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1 Supporting Information for

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3 Acid-induced disassemblable nanoparticles based on cyclic 4 benzylidene acetals-functionalized graft copolymer via 5 sequential RAFT and ATRP polymerization

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1 **Materials**

2 2,4,6-Trimethoxybenzylidene-1,1,1-tris(hydroxymethyl) ethane methacrylate (TTMA)
3 was synthesized according to a previous report by Grinstaff.[1] 2-(2-
4 Bromoisobutyryloxy) ethyl methacrylate (BMPEMA) was prepared by a reaction of a
5 α-bromoacid halide with 2-hydroxyethyl methacrylate in the presence of pyridine as
6 reported previously.[2] Byrene (99%), azobisisobutyronitrile (AIBN, 98%), and 2-
7 hydroxyethyl methacrylate (HEMA, 98%) were used as received from Aldrich. 2,2'-
8 Bipyridine (bpy, 99%, Beijing, Shiying Reagent Manufactory), copper (I) bromide
9 (CuBr, 98%, Fluka), and doxorubicin hydrochloride (DOX·HCl, >99%, Beijing
10 Zhongshuo Pharmaceutical Technology Development Co., Ltd.) were used as
11 received. Tetrahydrofuran (THF), diethyl ether, toluene, and dimethylsulfoxide
12 (DMSO) were provided by Jiangtian Chemical agent company (Tianjin, China). 2-
13 Cyanopropan-2-yl dodecyl trithiocarbonate (CPDTC) was synthesized according to
14 the described procedure.[3] AIBN was recrystallized twice from ethanol before use.

15 **Characterization**

16 Fourier transform infrared spectra (FT-IR) were recorded using KBr disks in the
17 region of 4000 ~ 500 cm^{-1} on BIO-RAD FT-IR 3000 spectrometer (BIO-RAD
18 Company, Hercules, USA). ^{1}H-NMR spectra were measured on a Varian INOVA 500
19 MHz spectrometer (Varian Inc., Palo Alto, USA) using deuterated chloroform
20 ($CDCl_3$) or dimethylsulfoxide (DMSO-d_6) as solvent and tetramethylsilane (TMS) as
21 an internal standard. The molecular weight (M_w, M_n) and molecular weight
22 polydispersity index (M_w/M_n) of the copolymers were determined by a Waters 1515
23 gel permeation chromatograph (GPC, Waters company, Milford, USA) instrument
24 equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard
25 column and a differential refractive-index detector. The measurements were

