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Research Article

Star Polymer-Drug Conjugates with pH-Controlled Drug Release and Carrier Degradation

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In this study, we describe the design, synthesis, and physicochemical and preliminary biological characteristics of new biodegradable, high-molecular-weight (HMW) drug delivery systems with star-like architectures bearing the cytotoxic drug doxorubicin (DOX) attached by a hydrazone bond-containing spacer. The star polymers were synthesized by grafting semitelechelic N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers on a 2,2-bis(hydroxymethyl)propionic acid- (bis-MPA-) based polyester dendritic core. The molecular weight of the star polymers ranged from 280 to 450 000 g/mol and could be adjusted by proper selection of the bis-MPA dendrimer generation and by considering the polymer to dendrimer molar ratio. The biodegradation of the polymer conjugates is based on the spontaneous slow hydrolysis of the dendritic core in neutral physiological conditions. Hydrazone spacers in the conjugates were fairly stable at neutral pH (7.4) mimicking blood stream conditions, and DOX was released from the conjugates under mild acidic conditions simulating the tumor cell microenvironment in endosomes and lysosomes (pH 5). Finally, we have shown the significant in vitro cytotoxicity of the star polymer-DOX conjugate on selected cancer cell lines with IC_{50} values almost comparable with that of the free drug and higher than that observed for a linear polymer-DOX conjugate with much lower molecular weight.

1. Introduction

So far, numerous polymer-based drug delivery systems (DDSs) have been designed, synthesized, and tested for their biological behavior. Many DDSs are based on water-soluble polymer conjugates of low-molecular-weight drugs, that is, cytostatic agents, covalently bound to the polymer via biodegradable spacers, enabling the controlled release of the active drug in tumor tissues [1–3]. Among them, *N*-(2-hydroxypropyl)methacrylamide copolymers (pHPMA) are some of the most often studied soluble polymeric DDSs [4–7]. pHPMA systems are water-soluble, nontoxic, and nonimmunogenic drug carriers that enable the controlled delivery of various biologically active molecules, for example, drugs, inhibitors, proteins, dyes, and radionuclides. Recently, pHPMA drug conjugates, in which the drug was attached via a pH-sensitive hydrazone bond, were described [8, 9]. The hydrazone bond-containing DDSs were relatively stable at neutral pH of 7.4, mimicking the pH of the bloodstream, and the drug was released in buffer solutions with mildly acidic pH (pH 5-6), mimicking the environment in a tumor tissue and in the endosomes and lysosomes of tumor cells. Moreover, pHPMA conjugates with various anticancer drugs, namely, docetaxel, paclitaxel, mitomycin C, dexamethasone, doxorubicin, or a combination of two drugs (e.g., doxorubicin with dexamethasone), showed superior in vivo antitumor activity in various tumor models in contrast to free drugs and other polymer-drug conjugates containing drugs attached via other biodegradable spacers, for example, lysosomotropic oligopeptides or cis-aconityl-based spacers [8, 10].

The antitumor activity of the frequently described linear pHPMA drug conjugates with M_w < 50 000 g/mol can be improved by using an HMW pHPMA carrier to enable increased accumulation in solid tumors. HMW pHPMA drug conjugates cannot easily be eliminated from organisms by glomerular filtration; thus, they circulate much longer in the bloodstream and are preferentially accumulating in solid tumors due to the enhanced permeability and retention (EPR) effect [11, 12].

In addition to tumor accumulation and site-specific and controlled drug release, the polymer carriers must fulfill other requirements, predominantly the safe elimination of the carrier from the body. Recently, we have shown that accumulation of the pHPMA copolymers in solid tumors via the EPR effect significantly depends not only on the molecular weight of the conjugates but also on their architecture [13]. It is well known that the backbones of pHPMA drug conjugates are nonbiodegradable. This limits pHPMA conjugates in vivo use to conjugates having polymer coil sizes in solution (molecular weight) below the renal threshold, which guarantees safe excretion of the carrier via glomerular filtration. The size of glomerular pores is approximately 8 nm in humans [14], which corresponds to approximately M_w = 50–70 00 g/mol for pHPMA copolymers as a maximum limit for their elimination [13, 15].

Several methods can be used to increase the molecular weight of pHPMA conjugates in order to enhance their accumulation in tumors. One widely used approach is grafting linear polymers to a dendrimer core. Dendrimers are nearly monodisperse, and their surface can be modified with various semitelechelic pHPMA copolymers [16]. The star-like pHPMA conjugate with DOX, based on the poly(amidoamine) (PAMAM) dendrimer and linear pHPMA, showed improved tumor accumulation and superior antitumor activity compared to the linear pHPMA-DOX conjugate.

Previously, we described pHPMA star-like conjugates based on PAMAM dendrimers containing enzymatically or reductively degradable spacers between the dendrimer core and polymer grafts [16]. The goal of obtaining degradable carrier systems able to be excreted from the body by glomerular filtration was achieved by using a quite complicated synthetic approach. The degradation of this system was induced by either enzymes or the reductive environment in tumors. In this study, we decided to simplify the synthesis of degradable star-like conjugates by changing the structure of the dendritic core. Instead of a nondegradable PAMAM core, a biodegradable 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) dendrimer core was used. Bis-MPA dendrimers with their polyester backbones have been suggested as suitable candidates for the synthesis of star-like polymer-dendritic hybrid systems for potential applications in drug delivery [14, 17]. Previously, studies from Parrott et al. [17] on the toxicity of bis-MPA dendrimers in rats revealed that higher generation bis-MPA dendrimers were rapidly removed from the blood stream via the kidneys and did not accumulate in any organs, suggesting good biocompatibility. The terminal functional groups, for example, hydroxyl, carboxyl, or azide groups, of the bis-MPA dendrimers can be easily modified with various types of polymers, enabling a suitable route for the synthesis of sophisticated HMW drug carriers.

