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Unimolecular micelles of Camptothecin-bonded hyperbranched star copolymers via *β*-thiopropionate linkage: synthesis and drug delivery

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In order to develop pH- and redox-responsive unimolecuar micelles composed of camptothecin (CPT)-conjugated hyperbranched star copolymers via acid-labile *β*-thiopropionate linkage, a new monomer, methacryloyloxy-3-thiohexanoyl-CPT is synthesized through conjugation of CPT with methacrylate via *β*-thiopropionate linkage, and then used in synthesis of the CPT-conjugated hyperbranched star copolymers by two steps of atom transfer radical polymerization (ATRP): self-condensation vinyl polymerization of the CPT-based monomer, 2-hydroxypropyl methacrylate and inimer, subsequent ATRP of oligo(ethylene glycol) methacrylate using the obtained hyperbranched polymers as macroinitiator. The obtained polymers dissolve in water to form unimolecular micelles, and their release behavior of CPT in water at various pHs and anticancer efficacy are studied. The CPT-loaded unimolecular micelles with diameters of 3.56-6.08 nm display quite stable under neutral environment, and are easily triggered by mild acidic pH, such as, 6.0 and 5.0. They can be easily internalized by the tumor cells, releasing the CPT. The CPT-conjugated unimolecular micelles via acid-labile *β*-thiopropionate linkage have potential for application of the tumor–targeted drug release systems.

Keywords : ATRP • camptothecin • hyperbranched star copolymer • pH-sensitive • control release • unimolecular micelles.

1. Introduction

Camptothecin (CPT), which is a cytotoxic quinoline alkaloid, shows remarkable anticancer activity in preliminary clinical trials because it is an inhibitor of the DNA-replicating enzyme topoisomerase I.[1] The CPT is isolated from the bark and stem of Camptotheca acuminate, a Chinese native tree used as cancer treatment in Traditional Chinese Medicine.[2] Nevertheless, low solubility of the CPT in water forced medicinal chemists to develop various derivatives for improving the performance of drug.[3-5] Currently, the polymeric carrier of CPT has been widely investigated, and there are two general methods for preparation of the CPT-loaded microspheres. One is encapsulation of the CPT within the hydrophobic core of micelles or nanoparticles,[6-8] the resultant microspheres enhance the stability of CPT in the active lactone form besides improvement of water-solubility. Another method is to graft the CPT onto hydrophobic cores of the micelles via a labile chemical bond, under external stimuli conditions, such as redox state of the medium or light, the labile bond can be cleaved and the CPT is released.[9-13] Controlled release of the free CPT from the nanostructured polymer materials was achieved via breaking of a photo-cleavable linker, such as 2-nitrobenzyl ester, under irradiation of 365 nm light,[9] or via breaking of a reducible disulfylbutyrate linker in the presence of glutathione.[10] The pH-responsive aggregates have attracted extensive attention because of their particular relevance in biological applications and significantly lower pH of the tumor cells than that of the normal cells.[14,15] Thus, study on grafting CPT onto the nanostructured polymer aggregates via acid-labile bond is particularly significant.

There are already many studies on pH-sensitive polymeric microspheres, and the acid-labile covalent linkages utilized to chemically link drugs onto the polymers or the assemblies include hydrazones,[16-18] acetal,[19-22] ketal,[23] and hydrazide.[24] The CPT can be linked onto the polymers by reaction of hydroxyl group in CPT with CHO group, however, besides the synthetic difficulty, the acetal linkage is extremely sensitive to acidic pH, and can be cleaved rapidly,[25] thus, controlled release of the drug in a sustained manner is difficult. The β-thiopropionate can hydrolyze in acidic solution, which is driven by the generation of a partial positive charge on the ester carbonyl carbon owing to the inductive effect of sulfur atom (**Scheme 1a**),[26,27] and this linkage undergoes acidic hydrolysis at relatively slow rate compare to the acetal linkage, so controlled drug release in a sustained manner is expected. This acid-labile β-thiopropionate linkage has been utilized in

pH-responsive polymer micelles,[28-30] and the pH-responsive polyion complex micelles were constructed by conjugation of siRNA to the lactosylated poly(ethylene glycol) through β-thiopropionate linkage. [31-33] However, study on drug release triggered by pH through β-thiopropionate is rare, only one article we found out in the literatures, the micelles are formed by dissolving the paclitaxel-conjugated linear block copolymers via β-thiopropionate linkage in an aqueous solution, and showed pH-triggered drug release property.[34] All the polymers used in these studies are linear, and their self-assembly formed microspheres. However, the results reported demonstrate that the branched polymeric architectures, such as dendrimers, hyperbranched polymers and dendronized polymers, possess features such as a number of surface groups and nanoscopic size, which make them attractive in drug delivery applications,[35,36] and the branched structures of sufficient size display extended *in vivo* circulation time in comparison with their linear counterparts.[37]
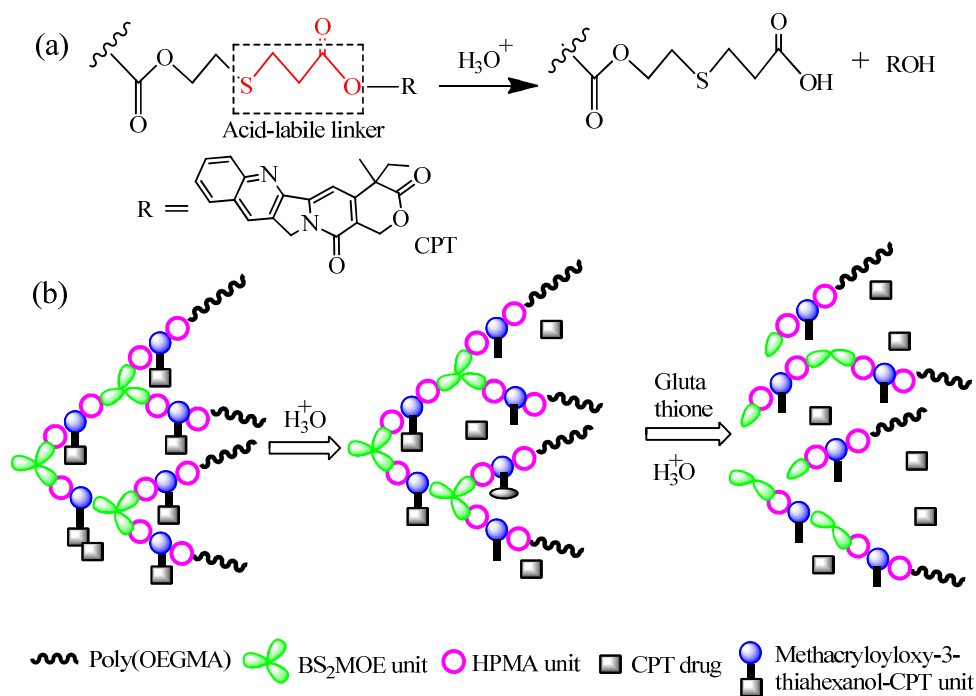
Scheme 1 (a) Structure of poly(methacryloyloxy-3-thiahexanoyl-CPT) and its acid-induced cleavage; (b) schematic representation of the drug release in acidic solution and decomposition of the hyperbranched star copolymer in the presence of glutathione.

Herein, we introduce a novel CPT-based hyperbranched star copolymer for application in drug delivery. **Scheme 1b** depicts the controlled release in acidic solution and decomposition of the

copolymers in the presence of glutathione. The core polymer is composed of BS_2MOE, HPMA and MACPT. The disulfide is a well-known reduction-responsive linkage,[38-40] thus, the BS_2MOE units in the core can ensure complete renal clearance after drug delivery.[9] By utilization of highly reactive bromine groups on the surface of hyperbranched polymers, a variety of targeting groups or bioactive compounds can be linked to their surface, forming various targeting drug delivery systems or improving blood compatibility. In this study, the highly hydrophilic poly[oligo(ethylene glycol) methacrylate] (POEGMA) chains were attached to the surface of hyperbranched polymers through ATRP method and the resultant hyperbranched star copolymers can form unimolecular micelles in aqueous solution, and the highly hydrophilic POEGMA chains are extended into aqueous solution shielding hydrophobic hyperbranched polymer core. Such polymers have potential applications in tumor–targeted drug release systems.