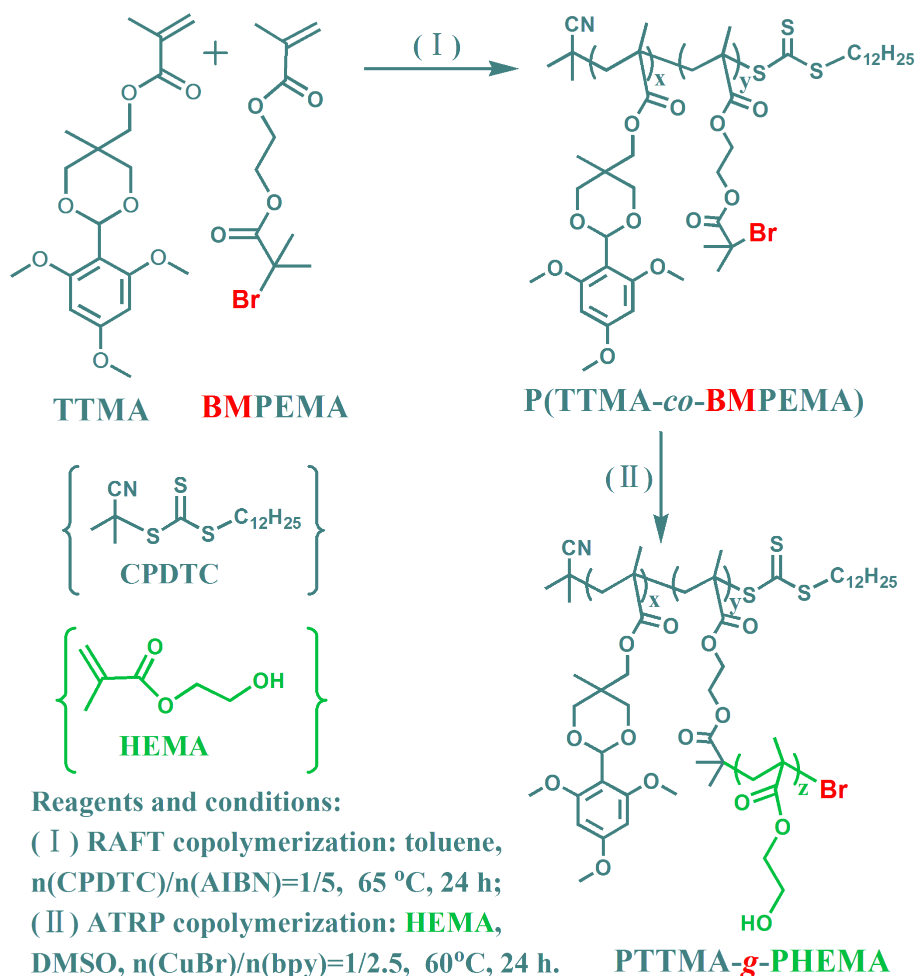
1 performed using DMF containing 0.05 M LiBr as an eluent at a flow rate of 1.0
2 mL/min at 25 °C and a series of narrow polystyrene standards for the calibration of
3 the columns. Dynamic laser scattering (DLS) measurements were performed on a
4 Brookhaven BI-200SM (Brookhaven Instruments Co., Holtsville, USA) at λ = 532 nm
5 with a fixed detector angle of 90°. Transmission electron microscopy (TEM)
6 measurements were performed on a JEOL JEM-1011 transmission electron
7 microscope with an accelerating voltage of 100 kV. A drop of the nanoparticles (NPs)
8 solution (1.0 mg mL^{-1}) was deposited onto a 230 mesh copper grid coated with carbon
9 and allowed to dry in air at 25 °C before measurements.

10 **Synthesis of P(TTMA-*co*-BMPEMA) macro-ATPR agent**

11 The P(TTMA-*co*-BMPEMA) copolymer bearing pendant 2-bromopropionyloxy
12 groups as macro-ATPR agent was facilely obtained by RAFT copolymerization of
13 TTMA and BMPEMA using CPDTC as a RAFT agent (Scheme S1). In a typical
14 example, TTMA (0.75 g, 2.05 mmol), BMPEMA (0.22 g, 0.8 mmol), CPDTC (19.7
15 mg, 0.10 mmol), AIBN (6.56 mg, 0.04 mmol) and toluene (3.0 mL) were added into a
16 10 mL Schlenk flask. The mixture was degassed through three freeze-pump-thaw
17 cycles. The polymerization tube was placed in a thermostatic water bath at 65 °C for
18 24 h under nitrogen atmosphere, which was finally quenched by immersing the tube
19 into liquid nitrogen. And then the reaction mixture was precipitated into an excess
20 of cold ether diethyl. The obtained product of pink powder was dried overnight in a
21 vacuum oven for 48 h at 25 °C, yield: 93.2 %.

22 M_n (^{1}H-NMR) = 9.82 kDa, M_n (GPC) = 10.5 kDa, M_w/M_n (GPC) = 1.21. FT-IR
23 (KBr): v(cm^{-1}) 2955, 748, and 654 (aromatic -C-H); 2843 and 1390 (methylic -C-H);
24 1732 (carbonyl –C=O); 1605 (aromatic -C-C-); 1465 (methylene -C-H); and 1030 (-
25 O-CH_3). ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.15 (aromatic protons), 5.78 (Ar-CH-),

1 3.74 (-OCH_2CCH_2O- and Ar-OCH_3), 1.94 (($(CH_3)_2C$- and -CH_2CCH_2-), 1.33 (-
2 $(CH_2)_{10}$-), 0.50 ~ 1.15 (CH_3CCOO- and CH_3C-). Elemental analysis: Br% = 6.45%.



