N-(2-Hydroxypropyl)methacrylamide-Based Polymer Conjugates with pH-Controlled Activation of Doxorubicin. I. New Synthesis, Physicochemical Characterization and Preliminary Biological Evaluation

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ABSTRACT: New method of synthesis of water-soluble polymer-drug conjugates, exhibiting remarkable anticancer activity in mice models, has been developed. In the conjugates, an anticancer drug doxorubicin (DOX) is attached to a polymer carrier based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer via a hydrolytically labile hydrazone bond. New methacrylamide-type comonomers, containing either hydrazide group or hydrazon of DOX, were used for copolymerization with HPMA. In contrast to the synthetic procedure described earlier the new method is simpler, cheaper, and results in a better-defined conjugate structure. The conjugates are fairly stable in buffer at pH 7.4 (model of blood stream) but release DOX under mild acid conditions modeling the tumor microenvironment. The conjugates showed significant *in vivo* antitumor activity in treatment of T-cell lymphoma EL-4 bearing mice with up to 100% long-term survivors. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 3050–3061, 2008

Key words: copolymerization; drug-delivery systems; water-soluble polymers; radical polymerization; doxorubicin

INTRODUCTION

Water-soluble synthetic polymer drug conjugates based on copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA) are a group of widely studied drug-delivery systems designed for delivering anticancer drugs specifically to tumors or tumor cells in experimental animal models as well as clinical trials.[1–6] The drug can be released from the polymer carrier either by enzymatic cleavage[7,8] or by pH-controlled chemical hydrolysis.[9,10] These polymer drugs have to fulfill a number of criteria to be effective as anticancer therapeutics. Ideally, they have to be inactive during their transport in the blood stream and, at the same time, release the drug only within a tumor tissue or inside a tumor cell. Moreover, the polymer molecules have to be small enough to be able to extravasate from circulation and large enough to accumulate within a solid tumor. These nanotherapeutics can be conjugated with monoclonal antibodies, lectins, or peptides, which target them on receptors of tumor cells.[11–13]

It was shown previously that HPMA-based polymer drugs fulfill the above-mentioned criteria, that is, they extravasate and accumulate in solid tumors as well as enter and kill the target cells.[14,15] Recently, we have described the synthesis of HPMA-based copolymers[10] containing the anticancer drug doxorubicin (DOX) bound to the polymer via a spacer containing pH-sensitive hydrazone linkage (hydrazone conjugate). We demonstrated that these conjugates exhibit a high level of cytostatic activity in several cancer cell lines and show a significant therapeutic effect in the treatment of mice-bearing EL4 lymphoma.[16–18] Furthermore, we improved the anticancer potential of the HPMA-based polymer prodrugs bearing anticancer drug doxorubicin by coupling them to human immunoglobulin.[19–21] In the syntheses of all the hydrazone conjugates, the polymer precursors with 4-nitrophenoxy[10] or Boc-protected hydrazide groups[16–18] were used as intermediates, and DOX was conjugated with the hydrazinolyzed or deprotected polymer precursors forming a conjugate composed of required HPMA and methacryloyl DOX hydrazone-derived monomer units but also of unwanted methacryloyl hydrazide, Boc-protected methacryloyl hydrazide, or methacryloylated carboxylic group-containing units.

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The new strategies of synthesis of the conjugates take advantage of using new types of monomers that both simplify the synthesis of hydrazone conjugates and produce them with well-defined structures. For direct introduction of hydrazide groups to the polymer precursor structure, the new monomers with free hydrazide groups were prepared and their ability to copolymerize with HPMA was studied. The new types of monomers designed for direct introducing of DOX to the polymer chain via copolymerization of HPMA with a DOX-containing monomer were also developed and tested. Accordingly to the previously published results[16,19] two different types of spacers in hydrazide group-containing monomers were used, residue of 6-aminohexanoic acid (ah) as model of nonbiodegradable spacer and biodegradable glycylphenylalanylleucylglycin tetrapeptide spacer (GFLG) susceptible to enzymatic degradation.

The use of polymer precursors based on highly hydrophilic poly(HPMA) polymers facilitates the synthesis of polymer conjugates with various contents of polymer-bound DOX, ranging from 5 to 23 wt %. The physicochemical behavior of the new conjugates in aqueous solutions was studied, and the results were compared with those obtained for the previously described conjugates. Furthermore, the rate of *in vitro* DOX release from the conjugates incubated under various conditions, *in vivo* rate of blood clearance of the conjugates and their accumulation in tumors as well as *in vitro* and *in vivo* cytostatic activities were studied.

EXPERIMENTAL

Chemicals

1-Aminopropan-2-ol, methacryloyl chloride, 2,2′-azobis(isobutyronitrile) (AIBN), 6-aminohexanoic acid (ah), methyl 6-aminohexanoate hydrochloride (ah-MeO), *N*,*N*′-dimethylformamide (DMF), *N*,*N*′-dicyclohexylcarbodiimide (DCC), leucylglycine, glycylphenylalanine, phthalaldehyde (OPA), *N*-ethyldiisopropylamine, dimethyl sulfoxide (DMSO), *tert*-butyl carbazate, hydrazine hydrate, trifluoroacetic acid (TFA), and DOX hydrochloride (DOX.HCl) were purchased from Fluka. 2,4,6-Trinitrobenzene-1-sulfonic acid (TNBSA) was purchased from Serva, Heidelberg, Germany.

Synthesis of monomers

N-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized as described in Ref. 3. M.P. 70°C; elemental analysis: calcd., C 58.72, H 9.15, N 9.78; found, C 58.98, H 9.18, N 9.82.

N-(*tert*-butoxycarbonyl)-*N*′-(6-methacrylamidohexanoyl)hydrazine (Ma-ah-NHNH-Boc) was prepared in two-step synthesis as described in Ref. 20. M.P. 110–114°C, elemental analysis: calcd. C 57.70 C, H 8.33, N 13.46; found C 57.96, H 8.64, N 13.25.