2. Experimental Section

2.1 Materials

CPT and SYTO-9 were respectively purchased from Aladdin and Invitrogen Corporation, and both were used as received. Methacryloyl chloride, acryloyl chloride, triethylamine (Et_3N) and 2-mercaptoethanol were distilled under reduced pressure. Hydroxypropyl methacrylate (HPMA, 97%, Aladdin) and oligo(ethylene glycol) methacrylate (OEGMA, M_n = 475 g/mol, Aldrich) were purified by passing through a neutral Al_2O_3 column to remove the inhibitor. The HPMA is an isomer mixture of 2-hydroxypropyl methacrylate (75 mol%) and 2-hydroxyisopropyl methacrylate (25 mol%). CuBr (98%, Aldrich) was purified as reported.[41] *N, N, N', N'', N'''*-pentamethyldiethylenetriamine (PMDETA, 99%, Aladdin) was deoxygenated before use by nitrogen bubbling for 2–3 h. 2-(2'-Bromoisobutyryloxy)ethyl- 2''-methacryloyl oxyethyl disulfide (BS_2MOE) was prepared according to our previous report.[42] ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 6.15 and 5.60 (s, 2H, $=CH_2$), 4.42 (dt, 4H, 2-O-CH_2), 3.02-2.94 (m, 4H, 2-S-CH_2), 1.95 (dd, 3H,-CH_3), and 1.94 (s, 6H, ($CH_3)_2$CBr). All other reagents with analytical grade were purchased from Shanghai Chemical Reagent and used without further purification.

2.2 Characterization

^{1}H NMR measurements were carried out on a Bruker AV300 NMR spectrometer using CDCl$_3$ or D$_2$O or DMSO-d$_6$ as solvent. High-performance liquid chromatography-mass spectrometry (LC-MS) analysis was conducted on a two-dimensional liquid chromatography-tandem mass spectrometry (2DLC-MS) from Thermo Fisher Scientific. Molecular weight and molecular weight distribution (M_w/M_n) were determined on Waters 150C gel permeation chromatography (GPC) equipped with two microstyragel column and RI 2414 detector at 30 °C, monodispersed polystyrene standards were used in the calibration of M_n, M_w, and M_w/M_n, and N, N'-dimethyl formamide (DMF) was used as eluent at a flow rate of 0.9 mL/min. UV/Vis measurement was performed on a Unico UV/vis2802PCS spectrophotometer. Cell imaging observations were conducted on a confocal laser scanning microscope (LSM510, Zeiss and Coherent) with a 63X objective. High performance liquid chromatography (HPLC) measurement was conducted on a Liquid Chromatograph (Agilent 1260). Dynamic light scattering (DLS) measurements were carried out on a DynaPro light scattering instrument (DynaPro-99E) at 25 °C with 824.3 nm laser, and the data was analyzed with DYNAMICS V6 software.

2.3 Synthesis of methacryloyloxy-3-thiohexanoyl-CPT (MACPT)

MACPT was synthesized through three steps including syntheses of acryloyl-CPT, 6-hydroxy-3-thiohexanoyl-CPT and MACPT (Scheme 2).

2.3.1 Acryloyl-CPT. Acryloyl chloride (0.447 g, 0.005 mol) was dropwise added into a suspension of CPT (0.348 g, 0.001 mol) and triethylamine (0.505 g, 0.005 mol) in dichloromethane (200 mL) in ice bath while stirring. The reaction mixture was then stirred at room temperature for 24 h. The solvent was removed under reduced pressure to yield yellow solid. Dilute ammonia water was added to dissolve the surplus acidoid while the acryloyl-CPT was insoluble in water and collected by filtration. The resultant product was further purified via silica column chromatography (ethyl acetate), and 0.35 g of light yellow solid was obtained in yield of 87%. ^{1}H NMR (300 MHz, CDCl$_3$, δ, ppm): 8.40 (m, 1H, ArH), 8.21 (m, 1H, ArH), 7.94 (m, 1H, ArH), 7.82 (m, 1H, ArH), 7.67 (m, 1H, ArH), 7.23 (m, 1H, ArH), 6.52 and 5.98 (d, 2H, =CH$_2$), 6.27 (dd, 1H, =CH), 5.71 and 5.43 (m, 2H, -O-CH$_2$), 5.29 (m, 2H, -N-CH$_2$), 2.41-2.16 (m, 2H, -CH$_2$), and 1.01 (t, 3H,-CH$_3$); MS: [M-H$^+$] = 403.12, found at 403.1286.

2.3.2 6-Hydroxy-3-thiohexanoyl-CPT. 2-Mercaptoethanol (0.340 g, 4.36 mmol) and

acryloyl-CPT (0.350 g, 0.87 mmol) were dissolved in THF (40 mL). The reaction mixture was stirred at 40 °C for 12 h. The solvent was removed under reduced pressure to yield yellow solid, and water was added to dissolve the surplus 2-mercaptoethanol while the 6-hydroxy-3-thiohexanoyl-CPT was insoluble in water and was collected by filtration (0.376 g, yield: 90%). ^{1}H NMR (300 MHz, CDCl$_3$, δ, ppm): 8.40 (m, 1H, ArH), 8.21 (m, 1H, ArH), 7.94 (m, 1H, ArH), 7.82 (m, 1H, ArH), 7.67 (m, 1H, ArH), 7.23 (m, 1H, ArH), 5.71 and 5.43 (m, 2H, -O-CH$_2$), 5.29 (m, 2H, -N-CH$_2$), 4.58 (s, 1H, -OH), 3.89 (dt, 2H, HO-CH$_2$-), 3.01-2.83 (m, 6H, -CH$_2$-), 2.41-2.16 (m, 2H, -CH$_2$), and 1.01 (t, 3H,-CH$_3$). MS: [M-H$^+$] = 481.14, found at 481.1420.

2.3.3 Methacryloyloxy-3-thiohexanoyl-CPT (MACPT). Methacryloyl chloride (0.409 g, 3.9 mmol) was dropwise added to a stirred suspension of 6-hydroxy-3-thiohexanoyl-CPT (0.376 g, 0.78 mmol) and triethylamine (0.395 g, 3.9 mmol) in dichloromethane (50 mL) in ice bath. The reaction mixture was then stirred at room temperature for 24 h. The solvent was removed under reduced pressure to yield dark yellow solid. Dilute ammonia water was added to dissolve the surplus acidoid while methacryloyl-3-thiohexanoyl-CPT was insoluble in water and can be collected by filtration. The resulted product was purified via silica column chromatography (ethyl acetate), and dark yellow solid was obtained (0.365 mg, yield: 85%). ^{1}H NMR (300 MHz, CDCl$_3$, δ, ppm): 8.40 (m, 1H, ArH), 8.21 (m, 1H, ArH), 7.94 (m, 1H, ArH), 7.82 (m, 1H, ArH), 7.67 (m, 1H, ArH), 7.23 (m, 1H, ArH), 6.18 and 5.55 (d, 2H, =CH$_2$), 5.71 and 5.43 (m, 2H, -O-CH$_2$), 5.29 (m, 2H, -N-CH$_2$), 4.28 (m, 1H, -O- CH$_2$), 3.01-2.77 (m, 6H, -CH$_2$-), 2.41-2.16 (m, 2H, -CH$_2$), and 1.91 (m, 3H, CH$_3$C=), 1.01 (t, 3H, -CH$_3$). MS: [M-H$^+$] = 549.16, found at 549.1686. Melting point = 196-198 °C.

^{1}H NMR spectra of acryloyl-CPT, 6-hydroxy-3-thiohextyoyl-CPT and MACPT are shown respectively in Figures S1-S3 of Supporting Information, and the characteristic signals are marked in these figures.

