCl was first converted to hydrophobic DOX by dissolving in DMF containing 2 equivalence of TEA and stirred overnight in the dark.[3] The hydrophobic DOX solution was then added to brush-chol-BCP-thiol solution in DMF and THF. The solutions were injected dropwise into distilled water, followed by the dialysis against distilled water for 48 h (MWCO: 6,000-8,000 Da). The resulting solutions were centrifuged at 3000 rpm for 10 min, followed by the filtration through 0.45 μm syringe to remove any precipitated free DOX. The final products were obtained by lyophilization.

Preparation of DOX-encapsulated AuNP-templated brush-chol-BCP-thiol nanoparticles in water (dual-encapsulated NPs)

To prepare dual-encapsulated NPs, the prepared hydrophobic DOX solution was added to an aliquot of AuNPs-templated brush-chol-BCPs solution in DMF and THF. The solutions were injected dropwise into distilled water, followed by the dialysis against distilled water for 48 h (MWCO: 6,000-8,000 Da). The resulting solutions were centrifuged at 11,000 rpm for 10 min, followed by the filtration through 0.45 μm syringe to remove any precipitated free DOX. The final products were obtained by lyophilization.

Characterization of nanoparticles

The average particle size and size distribution of blank NPs, AuNPs-encapsulated NPs, DOX-encapsulated NPs, and dual-encapsulated NPs (1 mg/mL) were measured using a dynamic light scattering (DLS) instrument (Malvern). The morphologies of the nanoparticles were imaged by Tecnai T12 TEM with accelerating voltage of 120 kV. Specimens were prepared by dropping solution of the nanoparticles on to copper grid coat with Formvar film, followed by air-drying. The EDX spectrums were observed and quantified the elemental composition of Au atom by Tecnai T12 TEM.

Critical micelle concentration (CMC) of brush-chol-BCP-thiol was determined by the fluorescence technique using pyrene as a hydrophobic probe. The pyrene solution ($3x10^{-4}$ M) in acetone was added into the test tubes, and followed by evaporation to remove the organic solvent. Then, various concentrations of the blank NPs solution in distilled water (10 mL) were added to the test tubes and sonicated for 3 h at 60 °C to equilibrate the pyrene and the nanoparticles. The concentration of sample solution was varied from 0.005 to 0.5 mg/mL. The final concentration of pyrene was $6.0x10^{-7}$ M. The emission spectra of pyrene were recorded in the range of 350-450 nm using a fluorescence spectrophotometer (Perkin Elmer LS-55B, USA) at the excitation wavelength of 336 nm. For the measurement of the intensity ratio of the first (374.5 nm) and the third highest energy bands (386 nm) in the pyrene emission spectra, the slit opening for the excitation and emission spectra was set at 2.5 nm.

The amount of DOX in the DOX-encapsulated NPs or dual-encapsulated NPs was determined by a colorimetric method. The lyophilized nanoparticles (0.5 mg) were dissolved in DMF (2 mL) to obtain clear solutions. The absorbance at 480 nm was detected with a UV-VIS spectrophotometer. DOX standard solutions were prepared at various concentrations and the absorbance at 480 nm was measured to generate the calibration curve for calculating the drug-

loading content. The drug-loading content (DLC) and encapsulation efficiency (EE) were calculated by the following equations:

$$DLC = \frac{\text{Amount of DOX in nanopartides}}{\text{Amount of DOX - encapsulated nanopartides}} \times 100$$

$$EE = \frac{\text{Amount of DOX in nanopartides}}{\text{Amount of DOX used for nanopartide preparation}} \times 100$$

Stability test and *in vitro* release of DOX from dual-encapsulated NPs

For stability test, lyophilized dual-encapsulated NPs (1 mg/mL) were suspended in the serum-containing phosphate-buffered saline (PBS) solution (50% FBS), followed by sonication for about 10 min and filtration through 0.45 μm syringe filter membrane. The particle size of the nanoparticles stored at 4 ºC was monitored over the storage time using a Malvern Zetasizer.

In vitro release of DOX from the nanoparticles was studied using a dialysis technique. Briefly, lyophilized DOX-encapsulated NPs and dual-encapsulated NPs (6 mg) were suspended in 3 mL of PBS (0.01 M, pH 7.4), followed by sonication for 10 min to give an optically clear solution. The solutions were introduced into dialysis bags (MWCO: 6,000-8,000 Da) and immersed in 20 mL of PBS at 37 °C in a shaking bath at 100 rpm. At selected time intervals, aliquots (10 mL) were removed from the dissolution medium and an equivalent volume of fresh medium was compensated. The concentration of DOX was immediately measured by UV at 480 nm. The amount of DOX released was calculated by comparing with standard.