6-Methacrylamidohexanohydrazide (Ma-ah-$NHNH_2$) was prepared by a two-step procedure as follows: methyl 6-aminohexanoate hydrochloride (30 g, 0.165 mol) was dissolved under stirring at room temperature in 350 mL of dichlormethane with addition of 4-(1,1,3,3-tetramethylbutyl)pyrocatechol as inhibitor. The solution was cooled down to 10°C, anhydrous sodium carbonate (50 g, 0.48 mol) was added, and temperature was decreased to 5°C. A solution of methacryloyl chloride (17.3 g, 0.165 mol) in 100 mL of dichloromethane was added dropwise under vigorous stirring at temperatures below 10°C. After adding the whole amount of methacryloyl chloride, the reaction mixture was stirred for 45 min at 15°C and then for 30 min at 25°C. After that, insoluble inorganic compounds were filtered off and dichloromethane was evaporated. The crude oily methyl 6-methacrylamidohexanoate was dissolved in 153 mL methanol, hydrazine hydrate (32 mL $\approx$ 33 g, 0.66 mol) was added, and the reaction mixture was gently stirred at 24°C. After 6 h, methanol was removed by distillation at reduced pressure, and an oily residue was dissolved in 150 mL of propan-2-ol. The solvent was again removed by distillation at reduced pressure, and the process was repeated twice with the aim to remove unreacted hydrazine hydrate. Then the product was dissolved in 300 mL of ethyl acetate, and the crude product was obtained by crystallization at −18°C. The crystals were isolated by filtration and washed with cold ethyl acetate. The product was recrystallized from a mixture dichloromethane/ethyl acetate (1 : 1). M.P. 79–81°C; elemental analysis: calcd., C 56.32, H 8.98, N 19.70; found, C 56.49, H 8.63, N 19.83. ^{1}H NMR 300 MHz ($CDCl_3$, 297 K): 1.35 m (2H, $CH_2(CH_2)_2$—N); 1.50–1.69 m (4H, $CH_2CH_2CH_2CH_2$—N); 1.95 dd (3H, CH_3); 2.17 t (2H, ((C=O)—CH_2); 3.26 dt (2H, *N*—CH_2); 3.91 s (2H, NH_2); 5.30 t (1H, C=CH_2 E); 5.67 t (1H, C=CH_2 Z); 6.10 s (1H, $NHNH_2$); 7.45 s (1H, NH—CH_2).

(*N*-Methacryloylglycyl)phenylalanylleucylglycinohydrazide (Ma-GFLG-$NHNH_2$) was prepared by a two-step procedure using the same conditions as described earlier for Ma-ah-$NHNH_2$. H-Gly-DL-Phe-Leu-Gly-OMe.TFA was prepared by a two-step synthesis described in Ref. 22. M.P. 160–162°C.

H-Gly-DL-Phe-Leu-Gly-OMe.TFA (22.32 g, 0.05 mol), dichloromethane 510 mL (270 + 240 mL), 4-(1,1,3,3-tetramethylbutyl)pyrocatechol (60 mg), sodium carbonate (15.1 g, 0.145 mol), and methacryloyl chloride (5.2 g, 0.05 mol) were used for the synthesis of methacryloylated oligopeptide methyl ester. Then,

TABLE I
Characteristics of Polymer Precursors

Polymer precursor	Spacer	Initiator (wt %)	Comonomer	M_w	M_w/M_n	Hydrazide content (mol %)
1	ah	1.0	Boc-hydrazide[a]	24200	1.72	5.3
2	ah	0.6	hydrazide[b]	30500	1.81	5.6
3	ah	0.8	hydrazide[b]	26300	1.85	5.7
4	ah	1.0	hydrazide[b]	17800	1.65	5.7
5	ah	1.0	hydrazide[b]	18000	1.71	11.2
6	GFLG	1.0	hydrazide[b]	18400	1.80	5.6

[a] MA-ah-NHNH-Boc.
[b] MA-ah-$NHNH_2$ or MA-GFLG-$NHNH_2$.

the methyl ester was used for hydrazinolysis with 10 g (0.2 mol) hydrazine hydrate and after 7 h of reaction, the unreacted hydrazine hydrate was removed by triple distillation with propan-2-ol, and the product was obtained by crystallization from a dichloromethane/ethyl acetate mixture. M.P. 139–140°C; elemental analysis: calcd., C 58.10, H 7.36, N 17.68; found, C 58.21, H 7.39, N 17.54. Amino acid analysis: Gly/L-Phe/D-Phe/L-Leu/D-Leu 2.03 : 0.5 : 0.47 : 1.00 : 0.01. HPLC showed two peaks of equal areas at 19.39 min (L-Phe peptide) and 19.91 min (D-Phe peptide).

6-Methacrylamidohexanohydrazide-DOX (Ma-ah-NHN = DOX) was prepared as follows: 6-methacrylamidohexanohydrazide (40 mg, 0.188 mmol) was dissolved at 25°C in 6 mL of methanol. DOX.HCl (115 mg; 0.198 mmol) was added to this solution, and the suspension was intensively stirred in the dark. After 5 min, 310 μL of acetic acid was added to the suspension, and the reaction mixture was stirred at 25°C for 24 h. The reaction was monitored by TLC (silica gel 60 F_{254}) (methanol : chloroform : acetic acid 2 : 8 : 1, R_f(DOX) = 0.75, R_f(Ma-ah-NHN = DOX) = 0.9). After 24 h, the reaction mixture was clarified. Hundred milligrams of the polymer-containing hydrazide groups (**4**) were added to the reaction mixture to remove unreacted DOX, and the mixture was stirred for another 4 h. The product was purified from the polymer and low-molecular-weight impurities by gel permeation chromatography (Sephadex LH20, column 1.5 × 60 cm, eluent methanol). The low-molecular-weight fraction containing the product was collected, methanol was evaporated, and the product was isolated by precipitation into diethyl ether.

Yield: 76%; M.P. 172–175°C; TLC on Silicagel 60 F_{254} (methanol : chloroform : acetic acid 2 : 8 : 1) one spot at R_f = 0.9. MALDI-TOF MS: 762.2 (M + Na).