2.4 Synthesis of HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)

Into a 2 mL polymerization tube, MACPT (75 mg, 0.137 mmol), HPMA (78 mg, 0.547 mmol), BS$_2$MOE (25.2 mg, 0.0679 mmol), CuBr (0.98 mg, 0.00679 mmol), PMDETA (2.35 mg, 0.0136 mmol) and THF (0.6 mL) were added. The mixture was degassed through three freeze-pump-thaw cycles, the polymerization tube was then sealed by flame under vacuum and the sealed tube was immersed into an oil bath thermostated at 50 °C. After 48 h, the

polymerization tube was cooled to room temperature rapidly, and then the mixture was dissolved in 50 mL of THF, and then passed through a short neutral alumina column for removal of metal salt. The final polymer (0.157 g, yield: 88%) was obtained by evaporating most of the solvent and precipitating into diethyl ether.

2.5 Synthesis of HP(HPMA-co-MACPT-co-BS$_2$MOE)(POEGMA)$_n$

Into a 5 mL polymerization tube, HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE) (0.100 g, 0.0381 mmol Br), OEGMA (0.362 g, 0.762 mmol), CuBr (0.55 mg, 0.00381 mmol), PMDETA (1.32 mg, 0.00762 mmol) and THF (2 mL) were added. The mixture was degassed through three freeze-pump-thaw cycles. The polymerization tube was then sealed by flame under vacuum, and the sealed tube was immersed into an oil bath at 50 °C. After 3 h, the polymerization tube was cooled to room temperature rapidly, and the mixture was dissolved into 50 mL of THF, and then passed through a short neutral alumina column for removal of the metal salt. The final polymer (0.195 g, yield: 26.3%) was obtained by evaporating most of the solvent and precipitating in n-hexane.

2.6 Preparation of unimolecular micellar solution in water

Hyperbranched star copolymer, HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)(POEGMA)$_n$ (10 mg) was dissolved in THF (1 mL). Under vigorously stirring, deionized water (9 mL) was added via a syringe pump at a rate of 3 mL/h. After the addition was completed, the dispersion was stirred for another 5 h. Subsequently, THF was removed by dialysis [molecular weight (M_w) cutoff: 35 kDa] against deionized water for 24 h, and the dialysis media was changed regularly.

The drug loading capacity (DLC) of the micelles was measured by ^{1}H NMR spectra and UV quantitative analysis. For UV method, a series of CPT solutions (0.005, 0.01, 0.05, 0.1, 0.25 mg/mL) in DMSO were prepared, and then UV absorbance of each solution was measured. The standard curve was obtained as shown in Figure S7. HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)(POEGMA)$_n$ (1 mg) was dissolved in DMSO (1 mL) and the CPT concentration was obtained based on the standard curve. The DLC was calculated according to the following formula:

DLC = (weight of loaded drug/weight of polymer) ×100%

2.7 Drug release

The concentration of the HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)(POEGMA)$_n$ micelles solution was adjusted to 1.0 mg/mL for subsequent *in vitro* drug release experiments. The drug release was performed under both reduction-insensitive (without DTT) and reduction-sensitive (with DTT) conditions. For each condition, the drug release was respectively performed pH = 5.0, 6.0 and 7.4. A typical procedure is as follows. An aqueous dispersion (3.0 mL) of CPT-loaded micelles (1.0 mg/mL) was transferred to a dialysis bag with a molecular weight cutoff of 3.5 kDa and then immersed in 60 mL of buffer solution with 10 mM DTT at pH = 5.0 or 6.0 or 7.4, which was purged with pure nitrogen for 30 min. The samples (3.0 mL) were taken at predetermined time intervals (2 h) to determine amount of the released drug by ultraviolet quantitative analysis, and the solution out of the dialysis bag was refilled with 3.0 mL of the fresh buffer solution. This procedure continued for 3 days, and the buffer solutions were collected. Finally, hydrolysis product was obtained by lyophilization for verification of its structure. The same method was employed for the reduction-insensitive experiment. UV absorbance of the dialysis solution was measured at 365 nm.

2.8 Cell viability assay

In vitro cytotoxicity was examined by MTT assay. HepG2 (or HeLa) cells were seeded in a 96-well plate with an initial density of 5000 cells/well in 100 μL of complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C under an atmosphere with 5% CO$_2$. Micellar solution of the HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)(POEGMA)$_n$ in phosphate buffered saline (PBS, 50 mM, pH 7.4) was added to achieve various polymer concentrations, while the plate with different concentrations of the unimolecular micelles was used and the plate without the polymers was used as control. After incubation for 24 h, MTT reagent (in 20 μL PBS buffer, 5 mg/mL) was added to each well, and the cells were further incubated with 5% CO$_2$ for 4 h at 37 °C. The culture medium in each well was removed and replaced by 100 μL of DMSO. The plate was gently agitated for 15 min and the optical densities (OD) were recorded at a wavelength of 490 nm upon using a Thermo Electron MK3 μm. The cell viability is calculated according to the following equation:

Cell viability %=(OD$_{sample}$/OD$_{control}$)× 100%

where OD_{sample} represents an OD value from a well-treated with samples and $OD_{control}$ from a well-treated with PBS buffer only. Each experiment was carried out in sextuplet. Mean value and the corresponding standard deviations (mean ±SD) were obtained. The same experiment were done using HeLa cells.

2.9 Cellular uptake and imaging

A cellular uptake and imaging assay of the CPT-loaded unimolecular micelles prepared from HP(HPMA-*co*-MACPT-*co*-BS₂MOE)(POEGMA)ₙ was carried out on the human hepatoma cells (HepG2). The HepG2 cells (5×10^4) were seeded on coverslip and then incubated in 500 µL of DMEM containing 100 µL of CPT-loaded HP(HPMA-*co*-MACPT-*co*-BS₂MOE)(POEGMA)ₙ micellar solution (3 mg/mL in deionized water), respectively, at 37 °C. After treated for 2 h, the culture medium was removed, and after being rinsed with PBS two times the cells were fixed with formaldehyde, and the cells were stained with STYO 9 (green). The cells were rinsed with PBS buffer and then were observed using a confocal laser scanning microscope (LSM510, Zeiss and Coherent) at 430 nm (E_x= 405 nm).

3. Results and discussion

As mentioned in the introduction, the *β*-thiopropionate linker is pH-sensitive and undergoes hydrolysis in acidic solution, which is essential for application in controlled drug release. Thus, we designed a new monomer, MACPT, in which CPT is linked to the methacrylate via acid-labile *β*-thiopropionate, for synthesis of the pH-sensitive CPT-loaded hyperbranched star copolymers, HP(HPMA-*co*-MACPT-*co*-BS₂MOE)(POEGMA)ₙ. As shown in **Scheme 2**, the monomer MACPT was synthesized through three steps: reaction of acryloyl chloride with hydroxyl group of CPT; Michael addition reaction between acryloyl-CPT and mercaptoethanol, and esterification reaction of 6-hydroxyl-3-thiohexanoyl-CPT with methacryloyl chloride. The structures of two intermediates are confirmed by ^{1}H NMR spectra (**Figs. S1** and **S2**). The proton signals of final product in **Fig. S3** prove the formation of MACPT: vinyl proton signals of the methacrylate at δ = 6.18 and 5.55 (n) ppm, methylene protons of the *β*-thiopropionate linker next to sulfur at δ = 2.9 ppm (k) and aromatic protons of CPT at δ = 7.2-8.8 ppm. Mass spectra also support successful synthesis of the