Here, we describe a simple and elegant synthetic method for a biodegradable star-like HMW drug carrier based on a bis-MPA dendrimer core. Bis-MPA dendrimer cores containing hydrolytically sensitive ester bonds were used and grafted with water-soluble pHPMA copolymers to increase the molecular weight of the carrier system, which facilitated higher accumulation in tumor tissue.

2. Material and Methods

2.1. Chemicals. 1-Aminopropan-2-ol, methacryloyl chloride, 2,2′-azobis(isobutyronitrile) (AIBN), 6-aminohexanoic acid (ah), tert-butyl carbazate (Boc), *N,N′*-dicyclohexylcarbodiimide (DCC), *N,N′*-diisopropylcarbodiimide (DIPCI), *N*-ethyldiisopropylamine (DIPEA), 2-thiazoline-2-thiol (TT), 2-cyano-2-propylbenzodithioate (CTA), *N*-(2-aminoethyl)maleimide trifluoroacetate (AEMI·TFA), sodium borohydride, tert-butyl alcohol, 4,4′-azobis(4-cyanopentanoic acid), hydrazine hydrate, *N,N′*-dimethylformamide (DMF), acetic acid, methanol, ethyl acetate, trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), 4-(dimethylamino)pyridine (DMAP), and 2,4,6-trinitrobenzene-1-sulfonic acid (TNBSA) were purchased from Fluka. Doxorubicin hydrochloride (DOX·HCl) was purchased from Meiji Seiko, Japan. Bis-MPA-24-carboxyl ($D_{24\text{-COOH}}$, G3) and bis-MPA-48-carboxyl ($D_{48\text{-COOH}}$, G4) dendrimers with carboxyl surface groups were purchased from Polymer Factory, Sweden.

2.2. Synthesis of Monomers. N-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized as described previously using K_2CO_3 [8] as a base. M.p. 69-70°C; purity > 99.8% (HPLC, 220 nm); elemental analysis: calc./found, C 58.72/58.98%, H 9.15/9.18%, N 9.78/9.82%.

N-(tert-Butoxycarbonyl)-N′-(6-methacrylamidohexanoyl)hydrazine (Ma-ah-NHNH-Boc) was prepared in two-step synthesis as described previously [10]. M.p. 130–134°C; purity (HPLC, 220 nm) > 99.5%; elemental analysis: calc./found C 57.70/57.96%, H 8.33/8.64%, N 13.46/13.25%.

2.3. Synthesis of Linear Polymer Precursors. The 2-thiazolidine-2-thione- (TT-) terminated semitelechelic statistical HPMA copolymer with Ma-ah-NHNH-Boc (**P1**) containing Boc-protected hydrazide groups was prepared by free radical polymerization (FRP) of HPMA (2.0 g, 0.014 mol) with Ma-ah-NHNH-Boc (378 mg, 1.21 mmol) in DMSO (14.4 mL) initiated with azo-initiator 3,3′-[azobis(4-cyano-4-methyl-1-oxobutane-4,1-diyl)]bis(thiazolidine-2-thione) (ABIC-TT, 0.76 g, 1.57 mmol), as described previously [18].

The amino group-terminated semitelechelic statistical copolymer **P2** containing protected hydrazide groups randomly distributed along the polymer chain was prepared by aminolysis of **P1** with an excess of ethylene diamine. Polymer **P1** (150 mg, 13.04 μmol) was dissolved in 1 mL of DMF, and a 10 molar excess of ethylene diamine (8.72 μL, 130.4 μmol) was added to the stirred solution. The reaction proceeded for 4 h at room temperature, and then the polymer precursor

P2 was purified from low-molecular-weight impurities by gel filtration (Sephadex LH-20, solvent methanol) and isolated by precipitation into ethyl acetate. The yield was 131 mg (83%).

The amino group-terminated semitelechelic HPMA copolymer **P3** with narrow molecular weight distribution was prepared by controlled radical RAFT polymerization of HPMA and Ma-ah-NHNH-Boc with 2-cyano-2-propylbenzodithioate as the chain-transfer agent, as described previously [16]. The amino end groups were introduced into the copolymer after the removal of the DTB end groups by reduction with $NaBH_4$, followed by reaction of the resultant sulfanyl groups with AEMI·TFA and by addition of an amount of DIPEA equivalent to the amount of TFA in AEMI·TFA.

The multivalent linear polymer precursor poly(HPMA-co-Ma-ah-$NHNH_2$) (**P4**) containing hydrazide groups randomly distributed along the polymer chain, which are intended for drug attachment, was prepared by free radical copolymerization using AIBN as the initiator, as previously described [10].

2.4. Synthesis of Star Polymer Precursors and Star Polymer-Drug Conjugates. The HMW star polymers **S1** and **S2** were prepared in two steps by grafting the amino group-terminated semitelechelic HPMA copolymer **P2** onto the $D_{24\text{-}COOH}$ or $D_{48\text{-}COOH}$ dendrimer using the carbodiimide coupling method. In the first step, the carboxyl groups of the bis-MPA dendrimer were activated with reactive 2-thiazoline-2-thione (TT) amides, which were subsequently aminolysed with the amino groups of the semitelechelic polymer precursor. In an example synthesis, the $D_{24\text{-}COOH}$ dendrimer (10.44 mg, 2.1 μmol, Figure 2) was dissolved in 80 μL of DMF and reacted with 2-thiazoline-2-thiol (6.0 mg, 50.33 μmol) and DIPCI (15.82 μL, 101.03 μmol) dissolved in 1.98 mL of DMF. After 1 h at room temperature, a solution of the polymer precursor **P2** (262.5 mg, 23.3 μmol) and DMAP (5.71 mg, 46.74 μmol) dissolved in 5.29 mL of DMF was added to the stirred mixture of the activated dendrimer. After 24 h, the reaction was terminated by adding 1-aminopropan-2-ol (5.23 mg, 69.57 μmol), and the product was purified from low-molecular-weight impurities by gel filtration (Sephadex LH-20, solvent methanol) and isolated by precipitation into ethyl acetate. The yield was 215.2 mg (78.8%).