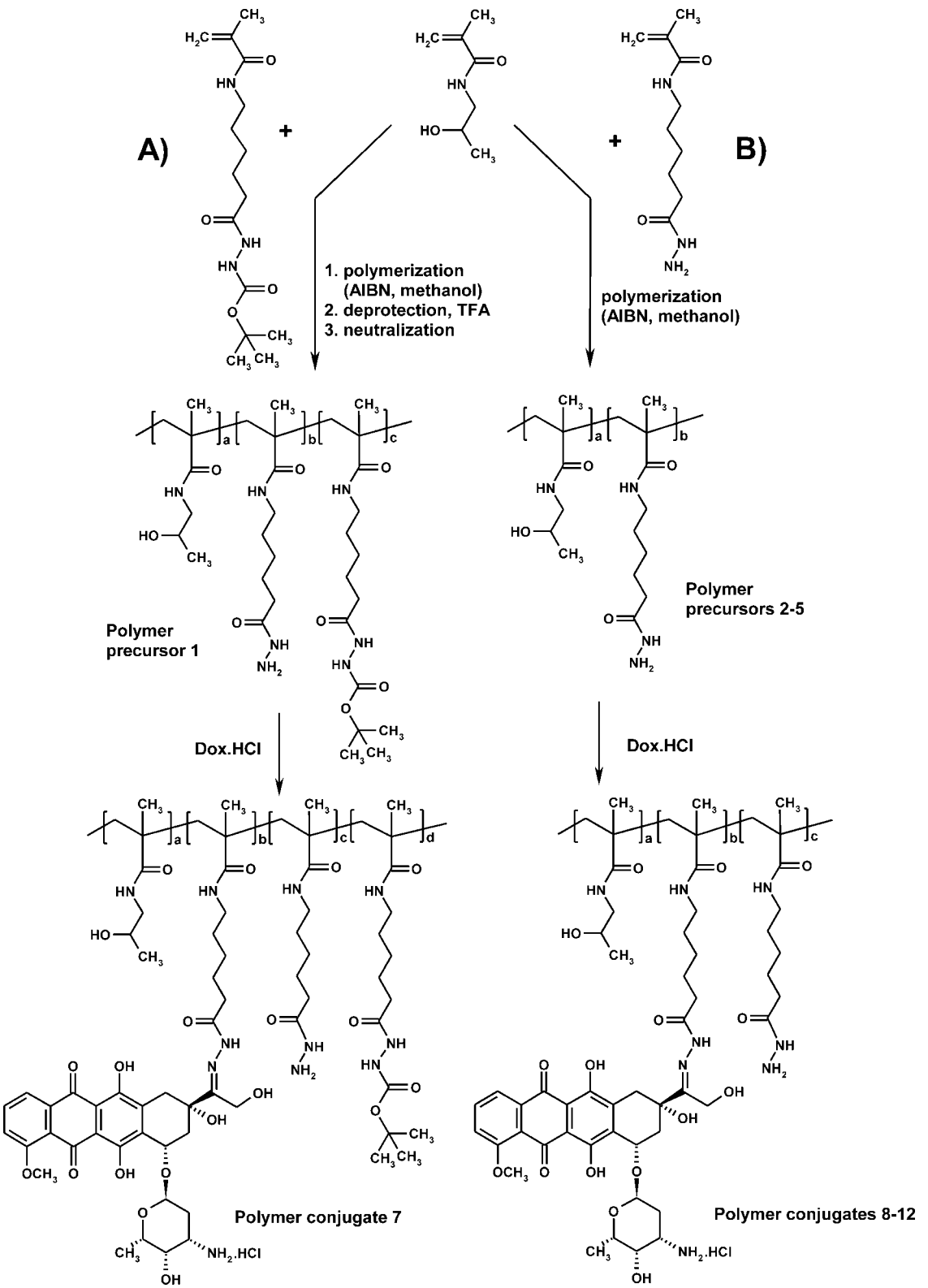
(*N*-Methacryloylglycyl)phenylalanylleucylglycinohydrazide-DOX (Ma-GFLG-NHN = DOX) was prepared by the same procedure as Ma-ah-NHN = DOX. Briefly (*N*-methacryloylglycyl)phenylalanylleucylglycinohydrazide (122 mg, 0,258 mmol) was dissolved at 25°C in 8 mL of methanol. DOX.HCl (157 mg; 0.271 mmol) was added to this solution, and the suspension was intensively stirred in the dark. After 5 min, 420 μL of acetic acid was added to the suspension, and the reaction mixture was stirred for 24 h at 25°C. Then, 130 mg of the polymer-containing hydrazide groups (**4**) was added to the reaction mixture to remove unreacted DOX, and the mixture was stirred for another 5 h. The product was purified (removing polymer and low-molecular-weight impurities) by gel permeation chromatography. The low-molecular-weight fraction containing the product was collected, methanol was evaporated, and the product was isolated by precipitation into diethyl ether. Yield: 74%; M.P. 179–182°C; TLC on silica gel 60 F_{254} (methanol : chloroform : acetic acid 2 : 8 : 1) one spot at R_f = 0.95. MALDI-TOF MS: 1023.5 (M + Na).

Purity of all monomers was examined by HPLC (LDC Analytical, USA) using a reverse-phase column Tessek SGX C_{18} (15 × 33 mm) with UV detection at 230 nm, eluent water–methanol with methanol gradient 50–100 vol %, flow rate 0.5 mL/min.

Synthesis of polymer precursors

Random copolymer of HPMA with Ma-ah-NHNH-Boc (polymer **1**, Table I, Scheme 1) was prepared by radical polymerization in methanol (AIBN, 1 wt %; monomer concentration 14 wt %; molar ratio HPMA : Ma-ah-NHNH-Boc 93 : 7; 60°C; 23 h) as described in Ref. 20. Free hydrazide groups of polymer **1** were prepared by deprotection of Boc-hydrazide in concentrated TFA.

Statistical copolymers of HPMA with Ma-ah-$NHNH_2$ or Ma-GFLG-$NHNH_2$ (polymers **2–6**, Table I, Scheme 1) containing free hydrazide groups were prepared by radical copolymerization in methanol (AIBN, 0.6–1.0 wt %; monomer concentration 18 wt %; molar ratio HPMA: Ma-ah-$NHNH_2$/Ma-GFLG-$NHNH_2$ 93 : 7 or 88 : 12; 60°C; 17 h). Example of polymerization: HPMA (2.0 g, 14 mmol), Ma-ah-$NHNH_2$ (227 mg, 1.06 mmol), and AIBN (96 mg,



Scheme 1 Scheme of the synthesis of polymer precursors and polymer conjugates. (A) former synthesis using Ma-ah-NHNH-Boc (protected hydrazide groups); (B) new synthesis using Ma-ah-$NHNH_2$.

0.58 mmol) was dissolved in methanol (12.7 mL). The solution was introduced into a polymerization ampoule, bubbled with nitrogen, and sealed. The polymerization was carried out at 60°C for 17 h. The polymer was isolated by precipitation into ethyl acetate and purified by reprecipitation from methanol

TABLE II
Characteristics of Polymer-DOX Conjugates

Polymer conjugate	Polymer precursor	Spacer	M_w	M_w/M_n	DOX (wt /mol %)	A_2[a]
7[b]	**1**	ah	25000	1.80	9.9/2.8	nd
8[b]	**4**	ah	19500	1.65	5.1/1.4	nd
9[b]	**4**	ah	20800	1.76	10.1/2.8	5×10^{-4}
10[b]	**4**	ah	21200	1.73	13.2/3.7	-8.1×10^{-6}
11[b]	**5**	ah	24200	1.84	16.7/4.7	-3.2×10^{-4}
12[b]	**5**	ah	25300	1.75	22.5/6.2	nd
13[b]	**6**	GFLG	21000	1.86	9.8/2.7	nd
14[c]	–	ah	34100	1.72	10.3/2.9	nd
15[c]	–	GFLG	32500	1.82	7.7/2.1	nd

[a] A_2 is a second virial coefficient.
[b] Polymer conjugate prepared from appropriate polymer precursor.
[c] Polymer conjugate prepared by copolymerization (MA-ah-NHN=DOX or MA-GFLG-NHN=DOX).

