New Binary Thermoresponsive Polymeric System for Local Chemoradiotherapy

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ABSTRACT: Radiolabeled thermoresponsive polymers (TRPs) with cloud-point temperatures between room temperature and body temperature may have an advantage for local radiotherapeutical applications because TRPs may be isotopically labeled in solution at room temperature and injected as a solution, and at the site of application, the polymers form a depo because of phase separation at body temperature. A new polymeric drug-delivery system designed for combined local chemoradiotherapy with an injectable TRP bearing a radionuclide and the hydrophobic moiety doxorubicin (DOX) was synthesized and characterized. In the system, DOX served as an antiproliferative agent with known synergic effects with ionizing radiation and the hydrophobic moiety controlling bioerosion and elimination of the system at the same time. DOX was bound to the polymer carrier by a hydrolytically labile *N*-glycosylamine bond. Hydrolysis of the *N*-glycosylamine bond thus controlled the DOX release and dissolution of the system in the model aqueous milieu. DOX was slowly released during incubation in aqueous milieu at 37°C, which caused complete dissolution of the bioerodable polymer within about 2 weeks. The model radionuclide iodine 125, bound to a small amount of poly(*N*-isopropylacrylamide-*co*-*N*-methacryloyl tyrosinamide), was retained in the separated phase and also slowly dissolved during the incubation. J Appl Polym Sci 111: 2220–2228, 2009

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INTRODUCTION

The development of "smart polymers" that respond to external stimuli such as temperature, light, and magnetic fields have recently attracted much attention.[1] Thermoresponsive polymers (TRPs; sometimes also called *thermosensitive polymers*) with lower critical solubility temperature (LCST) represent probably one of the most promising groups of such polymers for medical applications.[1,2] TRPs with LCST are soluble in aqueous milieu at lower temperatures (e.g., room temperature), and at elevated temperatures (above the LCST), their polymer coil in solution collapses because of desolvation and subsequent hydrophobization of the polymer chain.[3,4] Hydrophobization causes aggregation and macroscopic phase separation, which can be seen as precipitation. The temperature at which macroscopic precipitation occurs is the most often studied temperature point in TRP characterization and is called the *cloud-point temperature* (CPT). The CPT is generally dependent on polymer concentration, and CPT $\geq$ LCST. TRPs with CPTs near body temperature [37°C; e.g., poly(isopropyl acrylamide), poly(isopropyl methacrylamide), polyphosphazenes, poly (methyl vinyl ether)] have recently been studied extensively as potential candidates for the synthesis of systems suitable for biomedical applications. Thermoresponsive micelles, hyperthermia-targeted liposomes, water-soluble drug-delivery systems, *in situ* formed drug depoes, and so on[5] are representative of such systems.

The thermoresponsive behavior and the existence of LCST is given by the competition of polymer chain hydration and the formation of hydrogen

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bonds between the polymer and water, which force the polymer to dissolve, and hydrophobic interactions, which force the polymer to precipitate. Because each of these interactions has a different temperature dependence, there exists a temperature above which the hydrophobic interactions cannot be compensated by solvation interactions and phase separation occurs. If a more hydrophobic monomeric unit is introduced by copolymerization, the CPT decreases because of the increased strength of hydrophobic interactions and vice versa; the copolymerization of more hydrophilic monomeric units causes an increase in CPT.[1,6] If a hydrophobic but hydrolytically labile monomeric unit is introduced into the polymer chain and its hydrolytic degradation leads to hydrophilization (e.g., by hydrophobic moiety cleavage from the polymer chain), the CPT of such a copolymer increases during degradation.[6,7] One can thus set the CPT under body temperature before degradation and above body temperature after degradation, which leads to solubilization during degradation because of the reversibility of phase separation. This may significantly improve the elimination of such a system from the body via urine and bile after its task is fulfilled. Such polymeric systems have been described with monomeric units containing, for example, hydrolytically labile ester[7] or hydrazone[6] bonds. If the hydrophobic moiety to be hydrolytically cleaved is a hydrophobic drug,[8] controlled drug release may be synchronized with phase-separated polymer resolubilization.