intermediates and MACPT. However, the MACPT is hardly homo-polymerized probably due to its steric hindrance. So, HPMA was selected as a comonomer owing to its low toxicity. In order to prepare the unimolecular polymeric micelles in aqueous solution, a strongly hydrophilic shell, which can stabilize the micelles in water, is highly required. OEGMA has been widely used in preparation of the amphiphilic block copolymers due to its favorable hydrophilicity and very low cytotoxicity. Therefore, the hyperbranched star copolymers, HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)(POEGMA)$_n$s were synthesized according to **Scheme 3**. The hyperbranched star copolymers HP(HPMA-*co*-BS$_2$MOE)(POEGMA)$_n$s without the CPT were used as contrast.
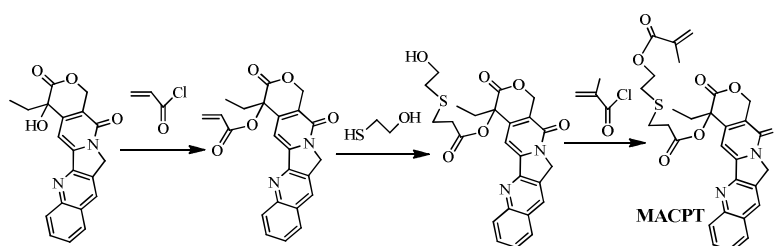
Scheme 2 Synthetic route of MACPT.
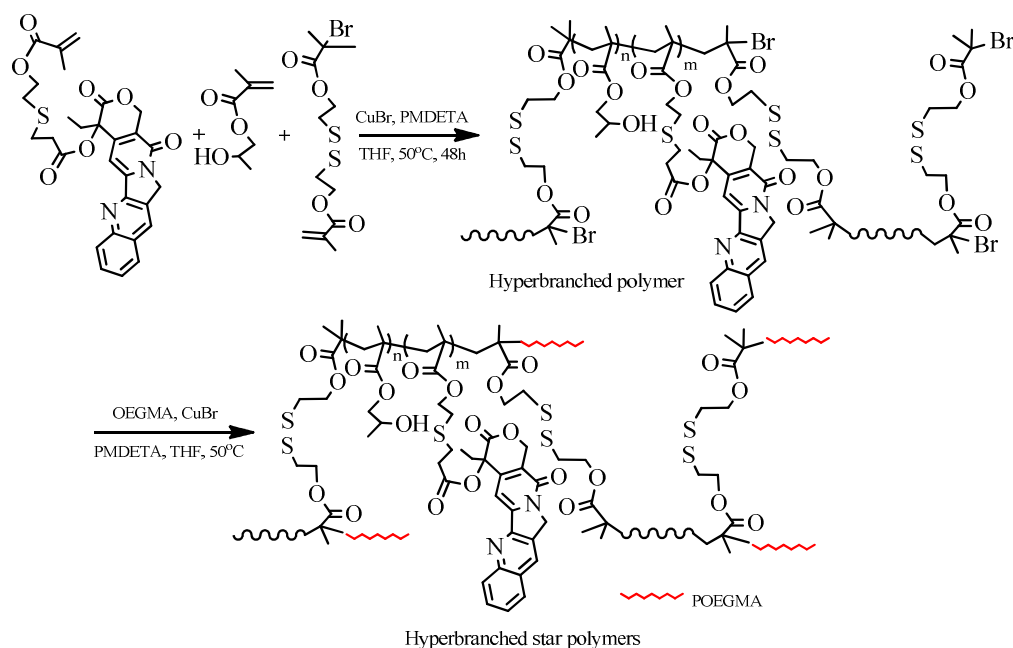
Scheme 3 Synthesis of hyperbranched copolymer, HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE) and hyperbranched star copolymer, HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)(POEGMA)$_n$

3.1 Synthesis and Characterization of HP(HPMA-*co*-MACPT-*co*-BS₂MOE)(POEGMA)ₙ

The first step for synthesis of the hyperbranched star copolymers is preparation of the macroinitiator, HP(HPMA–*co*–MACPT–*co*–BS₂MOE), which was synthesized by ATRP of HPMA, MACPT and the inimer BS₂MOE, the detailed polymerization conditions and the results are listed in **Table 1**. Their molecular weights and molecular weight distributions were measured by GPC, and the GPC curves are shown in **Figs. S5** and **S6**. Similar to the hyperbranched polymers prepared by self-condensing vinyl polymerization (SCVP), all the obtained polymers reveal broad molecular weight distribution (2.61~3.12).

Table 1. Preparation and results of the HP(HPMA-*co*-MACPT-*co*-BS₂MOE)s

Sample[a]	Inimer	Feed molar ratio of [M]/[H]/[B]/[CuBr]/[PMDETA][b]	Yield[c] (%)	M_n^d (g/mol)	M_w/M_n^d	Molar ratio of [M]/[H]/[B] in polymer[e]	NBU[f]
H0	BS₂MOE	0:1:0.1:0.01:0.02	90	16200	2.72	0:1:0.096	30
H1	BS₂MOE	1:4:0.5:0.05:0.1	88	11000	3.12	1:5.2:0.61	23
H2	BS₂MOE	1:8:0.9:0.09:0.18	85	14400	2.75	1:10.7:1.12	24
H3	BS₂MOE	1:20:2.1:0.21:0.42	87	15800	2.61	1:31:3.1	28

[a]H0, H1, H2 and H3 represent HP(HPMA-*co*-BS₂MOE) and HP(HPMA-*co*-MACPT-*co*-BS₂MOE) with various feed molar ratios. Polymerization temperature: 50 °C; time: 48 h. [b]M, H and B represent MACPT, HPMA and BS₂MOE, respectively. [c]Measured by gravimetry method. [d]Determined by GPC method. [e]Molar ratios of MACPT : HPMA: BS₂MOE were calculated according to the following equation: [MACPT] : [HPMA] : [BS₂MOE] = I_h/10 : I_d/2.75 : [(2I_a-3I_b)/12], where I_a, I_b, I_d and I_h are the integral values of the signals at δ = 0.5-1.3 ppm (a), and 1.6-2.1 ppm (b), 3.2-4.0 ppm (d) and 5.3-8.8 ppm (h), respectively. [f]NBU: average number of the branching unit per macromolecule is calculated according to the following equation: NBU = [(2I_a-3I_b)/6]/I_q, where I_q is the integral value of the signals at δ = 5.65 and 6.22 ppm (q)

   To verify structure of the obtained products, ¹H NMR spectra of the polymers were measured and a typical spectrum of the hyperbranched polymer H1 is shown in **Fig. 1a**. HPMA is composed of two isomers, 2-hydroxypropyl methacrylate and 2-hydroxyisopropyl methacrylate (HiPMA), the proton signal of ester methine in HiPMA unit appears at δ = 4.49 ppm (f); the proton signals of ester methylene, methylene and methine next to hydroxyl group appear at δ = 3.2-4.0 ppm (d); the signals

of aromatic protons and ester methylene in the MACPT units appear at δ = 7.2-8.8 and 5.6-5.9 ppm (h); the proton signals of methylene next to sulfur of BS$_2$MOE units appear at δ = 2.6-3.1 ppm (c). Compositions of the hyperbranched copolymers were calculated based on integral values of the proton signals at δ = 5.3-8.8 (h), δ = 3.2-4.0 ppm (d), the methyl and methylene proton signals in the main chains at δ = 0.5-1.3 (a) and 1.6-2.1 ppm (b), the results are listed in **Table 1**. The contents of MACPT in the polymers are lower than those of the corresponding recipe, indicating that the polymerization activity of MACPT is lower than that of HPMA, probably due to steric hindrance of the bulky side group CPT. Theoretically, every hyperbranched polymer prepared by SCVP of the inimers should contain one vinyl group, thus, the number of branched unit in every macromolecule (NBU) can be estimated based on the integral values of vinyl proton signals at δ = 5.65 and 6.22 ppm (I_q), and the methyl proton signal in the reacted initiating site of BS$_2$MOE [I_a-(3/2)I_b], and the results are listed in **Table 1**. The NBU ranging from 23 to 30 support hyperbranched structure of the resultant polymers. The methyl proton signal of bromoisobutyrate at δ =1.95 ppm is almost disappeared in **Fig. 1a**, indicating its high initiation efficiency, which is consistent with the reported result.[43] Therefore, the NBU is almost the same with the number of initiating site per macromolecule although probably the latter is slightly underestimated, and the hyperbranched polymers listed in **Table 1** were used as macroinitiator in the subsequent preparation of hyperbranched star polymers.

