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Synthesis of biodegradable multiblock copolymers by click coupling of RAFT-generated heterotelechelic polyHPMA conjugates

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ABSTRACT

A new strategy for the synthesis of biodegradable high molecular weight *N*-(2-hydroxypropyl)methacrylamide (HPMA)-based polymeric carriers has been designed. An enzyme-sensitive, alkyne-functionalized, chain transfer agent (CTA-GFLG-alkyne; N^{α}-(4-pentynoyl)-N^{δ}-(4-cyano-4-(phenylcarbonothioylthio)pentanoyl–glycylphenylalanylleucylglycyl)-lysine) was synthesized and used to mediate the reversible addition–fragmentation chain transfer (RAFT) polymerization and copolymerization of HPMA. Post-polymerization modification with 4,4′-azobis(azidopropyl 4-cyanopentanoate) resulted in the formation of heterotelechelic HPMA copolymers containing terminal alkyne and azide groups. Chain extension via click reaction resulted in high molecular weight multiblock copolymers. Upon exposure to papain, these copolymers degraded into the initial blocks. Similar results were obtained for copolymers of HPMA with *N*-methacryloylglycylphenylalanylleucylglycyl thiazolidine-2-thione and *N*-methacryloylglycylphenylalanylleucylglycyl-gemcitabine. The new synthetic method presented permits the synthesis of biocompatible, biodegradable high molecular weight HPMA copolymer–anticancer drug conjugates that possess long-circulation times and augmented accumulation in solid tumor tissue due to the enhanced permeability and retention effect.

1. Introduction

Poly[*N*-(2-hydroxypropyl)methacrylamide] (polyHPMA) is a nonimmunogenic, neutral, hydrophilic polymer currently employed in the delivery of anticancer drugs. Its physical properties and the synthetic flexibility have been proven to be very useful when combined with biological active agents [1,2]. HPMA copolymer–anticancer drug conjugates have been evaluated in numerous cancer models [3,4] and in clinical trials [5]. The necessity to limit the molecular weight distribution below the renal threshold (in order to secure elimination from the organism) resulted in short intravascular half-life and limited accumulation in the tumor. It is well known that accumulation of macromolecules in solid tumor is molecular weight-dependent [6]. Experimental evidence suggests that the higher the molecular weight of HPMA copolymer–drug conjugates, the higher the tumor accumulation with concomitant increase in therapeutic efficacy [7].

The emergence of living radical polymerization, especially reversible addition–fragmentation chain transfer (RAFT) polymerization, provided a powerful tool to prepare polyHPMAs with well-defined structures [8] as well as to introduce functional end-groups that can act as attachment points for various biomacromolecules [9].

Herein we describe the synthesis of biodegradable, biocompatible high molecular weight HPMA copolymer drug carriers that will be long circulating in the vasculature leading to enhanced tumor accumulation. To this end, we synthesized α-alkyne, ω-azido heterotelechelic polyHPMA using RAFT polymerization. A newly designed chain transfer agent (CTA-GFLG-alkyne) contains a degradable oligopeptide sequence, GFLG, and an alkyne group, which enabled a direct synthesis of alkyne-functionalized polyHPMA as well as HPMA copolymers with functional comonomers (*N*-methacryloylglycylphenylalanylleucylglycyl thiazolidine-2-thione) or polymerizable derivatives of anticancer drugs (*N*-methacryloylglycylphenylalanylleucylglycyl-gemcitabine). Post-polymerization modification of the other (non-alkynyl) chain end with an azido-group was achieved by the reaction of the polymer with azido-modified initiator V-501 (diazido-V-501; 4,4′-azobis(azidopropyl 4-cyanopentanoate)). Unlike the reported "clickable" RAFT agents [10], the new CTA contains an enzyme-sensitive oligopeptide (GFLG) sequence [11]. This feature makes the heterotelechelic polyHPMA a potentially biodegradable, long-circulation drug carrier. Addition of catalyst Cu(I) resulted in chain extension and formation of high molecular weight multiblock biodegradable HPMA copolymers. Exposure of the multiblock copolymer to papain, an enzyme with

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similar specificity as lysosomal cathepsin B, resulted in total biodegradation and formation of macromolecules with initial molecular weight.

2. Experimental

2.1. Materials

Side-chain protected Fmoc-amino acids, 2-(7-aza-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU), 4-dimethylaminopyridine (DMAP), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) were from AAPPTec (Louisville, KY). Diisopropylcarbodiimide (DIC), 4,4′-azobis(4-cyanovaleric acid) (V-501) and 2-mercaptothiazoline were from Fluka, 1-hydroxybenzotriazole (HOBt) was from AK Scientific (Mountain View, CA), and gemcitabine hydrochloride was from NetQem (Durham, NC). *N*,*N*-diisopropylethylamine (DIPEA; 99%) was from Alfa Aesar (Ward Hill, MA), diethyl ether and dichloromethane (DCM) were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Trifluoroacetic acid (TFA; 99%) was from Acros Organics (Morris Plains, NJ). 2,2′-azobis(isobutyronitrile) (AIBN) was from sigma–Aldrich (St. Louis, MO) and recrystallized in ethanol. HPMA [12], *N*-methacryloylglycylphenylalanylleucylglycyl thiazolidine-2-thione (MA-GFLG-TT) [13], and 4-cyanopentanoic acid dithiobenzoate [14] were synthesized as previously described. All other solvents were purchased from Sigma-Aldrich as the highest purity available and used as received.

2.2. Instrumentation

Mass spectra of all synthesized compounds were obtained using a mass spectrometer Voyager-DE (STR Biospectrometry Workstation, PerSeptiveBiosystems, Framingham, MA). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Mercury400 spectrometer in $CDCl_3$. Chemical shifts were reported in ppm (δ) relative to $CDCl_3$ (7.26 ppm for ^{1}H and 77.0 ppm for ^{13}C). Monomer conversion during polymerization was determined from the concentration of residual monomer using HPLC (Agilent Technologies 1100 series, Zorbax C8 column 4.6 × 150 mm) with gradient elution from 2% to 90% of Buffer B within 20 min and flowrate 1.0 mL/min (Buffer A: deionized water (DI H_2O) with 0.1% TFA, Buffer B: acetonitrile containing 0.1% TFA). Size-exclusion chromatography (SEC) was carried out on an ÄKTA FPLC system (Pharmacia) equipped with miniDAWN TREOS and OptilabEX detectors (Wyatt Technology, Santa Barbara, CA) with PBS (pH 7) as mobile phase. Superose 6/Superose 12 HR10/30 columns and Superose 6 HR16/60 column (Pharmacia) were used as needed. The molecular weight and molecular weight distribution were calculated using ASTRA software and calibration with polyHPMA fractions. UV–vis spectra were measured on a Varian Cary 400 Bio UV–visible spectrophotometer. FTIR spectra were obtained on Bio-Rad FTS 6000.