Cellular uptake of dual-encapsulated NPs

To observe the cellular uptake, HeLa cells were seeded at a density of 1.0×10^5 cells/well on 8-well chamber of a Lab-Tek II chamber slide and preincubated for 24 h at 37 °C, and 5 % CO_2.

Serum-free DMEM containing free DOX, DOX-encapsulated NPs, and dual-encapsulated NPs at 10 μg/mL DOX equivalence was added to each well, followed by the incubation for 2 h and 24 h at 37 °C. The cells were then rinsed with PBS, and fixed with 4% formaldehyde solution for 10 min. Cover glasses were then placed on the slide glasses. The cellular uptake was imaged by a confocal laser scanning microscope (Leica, England) at the excitation wavelength of 488 nm.

Cytotoxicity of dual-encapsulated NPs

HeLa cells (10,000 cells per well) were seeded on 96-well plates and cultured in 200 μL of DMEM supplemented with 10% FBS, 1% antibiotics, and 1% L-glutamine for 24 h at 37 °C, and 5% CO_2. After incubation, different concentrations of free DOX, DOX-encapsulated NPs, and dual-encapsulated NPs (1-50 μg/mL of DOX equivalence) dissolved in DMEM without supplements were added. After 24 h of incubation, cytotoxicity was determined using 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide dye (MTT dye, final concentration of 0.5 mg/mL) uptake at 540 nm on a microplate reader (Tecan group Ltd., Männedorf, Switzerland).

References

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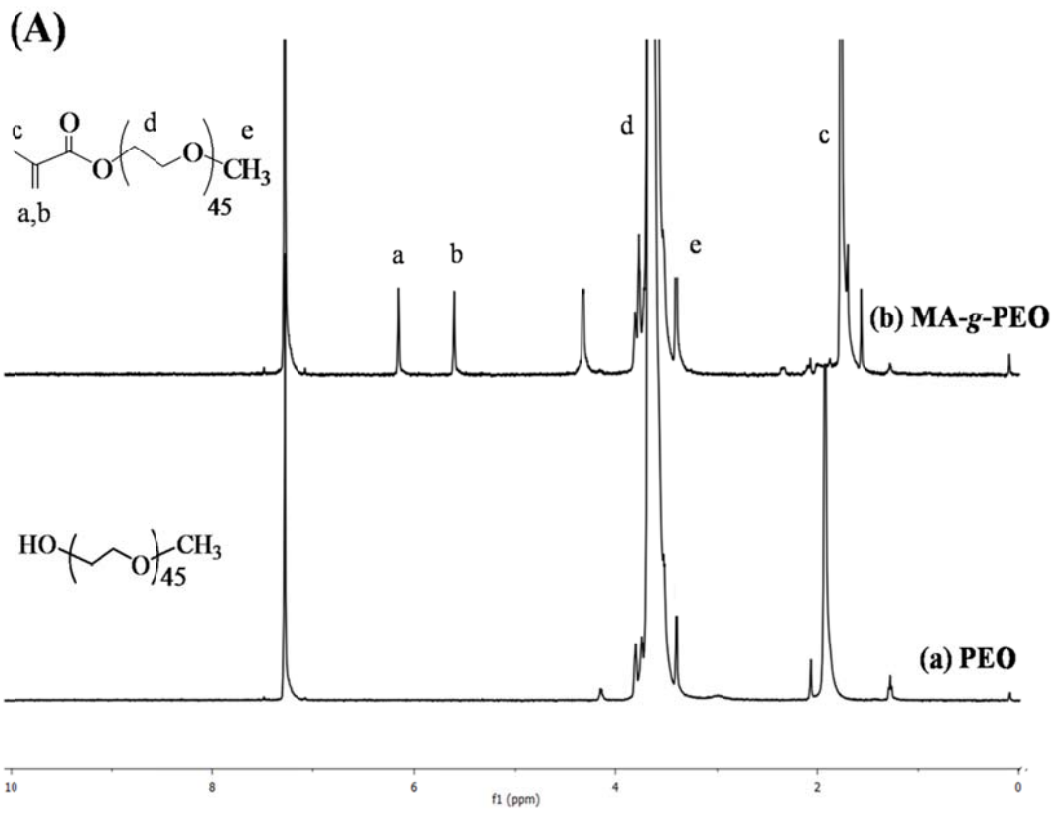
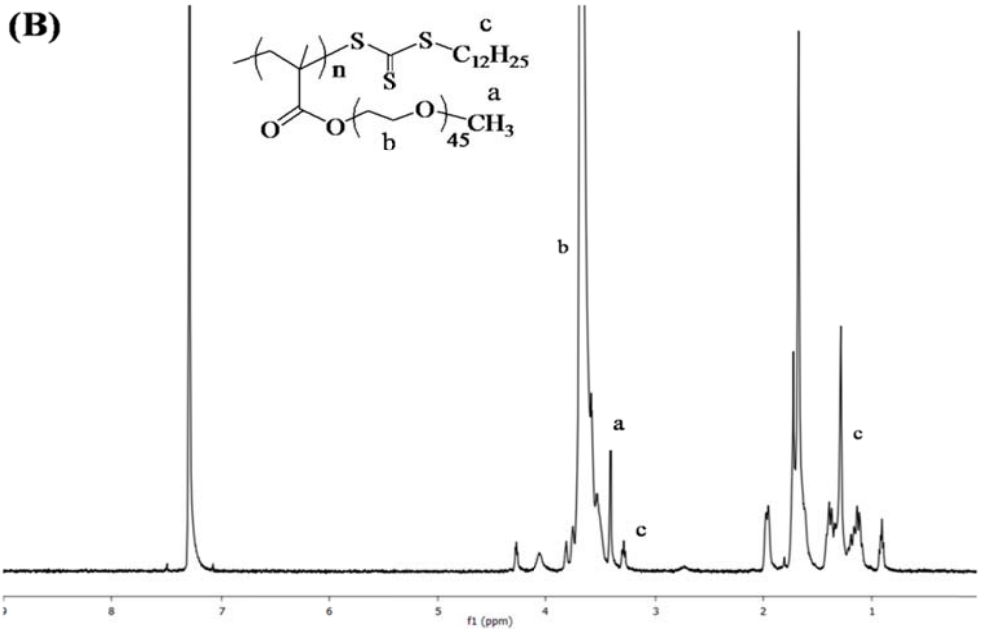
2. J. Lai, D. Filla and R. Shea, *Macromolecules* 2002, **35**, 6754-6756
3. L. Li, K. M. Huh, Y.-K. Lee and S. Y. Kim, *J. Mater. Chem.* 2011, **21**, 15288-15297