Table 2. Preparation and results of HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)](POEGMA)$_n$s

Sample[a]	Macro-initiator	M_n^b (g/mol)	M_w/M_n^b	Molar ratio of [M]:[H]:[B]:[O] in polymer[c]	DLC$_{UV}^d$ (wt%)	DLC$_{NMR}^e$ (wt%)	D[f] (nm)
H00	H0	36300	1.89	0:1:0.096:1.2	0	0	6.32
H4	H1	20300	1.98	1:5.2:0.61:2.0	16.7	14.1	3.56
H5	H2	26700	1.80	1:10.7:1.12:6.0	7.8	6.5	4.24
H6	H3	36000	1.87	1:31:3.1:46	1.3	1.2	6.08

[a]H00, H4, H5 and H6 represent HP(HPMA-*co*-BS$_2$MOE)(POEGMA)$_n$, and HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE) (POEGMA)$_n$1~3; All polymerizations with feed molar ratio of [Macroinitiator] : [OEGMA] : [CuBr] : [PMDETA] = 1 : 20 : 0.1 : 0.2 were performed at 50 °C for 3 h. [b]Determined by GPC, the M_n and M_w/M_n were calculated based on the polystyrene standards. [c]M, H, B and O refer to MACPT, HPMA, BS$_2$MOE and OEGMA, respectively. The molar ratios of [M] : [O] were calculated according to the equation: [MACPT]:[OEGMA] = (I_h/10):(I_i/3), where I_i and I_h are the integral values of the signals at δ = 3.2 ppm (i) and at δ = 5.3-8.8 ppm (h), respectively. Subsequently,

assuming that the compositions of HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)s does not change during the ATRP of OEGMA, the molar ratios of [M]:[H]:[B]:[O] were calculated. [d]DLC refers the drug loading capacity, that is, *wt*% of the CPT in the polymers determined by ultraviolet quantitative analysis. [e]Calculated based on the molar ratio of MACPT in HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE). [f]D refers to average diameter of the micelles dissolved in water, which was determined by DLS.
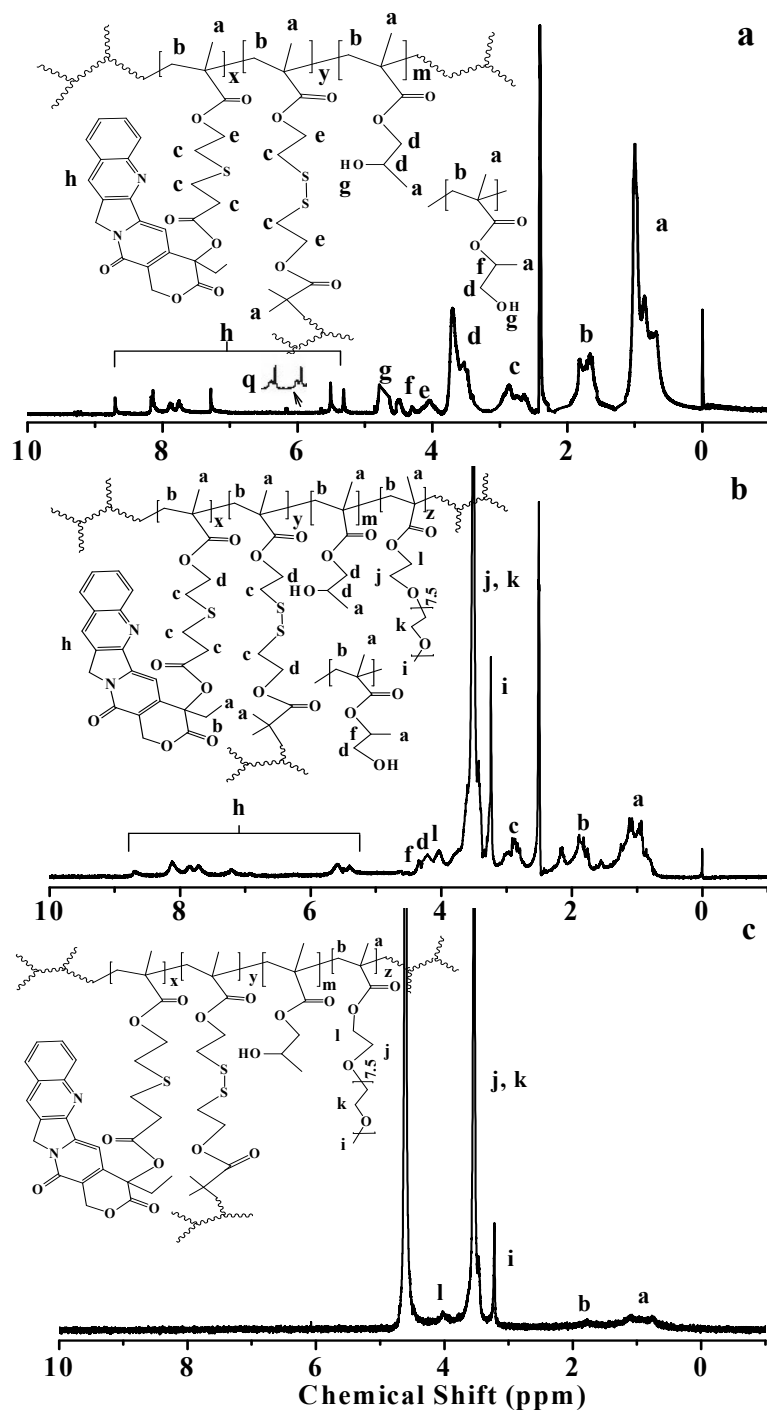
Fig. 1 ^{1}H NMR spectra of (a) the hyperbranched copolymer H1, HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE) recorded in DMSO-d$_6$; (b) the hyperbranched star copolymer H4, HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)(POEGMA)$_n$ recorded in DMSO-d$_6$; and (c) the same H4 recorded in D$_2$O.

Since the obtained hyperbranched polymers have a large quantity of reactive bromine groups on their surface, they can be conveniently modified based on requirement of their applications. High solubility of the hyperbranched copolymers in water is requisite for their application as drug carrier, owing to improving their water solubility, the POEGMA was selected as surface polymer of the hyperbranched star copolymers because it is water-soluble, nontoxic, and biocompatible.[44] The HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)(POEGMA)$_n$s were prepared by ATRP of OEGMA using HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE) as macroinitiator. Generally increasing molecular weight of the POEGMA chains will decrease the content of CPT in the resulted polymers, thus, the polymerization with a feed molar ratio of OEGMA/Br =20/1 was stopped at low conversion, and the detailed polymerization conditions and the results are listed in **Table 2**. To confirm the structures of products, also to estimate their composition, ^{1}H NMR measurements were conducted, and the resulted spectra are shown in **Fig. 1b** and **Fig. S4**. **Figure 1b** clearly shows the proton signals of OEGMA unit besides protons signals of HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE). The proton signals of ester methylene and ether methyl of the OEGMA units appear at $\delta = 4.0$ (l) and $\delta = 3.2$ (i) ppm, respectively, and the signal at $\delta = 3.3$-4.0 (j, k) ppm is significantly strengthened, indicating successful synthesis of the target polymers. Based on the integral value of the proton signals at $\delta = 3.2$ (i) and 5.3-8.8 (h) ppm, as well as the composition of their corresponding precursors, the compositions of resultant hyperbranched star copolymers, H00, H4, H5 and H6, which were prepared by ATRP of OEGMA respectively using H0, H1, H2 and H3 as macroinitiators, were calculated and the results are listed in **Table 2**. The M_w, M_n and M_w/M_n of the hyperbranched star copolymers were measured by GPC, and their GPC traces are shown in **Fig. S6**. Compare to the GPC traces of H0, H1, H2 and H3 in **Fig. S5**, all GPC traces of the H00, H4, H5 and H6 are shifted to the higher molecular weight region. Thus, the hyperbranched star copolymers, HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)(POEGMA)$_n$s were successfully synthesized and were used in the following studies.