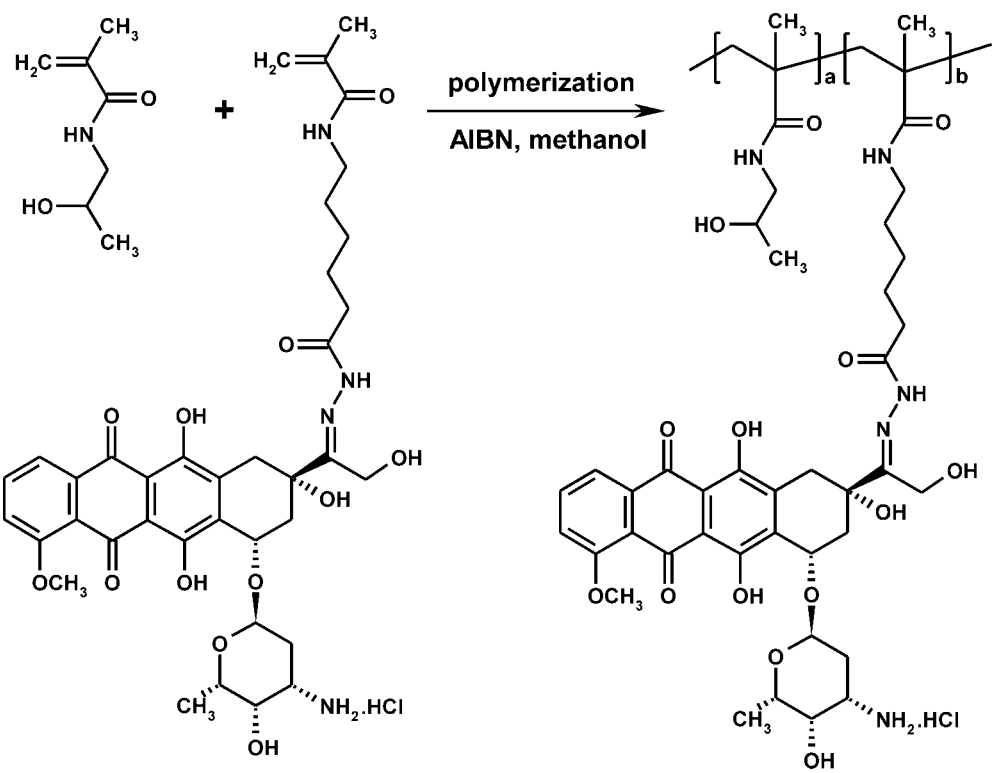
solution into ethyl acetate. The polymer was filtered off, washed with ethyl acetate, and dried in vacuum. The yield was 1.76 g (78.5%).

Attachment of DOX to polymer precursors

Polymer-DOX conjugates (conjugates **7–13**, Table II, Scheme 1) were prepared by the reaction of corresponding polymer precursors containing hydrazide groups with DOX.HCl in methanol in the dark as described.[10] The polymer-drug conjugates were purified removing low-molecular-weight impurities (DOX or its degradation products) by gel filtration using a Sephadex LH-20 column and methanol as eluent.

Synthesis of polymer conjugates via copolymerization with a DOX-containing monomer

Statistical copolymers poly(HPMA-*co*-Ma-ah-NHN = DOX) or poly(HPMA-*co*-Ma-GFLG-NHN = DOX) (polymers **14–15**, Table II, Scheme 2) containing DOX attached via hydrazone bond were prepared by radi-



Scheme 2 Scheme of the synthesis of polymer conjugate **14** via copolymerization with Ma-ah-NHN = DOX (drug-containing monomer).

cal copolymerization in methanol (AIBN, 0.8 wt %; monomer concentration 18 wt %; molar ratio HPMA : Ma-ah-NHN = DOX/Ma-GFLG-NHN = DOX 96.5 : 3.5; 60°C; 22 h). Example of polymerization: HPMA (840 mg, 5.87 mmol), Ma-ah-NHN = DOX (165 mg, 0.21 mmol), and AIBN (67 mg, 0.40 mmol) were dissolved in methanol (5.7 mL). The solution was placed into a polymerization ampoule, bubbled with nitrogen, and sealed. The polymerization was carried out at 60°C for 22 h. The polymer was isolated by precipitation into ethyl acetate and low-molecular-weight impurities and free DOX were removed by gel filtration (Sephadex LH 20, methanol). The polymer fraction was collected, and polymer conjugate was isolated by precipitation into ethyl acetate. The polymer was filtered off, washed with ethyl acetate, and dried in vacuum. The yield was 750 mg (75%).

Purification and characterization of polymers and conjugates

All the conjugates were characterized and tested for the content of the free polymer or free drug using a HPLC Shimadzu equipped with GPC columns Superose™ 6 or Superose™ 12 and TLC (Kieselgel 60 F_{254}). In addition, the content of free DOX was determined by HPLC Shimadzu after extraction of DOX from aqueous solution of the conjugate to chloroform or by GPC in aqueous methanol solution (Shimadzu HPLC system equipped with GPC column TSK gel G3000SWxl (300 × 7.8 mm; 5 μm); mobile phase 20% of 0.3*M* acetate buffer (CH_3COONa/CH_3COOH; pH = 6.5; 0.5 g/L NaN_3) and 80% of methanol; flow rate 0.5 mL/min) with UV–vis detection (Shimadzu SPD-10AVvp) (λ = 488 nm) from the area of the peaks corresponding to the free and polymer-bound DOX.

The total content of DOX in polymer conjugates was determined spectrophotometrically on Helios α (Thermochrom) spectrophotometer. Molar absorption coefficients of the free drug (ε_{488} = 11,500 L mol^{-1} cm^{-1} (water)) and modified DOX (Ma-ah-NHN = DOX: ε_{488} (water) = 9800 L mol^{-1} cm^{-1}; Ma-GFLG-NHN = DOX: ε_{488} (water) = 9600 L mol^{-1} cm^{-1}) were used for the calculation of DOX content.

Determination of molecular weight and polydispersity of conjugates was carried out with a HPLC Shimadzu system equipped with RI, UV, and multi-angle light scattering DAWN EOS (Wyatt, USA) detectors using 0.3*M* acetate buffer pH 6.5 and Superose 12 or Superose 6 column.

The content of hydrazide-terminated side chains in polymer precursors was determined by a modified TNBSA assay as described.[10] Molar absorption coefficient ε_{500} = 17,200 L mol^{-1} cm^{-1} (λ = 500 nm) estimated for the model reaction of MA-AH-$NHNH_2$ or MA-GFLG-$NHNH_2$ with TNBSA was used.

The static (SLS) and dynamic (DLS) light scattering of aqueous conjugate solutions were measured at the scattering angle 173° on a Nano-ZS, Model ZEN3600 (Malvern, UK) zetasizer. The apparent weight average of the molecular-weight (M_w^a) and the hydrodynamic radius (R_h^a) were determined by the DTS (Nano) program. The mean positions of the peaks in the intensity-hydrodynamic radius distribution were taken for R_h^a representation. The value dn/dc = 0.174 for poly(HPMA-*co*-MA-AH-NHN = DOX in the phosphate buffer (0.15*M*, pH = 7.4) was used in M_w^a calculations.

The static light scattering data were analyzed by a simplified Zimm plot procedure. The scattering angle dependence was neglected, which seems to be justified because of the low molecular weight of conjugates under investigation (M_w = 18,000–28,000). The weight–average molecular weight M_w and R_h of conjugates were obtained by linear extrapolation of $K_c/\Delta R$ (=$1/M_w^a$) and $1/R_h^a$ to zero concentration; ΔR is the Rayleigh ratio of the scattering intensity and K is the contrast factor containing the optical parameters.

In vitro release of DOX from conjugates

The rate of DOX release from conjugates (concentration equivalent to 0.5 m*M* DOX) was investigated in phosphate buffers at pH 5.0, 6.0, or 7.4 (0.1*M* phosphate buffer with 0.05*M* NaCl) at 37°C using extraction of released DOX into organic solvent followed by HPLC analysis as described previously[16] or using GPC in aqueous methanol solution as described earlier.