The star polymer **S3** was prepared by grafting the amino-terminated semitelechelic HPMA copolymer **P3** onto the $D_{24\text{-}COOH}$ dendrimer using a similar method to that described for star polymers **S1** and **S2**. The star polymer was separated from the unbound linear polymer by preparative gel filtration (Sephacryl S-300, eluent : acetate buffer pH = 4, 0.15 M NaCl). The star polymer fraction was purified from low-molecular-weight impurities using a centrifuge filtration tube (Amicon Ultra, 15 mL, Ultracel 5K) and freeze-dried. The yield was 64.3%.

Free hydrazide groups on the star polymer were obtained by removing the protective Boc groups from the hydrazides using concentrated TFA, as described previously [16].

Polymer-drug conjugates **P5** and **S4** containing DOX bound via a pH-sensitive hydrazone bond were prepared by reaction of the hydrazide groups of polymers **P4** and **S3** with DOX·HCl in methanol, as described previously [8]. The concentration of DOX was determined spectrophotometrically.

2.5. Characterization of the Polymer Products. Determination of the molecular weight and polydispersity of all polymers and conjugates was performed on an SEC/HPLC Shimadzu system equipped with UV (SPD-10AVvp Shimadzu, Japan), refractive index (Optilab-rEX), and multi-angle light scattering (DAWN HELLIOS II) detectors (both from Wyatt Technology Co., USA). The eluent for the TSkgel G3000SWxl/G4000SWxl column was a methanol-sodium acetate buffer (CH_3COONa/CH_3COOH; pH = 6.5) (80 : 20 vol%); the flow rate was 0.5 mL/min.

The polymer main-chain end-group functionality (F) was defined as the ratio between M_n obtained from the SEC and $M_{n,EG}$ calculated from the end-group content.

The star polymer conjugates were characterized by the amount of unbound polymer or free drug using the SEC method described above with either Superose™ 6 or TSkgel G4000SWxl columns. The concentration of amino groups on the polymer ends and hydrazide groups along the polymer chain was determined by a modified TNBSA assay, as described earlier [16]. Molar absorption coefficients of ε_{420} = 11 500 L mol^{-1} cm^{-1} and ε_{500} = 17 200 L mol^{-1} cm^{-1} were used. The content of TT end groups in the copolymers was determined spectrophotometrically in methanol, as described previously [16]. The molar absorption coefficient ε_{305} = 10 300 L mol^{-1} cm^{-1} was used.

The total content of DOX in the polymer conjugates was determined spectrophotometrically on a Helios α (Thermo-Spectronic) spectrophotometer using the hydrazone bond-bound DOX molar absorption coefficient in methanol of ε_{488} = 11 200 L mol^{-1} cm^{-1}.

The hydrodynamic radius (R_h) was estimated by the dynamic light scattering (DLS) of aqueous conjugate solutions (0.5 wt%) in phosphate buffer (pH 7.4) at 22°C at a scattering angle of 173° on Zetasizer Nano-ZS (Malvern, UK).

2.6. In Vitro Release of Doxorubicin. The DOX release rates from the linear polymer conjugate **P5** and star-like conjugate **S4** were investigated by incubating the conjugates in phosphate buffer at pH 5.0 or 7.4 (0.1 M phosphate buffer with 0.05 M NaCl) at 37°C. The concentration of the conjugate in the stock solution was equivalent to 0.5 mM DOX.

The amount of released DOX was determined by HPLC analysis after the extraction of DOX from the stock solution (200 μL) into chloroform, as previously described [8]. The analysis was performed using HPLC (Shimadzu, Japan) on a Chromolith Performance RP-18e, 100 × 4.6 column (using a water-acetonitrile eluent with an acetonitrile gradient of 0–100 vol% and a flow rate of 1 mL min^{-1}) with fluorescence detection (excitation at 488 nm and emission at 560 nm). The drug release data were expressed as the percentage of free drug relative to the total drug in the conjugates. All experiments were performed in triplicate.

2.7. In Vitro Degradation of Star Polymers in Buffer and in Plasma. The hydrolytic degradation of the selected star

Table 1: Physicochemical characteristics of linear polymer precursors and drug conjugate **P5**.

Polymer	M_w (g/mol)[a]	M_n (g/mol)[a]	$Đ$	F[b]	Side chain groups	DOX[c]
P1	25 100	16 700	1.5	1.4 (–TT)	–NHNH-Boc	—
P2	29 100	17 100	1.7	1.3 ($–NH_2$)	–NHNH-Boc	—
P3	23 300	21 200	1.1	0.9 ($–NH_2$)	–NHNH-Boc	—
P4	25 000	13 900	1.8	—	$–NHNH_2$	—
P5	27 000	14 200	1.9	—	—	9.8

[a]Molecular weight was determined by SEC using RI and MALS detection.
[b]Polymer functionality of end groups (ratio of M_n to $M_{n,EG}$ calculated from the content of end groups determined as described in Section 2.5).
[c]The DOX content was determined spectrophotometrically.

copolymers (**S1**, **S2**, and **S3**) was studied in sodium phosphate buffers (pH 7.4 or pH 5, 0.1 M sodium phosphate, and 0.05 M NaCl). The polymers (3 mg) were dissolved in 1 mL of phosphate buffer at pH 5 or 7.4, and the solutions were filtered with a 0.45 μm PVDF filter into a glass vial and incubated at 37°C. The molecular weight was measured at predetermined time intervals using a GPC/HPLC Shimadzu system (see above) with a built-in thermostat over a range of 0–14 days.