3.2 Dispersion behavior of hyperbranched star copolymers in water

The hyperbranched copolymers, HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)s are insoluble in water, due to highly hydrophilic POEGMA, the HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)(POEGMA)$_n$s are dissolved in water forming the unimolecular micelles with the HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE) core and the POEGMA shell. To validate the formation of core-shell micelles, the ^{1}H NMR measurements of the H4 in Table 2 were conducted in DMSO-d$_6$ and in D$_2$O, respectively. When DMSO-d$_6$ is used as solvent, the characteristic proton signals of HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)(POEGMA)$_n$ appear in its ^{1}H NMR spectrum of **Fig. 1b**. However, in D$_2$O solvent, only the characteristic proton signals of POEGMA chain appear, the proton signals of HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE) are absent as shown in **Fig. 1c**, demonstrating the formation of micelles with the hydrophobic HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE) core and the hydrophilic POEGMA shell. To further characterize the formed micelles, the DLS was used to detect size of the micelles, and the DLS curves are shown in **Fig. 2**, and their sizes are listed in **Table 2**. The results reveal that average diameters (*D*s) of the resultant H4, H5, H6 and H00 micelles are 3.56, 4.24, 6.08 and 6.32 nm, respectively, which are consistent with the order of their molecular weights. Thus, the hyperbranched star copolymers can form unimolecular micelles in water.
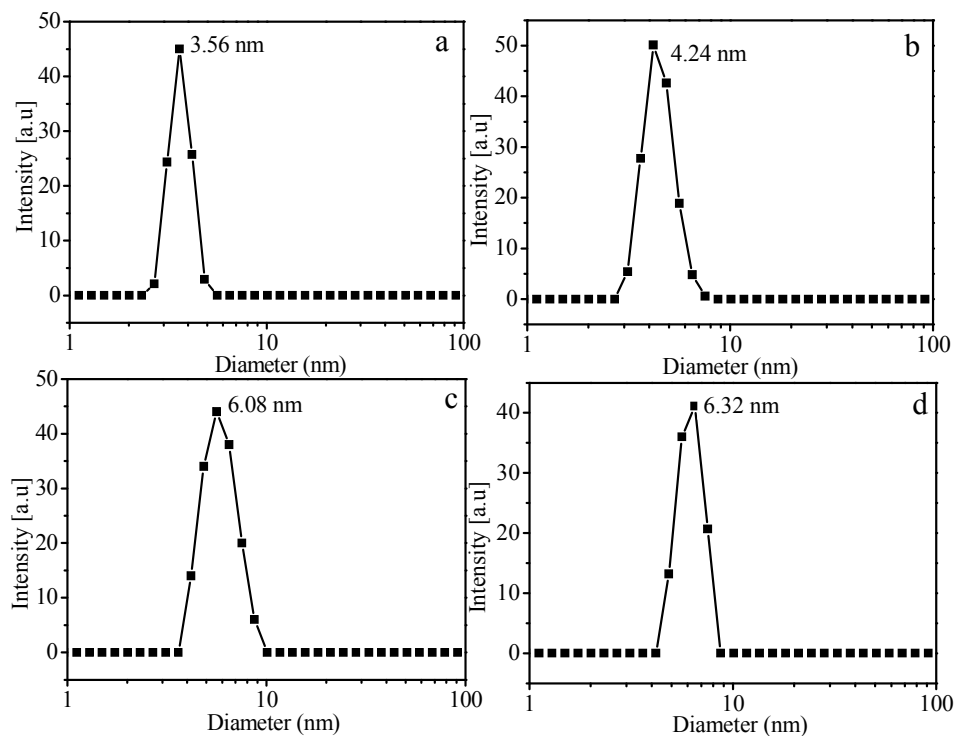
Fig. 2 DLS curves of the micelles prepared respectively from the hyperbranched star copolymers, H4 (a), H5 (b), H6 (c) and H00 (d).

The DLC of micelles is a very important factor for drug release. Since CPT has characteristic ultraviolet absorption at $v = 365$ cm^{-1}, the ultraviolet quantitative analysis was used to measure DLC of the resultant unimolecular micelles, and the standard curve in **Fig. S7** was used in estimation of DLC. The DLCs of H4, H5 and H6 unimolecular micelles are 16.7 wt%, 7.8 wt% and 1.3 wt%, respectively (**Table 2**), which is consistent with decrease of the MACPT content in the hyperbranched star copolymers from H4 to H6 (**Fig. S4**). The DLC was also estimated by ^{1}H NMR data, the results listed in **Table 2** reveal that the DLCs obtained by both methods are close to each other. Therefore, the unimolecular micelles with various DLCs have been successfully prepared, and their DLCs can be easily adjusted by varying the recipe. All these micellar samples in **Table 2** were used in the following study of drug release.

3.3 Release of CPT from unimolecular micelles

Since CPT is linked to the hyperbranched star copolymers via an acid-labile β-thiopropionate (**Scheme 3**), the unimolecular micelles are expected to have pH-sensitive property. In addition, the hyperbranched star copolymers may be redox-responsive also due to the disulfide linkage in the BS$_2$MOE branching units. The reducing agent, such as glutathione and DTT, can degrade the disulfide-containing polymers,[45,46] and this type of redox-sensitive polymers have been extensively used in drug delivery system.[40,47] The hyperbranched star copolymer (H4) was treated with an aqueous solution containing 10 mM DTT for 6 h, then the degraded products was analyzed by GPC. For comparison, GPC traces of both the H4 and its degraded products are shown in **Fig. 3**, and the molecular weight (M_n) of the products obtained after treated with DTT is significantly decreased from 20300 g/mol to 2100 g/mol due to cleavage of the disulfide linkage in the branching units. Therefore, the unimolecular micelles containing anticancer drug CPT are redox-sensitive, and the degraded products may be easily cleaned after drug delivery based on the previous results.[9]
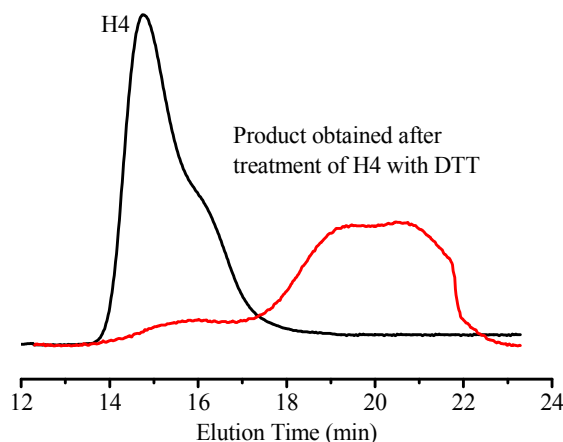
Fig. 3 GPC traces of HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE)(POEGMA)$_n$ (H4) with M_n = 20300 g/mol and M_w/M_n = 1.98 and the degraded products obtained after treatment of H4 in an aqueous solution containing 10 mM DTT for 6 h (M_n = 2100 g/mol, M_w/M_n = 8.15).

To understand drug release behaviors of the unimolecular micelles, we investigated the release of CPT from the micelles at different conditions, and the typical release profiles of the H4 micelles are shown in **Fig. 4**. When the release was conducted in an aqueous solution at pH = 7.4 with or without DTT, a few percents of the CPT in the drug carriers (9 wt% with 10 mM DTT and 8 wt% without DTT) are released, and obvious contribution of the reducing agent DTT to the drug release is not observed (**Fig. 4a** and **4b**) because the CPT is covalently bonded to the polymer chains via *β*-thiopropionate linkage, which is stable in the neutral solution. However, for control release of the drug in acidic solutions (pH=5 or 6) with and without 10 mM DTT, the drug release curves in **Fig. 4** exhibit similar behavior, at the beginning, the drug release rate is relatively fast, and after ca. 29 h, the drug is released at relatively slow rate. Different from the drug-conjugated micelles via other acid-labile linkages, such as acetal linkage that is extremely sensitive to the acid and can be rapidly cleaved to release the drug,[19,26] the CPT-conjugated unimoleclar micelles via *β*-thiopropionate linkage display controlled release of the drug in a sustained manner because this bond is hydrolyzed in acidic pH at a slow and sustained rate. As shown in **Fig. 4,** when the release is conducted in a solution at pH=6.0 without and with 10 mM DTT, the initial release rates are 3.27 and 3.69 μg/h, after ca. 29 h of drug release, the release rates decrease to 0.88 μg/h in the absence of DTT and 1.13 μg/h in the presence of DTT as shown in **Fig. 4c** and **4d**. When pH values of the solutions decrease to 5.0, the drug release rates significantly increase, and we also see a inflexion at approximately 29 h