Blood clearance and DOX content in tumor tissue

C57BL/6 (B/6) male mice were subcutaneously inoculated with 1 × 10^5 EL4 lymphoma cells. When the tumor reached a volume of 50–75 mm^3, the mice were intravenously injected either DOX.HCl (5 mg/kg) or the conjugate **9** (15 mg/kg). Then, blood, urine, and tumor tissue samples were taken at the following times after injection: 0.5, 1, 3, 6, 12, 24, 48, and 96 h (blood and urine), and 12, 24, 48, and 96 h (tumors). The blood samples were collected into heparinized tubes, and tumors were excised, weighed, and homogenized. The samples were tested for the total content of DOX, that is, the sum of free and polymer-bound DOX. Determination of the total DOX content was performed after quantitative acid hydrolysis in 1*M* HCl. After incubation for 1 h at 50°C, doxorubicinone (formed aglycon of DOX) was extracted with chloroform, the organic phase was evaporated to dryness, the remaining solid completely dissolved in methanol and analyzed using a gradient-based HPLC Shimadzu system equipped

with fluorescence detector (Shimadzu RF-10Axl) (λ_{exc} = 488 nm, λ_{em} = 560 nm). The calibration of the described method was carried out by injection of exact amounts of free DOX.HCl into blood, urine, and tumor samples obtained from untreated animals and then analyzed (homogenized, hydrolyzed, and extracted) as described earlier.

Cell lines

Two different cell lines—EL4 murine T cell lymphoma and Raji human B cell Burkitt's lymphoma—were used for *in vitro* studies. Both cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD). Raji human B cell lymphoma was cultivated in RPMI 1640 medium with L-glutamine (2 m*M*), sodium hydrogencarbonate (1.5 mg/mL), sodium pyruvate (110 μg/mL), 2-sulfanylethanol (5×10^{-5}*M*), HEPES (10 m*M*), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% v/v fetal bovine serum. Routine culture and *in vitro* experiments with T cell lymphoma EL4 were conducted in RPMI 1640 medium with extra L-glutamine (4 m*M*) and 2-sulfanylethanol (5×10^{-5}*M*); EL4 murine T cell lymphoma was used for *in vivo* studies as well.

In vitro cytotoxicity of conjugates

Cytotoxic potential of the drugs was assessed using MTT assay. Ninety-six-well flat-bottomed plates (NUNC) were seeded with 1×10^4 EL4 or Raji cells. The tested samples (in triplicates) were then added to the wells to achieve the desired concentrations (range, 0.0002–40.0 μg/mL). The plates were cultured in 5% CO_2 for 72 h at 37°C. A solution of a tetrazolium salt (MTT, 5 mg/mL, 20 μL) was added 3 h before the end of incubation. The insoluble purple formazan crystals produced by metabolically active cells were then dissolved in DMSO (200 μL per well), and the plates were read using an ELISA plate reader at a wavelength of 570 nm (reference wavelength 690 nm). The amount of color produced is directly proportional to the number of viable cells. Cells cultivated in fresh medium were used as controls.

Experimental animals

Inbred strain of mice C57BL/6 (H-2^b) male, aged 8–12 weeks, was purchased from the Animal Centre of the Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i. All mice were housed in accordance with approved guidelines and provided with food and water *ad libitum*. All studies were approved by the Institutional Review Board.

In vivo antitumor activity of the conjugates

C57BL/6 males were subcutaneously transplanted with 1×10^5 EL4 T cell lymphoma cells on the right flank on day 0. The mice that developed palpable tumors reaching a volume of 50–75 mm^3 within 10 days after the implantation were intravenously treated with the conjugates, diluted with PBS, as described in the Results. Controls were transplanted with the tumor cells and treated with PBS alone. The animals were observed three times a week searching for signs of tumor progression. The survival time, tumor size, and the number of long-term survivors were determined. Experimental groups were composed of eight animals.

RESULTS AND DISCUSSION

As it was shown previously,[10,16–18] HPMA-based copolymers containing the anticancer drug DOX bound to the polymer via a pH-sensitive hydrazone linkage (hydrazone conjugates) exhibit a high level of cytostatic activity in several cancer cell lines and show a significant therapeutic effect in an experimental model of EL4 lymphoma-bearing mice. These conjugates are quite stable in a phosphate buffer of pH 7.4, modeling conditions during transport in the blood stream, but release pharmacologically active-free DOX in buffers modeling mild acid environment in endosomes of target cells (pH 5–6).

Structure of the hydrazone conjugates synthesized by the new strategy described in this article is better defined than that of the conjugate prepared from Boc-protected polymer precursors. Polymer precursors prepared by direct copolymerization of HPMA with Ma-ah-$NHNH_2$ does not contain neither monomer units with carboxylic groups nor units with Boc protecting groups usually remaining in the polymer precursor and also in the final copolymer prepared by the original synthetic procedure. In case of HPMA copolymers prepared by copolymerization with DOX-containing comonomers, they do not contain even units with hydrazide groups always remaining in the conjugate prepared by one of the former methods (Scheme 1). Moreover, reproducibility of the new procedure is much higher than that of original method, the reaction encompasses less reaction steps and enable cheaper and reproducible high-scale production.

Synthesis of monomers and copolymers

The original synthesis[10] of hydrazone conjugates consists of preparation of polymers bearing in their side chains reactive 4-nitrophenyl esters, which are subsequently transformed to hydrazide groups by hydrazinolysis with hydrazine hydrate. This method,

however, has many drawbacks, for example, some of the reactive ester groups are hydrolyzed during the polymerization, and the resulting polymer precursor is therefore not well defined. Polymer precursors contained usually from 0.2 to 0.8 mol % monomer units with free carboxylic groups, which affect subsequently stability of polymer conjugates. Moreover, 4-nitrophenoxy groups affect polymerization by chain-transfer reaction and limit the molecular weight of polymers to 25,000 as maximum. Later on, synthesis using monomers with Boc-protected hydrazide group was developed.[20] This method partly overcame drawbacks of the original synthesis and enabled preparation of better-defined polymer (no hydrolysis of esters) precursors, which could be prepared with a broad range of molecular weights. Unfortunately, the last part of this synthesis, deprotection of hydrazide groups in TFA, was rather a troublesome step. After the deprotection was carried out in a high excess of TFA, removal of a bound and adsorbed TFA and its anions from the polymer was a difficult, time-consuming, and scale-limiting procedure. Moreover, NMR data of the copolymer suggest that the deprotection was not complete. After the removal of TFA by codistillation with solvents, followed by neutralization, purification by gel filtration and freeze-drying there were still present traces of remaining TFA influencing consecutive reactions.