The in vitro degradation of star polymer **S3** in plasma was conducted in human plasma solution (plasma : PBS buffer = 80 : 20, pH 7.5) at 37°C with a concentration of 3 mg/mL of star polymer. At selected time intervals, a 250 μL aliquot of the solution was extracted, and 1 mL of cold MeOH (for protein aggregation) was added. The solution was centrifuged, and the molecular weights of the star polymer and polymer degradation products in supernatant were determined by GPC using a Superose 6 column with DAWN 8 and Optilab-rEX detectors (acetate buffer, pH 6.5). All experiments were performed in triplicate.

2.8. In Vitro Cytotoxicity. The conjugate cytostatic potential was assessed using a [^{3}H]-thymidine incorporation assay. Nunc 96-well flat-bottom plates were seeded with 5×10^3 cancer cells per well. The samples were then added at the desired concentration in triplicate or quadruplicate. The plates were cultured at 37°C in 5% CO_2 for 72 h, and afterwards 18.5 kBq (0.5 mCi) [^{3}H]-thymidine per well was added for the final 6 h of incubation. The cells were then collected onto glass fiber filters (Filtermat, Wallac, Finland) using a cell harvester (Tomtec, Orange, CT), and the cellular radioactivity was measured via scintillation (1450 MicroBeta TriLux, Wallac, Finland). Cells cultivated in drug-free media were used as controls. All IC_{50} values (the drug concentration that inhibits the growth of 50% of the cells) were obtained from at least three independent experiments.

3. Results and Discussion

In this paper, the synthesis and physicochemical and preliminary biological properties of novel biodegradable HMW drug carriers and conjugates are described. HMW polymer systems are water-soluble polymers with star-like architectures based on linear pHPMA copolymer grafted onto 3rd and 4th generation bis-MPA dendrimers. HMW star polymers are designed to be slowly degradable in the environment of living organisms due to the hydrolytic instability of the ester bonds in the bis-MPA dendrimer core. This hydrolysis is effective at neutral pH (7.4), mimicking the pH of physiological environments. We focused on improving the properties of biodegradable HMW drug delivery systems by designing new biodegradable structures of the star pHPMA-DOX conjugates with well-defined architecture and proper control of molecular weight (with preservation of low polydispersity). In addition, we aimed to synthesize polymer structures that efficiently accumulated in solid tumors and then were excreted from the body after the drug load was released, and the polymer carrier was subsequently degraded under physiological conditions.

3.1. Synthesis of Linear Polymer Precursors and Drug Conjugate. The semitelechelic statistical pHPMA copolymer **P1** (Table 1) with TT end groups (F = 1.4) containing Boc-protected hydrazide groups located randomly along the polymer chain was prepared by FRP initiated by an azo-initiator containing reactive TT groups. Polymerization conditions were chosen to keep the molecular weights of the copolymers under the renal threshold, which is crucial for the subsequent elimination of the polymer from the body via glomerular filtration.

The polymer precursor **P2**, bearing Boc-protected hydrazide groups, was prepared by reaction of the **P1** TT end groups with an excess of ethylene diamine. The excess was used with the aim to avoid crosslinking of the polymer precursor and to introduce main end-chain amino groups. The functionality of copolymer **P2** (F = 1.3) was similar to the functionality of **P1**. The molecular weight and polydispersity of the **P2** copolymer did not change after reaction. Polymers **P1** and **P2** showed slightly broader dispersities, as they were prepared by FRP. The semitelechelic polymer precursor **P3** with a narrow molecular weight distribution was prepared by controlled radical RAFT polymerization carried out in *tert*-butyl alcohol. The RAFT polymerization technique using a dithiobenzoate-based chain-transfer agent was successfully utilized for the preparation of nearly monodisperse PHPMA precursors with yields comparable to those obtained in the free radical polymerization technique. The dithiobenzoate end groups of copolymer **P3** were removed by reduction with $NaBH_4$, and the subsequently formed thiol groups were used in situ for the introduction of amino groups by reaction with AEMI·TFA. The low dispersity (1.1) and the

Table 2: Physicochemical characteristics of HMW copolymers and drug conjugate.

Star polymer	Polymer precursor	Dendrimer	Molar ratio polymer : dendrimer	M_w (g/mol)[a]	$Đ$	R_h (nm)[b]	Yield of star polymer (%)
S1	**P2**	$D_{24\text{-COOH}}$, G3	11 : 1	280 000	1.74	15.2	82
S2	**P2**	$D_{48\text{-COOH}}$, G4	19 : 1	450 000	1.71	17.5	69
S3	**P3**	$D_{24\text{-COOH}}$, G3	12 : 1	280 000	1.27	11.7	97
S4[c]	**S3**	$D_{24\text{-COOH}}$, G3	—	320 000	1.38	12.3	—

[a]Molecular weight was determined by SEC using RI and MALS detection.
[b]Hydrodynamic radius was determined by DLS.
[c]Star polymer-DOX conjugate containing 10.4 wt% DOX.

functionality of the amino end groups (0.9) close to the unity predicted copolymer **P3** to be effective and highly valuable in the synthesis of HMW star polymers. The functionality of the semitelechelic copolymer **P2**, prepared by FRP, was significantly higher than 1, which indicated the presence of telechelic copolymers bearing amino groups on both polymer chain ends. For that reason, a small amount of crosslinked structures were also found after the grafting of polymer **P2** to the dendrimer core. The functionality of copolymer **P3**, prepared by RAFT polymerization, close to unity minimized the possibility of crosslinking reactions during the formation of the star copolymer and therefore led to the preparation of star polymer precursor with low dispersity.

The linear polymer precursor **P4** with hydrazide groups was prepared by FRP and used as a precursor for the synthesis of a control polymer-DOX conjugate **P5**. The concentration of DOX (9.8 wt%) in polymer conjugate **P5** was sufficient for the following in vitro study. The linear conjugate **P5** was purified by gel filtration and contained less than 0.2% of free DOX. All physicochemical characteristics of the prepared polymer precursors are summarized in Table 1.