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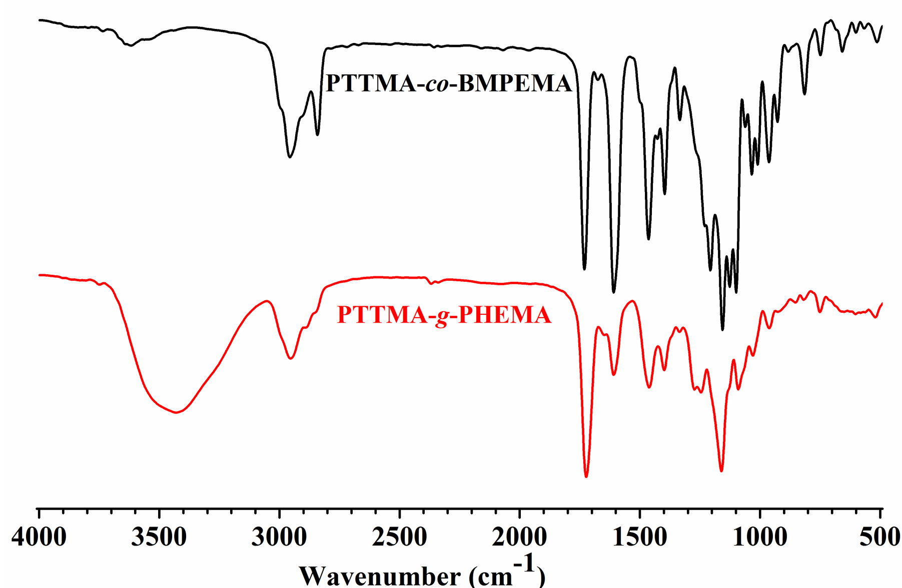
4 Scheme S1. Synthesis of P(TTMA-*co*-BMPEMA) macro-ATPR agent by RAFT
5 polymerization (I) and amphiphilic graft copolymer PTTMA-*g*-PHEMA by
6 sequential ATRP polymerization of HEMA using P(TTMA-*co*-BMPEMA) as a
7 macro-ATRP agent (II).

8 **Synthesis of PTTMA-*g*-PHEMA graft copolymer by ATRP polymerization**

9 PTTMA-*g*-PHEMA graft copolymer was prepared by ATRP polymerization of
10 HEMA using P(TTMA-*co*-BMPEMA) as a macro-ATRP agent (Scheme S1). Briefly,
11 under a nitrogen atmosphere, P(TTMA-*co*-BMPEMA) (0.30 g, 0.03 mmol) was
12 dissolved in DMSO (10.0 ml) in a Schlenk tube at room temperature. Then, HEMA

1 (0.32 g, 2.77 mmol), CuBr (34.4 mg, 0.24 mmol) and bpy (93.8 mg, 0.60 mmol) were
2 added and the mixture was thoroughly degassed. The polymerization tube was sealed
3 and placed into an oil bath thermostatted at 60 °C. The mixture proceeded with
4 magnetic stirring for 24 h. The product was dialyzed using Spectra/Pors membrane
5 (MWCO: 8 000 Da) against distilled water for 36 h to remove copper catalyst and
6 residual monomer, and then lyophilized to obtain PTTMA-*g*-PHEMA copolymers.
7 Yield: 92.5 %.