of the drug release in the curves e and f of **Fig. 4**, the release rates before and after 29 h of the CPT release are respectively 6.39 and 1.06 μg/h under reduction-insensitive condition, and 7.67 and 1.43 μg/h under reduction-sensitive condition. Relatively high drug release rates in the initial 29 h are probably due to relatively easy attack of the H_3^+O on the surface β-thiopropionate linkages of the core, and slow hydrolysis of the β-thiopropionate linkages inside the core may lead to slow release of the CPT. After the control release without and with 10 mM DTT is conducted for 5 days, approximately 58% and 65% of the CPT in the micelles are respectively released, and the reason for slight increase of the released drug in the presence of DTT is mainly that degradation of the polymer chains benefits attack of the H_3^+O to β-thiopropionate linkage.
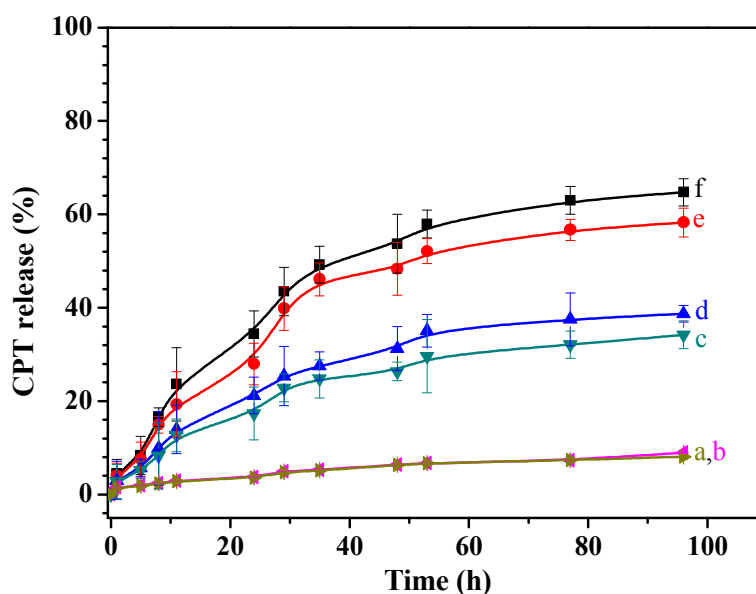
Fig. 4 CPT release profiles of the hyperbranched star copolymer H4 micelles in the aqueous buffer solution under different conditions: at pH = 7.4 without DTT (a); pH = 7.4 with 10 mM DTT (b); pH = 6.0 without DTT (c); pH = 6.0 with 10 mM DTT (d); pH = 5.0 without DTT (e); pH = 5.0 with 10 mM DTT (f)

Another problem we concern is whether structure of the released CPT is the same as the original drug because the structure change will diminish, even lose antitumor activity, for example, an intact hydroxyl lactone is essential for antitumor activity.[3] Besides ^{1}H NMR spectra of the released and the original CPT, the HPLC was used to characterize the released CPT, and the HPLC curves of the pure

CPT and the released molecules are showed in **Fig. 5**. Both the pure CPT and the released CPT from H4 micelles have the same retention time, demonstrating that the drug CPT is not changed during the release process, which is consistent with the result of ^{1}H NMR spectra. Therefore, the drug vector obtained can unload original drug strictly according to the reaction shown in **Scheme 1a** without superfluous reaction.
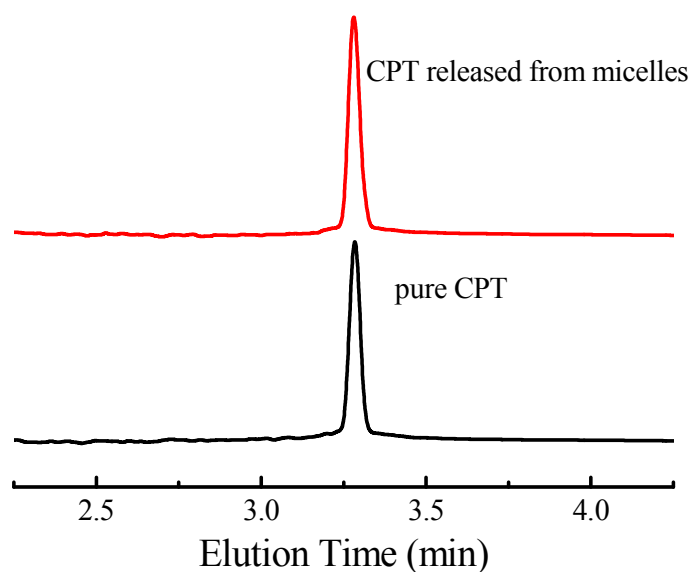

**Fig. 5** HPLC curves of the pure CPT and the CPT released from H4 micelles under pH = 5.0.

3.4 Cytotoxicity of unimolecular micelles

The cytotoxicity of H00 micelles was evaluated by MTT assay using HepG2 and Hela cells. The cells were incubated with various concentrations of micellar solutions at 37°C for 24 h, while the cells treated with PBS solution without the micelles were used as a control. The results in **Fig. 6** demonstrate that the micelles of HP(HPMA-*co*-BS$_2$MOE)(POEGMA)$_n$ without CPT display low toxicity up to 2 g/mL of the hyperbranched star copolymer. **Fig. 7** shows relationship of the free CPT and the CPT-loaded H4 micelles with cell viability, and the results reveal that in the solution at pH = 7.4, the free CPT displays similar dose-responsive curves to the HepG2 and HeLa cells, and the CPT concentrations for 50% cell viability (IC$_{50}$) of the HepG2 and the HeLa cells are ~1.36 mg/mL (HepG2) and ~1.24 mg/mL (HeLa), respectively. The CPT-loaded H4 unimolecular micelles have almost the same dose-responsive curves to the HepG2 and Hela cells also, and their IC$_{50}$ values are ~2.62 mg/mL (HepG2) and ~2.68 mg/mL (HeLa), which are very close. Thus, the CPT-loaded

micelles show 1.93- (HepG2) or 2.16-fold (HeLa) lower cytotoxicity than the free CPT drug, which is ascribed to controlled release of the CPT from the unimolecular micelles, and this explanation is confirmed by the cell imaging in the following section.
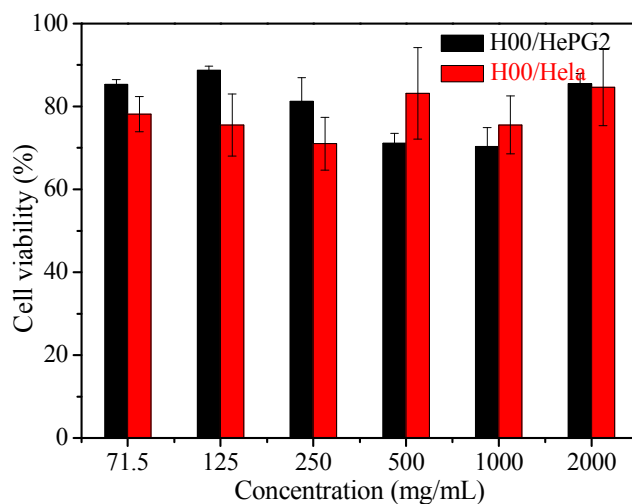