3.2. Synthesis of HMW Polymer Precursors and Conjugate. HMW polymers suitable for the passive targeting of tumor tissue and delivery of anticancer drugs into tumors and tumor cells were designed and synthesized. Semitelechelic polymers containing amino end groups were used as polymer grafts attached via amide bonds to the bis-MPA dendrimer core to form star-like molecular structures with synthesis-controlled molecular weights. The molecular weights of the synthetized HMW star polymers were in the range of 280–450 kg/mol with polydispersities corresponding to that of the polymer precursors (Table 2). The HMW copolymers **S1** and **S2** were synthesized by coupling the semitelechelic HPMA copolymer **P2** with amino end group onto the G3 or G4 generation of bis-MPA cores that contained 24 (**S1**) or 48 (**S2**) carboxyl groups terminating each of the dendrimer arms. The molar ratio of polymer **P2** to dendrimer in the conjugation reaction was 11 : 1 and 19 : 1 in the synthesis of **S1** and **S2**, respectively. The content of star polymer in the final crude product was approximately 80% for **S1** and 70% for **S2**. The lower yield in the reaction of **S2** was likely caused by steric hindrance of the polymer molecules accessing the relatively dense, carboxyl-containing dendrimer core. For this reason, only approximately 50% of the carboxyl groups from the G3 dendrimer and 40% of those from the G4 dendrimer were able to be modified with polymer grafts. The molecular weights of star polymers **S1** and **S2** reflect the idea that 9 polymer chains were grafted in the polymer conjugate of **S1** and approximately 13-14 polymers were grafted in that of **S2**. The genesis of the HMW carrier led to a 3-fold increase in the hydrodynamic radius (R_h) from approximately 4-5 nm, corresponding to the linear polymer precursor, to 12–17 nm for the star polymers. The Boc-protected hydrazide groups in the star copolymers were deprotected with TFA, and we confirmed that neither R_h nor M_w changed during deprotection. The size of the star polymers fulfilled the prerequisite criteria mentioned in literature that are necessary for the achievement of enhanced passive accumulation of polymers in solid tumors due to the EPR effect.

Star polymer **S3** was synthesized in the same manner as previously described for star polymer **S1** using semitelechelic polymer **P3** prepared by controlled RAFT polymerization and a ratio of polymer to dendrimer core of 12 : 1. The molecular weight of HMW copolymer **S3** was determined as 280 000 g/mol, implying that approximately 10-11 polymer chains were attached to the dendrimer core. Unreacted linear precursor in the star polymers was removed by preparative gel filtration, and the content of the remaining linear precursor was less than 3%. The dispersity of polymer **S3** was below 1.3, which in contrast to the dispersity of star polymers prepared from FRP precursors clearly demonstrates the advantage of the polymer system prepared by controlled RAFT polymerization. As expected, R_h of **S3** of 12 nm was similar to that observed for **S1** and was again 3 times higher than that of the linear polymer **P3**. The removal of the Boc-protecting groups by TFA solution influenced neither the molecular weight nor the dispersity of HMW star carrier **S3**. Thus, a hydrazide group-containing HMW star polymer carrier with low dispersity was successfully synthesized.

The HMW star polymer-drug conjugate **S4** was subsequently prepared for forthcoming physicochemical and biological experiments, namely, in vitro studies. The deprotected hydrazide groups of the star polymer precursor **S3** were used for the attachment of DOX via pH-sensitive hydrazone bonds in the presence of acetic acid. The conversion of the conjugation reaction was almost quantitative, reaching 98%. Unbound DOX was removed from the star polymer conjugate by gel filtration, and the final product contained less than 0.2% of free DOX. As a control for the in vitro studies, the linear polymer-DOX conjugate (M_w = 27 000 g/mol),

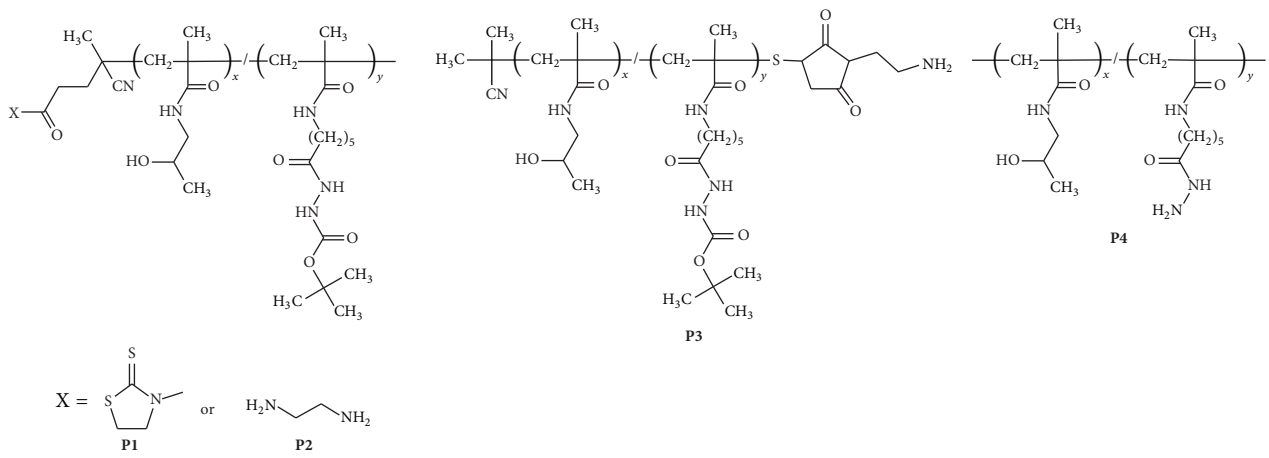

Figure 1: Schematic structure of statistical copolymer **P4** and semitelechelic polymer precursors **P1**–**P3** with different end groups.

polymer **P5**, was synthesized. The structures of the polymer precursors and star-like polymer conjugates are given in Figures 1 and 2. All physicochemical characteristics of the synthetized HMW polymers are summarized in Table 2.