Here, we describe a new method of synthesis overcoming the above-mentioned cumbersome step by use of new types of monomers. The monomers with free hydrazide group (Ma-ah-$NHNH_2$ or Ma-GFLG-$NHNH_2$) were prepared, and their ability to copolymerize with HPMA was studied (Table I). The monomers were prepared by a two-step procedure in high yield (around 70%). First, methyl ester of 6-aminohexanoic acid or a tetrapeptide was methacryloylated with methacryloyl chloride, and the resulting methyl ester was hydrazinolysed with hydrazine hydrate. This synthesis enables easy preparation of the monomer and simplifies the synthesis of polymeric precursors because the deprotection step is not needed in this case. The synthesis of monomers Ma-ah-$NHNH_2$ and Ma-GFLG-$NHNH_2$ also allows the improvement of the analytical method used earlier[10] for the determination of hydrazide groups content in the copolymer. A new improved method of analysis was developed. In this analysis, despite a molar absorption coefficient obtained in the reaction of TNBSA with ethyl carbazate as described in a literature, the new molar absorption coefficients were obtained by the reaction of TNBSA with the respective new monomers [ε_{500} (Ma-ah-$NHNH_2$) = 17,200 L mol^{-1} cm^{-1} and ε_{500} (MA-GFLG-$NHNH_2$) = 17,800 L mol^{-1} cm^{-1}] and used for the determination of hydrazide groups content in respective polymers.

Molecular weights, polydispersity, and the content of hydrazide groups in copolymers are shown in Table I. The copolymers prepared from the new hydrazide group-containing monomers have similar characteristics as the copolymer prepared from the respective Boc-protected monomer. In addition to the simpler synthetic procedure, molecular weights and the hydrazide groups content only hardly controllable in the original synthesis (due to incomplete deprotection reaction) can now be sensitively controlled by changing the composition of polymerization mixture, for example, by changing initiator concentration as it is shown in Table I (samples 2–4). Moreover, in contrast to the previously described methods of synthesis, new polymer precursors are better defined because their polymer chains contain only monomer HPMA and Ma-ah-$NHNH_2$/MA-GFLG-$NHNH_2$ units without contamination with carboxylic groups originated by hydrolysis during hydrazinolysis, remaining Boc-protected hydrazide groups, and undefined amount of TFA adsorbed on the polymer. In the original synthesis, these contaminating structures with hardly to determine content also decreased yield of the DOX-binding reaction (about 90%).

Attachment of DOX to polymer precursors

The use of polymer precursors based on highly hydrophilic poly(HPMA) copolymers facilitated the synthesis of polymer conjugates with a wide range of drug content. Attachment of DOX to the polymer precursors was performed in methanol in the presence of acetic acid by a procedure described earlier.[10] Depending on the structure of the copolymers used for the synthesis and amounts of DOX, conjugates with the DOX content ranging from 1.5 to 7 mol %, that is, from 5 to 23 wt %, were prepared. All polymer conjugates with different loading of the drug were well soluble in physiological saline solution giving even high concentrations, above 150 mg/mL. In all cases, the attachment of DOX had no dramatic influence on the molecular weight and distribution of molecular weights of the polymer conjugates. Detailed analysis of the reaction products revealed that this method of DOX attachment proceeds high yields, in most cases reaching 99%. As can be seen from the content of hydrazide groups in polymer precursors and in final conjugates (Tables I and II), there are some free hydrazide groups remaining in the conjugate structure (2–5 mol %) after conjugation with DOX. These groups can be used, if need be, for subsequent attachment of targeting moiety or preparation of grafted high-molecular-weight structures (paper in preparation for *J Controlled Release*). Nevertheless, previous physicochemical studies and evaluation of biological properties of the hydrazone HPMAcopolymer-DOX conjugates

revealed[16–20] that the presence of small amount of free hydrazide groups in a copolymer (up to 5 mol %) do not influence significantly neither its solution properties nor *in vitro* and *in vivo* behavior.

All the above-mentioned results allow us to assume that HPMA-based polymers containing hydrazide groups are useful candidates for a large-scale synthesis of DOX-containing water-soluble drug-delivery systems.

Synthesis of polymer conjugates via copolymerization with DOX-containing monomers

Another method of the synthesis of hydrazone conjugates consists of direct copolymerization of HPMA with DOX-containing monomer. Polymer conjugates **14** and **15** (Table 2, Scheme 2), prepared by direct copolymerization, showed a well-defined structure consisting only of HPMA and drug-bearing monomer units, and no free hydrazide groups were observed in the structure of polymer conjugates **14–15**. Molecular weights of polymer conjugates **14–15** were slightly higher than those of conjugates prepared by the reaction of hydrazide groups of polymer precursors with DOX, but still remained below the limit of renal threshold for HPMA-based polymer conjugates ($\sim$ 50,000 g/mol).[23] This method of synthesis enables synthesis of well-defined structure but, probably, it is not suitable for large-scale production due to the high loss of expensive DOX because of lower yield of copolymerization (65–70%).

In addition, the synthesis of monomers Ma-ah-NHN = DOX or Ma-GFLG-NHN = DOX enabled us to improve the analytical method of the determination of drug content in the conjugates. Usually, the content of DOX was determined spectrophotometrically using the molar absorption coefficient estimated for free DOX.HCl [ε_{488} (water) = 11,500 L mol^{-1} cm^{-1} or ε_{488} (methanol) = 13,500 L mol^{-1} cm^{-1}). It was found that the absorption coefficient of monomeric DOX is lower than that of free DOX. The UV–vis spectra of both free and hydrazone-bound DOX were similar, but their intensity was lower in the case of the bound drug. The absorption coefficient is only slightly dependent on the detailed structure of the monomer, for MA-ah-NHN = DOX, we found ε_{488} (water) = 9800 L mol^{-1} cm^{-1} or ε_{488} (methanol) = 11,200 L mol^{-1} cm^{-1} and for MA-GFLG-NHN = DOX ε_{488} (water) = 9600 L mol^{-1} cm^{-1} or ε_{488} (methanol) = 10,900 L mol^{-1} cm^{-1}. As a control, the content of DOX in the conjugates was also measured as an amount of doxorubicinone (aglycon of DOX) originating after acid hydrolysis of glycoside bond in DOX. Both the methods showed similar values of the DOX content. The UV–vis spectrophotometrical method using the new exact molar absorption coefficients proved to be a suitable, easy, and precise method for determination of DOX content in HPMA-based polymer conjugates.

Physical characterization of solution behavior of conjugates

To further characterize solution behavior of the conjugates, we provide $K_c/\Delta R$ values obtained from the SLS measurements, which are plotted as a function of the conjugate concentration c for three conjugates (**9, 10,** and **11)** differing in the molar content of DOX (Fig. 1).

The second virial coefficients A_2 calculated from the slopes of light scattering curves in Figure 1 are given in Table II. The A_2 values range from positive at lower molar contents of DOX (conjugate **9**) to negative value at higher molar content of DOX (conjugate **11**), which reflects growing hydrophobicity of the conjugates with increasing content of DOX attached to an originally highly hydrophilic HPMA copolymer. The hydrophobic character of DOX bound to the conjugates becomes significant after drug loading exceeds 13 wt %. The M_w values obtained from the light-scattering experiment (not shown) differ from those obtained by GPC in aqueous solutions by 10%.

The R_h values obtained by a linear extrapolation to zero concentration showed no significant increase with increasing amount of attached hydrophobic drug (4.2 nm for conjugate **7**; 4.2 nm for conjugate **10**; and 4.6 nm for conjugate **11**). The small increase in R_h found for conjugate **11** could be caused by the presence of a very small amount of aggregates or micelles formed in solution due to hydrophobic interactions of polymer chains.