2.3. Synthesis of N^{α}-(4-pentynoyl)-N^{δ}-(4-cyano-4-(phenylcarbonothioylthio)pentanoyl-glycylphenylalanylleucylglycyl)-lysine (**1**, CTA-GFLG-alkyne)

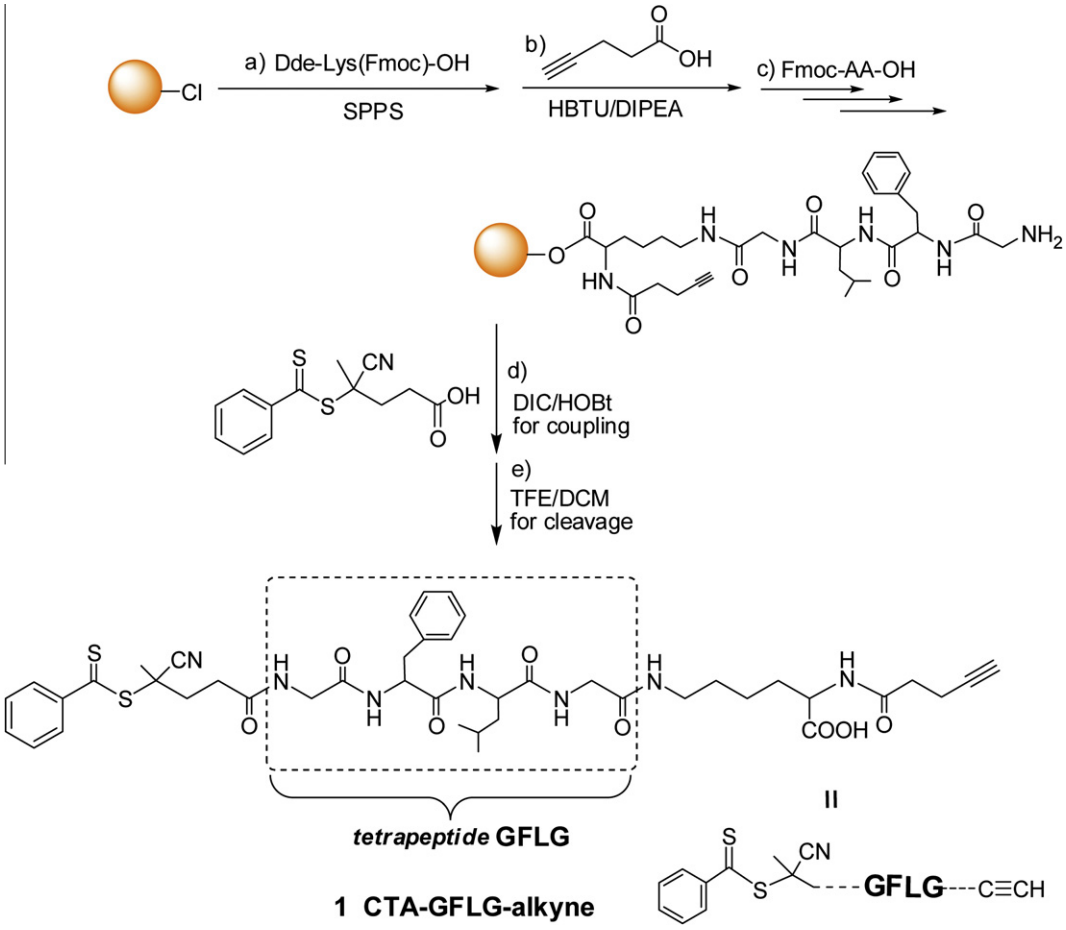
The CTA-GFLG-alkyne was synthesized in several steps (Scheme 1) using solid phase peptide synthesis (SPPS) methodology and manual Fmoc/tBu strategy. Dde-Lys(Fmoc)-OH was chosen as a linker in which two protecting groups (Fmoc- and Dde-) can be removed selectively: one deprotected amino group was used to introduce the alkyne into the peptide structure, the other functioned in constructing the peptide sequence. A detailed experimental procedure is shown below. Dde-Lys(Fmoc)-OH (351 mg, 0.64 mmol) and DIPEA (285 μL 1.61 mmol) were dissolved in DCM (10 mL) and added to 2-chlorotrityl chloride resin (1.0 g, 1.29 mmol/g). The vial was kept gently shaking for 1.5 h (*Step a*). The resin was transferred to a polypropylene tube, rinsed with mixture of DCM:MeOH:DIPEA (17:2:1) (20 mL × 4), followed by washing three times with DCM and DMF, respectively. After removal of Fmoc-group with 10 mL 20% piperidine in DMF (5 min × 3), 4-pentynoic acid (2.5×, 1.6 mmol) was reacted with α-NH_2 of Lys using HBTU (2.4×, 1.55 mmol, 586 mg) and DIPEA (5×, 3.2 mmol, 570 μL) as coupling agents to introduce the alkyne group (*Step b*). Ninhydrin test (also known as Kaiser test) was used to confirm the completion of each coupling step. Dde group was removed with 10 mL 3% hydrazine in DMF (5 min × 3), and elongation of tetrapeptide GFLG was accomplished by sequentially coupling Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, and Fmoc-Gly-OH using the HBTU/DIPEA procedure, (*Step c*). The *N*-terminus of the peptide was capped with 4-cyanopentanoic acid dithiobenzoate in the presence of DIC/HOBt (*Step d*). The product was cleaved from the resin with TFE/DCM (3:7 v/v) at room temperature for 2 h (*Step e*). The resin was removed by filtration. The filtrate was condensed under reduced pressure, purified by precipitation of the solution in methanol into ether. Pink powder was obtained. Yield: 339 mg (61%). The structure was verified by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). $[M+H]^+$ 862.38. ^{1}H NMR (400 MHz, Methanol-d4, δ, ppm): 7.84–7.32 (m, 5 H, Ph-***H***), 7.20–7.11 (m, 5 H, Ph-***H***), 4.49 (m, 1 H, α-Phe-***H***), 4.30 (m, 1 H, α-Leu-***H***), 4.15 (m, 1 H, α-Lys-***H***), 3.85–3.70 (m, 4 H, NH–C***H***$_2$–CO), 3.20–3.03 (m, 3 H, $CHCH_2CH_2CH_2C\boldsymbol{H}_2NH$, Ph-C***H***H), 2.85 (m, 1 H, Ph-CH***H***), 2.50–2.24 (m, 8 H, $COC\boldsymbol{H}_2CH_2C$, $COCH_2C\boldsymbol{H}_2C$ and $HCCC\boldsymbol{H}_2C\boldsymbol{H}_2CO$), 2.15 (s, $\boldsymbol{H}CCCH_2$), 1.84 (s, 3 H, (CN)C–C***H***$_3$), 1.80–1.27 (m, 9 H, $CHC\boldsymbol{H}_2C\boldsymbol{H}_2C\boldsymbol{H}_2CH_2NH$, $CHC\boldsymbol{H}_2C\boldsymbol{H}(CH_3)_2$), 0.83–0.77 (m, 6 H, Leu-($C\boldsymbol{H}_3$)$_2$); ^{13}C NMR (100 MHz, Methanol-d4, δ, ppm):225.04, 175.30, 174.88, 174.20, 173.89, 173.81, 172.13, 171.38, 146.10, 138.16, 134.27, 130.32, 129.78, 127.91, 119.84, 83.58, 70.40, 56.42, 53.78, 53.51, 47.39, 47.37, 43.93, 43.89, 43.51, 40.92, 40.15, 38.21, 36.06, 34.76, 32.23, 31.83, 29.82, 25.66, 24.34, 24.31, 24.15, 23.64, 21.83, 15.79. The purity was determined by RP-HPLC (>95%).

2.4. Synthesis of 4,4′-azobis(azidopropyl 4-cyanopentanoate) (**2**, diazido-V-501)

4,4′-Azobis(4-cyanovaleric acid) (V-501, 1 g, 3.6 mmol), 3-azidopropanol (1.08 g, 10.7 mmol) and DMAP (0.35 g, 2.9 mmol) were dissolved in DCM/THF (1:1 v/v, 25 mL) and cooled to 4 °C. DCC (1.62 g, 7.9 mmol) in 10 mL of DCM was added dropwise. The reaction mixture was stirred at 4 °C overnight, then at room temperature for 1 h. After completion of the reaction, two drops of acetic acid were added to the reaction mixture, and stirring continued for 30 min. Dicyclohexylurea (DCU) was removed by filtration and the solvent was removed by rotary evaporation. The residue was purified by silica gel chromatography (silica gel 60 Å, 200–400 mesh, ethyl acetate/hexane 1/1); yield 1.05 g (66%). The structure was confirmed by ^{1}H NMR and ^{13}C NMR spectroscopy. ^{1}H NMR ($CDCl_3$, δ, ppm): 4.17 (m, 4H, –COOC***H***$_2$); 3.37 (m, 4H, $N_3C\boldsymbol{H}_2$); 2.60–2.30 (m, 8H, CO–C***H***$_2$C***H***$_2$–C); 1.89 (m, 4H, C–C***H***$_2$–C); 1.68 (d, 6H, C***H***$_3$). ^{13}C NMR ($CDCl_3$, δ, ppm): 171.16; 117.42; 71.76; 62.01; 48.07; 33.05; 29.01; 27.97; 23.86.