Fig. 6. Relative cell viability of the Hela and HePG2 cells evaluated by MTT assay after incubation with various concentrations of the HP(HPMA-*co*-BS$_2$MOE)(POEGMA)$_n$ (H00) micellar solution at pH=7.4;
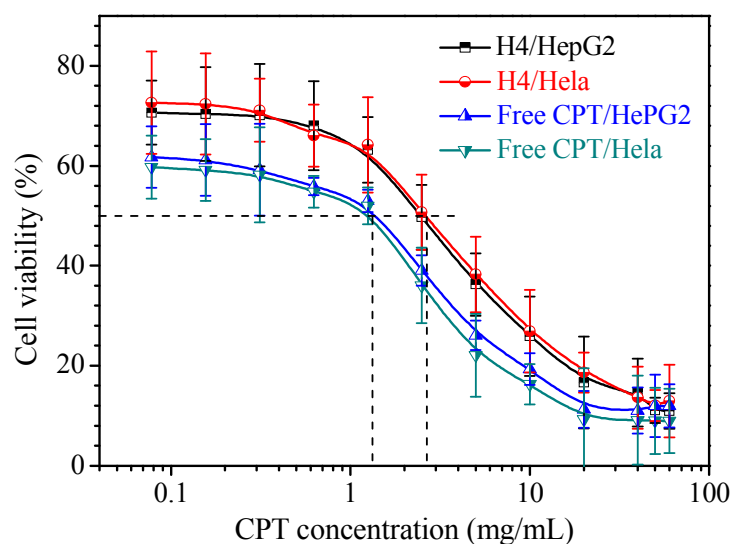
Fig. 7. Relative cell viability of HePG2 cells and Hela cells evaluated by the MTT assay after incubation with the micelles solution of CPT-loaded HP(HPMA-*co*-MACPT-*co*-BS$_2$MOE) (POEGMA)$_n$ (CPT-loaded H4) at pH=7.4; and free DOX at pH=7.4. Incubating temperature: 37 °C; time: 24h.

3.5 Cellular Uptake of unimolecular micelles and cell imaging

In order to investigate whether the unimolecular micelles can be successfully internalized by the HepG2 cell through endocytosis, we used confocal laser scanning microscope (CLSM) to study cellular uptake assay of the CPT solution or the CPT-loaded H4 micelles. After the HepG2 cells were respectively incubated with DMEM medium containing the CPT-loaded H4 micelles or containing free CPT solution for 2 h, and then stained with STYO 9 (green), the fluorescence images of the HepG2 cells were acquired under UV irradiation at 405 nm or 483 nm. Because the CPT emits blue fluorescence, the STYO 9 stain, which has a high affinity for DNA, was selected for staining the cell nuclei. The CLSM images in **Figure 8** reveal that the CPT blue fluorescence appears mainly at the perinuclear region of the HepG2 cells in both cases, but the cells including nuclei exhibit the STYO 9 green fluorescence. When we carefully analyze the CLSM images, the cell nuclei incubated with the CPT-loaded H4 micelles display relatively weaker CPT blue fluorescence as shown in Figure 8B, compare to the images of the HepG2 cells treated with free CPT in **Figure 8A**, probable reason is controlled release of the CPT from the unimolecular micelles. The results demonstrate that the CPT-loaded H4 micelles have been successfully internalized by the HepG2 cells and the CPT is released from the micelles at a controlled rate, which is consistent with the results of cytotoxicity.
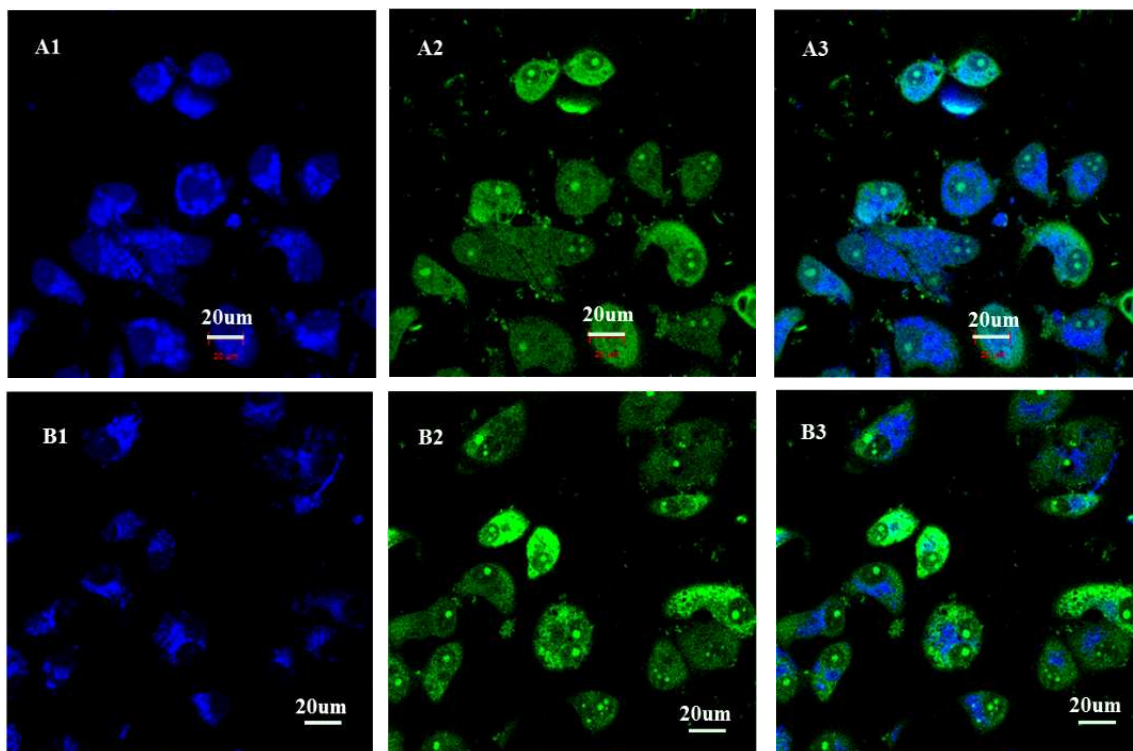
Figure 8. Confocal laser scanning microscope images of the HepG2 cells treated with the free CPT solution (A) and the H4 unimolecular micellar solution (B) at 37 °C for 2 h. The images from left to right show CPT (blue), SYTO 9 (green) and merge of the two images..

4. Conclusions

A new monomer, MACPT, in which the methacrylate group and the anticancer drug CPT are linked via acidic-labile β-thiopropionate linkage, has been successfully synthesized. Since the disulfide bond in the BS$_2$MOE monomer is redox-responsive, the hyperbranched star copolymers (HSPs), HP(HPMA–co–MACPT–co–BS$_2$MOE)(POEGMA)$_n$s possess pH– and redox–responsive properties. Owing to highly hydrophilic property of the POEGMA, the HP(HPMA–co–MACPT–co–BS$_2$MOE)(POEGMA)$_n$s are well dissolved in water to form unimolecular micelles with diameters of 3.56-6.08 nm upon their molecular weights. Controlled release of CPT from the micelles is conveniently triggered by mild acidic pH (6.0 or 5.0), and the released CPT remains its anticancer ability because no change of the CPT structure occurs during the release process. Due to hydrolysis of the β-thiopropionate linkage in acidic pH at a sustained rate, the control release of the CPT is released from the micelles in a sustained manner, and the control release of CPT is pH dependent, the release rate is much faster in an acidic buffer solution of pH = 5.0 than that in a neutral solution. The HSPs micelles is of low cytotoxicity, and the CPT-loaded micelles display low cytotoxicity (IC$_{50}$ = ~2.62 mg/mL for HepG2 and ~2.68 mg/mL for HeLa) than the free CPT (IC$_{50}$ = ~1.36 mg/mL for HepG2 and ~1.24 mg/mL for HeLa) owing to controlled release of the CPT. The CPT-loaded micelles can be successfully internalized by the HepG2 cells. Since extracellular pH at a solid tumor site is usually weak acidic, the micelles we prepared have potential application in the tumor–targeted drug release systems.

Electronic Supplementary Information (ESI) available: ^{1}H NMR spectra of methacryloyloxy 3-

hiahextyloyl-CPT and its intermediates, three hyperbranched star polymers; GPC curves of the hyperbranched polymers and the hyperbranched star polymers; as well as standard curve of CPT.

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Unimolecular micelles of Camptothecin-bonded hyperbranched star copolymers via *β*-thiopropionate linkage: synthesis and drug delivery

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The pH- and redox-sensitive camptothecin-loaded unimolecular micelles display low cytotoxicity, controlled drug release in a sustained manner.