3.3. In Vitro Release of DOX. The results of the in vitro drug release experiments showed that the hydrazone bond used for DOX attachment in copolymer **P5** and in HMW star conjugate **S4** was fairly stable in buffer solution at pH 7.4 and 37°C (Figure 3), modeling the blood environment. Only a slight release of DOX, up to 4%, was observed within 24 h of incubation for **P5** and **S4** at pH 7.4. By contrast, approximately 90% of the DOX was released in the same time period from both conjugates after incubation in buffer at pH 5 (37°C), which simulated conditions in endosomes of tumor cells. In agreement with previous conclusions [13], it was confirmed that steric hindrance did not influence the drug release rate from soluble HMW pHPMA conjugates. Both conjugates (linear and HMW star) fulfill the drug release requirements for effective anticancer drug delivery systems, that is, high stability in blood circulation conditions and release of the active drug after entering the tumor cell.

3.4. In Vitro Degradation of Star Polymers in Buffers and Plasma. In this study, the biodegradable HMW star copolymers with M_w above the renal threshold are composed of pHPMA precursors with M_w of approximately 27 000 g/mol coupled via amide bonds to a bis-MPA core containing hydrolytically degradable ester bonds. The stability of the HMW polymers was tested by incubation in phosphate buffer (pH 5.0 and pH 7.4), mimicking either the pH of the hypoxic environment of tumors and tumor cells (5.0) or the physiological pH of normoxic body tissue (7.4). The selected HMW copolymer was also degraded in human plasma.

The degradation studies of HMW copolymers **S1**–**S3** showed high stability in phosphate buffer at pH 5.0; at this pH, the HMW copolymer was nondegradable because the ester bonds in the dendritic core are stable in slightly acidic conditions. Neither molecular weight nor the hydrodynamic radius changed within 12 days of incubation (data not shown).

In contrast, the slow hydrolysis of ester bonds in HMW copolymers **S1** and **S3** was observed in phosphate buffer at the normoxic physiological pH of 7.4. This hydrolysis resulted in a gradual decrease in the amount of HMW copolymer **S1** (dashed line, Figure 4) and copolymer **S3** (full line, Figure 4) with the simultaneous release of linear polymers. Thus, linear polymer grafts with molecular weights under the limit of the renal threshold were formed, and biodegradability in the physiological conditions was proven.

The degradation half-life of star polymer **S1**, prepared by free radical polymerization, was reached after 8 days of incubation compared to 6 days for star polymer **S3**, prepared by controlled RAFT polymerization (Figure 4). With increasing degradation time, the molecular weight of HMW star polymers simultaneously decreased.

It was previously reported that the first sign of hydrolytic degradation of bis-MPA dendrimers in physiological conditions (pH 7.4) was observed approximately 6 h after being dissolved in aqueous media. Once the first segments were cleaved, the diffusion of water to the internal ester bonds was facilitated, and the degradation process became faster. The results indicated that the degradation progressed from the periphery to the core, releasing building unit after building unit through a mechanism similar to depolymerization [19]. This observation was also confirmed in our carriers by DLS measurement, as seen by decreasing values of the hydrodynamic radius of star-like carriers in a buffer of pH 7.4, modeling physiological conditions (Figure 5). Figure 5 clearly shows that the R_h value of star polymer carrier **S1** decreases rapidly to less than half radius within one week at pH 7.4, whereas at pH 5 the R_h value does not change within this time period.

Degradation studies of star polymer **S2**, which had the highest molecular weight (450 kg/mol), were performed in phosphate buffer at pH 5 and 7.4 and in plasma. In buffer at pH 5, the hydrolysis of ester bonds in the dendrimer core was not observed, and therefore neither the molecular weight nor the hydrodynamic radius changed (data not shown). Conversely, in phosphate buffer at pH 7.4 and in plasma (pH 7.5), the ester bonds slowly hydrolyzed, and