2.5. Synthesis of N-methacryloylglycylphenylalanylleucylglycyl-gemcitabine (**3**, MA-GFLG-gemcitabine)

MA-GFLG-TT (90 mg, 0.16 mmol) and gemcitabine hydrochloride (44.95 mg, 0.15 mmol) were dissolved in pyridine (5 mL) under nitrogen atmosphere. The solution was stirred at 50 °C for 12 h and then the solvent was removed by rotary evaporation. The crude



Scheme 1. Synthesis of N^{α}-(4-pentynoyl)-N^{δ}-(4-cyano-4-(phenylcarbonothioylthio)pentanoyl-glycylphenylalanylleucylglycyl)-lysine (CTA-GFLG-alkyne).

product was purified by column chromatography (silica gel 60 Å, 200–400 mesh, DCM: CH_3OH = 6:1) to give white solid in 66.1% yield. The structure was confirmed by ^1H NMR spectroscopy and MALDI-TOF MS. MALDI-TOF MS: m/z 706.22 $(M + H)^+$, 728.21 $(M + Na)^+$, 744.19 $(M + K)^+$. ^1H NMR: 8.20 (d, 1 H, *J* = 7.6 Hz, *H*-6), 7.84 (s, 2 H, N*H*), 7.31 (d, 1 H, *J* = 7.6 Hz, *H*-5), 7.06 (m, 5 H, Ph-*H*), 6.97 (s, 1 H, N*H*), 6.12 (t, *J* = 7.6 Hz, *H*-1′), 5.64 (s, 1 H, CH_3–CH(R)–CH_2–H^a), 5.24 (s, 1 H, CH_3–CH(R)–CH_2–H^b), 4.52 (m, 1 H, α-*H*), 4.23 (m, 1 H, *H*-3′), 4.15 (m, 1 H, α-*H*), 3.97 (m, 1 H, *H*-4′), 3.83 (m, 4 H, NH–CH_2–CO), 3.66–3.80 (m, 2 H, *H*-5′, *H*-5″), 3.06 (m, 2 H, Ph-CH_2), 2.04 (s, 1 H, O*H*), 1.85 (s, 1 H, O*H*), 1.78 (s, 3 H, CH_3), 1.52 (m, 3 H, $CHCH_2CH(CH_3)_2$), 0.79 (m, 6 H, $CHCH_2CH(CH_3)_2$).

2.6. RAFT polymerization of HPMA mediated by CTA-GFLG-alkyne

HPMA homopolymerizations were performed in either DI H_2O at 70 °C using V-501 as the initiator or methanol at 60 °C using AIBN as the initiator. A typical polymerization procedure in methanol was as follows: An ampoule containing HPMA (0.5 g, 3.5 mmol) and CTA-GFLG-alkyne (8.6 mg, 0.01 mmol) was attached to the Schlenk-line. After three vacuum–nitrogen cycles to remove oxygen in the ampoule, 2.5 mL degassed methanol was added, followed by addition of AIBN solution in methanol (0.55 mg in 0.1 mL) via syringe. The ampoule was sealed and polymerization was performed at 60 °C for 20 h. The polymer was obtained by precipitation into acetone and purified by re-dissolving in methanol and precipitation in acetone two more times. PolyHPMA with pink color was dried under vacuum. Yield 70%.

Similarly, the polymerization in DI H_2O was conducted at 70 °C using V-501 as the initiator.

For kinetic studies, stock solutions were prepared as described above but in a vial equipped with a septum. Aliquots were transferred via syringe to six different ampoules; each one was attached to the Schlenk-line and had been purged with nitrogen. The ampoules were sealed under N_2 atmosphere followed by placement in a 60 °C oil bath. At different time points, the samples were taken out and quenched in liquid nitrogen. The ampoule was opened just prior to measurement. Twenty microliter polymer solution was diluted into 1 mL and analyzed by HPLC. The monomer conversion was calculated by comparison of the remaining monomer concentration with initial concentration. The molecular weight of the sample was measured using SEC after precipitation of the polymer solution into cold anhydrous ether.

2.7. Synthesis of HPMA copolymer containing thiazolidine-2-thione (TT) reactive groups (P-TT)

HPMA copolymer containing side-chain reactive group was produced by copolymerization of HPMA with MA-GFLG-TT. An ampoule containing HPMA (164.5 mg, 1.15 mmol), MA-GFLG-TT (34 mg, 0.06 mmol), CTA-GFLG-alkyne (3 mg, 3.46 μmol) was attached to the Schlenk-line. After three vacuum–nitrogen cycles to remove oxygen, 1 mL degassed methanol was added, followed by addition of AIBN solution in methanol (0.2 mg in 0.1 mL, 1.12 μmol) via syringe. The ampoule was sealed and copolymerization was performed at 60 °C for 24 h. The polymer was obtained by precipitation into acetone and purified by re-dissolving in methanol and

precipitation in acetone two more times. The copolymer (P-TT) was dried under vacuum. Yield: 108 mg (54%). The content of TT in the copolymer was determined by UV spectrophotometry in methanol (ε_{305} = 10,800 L mol^{-1} cm^{-1}).

2.8. Synthesis of HPMA copolymer containing anticancer drug gemcitabine (P-gemcitabine)

HPMA copolymer containing anticancer drug gemcitabine (P-gemcitabine) was produced by copolymerization of HPMA with a polymerizable derivative of the drug. An ampoule containing HPMA (160 mg, 1.12 mmol), MA-GFLG-gemcitabine (40 mg, 0.057 mmol), and CTA-GFLG-alkyne (3 mg, 3.46 μmol) was attached to the Schlenk-line. After three vacuum–nitrogen cycles to remove oxygen, 1 mL degassed MeOH were added, followed by addition of initiator AIBN solution in methanol (0.2 mg in 0.1 mL, 1.12 μmol) via syringe. The ampoule was sealed, and copolymerization was performed at 60 °C for 24 h. The polymer was obtained by precipitation into acetone and purified by re-dissolving in methanol and precipitation in acetone two more times. The copolymer (P-gemcitabine) was isolated as a pink powder and dried under vacuum. Yield: 136 mg (68%). The content of gemcitabine was estimated by UV in methanol (ε_{300} = 5710 L mol^{-1} cm^{-1}).

2.9. Post-polymerization chain end modification

The dithiobenzoate group was replaced with an azido group by the reaction of the RAFT-generated polymer with diazido-V-501 (Scheme 2). Briefly, polyHPMA (125 mg, Mn = 36 kDa, 3.5 μmol) was added into an ampoule. Diazido-V-501 (20 ×, 32 mg, 70 μmol) solution in methanol was then added via syringe. The solution was bubbled with N_2 for 30 min, sealed and kept stirring at 70 °C for 3 h. The polymer, heterotelechelic N_3–polyHPMA–C≡CH, was purified by precipitation into ether and dried under vacuum (yield 104 mg).