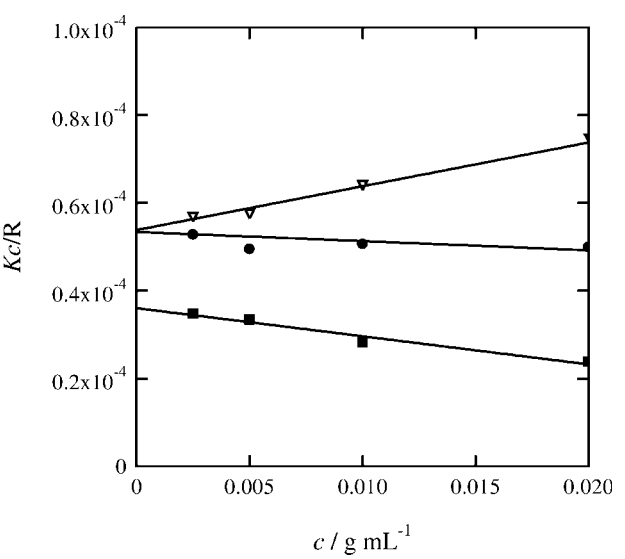


Figure 1 Zimm plot of scattering curves of the conjugates. Full lines are linear fits to the light scattering curves; (▲) conjugate **9**, (●) conjugate **10**, and (■) conjugate **11**.

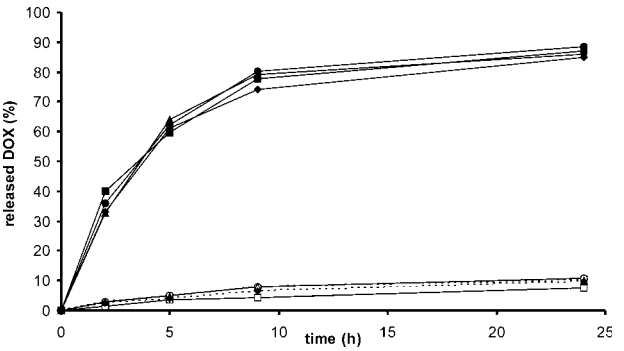


Figure 2 Release profiles of DOX from polymer-DOX conjugates incubated in phosphate buffers at 37°C. (■ —) conjugate **8**, pH 5; (● —) conjugate **9**, pH 5; (▲ —) conjugate **12**, pH 5; (◆ —) conjugate **14**, pH 5; (□ —) conjugate **8**, pH 7.4; (○ —) conjugate **9**, pH 7.4; (Δ —) conjugate **12**, pH 7.4; and (◆ —) conjugate **14**, pH 7.4.

Release of DOX from the conjugates

The measurements of DOX release *in vitro* showed that all conjugates are quite stable in buffer solutions at pH 7.4 and 37°C (Fig. 2 and Table III), with only a minor release of DOX being observed after 24 h of incubation. On the contrary, ~ 80–90% of DOX was released within 24 h in a buffer of pH 5.0 (37°C) and the halftime of hydrolysis (i.e., the time when 50% of the bound DOX was released) was ~ 50–56 h in a buffer of pH 6 (37°C). The rate of DOX release in a buffer of pH 5, 6, or 7.4 depends only slightly on the detailed structure and the DOX content in the conjugates. The observed slower release of DOX from conjugates containing GFLG spacer in a buffer of pH 5 corroborated our previously published results.[16] As we showed earlier, in the case of conjugates with GFLG spacer, the lower rate of chemical hydrolysis could be compensated by enzymatic degradation of the spacer in secondary lysosomes. We can conclude that neither the amount of drug attached to the polymer carrier nor the method of synthesis (copolymerization of DOX-containing monomers or monomers containing free hydrazide groups) significantly affect the rate of the drug release. To resume, all prepared conjugates fulfill the criteria for anticancer drug-delivery systems, that is, stability in blood circulation and release of active drug after entering tumor cell/tissue due to a pH decrease.

TABLE III
Stability of Polymer-DOX Conjugates at pH 5.0, 6.0, and 7.4 in Phosphate Buffers at 37°C

Conjugate	t_{50} (h)[a] at pH 5.0	t_{50} (h)[a] at pH 6.0	Stability at pH 7.4[b]
7	~ 4.8	~ 52	8.8
8	~ 3.5	~ 50	8.4
9	~ 3.6	n.d.	8.9
10	~ 3.5	~ 49	9.7
11	~ 3.6	n.d.	10.3
12	~ 3.5	~ 50	10.0
13	~ 6.1	~ 56	9.7
14	~ 3.8	n.d.	9.8
15	~ 6.5	n.d.	9.9

[a] Time after which the amount of released DOX is 50% of its initial value in the conjugate.
[b] The amount of released DOX from the conjugate after 24 h.

Blood clearance, tumor accumulation, and urine excretion

Blood, urine, and subcutaneous tumors of B/6 mice bearing EL4 lymphoma were collected at different time intervals after i.v. injection of free or polymer-bound DOX (conjugate **9**). The total DOX content in samples was determined by HPLC after their homogenization and hydrolysis with 1*M* HCl, followed by chloroform extraction of the DOX aglycon. The obtained data are given in Figure 3. As anticipated, the conjugates circulated in the bloodstream substantially longer than free DOX. The hydrazone conjugates show a biphasic clearance, with the half-time several times higher than that of free DOX 30 min after i.v. injection, the concentration of free DOX in blood was less than 2% of the initial dose but about 25% of polymer-bound DOX was still circulating in blood of mice. The results of determination of the DOX amount accumulated in the tumors were in good agreement with the above-discussed blood clearance profiles. We observed that the amount of DOX in tumors after application of the free drug was quite small and rapidly decreasing during the time. Actually, the intratumoral concentration was the same as the concentration of the drug in blood. In contrast, after application of the conjugate, we found a much higher amount of DOX in the tumors.

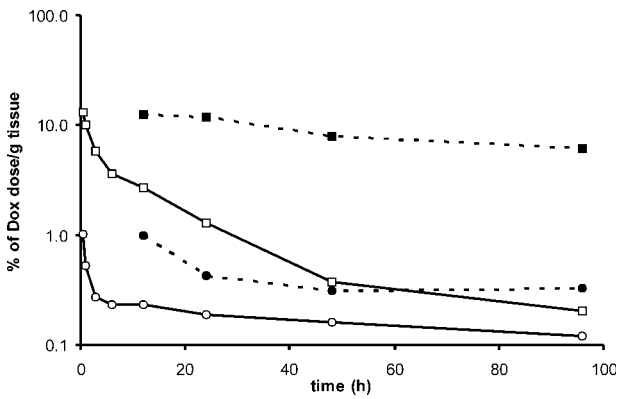


Figure 3 Blood clearance and tumor accumulation of free DOX HCl and polymer-DOX conjugate 9 in mice bearing EL4 lymphoma after i.v. injection; blood: (○ —) DOX HCl, (□ —) conjugate **9**; tumor: (● —) DOX HCl, and (■ —) conjugate **9**.