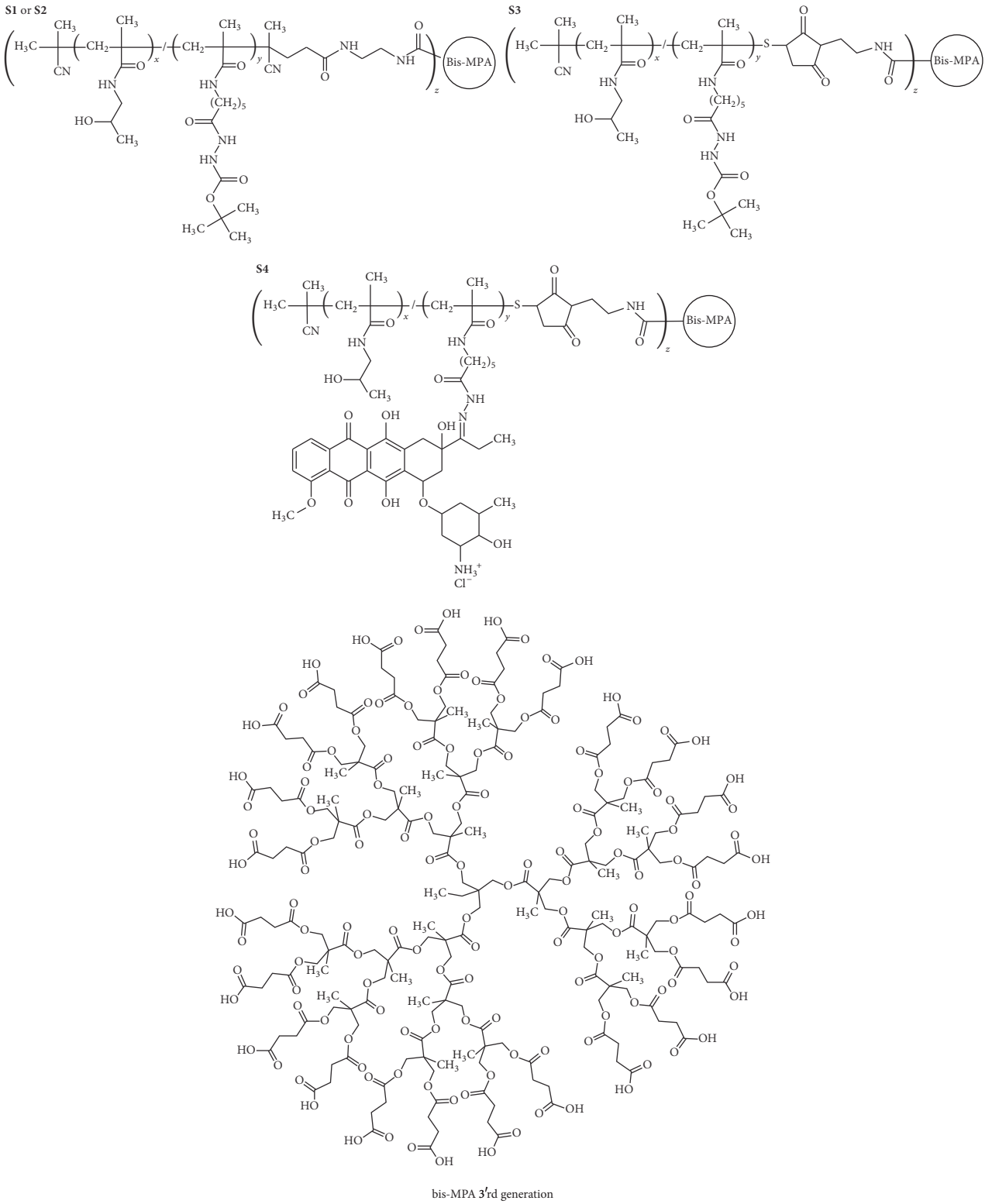

Figure 2: Schematic structures of the HMW star polymers, HMW star polymer-DOX conjugate, and bis-MPA dendrimer.

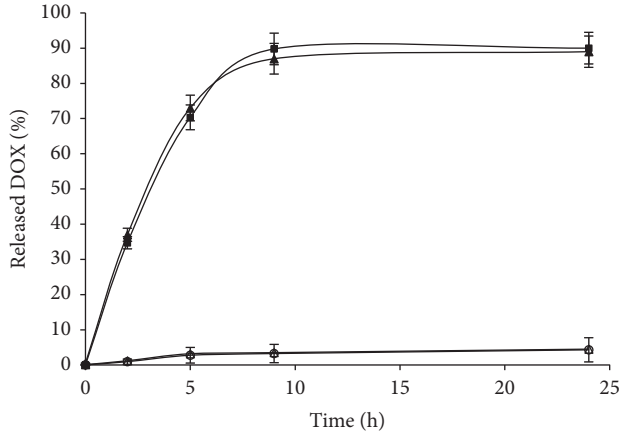

Figure 3: Release of DOX from linear polymer conjugate **P5** and star copolymer **S4** incubated in phosphate buffer at 37°C. Linear polymer **P5** at (▲) pH 5 and (Δ) pH 7.4. Star-like polymer **S4** at (■) pH 5 and (○) pH 7.4.

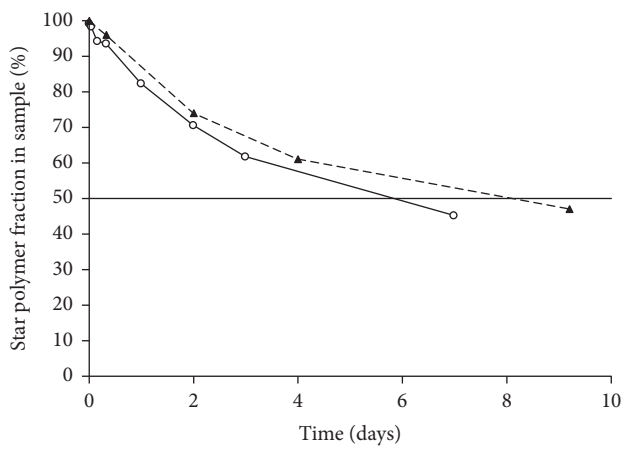

Figure 4: Comparison of the degradation of HMW copolymer **S1** (- - -▲) and **S3** (—○) in phosphate buffer at pH 7.4, plotted as % of star polymer in the incubation media.

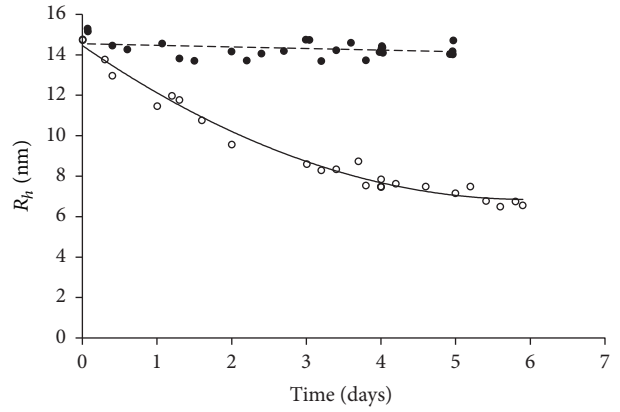

Figure 5: Degradation study of HMW polymer **S1** in phosphate buffer at pH 5 (●, trend line - - -) and pH 7.4 (○, trend line —), measured by DLS and plotted as the dependence of the R_h value of the star polymer on time.