2.10. Synthesis of biodegradable multiblock polyHPMA by click reaction

Heterotelechelic N_3–polyHPMA–C≡CH (40 mg, 1.1 μmol) and sodium ascorbate (4.4 mg, 22 μmol) were added to a vial and then attached to Schlenk-line. The vial was evacuated and refilled with nitrogen three times before adding 200 μL of degassed DI H_2O. The solution was bubbled with N_2 for 30 min. The click reaction was initiated by adding 50 μL $CuSO_4$ (1.8 mg, 11 μmol) aqueous solution via syringe. The reaction mixture was kept at RT for 12 h. The polymer was precipitated into ether and dried under vacuum. Yield: 32 mg (80%). The removal of copper salts was accomplished when the product was fractionated by size-exclusion chromatography on a Superose 6 preparative column using PBS as the eluent. The fractions were dialyzed against H_2O to remove salts using a 6–8 kDa cutoff membrane and freeze-dried.

2.11. Synthesis of multiblock HPMA copolymers containing anticancer drug gemcitabine

Multiblock HPMA copolymer–gemcitabine conjugates were synthesized following a similar procedure as described in section 2.10. Briefly, N_3–P-gemcitabine–C≡CH (40 mg, Mn 28.5 kDa, 1.4 μmol) and sodium ascorbate (5.5 mg, 28 μmol) were dissolved in 200 μL of degassed DI H_2O. The solution was bubbled with N_2 for 30 min; then, the click reaction was initiated by adding 50 μL $CuSO_4$ (2.23 mg, 14 μmol) aqueous solution via syringe. The reaction mixture was kept stirring at RT for 12 h. The polymer was precipitated into ether and dried under vacuum. Yield: 29 mg (72%). The removal of copper salts was accomplished when the product was fractionated by size exclusion chromatography on a Superose 6 preparative column. The fractions were dialyzed against H_2O to remove salts using a 6–8 kDa cutoff membrane and freeze-dried.

2.12. Cleavage of multiblock polyHPMA and multiblock HPMA copolymer containing anticancer drug gemcitabine

The degradation of multiblock polyHPMA and multiblock HPMA copolymer–gemcitabine conjugate was performed in McIlvaine's buffer (50 mM citrate/0.1 M phosphate) at pH 6.0,37 °C using papain as model enzyme. Papain, at concentration of 0.29 mg/mL (determined by UV at 280 nm, with absorbance of 25 at 1%), was reduced with 5 mM glutathione for 5 min before addition to polymer (3 mg polymer/mL enzyme solution). The medium was analyzed with size-exclusion chromatography using a Superose 6 HR/10/30 column with PBS (pH 7) as the mobile phase. In addition, for HPMA copolymer–gemcitabine conjugate, the medium was also analyzed with HPLC to detect the cleaved gemcitabine.

3. Results and discussion

3.1. Design and synthesis of functional biodegradable RAFT agent, CTA-GFLG-alkyne

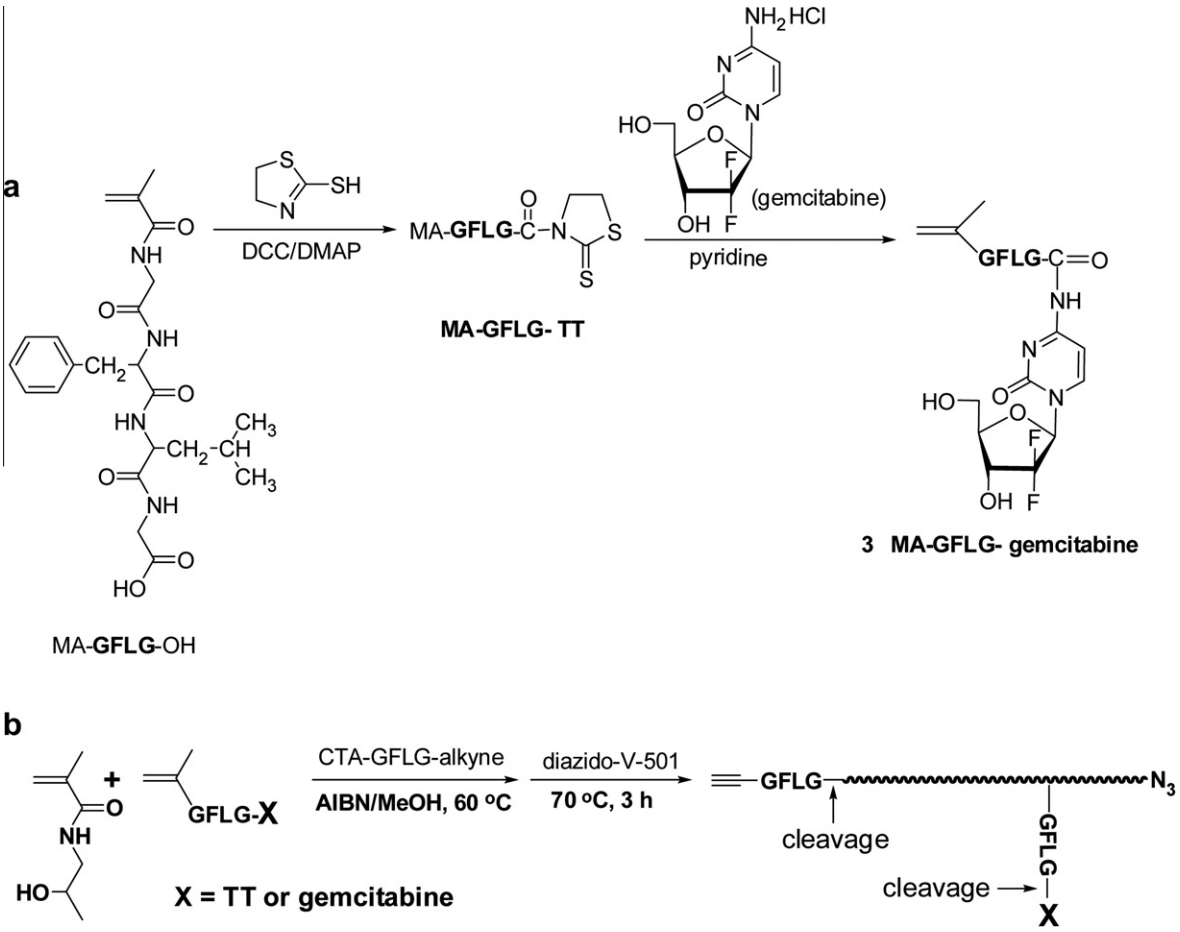
In RAFT polymerizations, chain transfer agents play a crucial part, so they must be chosen carefully [15]. 4-Cyanopentanoic acid dithiobenzoate was the first chain transfer agent used for HPMA RAFT polymerization [8]. Due to the high activation of the thiocarbonyl function by the phenyl substituent, it has been one of the most commonly used RAFT agents [16]. Numerous publications indicated that modifications of carboxy group in this chain transfer agent could be made without loss of fragmentation ability. Reactive or potentially reactive groups such as azido-, trimethylsilane protected alkyne-, TT-, α-pentafluorophenyl ester have been introduced into the chain transfer agent structure; this provided a pathway for direct synthesis of α-functionalized polymers [9,10,17–20].

In this study, a new functional dithiobenzoate derivative was designed and synthesized as shown in Scheme 1. An alkyne group was incorporated at the R-group (S=C(**Z**)-S**R**) without protection. The important feature of the new chain transfer agent is the insertion of an enzyme-degradable peptide linker (GFLG), which makes it a versatile tool for the synthesis of polymers with biodegradable backbones. The successful synthesis of the new CTA-GFLG-alkyne was validated by MALDI-ToF-MS, ^{1}H NMR, and ^{13}C NMR. The molar absorption coefficient in methanol was measured as ε_{302} = 15340 M^{-1} cm^{-1}.