(A)
c
O
d
e
O
O
CH3
45
a,b
a
b
d
e
c
(b) MA-g-PEO
HO
O
CH3
45
(a) PEO
10
8
6
4
2
0
f1 (ppm)

206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S
c
C12H25
n
S
a
O
O
O
CH3
b
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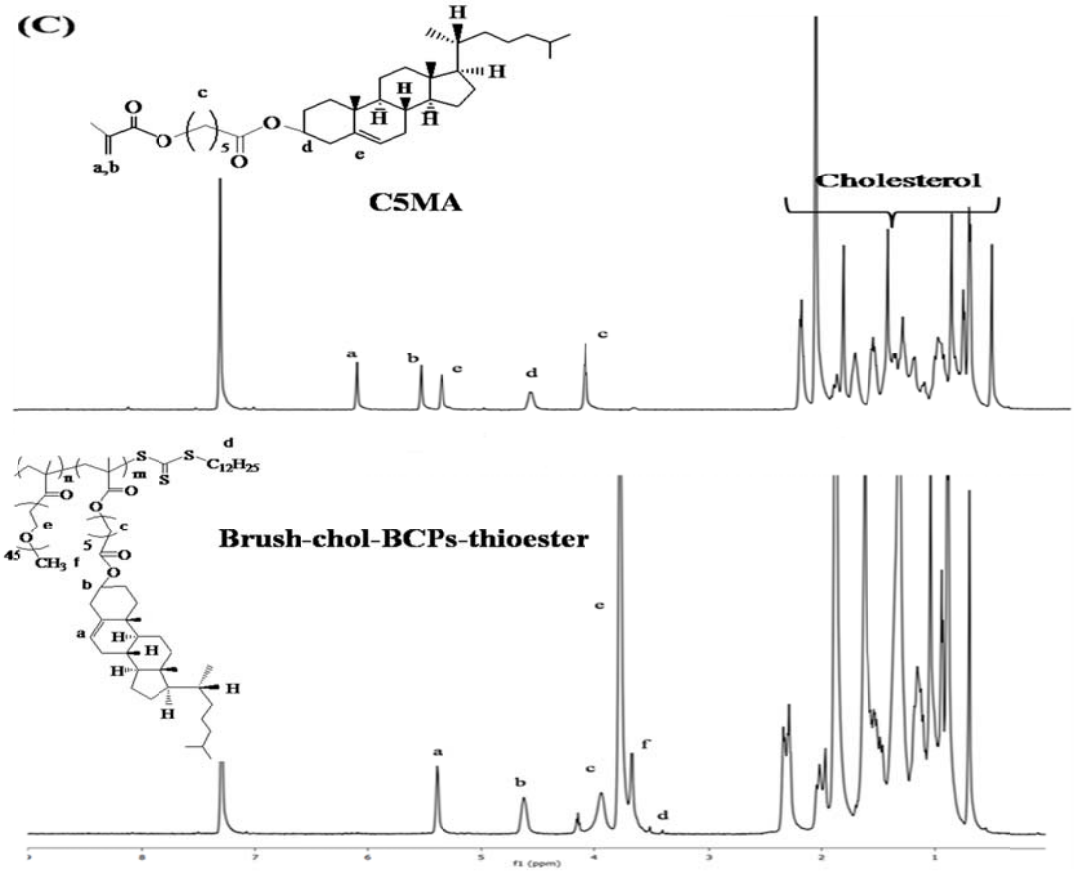
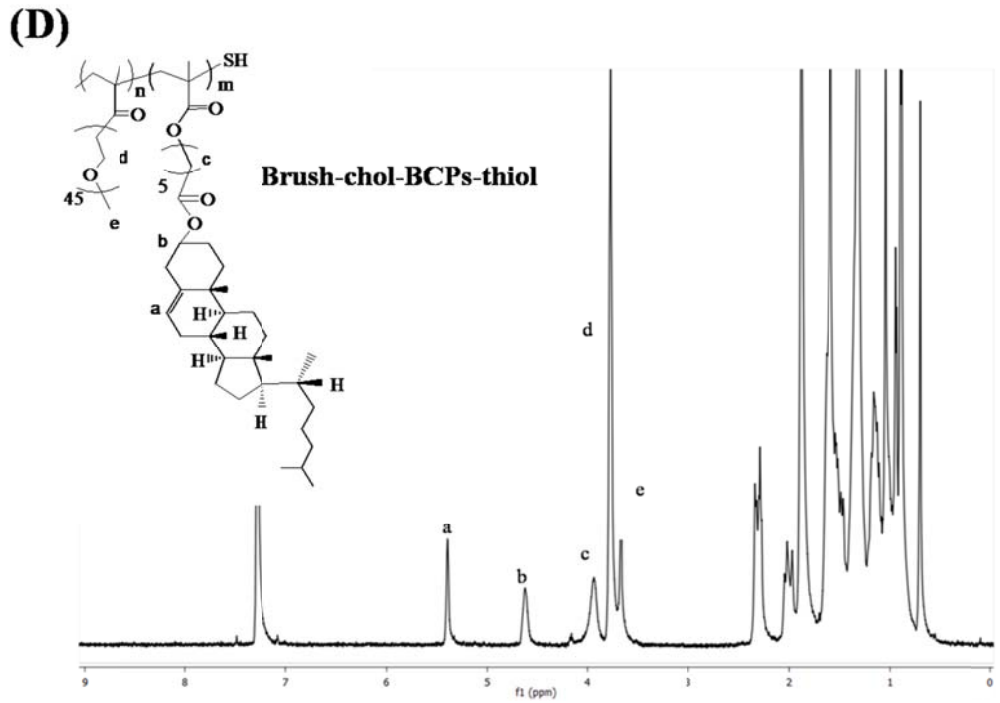
b
a
c
c
8
7
6
5
4
3
2
1
f1 (ppm)

207


(C)
C5MA
Cholesterol
Brush-chol-BCPs-thioester
f1 (ppm)

208


(D)
Brush-chol-BCPs-thiol
f1 (ppm)

209

Figure S1: ^{1}H-NMR spectra of (A) MA-*g*-PEO; (B) PMA-*g*-PEO-thioester macro chain transfer agent; (C) C5MA monomer (a), Brush-chol-BCP-thioester (b); and (D) Brush-chol-BCP-thiol in $CDCl_3$ at room temperature.