TABLE 4
Tumor-to-Blood Ratio of Free DOX.HCl and Polymer–DOX Conjugate 9 in Mice-Bearing EL4 Lymphoma

Time (h)	Tumor-to-blood ratio	
	Conjugate **9**	DOX.HCl
12	4.6	2.2
24	9.7	1.1
48	20.9	1.1
96	28.7	1.0

This amount was only slowly decreasing during the time interval of 12–96 h after the conjugate injection, and it was more than 10–15 times higher than in the case of free DOX injection. In fact, the concentration of the drug within tumor was 5–30-fold higher compared to blood. In other words, the tumor-to-blood ratio was increasing with time, which is an evidence that the prodrugs are passively accumulated within a tumor mass due to the enhanced permeability and retention effect (Table IV). The amount of the drug excreted with urine was also monitored: up to 80% of the injected conjugate is excreted within the first 12 hours after application. If the excreted amount of the drug is taken into consideration, it becomes evident that a significant proportion of the circulating drug is taken up and retained within the tumor (up to 8% of the drug remained in the body, that is, up to 60% of the drug remained in the body per gram of tumor tissue). It can be therefore concluded that the conjugates show a very favorable pharmacokinetic profile (a decreased rate of blood clearance), which together with the increased amount of drug retained within the tumor makes the described system a promising antitumor agent.

Antitumor efficacy of the conjugates—Preliminary results

The antitumor potential of the conjugates was determined both *in vitro* and *in vivo*. The cytotoxic properties *in vitro* were evaluated by MTT assay, and the inhibition of tumor cell growth was expressed as IC50, that is, the concentration of DOX or DOX equivalent that inhibits the cell growth by 50%. We found that all conjugates were highly toxic for both cell lines tested (see Table V)—Raji human B cell lymphoma and mouse EL4 T cell lymphoma—and that there were no significant differences between conjugates with different drug amounts or conjugates prepared by different syntheses. Generally, the cytotoxic activity of the hydrazone conjugates was approximately by one to two orders of magnitude lower than that of the free drug. This is not surprising as it was repeatedly demonstrated that conjugation of any low-molecular-weight drug to a polymer decreases its *in vitro* activity.[24]

TABLE V
Antitumor Cytotoxic Activity of the Drugs *In Vitro*

	EL 4 mouse T cell lymphoma	Raji human B cell lymphoma
Conjugate **8**	0.166 ± 0.130	0.271 ± 0.024
Conjugate **9**	0.106 ± 0.028	0.188 ± 0.089
Conjugate **10**	0.116 ± 0.032	0.232 ± 0.133
Conjugate **11**	0.071 ± 0.065	0.374 ± 0.358
Conjugate **12**	0.039 ± 0.014	0.122 ± 0.050
Conjugate **14**	0.108 ± 0.016	0.177 ± 0.075
DOX.HCl	0.008 ± 0.002	0.002 ± 0.001

Cytotoxicity *in vitro* was assessed by MTT assay, and the inhibition of tumor cell growth was expressed as IC_{50} (μg/mL).

The *in vivo* therapeutic effect of the hydrazone conjugates was evaluated on the EL4 T cell lymphoma-bearing C57BL/6 mice. Weight of animals, growth of established tumors, and overall survival of tumor-bearing mice were tested. Based on previous experiments,[18] the conjugates **7** and **9** were given at a dose of 1 × 15 mg/kg of conjugated DOX (on day 9), that is, a dose that is far from the MTD of the conjugate **7** and suboptimal for the treatment of established EL4 lymphoma. However, it was chosen to reveal possible differences in therapeutic efficacy of the conjugates **7** and **9**, that is, to reveal whether

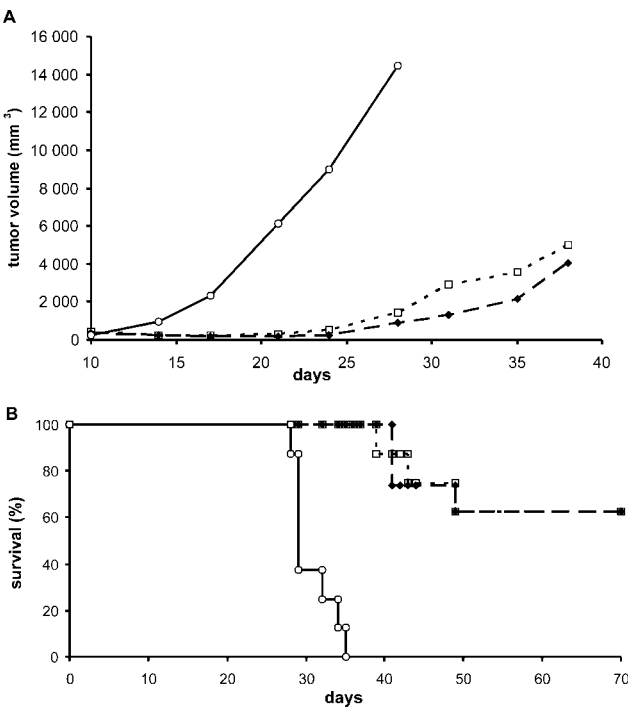


Figure 4 Effect of HPMA copolymer-doxorubicin conjugate with pH-controlled release of doxorubicin on the tumor growth (A) and overall survival (B) of C57BL/6 mice bearing EL4 lymphoma; (□ —) conjugate **7**; (◆ — —) conjugate **9**; (○ —) control (physiological solution). All treated groups survived significantly longer compared with untreated control (Student's *t*-test; $P < 0.05$).

the method of synthesis influences the therapeutic activity of the conjugate. Mice treated with saline were used as a control.

We have demonstrated that both conjugates are very potent in inhibition and retardation of tumor growth, substantially prolonged the overall survival of treated animals and cured more than 60% of treated animals (Fig. 4). Both conjugates caused the inhibition of tumor growth to the similar extent and cured the same proportion of the animals. In conclusion, *in vivo* experiments showed that the conjugates are highly therapeutically effective anticancer agents, and the method of synthesis does not significantly influence their antitumor activity. Detail experiments of *in vivo* antitumor activity are under way, and the results will be published in the near future.

CONCLUSIONS

A new method of synthesis of efficient anticancer drugs, HPMA copolymer, DOX conjugates (hydrazone conjugates) has been developed. In the synthesis, a copolymerization of HPMA with new methacrylamide-type monomers containing either hydrazide group or DOX was used. In comparison with earlier published synthesis, the new methods represent simple synthetic ways resulting in a polymer drugs with well-defined structures and allowing in the preparation of the polymer DOX conjugates with a broad range of molecular weights and content of attached drug. Copolymerization of HPMA with methacrylamide-type monomers containing hydrazide group also enable high-scale production of the polymer precursors and also drug conjugates, even in kilogram amounts. These conjugates are highly cytotoxic *in vitro*, which together with their favorable pharmacokinetic profile and enhanced accumulation within solid tumor tissue make them highly efficient also in a treatment of animal tumor model (mice, EL4 T cell lymphoma). The hydrazone conjugates prepared by herein introduced new method of synthesis are promising anticancer agents with high potential preferably for the treatment of solid tumors.

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