Local brachytherapy with surgically implanted radioactive emitters is a very widely used method for the treatment of localized cancer lesions, especially in the case of prostate cancer but also for breast, ovarian, and other cancers.[9,10] Local application largely eliminates the radiation burden of healthy tissues when high radiation doses are deployed to the site of implantation. The odds are that the enclosed emitter must be surgically implanted and, after treatment, surgically removed. Radiolabeled TRPs with CPTs between room temperature and body temperature may have an advantage for such applications in that TRPs may be isotopically labeled in solution at room temperature and injected as a solution, and at the site of application, the polymers form depoes because of precipitation at body temperature.[6] If such a polymer is biosolubilizable (see the previous discussion) and readily eliminable from the body after solubilization, such depoes may dissolve after the decay of the radionuclide, and both surgeries, during implantation and removal, may be avoided.[6] The polymer should thus have a proper hydrolytic degradation rate with respective to the radionuclide decay half-life ($T_{1/2}$), be biocompatible and nonirritating, have no significant specific organ deposition, and be rapidly eliminable by the kidneys if in solution [above all, it should have a molar weight below the renal threshold, which is about 40 kDa for (meth)acrylamide polymers].[11]

Numerous anticancer drugs, for example, doxorubicin (DOX), show synergic cytotoxic effects with ionizing radiation;[12] this gives a special advantage to the connection of local chemotherapy and local radiotherapy with an injectable TRP with a radionuclide and anticancer drug, which serves as an antiproliferative agent and hydrophobic moiety controlling bioerosion in the same time. We chose DOX as a model drug for this study because it is a potent cancerostatic with known strong synergy with ionization radiation[12] and it is sufficiently soluble in water to readily diffuse from the degrading polymer and sufficiently hydrophobic to allow efficient control over CPT at the same time. As stated previously, the bond by which DOX is attached to the polymer should be cleavable at a proper rate corresponding to the $T_{1/2}$ of the radionuclide used that is on the order of days for common therapeutic radionuclide (e.g., 8.040 days for ^{131}I, 1.117 days for ^{166}Ho, 2.67 days for ^{90}Y, 6.71 days for ^{177}Lu). The bond also should not have a strong pH dependence on the drug release rate because inflamed or cancer tissues have often lower pH values than blood (pH 7.4).[13] The high pH dependence of the degradation rate may thus lead to uncertainty in the degradation of such a polymer because the pH of the application site is case-to-case different and also changes in time. We developed a new type of DOX attachment to the polymer for this purpose,[14] which is described in this article, the *N*-glycosylamine bond. The *N*-glycosylamine bond is readily formed from a reducing saccharide and a primary amine and is hydrolytically labile under neutral and slightly acidic pH value. We thus describe a new type of such system in this article, which uses DOX bound by a hydrolytically labile *N*-glycosylamine bond to the polymer support to provide both solubilization control and potentially additional chemotherapeutic action to the polymer-bound therapeutic radionuclide.

EXPERIMENTAL

Materials

N-Isopropylmethacrylamide (NIPMA) and *N*-isopropylacrylamide (NIPAA) were purchased from Aldrich (Sigma-Aldrich Co., Czech Republic) and were twice recrystallized from hexane. 2,2′-Azobisisobutyronitrile (AIBN) was purchased from Fluka (Sigma-Aldrich Co., Prague, Czech Republic) and crystallized from acetone. 1,2 : 3,4-Di-*O*-isopropylidene-α-D-galactopyranose (DIGA) was obtained from Sigma-Aldrich and was used without additional purification. No-carrier-added Na^{125}I solution (370 MBq

in 500 μL) was purchased form Lacomed, Ltd. (Křež, Czech Republic). All other chemicals were obtained from (SIGMA-ALDRICH) and were used without additional purification.