3.2. RAFT polymerization of HPMA mediated by CTA-GFLG-alkyne

The results of the RAFT polymerization of HPMA mediated by CTA-GFLG-alkyne are shown in Table 1 and Fig. 1. The agreement between theoretical and experimental molecular weights over the course of polymerization and the narrow molecular weight distribution (Table 1) indicated that a well-defined polymer structure was achieved. Fig. 1A shows the kinetic plots of HPMA polymerization in water and methanol. In both solvents pseudo-first-order kinetics was observed. Fig. 1B presents the evolution of the molecular weight distribution with time for HPMA homopolymerization in methanol at 60 °C. The peaks are unimodal; their shift toward higher molecular weights with time further indicates the controlled nature of the polymerization process. Fig. 1C shows that the polymerization proceeded in a controlled manner in the whole range of evaluated conversions, i.e. up to 60%.

The living feature of the system was further confirmed by evolution of molecular weight when isolated polyHPMA from one



Scheme 2. Schemes of: (a) synthesis of comonomers MA-GFLG-TT and MA-GFLG-gemcitabine; and (b) RAFT copolymerizations of HPMA with MA-GFLG-TT/MA-GFLG-gemcitabine.

Table 1
Summary of HPMA polymerization mediated by CTA-GFLG-alkyne.

Entry	$[M]_0/[CTA]_0/[I]_0$	Initiator	Solvent	Temp °C	Time h	% conv[a]	M_{th}[b]	Mn_{SEC}	Mw/Mn
1	350:1:0.2	V-501	H_2O	70	1.3	20.9	11,300	12,800	1.07
2	350:1:0.2	V-501	H_2O	70	2.8	43.5	22,700	24,200	1.04
3	350:1:0.2	V-501	H_2O	70	4.5	55.1	28,400	30,500	1.02
4	350:1:0.2	V-501	H_2O	70	7	70.2	35,100	36,000	1.06
5	350:1:0.33	AIBN	CH_3OH	60	8	23.5	11,800	12,300	1.04
6	350:1:0.33	AIBN	CH_3OH	60	17	42.2	22,000	22,300	1.06

[a] The monomer conversion was measured by HPLC and calculated from HPMA calibration curve.
[b] Theoretical value (Mth) calculated from the equation: Mth = {([M]0/[CTA]0)*conversion* wHPMA + MwCTA}.

experiment was used as macro-CTA to mediate a new polymerization of HPMA (Fig. 1D). The obtained "diblock" polyHPMA showed the expected value of molecular weight and narrow molecular weight distribution, indicating that the dithiobenzoate end group remained functional in polyHPMA as previously shown with other chain transfer agents [8].

3.3. RAFT copolymerization of HPMA

Next, we evaluated HPMA copolymerization using a functional monomer (MA-GFLG-TT) that provided groups suitable for polymeranalogous attachment of anticancer drugs or a polymerizable derivative of drug (**3**, MA-GFLG-gemcitabine) that incorporated the drug directly by copolymerization (Scheme 2).

The results of copolymerization of HPMA with MA-GFLG-TT in methanol at 60 °C mediated by CTA-GFLG-alkyne are shown in Fig. 2. The linear increase of molecular weight with conversion and the low polydispersity at all time points indicate that the copolymerization was controlled similarly to the HPMA homopolymerization.

The copolymerization of HPMA with MA-GFLG-gemcitabine also proceeded in a controlled manner (results not shown). Gemcitabine hydrochloride shows absorbance maxima at 232 and 263 nm. These peaks shift to 248 nm and 300 nm, respectively, after attachment to MA-GFLG-OH. No further changes in absorbance maxima have been detected after polymerization. Therefore, the content of gemcitabine in the HPMA copolymers was estimated by UV as previously reported [21] (Table 2).

3.4. Synthesis of α-alkyne, ω-azido heterotelechelic HPMA (co)polymers

The polymers produced by RAFT polymerization mediated by CTA-GFLG-alkyne contain an alkyne group at one chain end and the dithiobenzoate group at the other end. To produce α-alkyne,

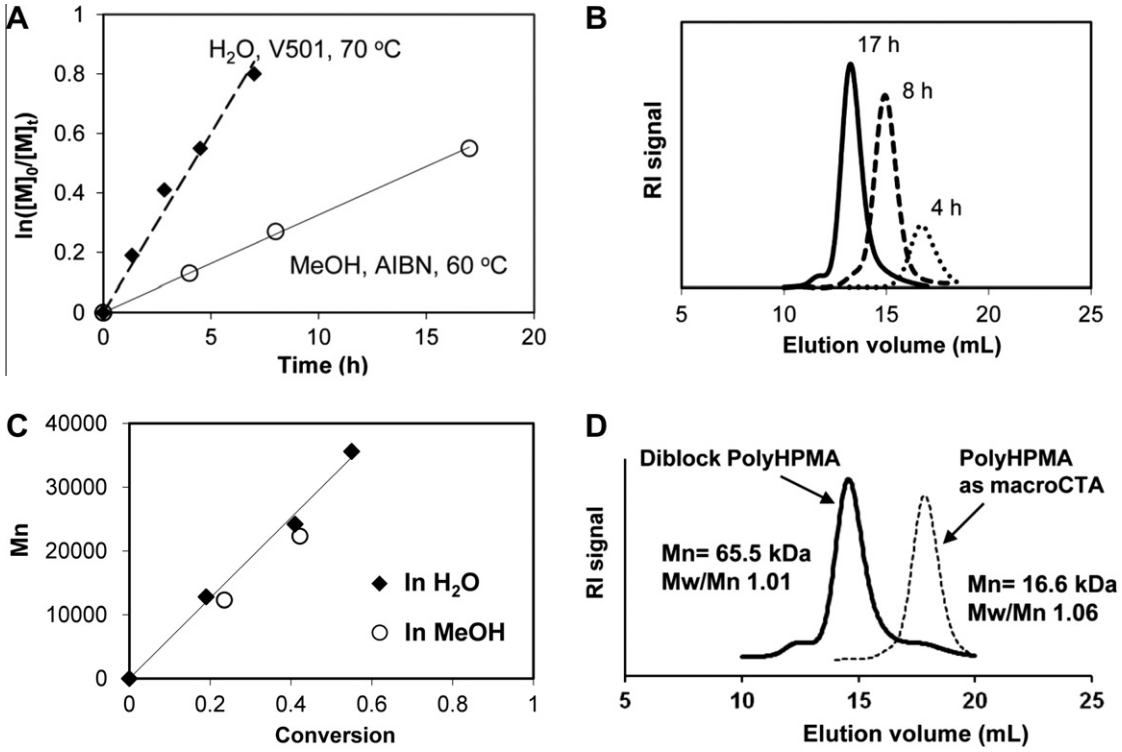


Fig. 1. RAFT polymerization of HPMA mediated by CTA-GFLG-alkyne: (A) pseudo first-order rate plots for polymerizations in methanol at 60 °C and in water at 70 °C, (B) evolution of molecular weight (RI detection; Superose 12 column) with time for HPMA polymerization in methanol at 60 °C, (C) plot of Mn versus conversion for HPMA polymerization, (D) SEC chromatogram (RI detection; Superose 6 column) of RAFT polymerization of HPMA in water at 70 °C using polyHPMA as macro-CTA, $[M]_0/[\text{macro-CTA}] = 500$, $M_{th} = 66{,}700$ ($\rho = 70\%$) and showing the evolution of molecular weight.

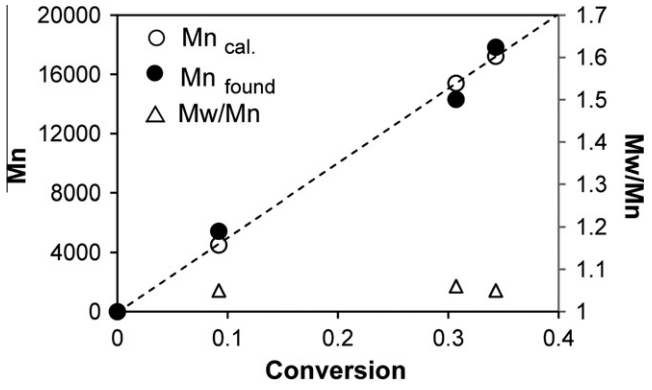


Fig. 2. RAFT copolymerization of HPMA with 5 mol% MA-GFLG-TT in methanol at 60 °C ($([M]_0/[\text{CTA-GFLG-alkyne}]_0 = 350, [\text{CTA-GFLG-alkyne}]_0/[\text{AIBN}]_0 = 3$). Evolution of molecular weight and polydispersity (Mw/Mn) with conversion.