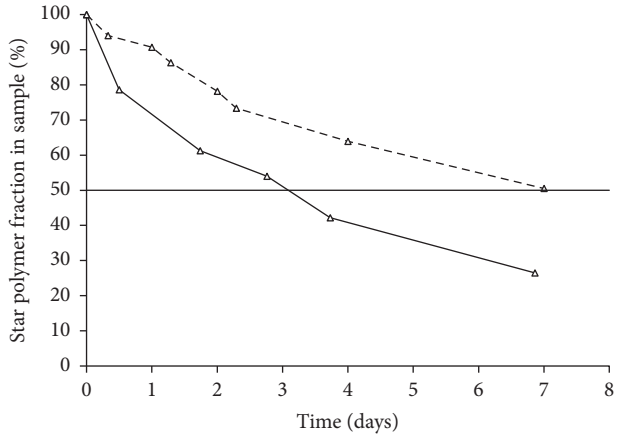

Figure 6: Comparative degradation study of star copolymer **S2** in phosphate buffer at pH 7.4 (- - -Δ) and in plasma (—Δ), plotted as % of star polymer in the sample versus time.

the HMW star polymer degraded within several days. The rate of degradation of star copolymer **S2** in plasma was approximately 2 times faster than that in the plasma-free buffer at pH 7.4 (Figure 6). The half-life of the **S2** copolymer was 3 days during incubation in plasma and 7 days during incubation in buffer at pH 7.4. We hypothesize that the increased rate of degradation of **S2** in plasma compared to that in buffer was most likely due to the presence of various esterases located in the plasma. These enzymes can increase the rate of degradation of star polymers, resulting in the linear precursor being excreted from the body by renal filtration.

The results of the hydrolytic degradation studies of HMW star polymers demonstrated that the copolymers **S1**–**S3** underwent slow and time-dependent degradation during incubation in the buffer modeling normoxic physiological conditions in the body. The degradation of the star copolymer was accomplished much faster in plasma than in the buffer with similar pH, which is likely due to the presence of esterases and, perhaps, other enzymes in plasma. The degradation proceeded more rapidly in the buffer modeling physiological conditions for the less dispersed star polymer **S3** (half-life approximately 5-6 days) than for copolymers **S1** and **S2** prepared from FRP precursors (half-life approximately 7–9 days). We suppose that the slower rate of degradation of the more dispersed and less defined **S1** and **S2** polymers was most likely caused by the higher incidence of linear telechelic polymers, which resulted in both polymer ends grafting to the dendrimer and thus creating partial crosslinking; such structures required more time for complete degradation. It seems that, for the practical use of the star polymer carriers, the better defined and less disperse structure obtained by grafting with semitelechelic polymers prepared by RAFT is more suitable.

In sum, the described star polymer system can serve as an HMW polymer carrier, offering prolonged blood circulation and enhanced tumor accumulation via a mechanism based on the EPR effect, as recently described for similar star

Table 3: IC_{50} values of polymer-DOX conjugates determined on two different cell lines.

Sample	EL4 (μg/mL)	LL2 (μg/mL)
P5	0.056 ± 0.016	0.210 ± 0.014
S4	0.027 ± 0.018	0.075 ± 0.007
DOX·HCl	0.006 ± 0.004	0.050 ± 0

systems [16]. Indeed, star polymers show stimuli-responsive behavior, as they release the drug after reaching the hypoxic tumor environment and are afterwards slowly degraded in predominantly normoxic physiologic conditions. We strongly believe that these unique properties are highly favorable and make the star polymers suitable candidates for further biomedical research.

3.5. In Vitro Cytotoxicity. The cytostatic activities of free DOX and polymer conjugates **P5** and **S4** containing DOX were evaluated on two different murine cancer cell lines, including EL4 T-cell lymphoma cells and LL2 Lewis lung carcinoma cells, where the EL4 cell line is much more sensitive to DOX compared to the LL2 cell line. As mentioned in our recent reports, the cytotoxicity of the polymer conjugates is always lower than the cytotoxicity of the free drug. In contrast to the low-molecular-weight drug, which entrances cells quite rapidly by diffusion, the polymer conjugates reach cells via the slower process of endocytosis. Similar observations were also seen in the case of the described star polymers, as the cytotoxicities of the polymer conjugates were always lower than that of free DOX. The values of IC_{50}, the concentration of drug that inhibits cell proliferation by 50%, for both cell lines and both DOX conjugates are summarized in Table 3. We observed a slightly higher cytotoxicity of the star polymer conjugate compared to the linear polymer conjugate, but it is statistically significant only for LL2 cells. The cytotoxicity of the star polymer conjugate was almost comparable to the cytotoxicity of free DOX on the DOX less-sensitive LL2 cell line.

We have performed confocal microscopy measurements using fluorescently labeled polymer conjugates on both cell lines; however, we have not found statistically significant difference between the rate of internalization of the star and linear polymer conjugates. Thus, we hypothesize that the increase in cytotoxicity of the star polymer conjugate compared to the linear polymer conjugate is caused by the increased amount of carried drug within each polymer carrier. Thus, a larger amount of active drug could be released after a single star polymer conjugate entered the cell. Moreover, the almost comparable cytotoxicity of the star polymer with free DOX makes the described star polymer a suitable candidate for further pharmacological development as a highly potent biodegradable stimuli-sensitive drug delivery system.

4. Conclusions

We have designed and described the complex synthesis of a water-soluble biodegradable HMW star polymer-drug system designed for tumor-specific drug delivery. The HMW star system containing hydrolytically degradable ester bonds on a bis-MPA core was constructed as a long-circulating polymer carrier, enabling prolonged drug circulation with highly enhanced accumulation in solid tumors. The major advantage of these systems is the combination of the hydrolytically degradable bis-MPA dendrimer core and the nontoxic, nonimmunogenic and completely water-soluble grafts based on HPMA copolymers. The time-dependent hydrolytic biodegradation of the HMW system in normoxic physiologic conditions in model buffers and human plasma ensures the safe elimination of polymer carriers from the body after fulfilling their function. Moreover, the pH-sensitive release of the active drug in a hypoxic tumor microenvironment showed the stimuli-responsive behavior of the star polymer conjugates. The star polymer conjugate with DOX inhibited proliferation of tumor cells in vitro significantly more than the linear polymer-DOX conjugate and to a similar extent as free DOX. We believe that the described biodegradable star polymer conjugates are promising candidates for the highly efficient therapy of solid tumors.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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