8 M_n (^{1}H-NMR) = 20.2 kDa, M_n (GPC) = 20.9 kDa, M_w/M_n (GPC) = 1.29. FT-IR
9 (KBr, Figure S1): *v*(cm^{-1}) 3650 ~ 3050 and 1033 (-O-H); 2955, 748, and 654
10 (aromatic -C-H); 2843 and 1390 (methylic -C-H); 1732 (carbonyl –C=O); 1605
11 (aromatic -C-C-); 1465 (methylene -C-H); and 1030 (-O-CH_3). ^{1}H NMR (500 MHz,
12 $CDCl_3$): δ 6.15 (aromatic protons), 5.78 (Ar-CH-), 4.84 (-OH); 3.89 and 3.58 (-O-
13 CH_2CH_2-O- of PHEMA), 3.74 (-OCH_2CCH_2O- and Ar-OCH_3), 1.94 (($CH_3)_2C$- and
14 -CH_2CCH_2-), 1.33 (-$(CH_2)_{10}$-), and 0.50 ~ 1.15 (CH_3CCOO- and CH_3C-).



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16 Fig. S1 FT-IR spectra of P(TTMA-*co*-BMPEMA) and PTTMA-*g*-PHEMA.

1 Table S1 Characterization of P(TTMA-*co*-BMPEMA) and PTTMA-*g*-PHEMA

Copolymer	M_n (kDa)			M_w/M_n [d]
	Design	M_n [c]	M_n [d]	
P(TTMA-*co*-BMPEMA)[a]	10.0	9.82	10.5	1.21
PTTMA-*g*-PHEMA[b]	20.8	20.2	20.9	1.29

2 [a)]RAFT polymerization conditions: [TTMA]/[BMPEMA]/[CPADN]/[AIBN] =
3 20.5/8.0/1.0/0.2 (mol/mol), toluene, 65 °C, 24 h; [b)] ATRP polymerization conditions:
4 [HEMA]/[P(TTMA-*co*-BMPEMA)][bpy]/[CuBr] = 84.5/1.0/20.0/8.0 (mol/mol),
5 DMSO, 60 °C, 24 h. [c)] Determined by ^{1}H-NMR analysis by comparing the intensities
6 of signals at δ 1.33 (methylene protons in CPADN), 6.15 (benzene methylene protons
7 in PTTMA) and 4.84 (hydroxyl protons in PHEMA); [d)] Determined by GPC (DMF
8 containing 0.05 M LiBr as an eluent, at a flow rate of 1.0 mL/min, 25 °C, polystyrene
9 standards).

10 **Critical micelle concentration (CMC) measurement**

11 CMC was measured by steady-state fluorescent-probe methodology using pyrene as a
12 probe on a Varian fluorescence spectrophotometer at 25 °C.[4] Sample solutions for
13 fluorescence investigation were prepared as described previously. The copolymer
14 concentrations in this experiment varied from 1.0×10^{-6} to 1.0 mg mL^{-1}. The final
15 pyrene concentration in copolymer solution was kept at 6.0×10^{-7} mol L^{-1}. These
16 solutions were shaken vigorously and then allowed to equilibrate at 25 °C for at least
17 24 h. The excitation spectra of pyrene with various copolymer concentrations were
18 measured at the detection emission wavelength (λ_{em}= 373 nm). The CMC value was
19 obtained from the intersection of the tangent to the horizontal line of I_{337}/I_{333} with
20 relative constant value and the diagonal line with rapidly increased I_{337}/I_{333} ratio.

21 **Formation and characterization of NPs**

22 PTTMA-*g*-PHEMA NPs were prepared by a solvent exchange method. Briefly, to a
23 stirred DMSO solution (2.0 mL) of PTTMA-*g*-PHEMA (10.0 mg) was dropwise

1 added 8.0 mL of double distilled water. The mixture was sonicated for 10 min and
2 then extensively dialyzed against double distilled water for 24 h (MWCO 3500 Da) at
3 room temperature. The NPs suspension was filtered through a 450 nm syringe filter
4 before measurements to remove dust and other adventitious impurities. The size, size
5 distribution and morphology of the NPs were determined by DLS and TEM,
6 respectively.