PD-10 desalting columns were obtained from the Amersham Biosciences (Uppsala, Sweden). Spectrapor tubing (molecular weight cutoff = 3500 Da) was obtained from Fischer Scientific (Czech Republic).

Methods

Synthesis of 6-*O*-methacryloyl-1,2 : 3,4-di-*O*-isopropylidene-α-D-galactopyranose (MADIGA)

MADIGA was prepared by a modified procedure according to ref. 15. In brief, DIGA (12.6 g, 48.4 mmol) was dissolved in dry dichloromethane (40 mL), and triethylamine (10.1 mL, 72.6 mmol) was added. The solution was then cooled to 0°C, and methacryloyl chloride (5.05 mL, 52.5 mmol) was dropwise added with stirring and cooling in an ice-water bath so that the temperature did not exceed 10°C. After the addition of all of the methacryloyl chloride, the cooling bath was removed, and the mixture was left for 2 h at room temperature. The mixture was then filtered, and the filtrate was subsequently washed twice with 0.5*M* aqueous sodium carbonate (100 mL) and twice with water (100 mL) and dried with anhydrous magnesium sulfate. All volatiles were then removed *in vacuo,* and the solid residue was crystallized from ethanol.

Yield = 13.8 g (87%) of yellowish crystals; mp = 63°C. Calcd for CHN: C, 58.53%; H, 7.37; N, 0. Found C, 58.46%; H, 7.41%; N, 0%. ^{1}H-NMR ($CDCl_3$): σ = 1.32 (3 H, s), 1.33 (3 H, s), 1.45 (3 H, s), 1.49 (3 H, s), 1.94 (3 H, s), 4.07 (1 H, m), 4.28 (4 H, m), 4.61 (1 H, m), 5.52 (1 H, d), 5.56 (1 H, s), 6.12 (1 H, s). IR (cm^{-1}): 1718 (C=O).

Synthesis of the polymers

NIPMA copolymers with a variety of comonomers were prepared with the following general procedure. A mixture of monomers (Σ = 3.93 mmol) and AIBN (100 mg) were dissolved in anhydrous tetrahydrofuran (2.5 mL), and the mixture was polymerized under a nitrogen atmosphere at 60°C for 16 h. The polymer was then precipitated in diethyl ether (50 mL), filtered off, dissolved in chloroform (5 mL), precipitated again in diethyl ether (150 mL), filtered off, and vacuum-dried (yield ≈ 65%). For the preparation of the NIPMA copolymers with MADIGA, 0, 5, 10, and 15 mol % MADIGA in the polymerization mixture was used. For the preparation of the NIPMA copolymers with NIPAA, 0, 5, 10, 15, 25, 50, and 100 mol % NIPAA in the polymerization mixture was used. In the case of the copolymers to be isotopically labeled, 0.5 mol % *N*-methacryloyl tyrosinamide (prepared according to ref. 11) was added to the polymerization mixture.

The MADIGA monomeric unit content in the copolymers with NIPMA and NIPAA was assayed by ^{1}H-NMR in $CDCl_3$ according to the following equation:

$$w_{\mathrm{MADIGA}} = S_{4.59}/(S_{4.59} + S_{3,90}) \times 100\%$$

where $S_{4.59}$ is the integral signal of the —C*H*(OC_{acetal})— signal of hydrogen nucleus on the MADIGA monomeric unit at σ = 4.59 ppm, $S_{3.90}$ is the integral signal of the —CO—NH—C*H*$(CH_3)_2$ nuclei in the NIPMA and NIPAA monomeric units, and w_{MADIGA} is the MADIGA monomeric unit content (mol % of monomeric units).

The content of the NIPAA monomeric unit in the NIPAA–NIPMA copolymers was assayed by ^{1}H-NMR in $CDCl_3$ according to the following equation:

$$w_{\mathrm{NIPAA}} = [650(S_{3,90}/S_{\Sigma}) - 50]/(S_{3,90}/S_{\Sigma})$$

where S_{Σ} is the integral signal of all of the hydrogen nuclei in the polymer (contributed by 11 H nuclei per NIPAA monomeric unit and by 13 hydrogen nuclei per NIPMA monomeric unit), $S_{3,90}$ is the integral signal of the —CO—NH—C*H*$(CH_3)_2$ nuclei in the NIPMA and NIPAA monomeric units, and w_{NIPAA} is the NIPAA monomeric unit content (mol % of monomeric units).