Table 2
Characterization of HPMA copolymers[a].

Entry	Polymer	TT- or gem-content		% Yield[b]	Mn_{SEC}	Mw/Mn
		mo1%	wt.%			
1	P-TT	2.35	N/A	53.8	33,900	1.03
2	P-gemcitabine	2.24	4.12	68.0	28,500	1.02

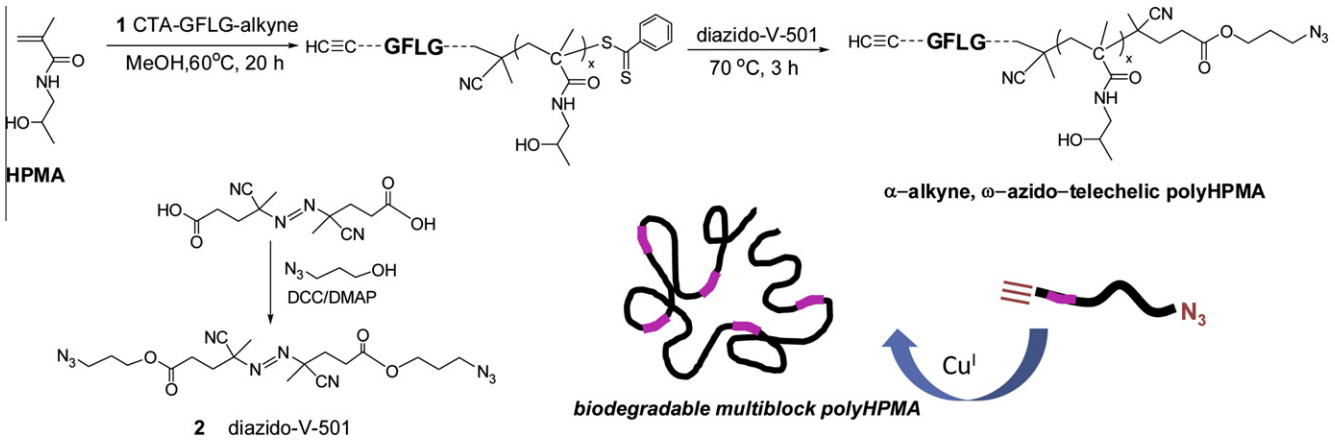
[a] Polymerizations were carried out in CH_3OH at 60 °C for 24 h, using AIBN as initiator, $[M]_0 = 1$ M, $[M]_0/[CTA]_0/[AIBN] = 350:1:0.33$.
[b] Yield was determined gravimetrically.

ω-azido heterotelechelic polyHPMA, a radical reaction of the initial polymer with an excess of diazido-V-501 was performed (Scheme 3). The radical-induced removal of thiocarbonylthio end group from RAFT-generated polymers has been studied previously [20,22,23]. For example, Goldmann et al. have shown the replacement of end dithiobenzoate groups on polystyrene with alkyne using propargyl alcohol modified V-501 (an azo-compound) resulted in total conversion. A heterotelechelic α-azido, ω-alkyne polystyrene was obtained. At low polymer concentrations, polystyrene macrocycles were produced via click reaction [20].

In this study, diazido-V-501 was synthesized and 20-fold molar excess was used for end-modification. The complete removal of dithiobenzoate group was demonstrated by UV/Vis spectroscopy (Fig. 3A; no detectable dithiobenzoate group absorbancy at 300 nm) and by ^{1}H NMR (absence of detectable dithiobenzoate group in $\delta = 7.3$–7.9 ppm, 5H; not shown). The presence of azido group was confirmed by the appearance of a new peak at 2100 cm^{-1} (azide) [20] in FTIR spectra (Fig. 3B). SEC analysis showed that there was no significant change in the molecular weight and molecular weight distribution during end-group modification (Fig. 3C). Nonetheless, the appearance of a small higher molecular weight shoulder (indicated by arrow) suggests that recombination of radicals might be a minor side reaction.

3.5. Synthesis of biodegradable multiblock polymer by Cu (I) catalyzed click reaction of α-alkyne, ω-azido heterotelechelic polyHPMA

To validate our hypothesis that the new design of polymeric drug carriers will result in degradable mulitblock copolymers, the copper^{I}-catalyzed Huisgen 1,3-dipolar cycloaddition of α-alkyne, ω-azido heterotelechelic polyHPMA into high molecular weight multiblock polymers (Scheme 3) was studied, and their degradation into initial polymer that possess a molecular weight below the renal threshold was also investigated. The advantage of this system is the presence of two orthogonal end groups on each macromolecule, which facilitates the stoichiometric azide–alkyne click reaction. The effects of concentration, the type and ratio of catalyst,



Scheme 3. General approach to the synthesis of biodegradable multiblockpolyHPMA via RAFT polymerization and click chemistry.

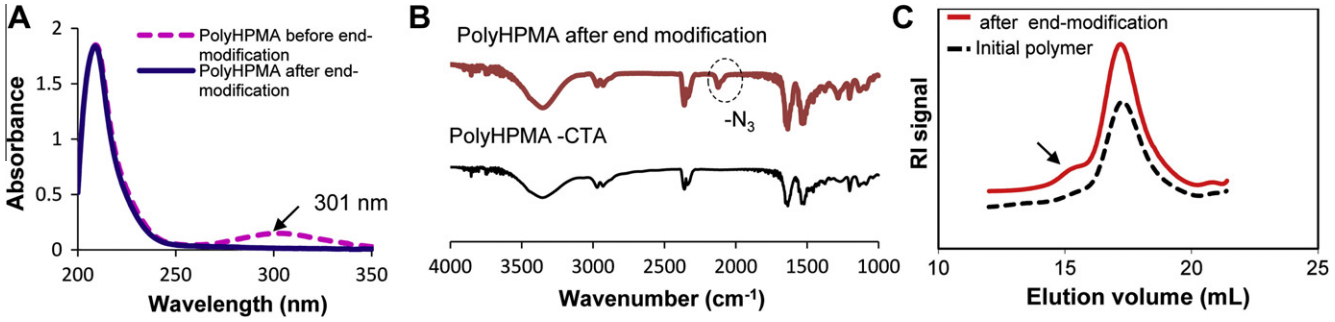


Fig. 3. (A) UV spectra of polyHPMA containing an alkyne end group and a dithiobenzoate end-group (polyHPMA-before end-modification) and of heterotelechelicpolyHPMA containing an azido and an alkyne end-group (polyHPMA after end-modification) – note the difference at absorbency at 300 nm; (B) FTIR spectra demonstrating the appearance of the azido peak at 2100 cm^{-1} in the end-modified polyHPMA; (C) SEC chromatograms of copolymer of HPMA with MA-GFLG-TT: comparison of profiles before and after end-group modification.

and different solvents have been examined. To avoid formation of cyclic polymers, high concentrations of functional groups and of the initial polymer were used; the polymer was dissolved in the minimal amount of solvent needed for dissolution. The click reaction was first conducted in DMF and methanol using CuBr/L-ascorbic acid as catalyst system as previously reported [24]. However, polyHPMA is a water-soluble polymer and frequently it is conjugated to bioactive molecules. Consequently, we chose DI H_2O as solvent and $CuSO_4$/sodium ascorbate as catalyst. There was no significant difference between the solvent/catalyst systems used; as expected, a high polymer concentration favored the click reaction (data not shown). Typical results are shown in Fig. 4. An initial polyHPMA (Mw 41.8 kDa; PDI 1.05) was exposed to in situ formed copper catalyst ($CuSO_4$/sodium ascorbate) resulting of chain extension into multiblock polymer. The multiblock polymer was fractionated on a Superose 6 preparative column. The selected fraction of multiblock polymer (Mw 291 kDa; PDI 1.11) was exposed to a solution of papain, a thiol proteinase with specificity similar to lysosomal cathepsin B. The degradation product was a polymer with a molecular weight distribution close to the original one (Mw 41.7 kDa, PDI 1.04). Thus the synthetic design which includes the insertion of an enzymatically cleavable sequence into the chain transfer agent, end-group modification and chain extension appears to be a new and efficient pathway for the synthesis of new backbone degradable polymeric drug carriers.