7 **pH-Triggered hydrolysis of CBAs groups in the NPs**

8 The CBAs hydrolysis was followed by UV/vis spectroscopy by measuring the
9 absorbance at 292 nm, according to the previous reports.[5] The PTTMA-*g*-PHEMA
10 NPs solutions (1.0 wt%) were prepared and divided into three aliquots (2.0 mL). Their
11 pHs were adjusted to 4.0 and 5.0 by addition of 4.0 M pH 4.0 and 5.0 acetate buffer or
12 maintained at pH 7.4 using phosphate buffer, respectively. The solutions were shaken
13 at 37 °C. At desired time intervals, 80 μL aliquot was taken out and diluted with 3.5
14 mL phosphate buffer (10 mM, pH 7.4). The absorbance at 292 nm was monitored. At
15 the end, all the samples were completely hydrolyzed by the addition of two drops of
16 concentrated HCl and were measured again to determine the absorbance at 100 %
17 hydrolysis, which was used to calculate the degree of CBAs hydrolysis.

18 **Size changes of NPs in response to CBAs hydrolysis**

19 The changes in size of PTTMA-*g*-PHEMA NPs in response to CBAs hydrolysis were
20 followed by DLS. The NPs solutions under pH 7.4 and 5.0 were prepared as above.
21 The samples were gently stirred at 37 °C. The changes in NPs size were monitored in
22 time by DLS.

23 **CBAs hydrolysis tracked by steady-state fluorescent-probe methodology**

24 CBAs hydrolysis of PTTMA-*g*-PHEMA NPs was further tracked by steady-state
25 fluorescent-probe methodology using pyrene as a probe on a Varian fluorescence

1 spectrophotometer at 25 °C. Firstly, pyrene was encapsulated into PTTMA-*g*-PHEMA
2 NPs (10 ml, 1.0 mg mL^{-1}) and the final pyrene concentration in NPs solution was 11.2
3 μg mL^{-1}. The fluorescence intensity was measured in time by a Varian fluorescence
4 spectrophotometer under pH 5.0 condition.

5 **Drug loading and *in vitro* pH-triggered release of DOX**

6 DOX-loaded PTTMA-*g*-PHEMA NPs were prepared by a dialysis method. Typically,
7 copolymer (10 mg) and DOX (0.50 mg) were dissolved in 2.0 mL of DMSO and
8 added dropwise to 8.0 mL double distilled water, after that, the solution was
9 transferred to an Spectra/Pors membrane (MWCO 3500 Da) dialysis bag and dialyzed
10 for 12 h to remove the organic solvents and free DOX. The whole procedure was
11 performed in the dark. Then, the solution was filtered and lyophilized. For
12 determination of drug loading content (DLC) and drug loading efficiency (DLE), the
13 drug loaded NPs was dissolved in DMSO and the absorbance at 500 nm was
14 determined by UV−visible spectrophotometer (TU-1900, China). A calibration curve
15 was constructed using different concentrations (1 ~ 50 μg mL^{-1}) of free DOX in
16 DMSO. The DLC and DLE of the drug loaded NPs were calculated according to
17 equation (1) and (2), respectively:

18 DLC (%) = (amount of loaded drug)/(amount of drug-loaded NPs) ×100 %   (1)

19 DLE (%) = (amount of loaded drug)/(total amount of feeding drug) × 100 %   (2)

20 Table S2 Characterization of DOX-loaded PTTMA-*g*-PHEMA NPs

Enry	DLC[a] (%)	DLC[b] (%)	DLE[b] (%)	Size[c] (nm)	PDI[c]
1	5	4.75	94.5	90.2	0.18
2	10	9.04	90.4	130.4	0.21
3	20	17.4	87.0	161.7	0.25

21 [a] Theoretical value; [b] Determined by UV spectrophotometry; [c] Measured by DLS at a
22 concentration of 1.0 mg mL^{-1} in phosphate buffer (10 mM, pH 7.4) at 25 °C.