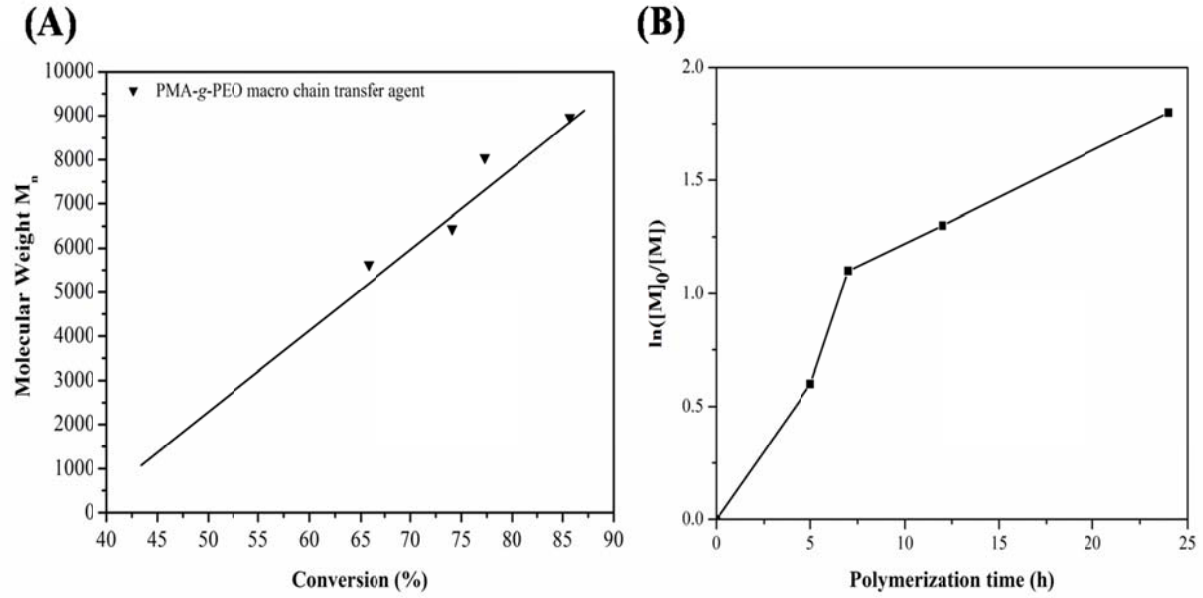
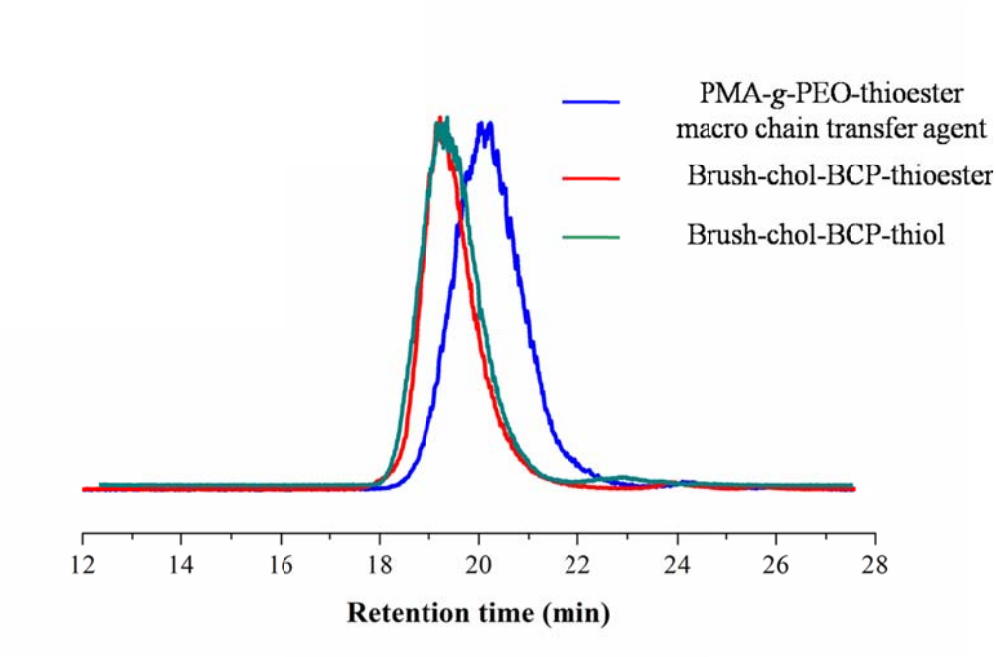


Figure S2. Polymerization kinetic of PMA-*g*-PEO-thioester macro chain transfer agent: (A) Plot of conversion vs molecular weight, and (B) rate of polymerization determined by slope of line in plot of $\ln[(M)_0/(M)]$ vs time.