3.6. Chain extension and degradation of HPMA copolymers

Our ultimate goal is to synthesize relatively high molecular weight, degradable polyHPMA–drug conjugates. To demonstrate that the same click chemistry that was used for polyHPMA can also be used to prepare multiblock HPMA copolymer–drug conjugates, we chose to evaluate gemcitabine, an FDA approved anticancer drug.

It is important that the chain extension and degradation of the drug (gemcitabine)-containing HPMA copolymer proceeded similarly to the HPMA homopolymer (Fig. 4). An aqueous solution of P-gemcitabine (Mw 28.5 kDa; PDI 1.02) was exposed to copper catalyst ($CuSO_4$/sodium ascorbate) resulting in chain extension into multiblock copolymer. As shown in Fig. 5A and B, about 20% of the heterotelechelic polymer formed a dimer and 40% formed higher oligomers. The multiblock copolymer was fractionated on a Superose 6 preparative column. The selected fraction of multiblock P-gemcitabine (Mw 224 kDa; PDI 1.09) was exposed to a solution of papain. The degraded product showed very narrow polydispersity and the molecular weight close to the original heterotelechelic copolymer (Mw 33 kDa, PDI 1.04) (Fig. 5C). Moreover, compared with the free gemcitabine, the identical elution time of the cleaved gemcitabine from both the monomer and the multiblock polymer–drug conjugate upon exposure to papain indicated that conjugation and click reaction did not distort the structure of gemcitabine (Fig. 5D). Detailed evaluation of drug release from such multiblock conjugate and its biological effect is currently on progress.

3.7. General comments

The combination of living radical polymerization and click reaction for the synthesis of diblock and multiblock copolymers has been studied extensively. Several approaches have been evaluated: the coupling of telechelic copolymers with low molecular weight compounds, such as the reaction of diazido terminated polystyrene

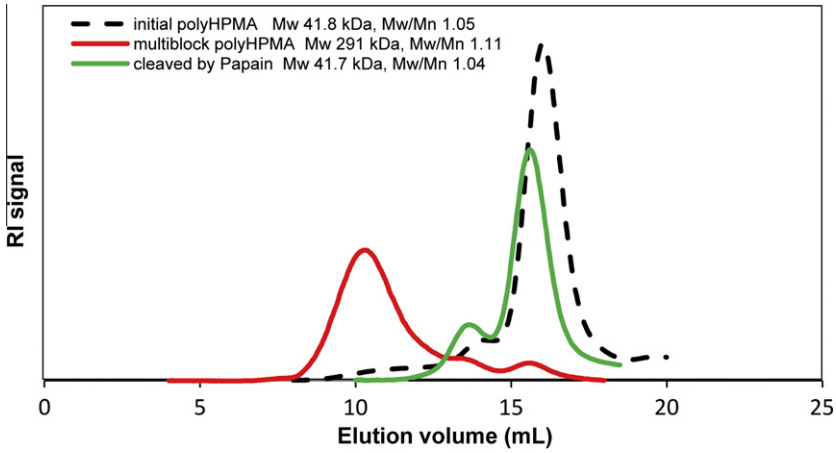


Fig. 4. Chain extension of heterotelechelic polyHPMA (Mw = 41.8 kDa, Mw/Mn = 1.05) by copper catalyzed alkyne and azide 1,3-dipolar cycloaddition into a multi block copolymer. Incubation of the multiblock copolymer (selected fraction; Mw = 291 kDa, Mw/Mn = 1.11) with 0.14 mg/mL papain in citrate–phosphate buffer (pH 6.0) at 37 °C resulted in total degradation of the oligopeptide sequences in multiblock copolymer backbone and formation of the polymer with initial molecular weight (Mw = 41.7 kDa, Mw/Mn = 1.04).