1 The release profiles of DOX from PTTMA-*g*-PHEMA NPs were studied using a
2 dialysis tube (MWCO: 8 000 Da) at 37 °C in three different media, i.e., phosphate
3 buffer (10 mM, pH 7.4), acetate buffer (10 mM, pH 5.0) and acetate buffer (10 mM,
4 pH 4.0). In order to ensure sink conditions, drug release studies were performed at
5 low drug loading contents (final concentration about 30.0 μg mL^{-1}) and with 5.0 mL
6 of NPs suspension dialysis against 15 mL of the same medium. At desired time
7 intervals, 4.0 mL of release media was taken out and replenished with an equal
8 volume of fresh media. The amount of DOX released was calculated based on the
9 absorbance intensity at 500 nm with UV/vis spectroscopy. The release experiments
10 were conducted in triplicate. The results presented are the average data with standard
11 deviations.

12 **Cell viability assays**

13 The cytotoxicity of blank PTTMA-*g*-PHEMA NPs, DOX-loaded NPs and free DOX
14 was examined by MTT assays on human hepatoma (HepG-2) cells. In brief, the cells
15 were seeded in 96-well plates at 8 000 cells per well in 100.0 μL of complete
16 Dulbecco's modified Eagle's medium (DMEM) and incubated at 37 ^{o}C in a 5 % CO_2
17 atmosphere for 24 h, followed by removing the culture medium and adding blank NPs,
18 free DOX or DOX-loaded NPs solutions at different concentrations. The cells were
19 subjected to MTT assay after being incubated for another 48 h. The culture medium
20 was then replaced with 100 μL fresh DMEM, followed by the addition of 20 μL MTT
21 solutions (5 mg mL^{-1} in phosphate buffer), and incubated for 4 h. Afterward, the
22 medium was completely removed and 150 μL of DMSO was added to each well to
23 extract the formazan products formed by viable cells. The absorbances of the
24 solutions were measured on a Bio-Rad 680 microplate reader at 570 nm. The results
25 were expressed as a percentage of the absorbance of the blank control.

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Cell Uptake Studies

The cellular uptake of DOX was studied on HepG-2 cells using fluorescence microscopy. HepG-2 cells were seeded onto glass coverslips in a six-well culture plate at a density of 5 × 10^4 cells $well^{-1}$. After 24 h incubation, the cells were treated with free DOX (10 μg mL^{-1}) or DOX-loaded PTTMA-*g*-PHEMA NPs (10 μg mL^{-1} equivalent DOX concentration) for 2 h. After incubation, the cell monolayers were rinsed three times with 1 mL phosphate buffer (10 mM, pH 7.4) to remove excess NPs or free DOX. Fresh phosphate buffer (10 mM, pH 7.4) was added to the plates and the cells were viewed and imaged under a confocal laser scanning microscope (CLSM, Leica AF 6500, Leica Microsystems GmbH, Germany). Similarly, for time dependant cellular uptake studies of free DOX and DOX-loaded NPs, the glass coverslips were removed from the incubator at predetermined time intervals and the cells were processed using the above confocal studies protocol.

References

1 A. P. Griset, J. Walpole, R. Liu, A. Gaffey, Y. L. Colson, and M. W. Grinstaff, *J. Am. Chem. Soc.* **2009**, *131*, 2469.

2 H. Mori, A. Böker, G. Krausch, and A. H. E. Müller, *Macromolecules* **2001**, *34*, 6871.

3 Y. K. Chong, G. Moad, E. Rizzardo, and S. H. Thang, *Macromolecules* **2007**, *40*, 4446.

4 L. Chang, L. Deng, W. Wang, Z. Lv, F. Hu, A. Dong, and J. Zhang, *Biomacromolecules* **2012**, *13*, 3301.

5 W. Chen, F. Meng, F. Li, S. J. Ji, and Z. Zhong, *Biomacromolecules* **2009**, *10*, 1727.