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225 **Figure S3**. GPC traces of the prepared (PMA-*g*-PEO)-*b*-PC5MA brush-chol-BCP.

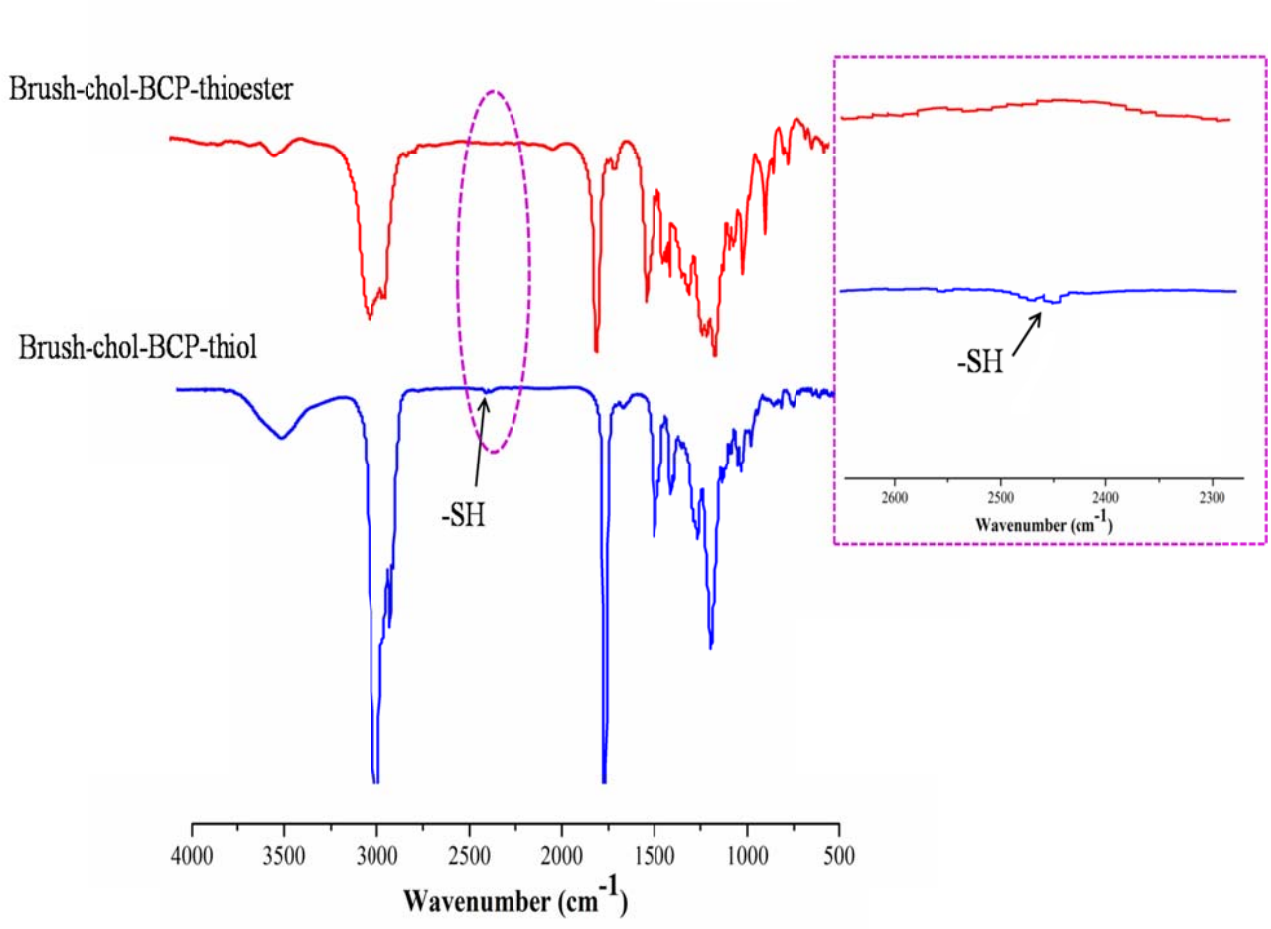

Figure S4. FT-IR spectra of brush-chol-BCP-thioester and brush-chol-BCP-thiol.