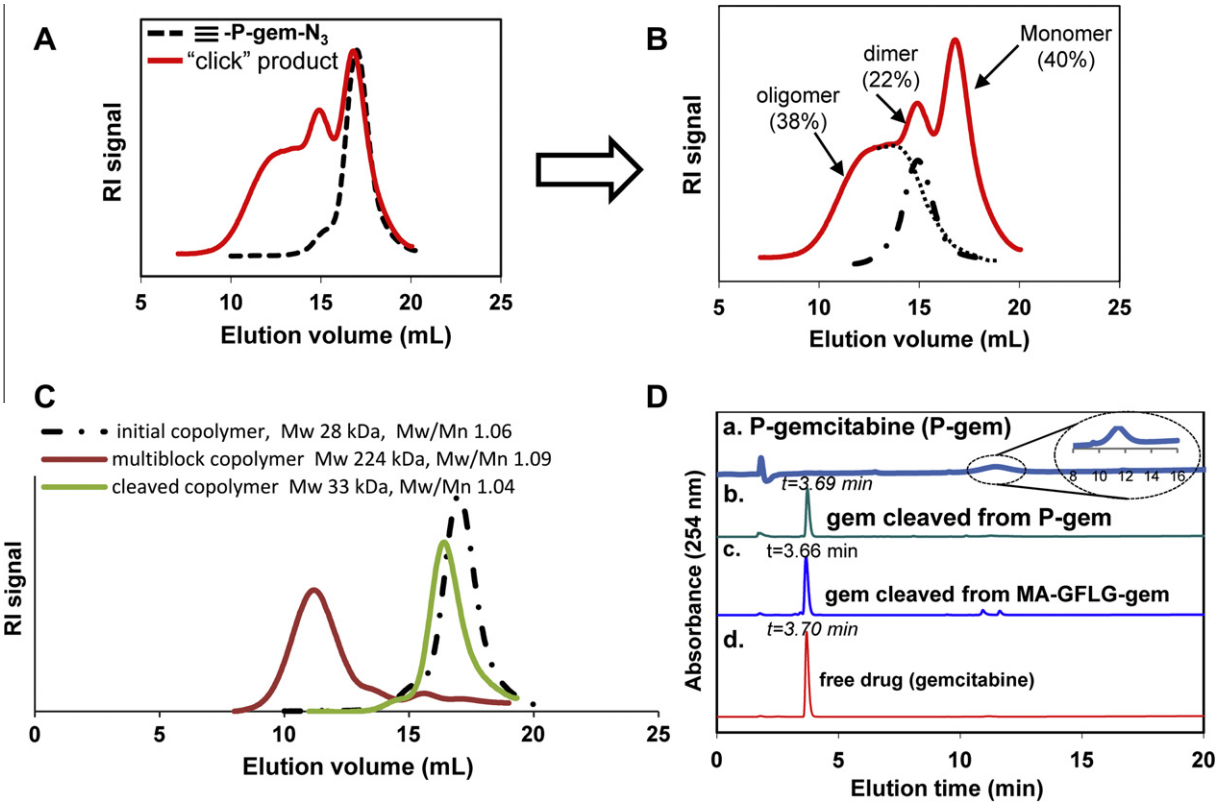


Fig. 5. (A) SEC chromatogram (RI detection; Superose 6 column) of initial P-gemcitabine conjugate (dashed line) and the multiblock copolymer after chain extension (full line); (B) SEC chromatogram (RI detection; Superose 6 column) of the multiblock copolymer after chain extension after Astra software multipeak fitting; (C) chain extension of heterotelechelic copolymer of HPMA with MA-GFLG-gemcitabine (Mw = 28 kDa, Mw/Mn = 1.06) by copper catalyzed alkyne and azide 1,3-dipolar cycloaddition into a multi block copolymer (selected fraction of Mw = 224 kDa, Mw/Mn = 1.09). Incubation of the multiblock copolymer (selected fraction; Mw = 224 kDa, Mw/Mn = 1.09) with 0.14 mg/mL papain in citrate–phosphate buffer (pH 6.0) at 37 °C resulted in total degradation of the oligopeptide sequences in multiblock copolymer backbone and formation of the polymer with close to initial molecular weight (Mw = 33 kDa, Mw/Mn = 1.04), (D) HPLC traces of: (a) multiblock high molecular weight copolymer of HPMA–gemcitabine conjugate (P-gem, fraction of Mw = 224 kDa, Mw/Mn = 1.09; (b) gemcitabine cleaved from multiblock high molecular weight copolymer (P-gem); (c) gemcitabine cleaved from monomer, MA-GFLG-gemcitabine, and (d) free gemcitabine.

[25,26] or diazido terminated triblock and pentablock copolymers with propargyl alcohol [27]; the coupling of two polymers with different end functionalities such as coupling of azido- and alkyne-terminated semitelechelic polymers [28]; and attachment of polymers with dissimilar reactivities [10]. These studies clearly demonstrated the feasibility to prepare multiblock copolymers using the combination of living radical polymerization and click reactions. Polymers with different functionalities, different reactivities, and those prepared by different polymerization techniques have been combined into multiblock copolymers (reviewed in [29]). The contribution of this study is to expand the chain extension to heterotelechelic polymers and anticancer drugs-containing heterotelechelic copolymers,

as well as to introduce a new RAFT chain transfer agent that contains an enzymatically degradable sequence. In addition, with biomedical application in mind, the initial heterotelechelic polymers possessed a higher molecular weight than polymers used in the majority of studies. Consequently, multiblock copolymers with molecular weight >200 kDa have been prepared.

4. Conclusions

We have presented a new design for the synthesis of high molecular weight biodegradable multiblock HPMA (co)polymers that consists of three steps: (a) RAFT (co)polymerization mediated by an enzyme-sensitive RAFT agent in both aqueous and organic media; (b) post-polymerization modification; and (c) click reaction. The versatility of this strategy permits facile incorporation of hydrophilic/hydrophobic comonomers into the polyHPMA structure. As an example, a heterotelechelic polyHPMA precursor with pendant TT reactive groups was synthesized, which can be further conjugated with amine-containing bioactive agents. More importantly, multiblock biodegradable HPMA copolymer conjugate bearing anti-cancer drug gemcitabine with a molecular weight over 200 kDa (the renal threshold is 40–45 kDa) was obtained by click coupling of heterotelechelic α-alkyne, ω-azido HPMA copolymer–gemcitabine conjugate (initial mol. wt. 28.5 kDa). Exposure to papain demonstrated the degradability of the HPMA copolymer backbone and the release of intact gemcitabine. The results indicate the potential of this HPMA-based drug delivery system for enhanced intravascular half-life with concomitant improvement in cancer therapeutic efficacy.

Acknowledgements

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References

[1] J. Kopeček, Polim. Med. 7 (1977) 191–221.
[2] J. Kopeček, P. Kopečková, Adv. Drug Deliv. Rev. 62 (2010) 122–149.
[3] T. Minko, P. Kopečková, J. Kopeček, Int. J. Cancer 86 (2000) 108–117.
[4] E. Segal, H. Pan, P. Ofek, T. Ugadawa, P. Kopečková, J. Kopeček, R. Satchi-Fainaro, PLoS ONE 4 (4) (2009) e5233.
[5] P.A. Vasey, S.B. Kaye, R. Morrison, C. Twelves, P. Wilson, R. Duncan, A.H. Thomson, L.S. Murray, T.E. Hilditch, T. Murray, S. Burtles, D. Fraier, E. Frigerio, J. Cassidy, Clin. Cancer Res. 5 (1999) 83–94.
[6] H. Maeda, Bioconjugate Chem. 21 (2010) 797–802.
[7] J.-G. Shiah, M. Dvořák, P. Kopečková, Y. Sun, C.M. Peterson, J. Kopeček, Eur. J. Cancer 37 (2001) 131–139.
[8] C.V. Scales, Y.A. Vasilieva, A.J. Convertine, A.B. Lowe, C.L. McCormick, Biomacromolecules 6 (2005) 1846–1850.
[9] L. Tao, J. Liu, J. Xu, T.P. Davis, Org. Biomol. Chem. 7 (2009) 3481–3485.
[10] D. Quémener, T.P. Davis, C. Barner-Kowollik, M.H. Stenzel, RAFT and click chemistry: a versatile approach to well-defined block copolymers, Chem. Commun., 2006, pp. 5051–5053.
[11] J. Kopeček, P. Rejmanová, Enzymatically degradable bonds in synthetic polymers, in: S.D. Bruck (Ed.), Controlled Drug Delivery, vol. I, CRC Press, Boca Raton, FL, 1983, pp. 81–124.
[12] J. Kopeček, H. Bažilová, Eur. Polym. J. 9 (1973) 7–14.
[13] V. Šubr, K. Ulbrich, React. Funct. Polym. 66 (2006) 1525–1538.
[14] Y. Mitsukami, M.S. Donovan, A.B. Lowe, C.L. McCormick, Macromolecules 34 (2001) 2248–2256.
[15] D.B. Thomas, B.S. Sumerlin, A.B. Lowe, C.L. McCormick, Macromolecules 36 (2003) 1436–1439.
[16] A. Favier, M.T. Charreyre, Macromol. Rapid Commun. 27 (2006) 653–692.
[17] S.R. Gondi, A.P. Vogt, B.S. Sumerlin, Macromolecules 40 (2007) 478–481.
[18] P.J. Roth, F.D. Jochum, R. Zentel, P. Theato, Biomacromolecules 11 (2010) 238–244.
[19] J. Xu, L. Tao, C. Boyer, A.B. Lowe, T.P. Davis, Macromolecules 43 (2010) 20–24.
[20] A.S. Goldmann, D. Quémener, P.-E. Millard, T.P. Davis, M.H. Stenzel, C. Barner-Kowollik, A.H.E. Müller, Polymer 49 (2008) 2274–2281.
[21] T. Lammers, Drug Targeting to Tumors using HPMA Copolymers, in: B.V. Ipskamp Drukkers, Enschede, The Netherlands, 2009, p. 159.
[22] Y.K. Chong, G. Moad, E. Rizzardo, S. Thang, Macromolecules 40 (2007) 4446–4455.
[23] M. Chen, G. Moad, E. Rizzardo, J. Polym. Sci. Part A 47 (2010) 6704–6714.
[24] J. Yang, M.T. Jacobsen, H. Pan, J. Kopeček, Macromol. Biosci. 10 (2010) 445–454.
[25] N.V. Tsarevsky, B.S. Sumerlin, K. Matyjaszewski, Macromolecules 38 (2005) 3558–3561.
[26] P.L. Golas, N.V. Tsarevsky, B.S. Sumerlin, K. Matyjaszewski, Macromolecules 39 (2006) 6451–6457.
[27] P.L. Golas, N.V. Tsarevsky, B.S. Sumerlin, L.M. Walker, K. Matyjaszewski, Austr. J. Chem. 60 (2007) 400–404.
[28] J.A. Opsteen, J.C.M. van Hest, Chem. Commun. (2005) 57–59.
[29] P.L. Golas, K. Matyjaszewski, Chem. Soc. Rev. 39 (2010) 1338–1354.