The molecular weights of the polymers described in this article were determined by gel permeation chromatography in a mixture of acetate buffer (pH 6.5, 0.3 mol/L) and methanol (20 : 80 v/v) as a mobile phase on a TSK 4000 column (Polymer Laboratories, Ltd., Church Stretton, UK) with an high performance liquid chromatography (HPLC) system ÄKTA Explorer (Amersham Biosciences, Uppsala, Sweden) equipped with RI, ultraviolet (UV), and multiangle light-scattering DAWN DSP-F (Wyatt, Santa Barbara, CA) detectors.

Deprotection of the acetal groups from the polymers containing the MADIGA monomeric unit

The acetal groups were deprotected from the MADIGA monomeric units in the copolymers of MADIGA with NIPMA and NIPMA + NIPAA + *N*-methacryloyl tyrosinamide with 80% (v/v) aqueous trifluoroacetic acid. Thus, the polymer (1.00 g) was dissolved in the mixture of trifluoroacetic acid (20 mL) and water (5 mL), and the solution was stirred at room temperature for 1.5 h. Then, trifluoroacetic acid and water were evaporated on a rotary vacuum evaporator. The solid residue was dissolved in water (20 mL), and the polymer was isolated on a Sephadex G-25 column (120-mL bed volume) with water

as the eluent with conductometric detection and freeze-dried (yield ≈ 90%).

The complete deprotection of the acetals of the MADIGA monomeric units to 6-*O*-methacryloyl-D-galactose (MAGA) monomeric units was confirmed by ^{1}H-NMR spectrometry in CD_3OD (complete disappearance of the acetal —CH_3 proton signals in the range σ = 1.32–1.49 ppm).

Conjugation of DOX

The copolymer containing MAGA monomeric groups (200 mg), anhydrous sodium acetate (146 mg, 1.8 mmol), acetic acid (108 μL, 1.8 mmol), and DOX hydrochloride (5, 10, and 25 wt % of the MAGA copolymer, respectively) were dissolved in a mixture of dimethyl sulfoxide (DMSO; 12.5 mL) and methanol (25 mL), and anhydrous sodium sulfate (200 mg) was added. The mixture was stirred in the dark under nitrogen at room temperature for 3 days. Then, the mixture was filtered, and the filtrate was concentrated *in vacuo* to about 15 mL and then separated on a Sephadex LH-20 column (120-mL bed volume) in methanol. Methanol was evaporated from the polymer fraction, and the remaining solid was dissolved in chloroform (5 mL), precipitated in diethyl ether (50 mL), filtered off, and dried *in vacuo* (yield ≈ 75%).

The DOX content in the polymer was assayed by UV–visible spectrophotometry (λ = 480 nm, ε = 9800 mol L^{-1} cm^{-1}) in a methanol–acetic acid mixture (98 : 2 v/v, polymer concentration = 0.5 mg/mL).

CPT determination

The CPTs of the polymers in this study were determined in a solution of the particular polymer (25 mg/mL) in phosphate buffered saline (PBS) at pH 7.4 at a heating rate of 2°C/min and by visual detection.

Dynamic light scattering

The apparent hydrodynamic diameter, polydispersity index, and light-scattering intensity at 173° (I_S) of the copolymers dissolved in 0.15 mol/L aqueous sodium chloride (polymer concentration = 2.0 mg/mL) were determined at different temperatures with a Nano-ZS model ZEN 3600 zetasizer (Malvern Instruments, Malvern, UK). The DTS (Nano) program was used for data evaluation.

DOX release

The DOX release from the optimized NIPMA–NIPAA–MAGA–DOX copolymer during incubation in PBS was studied as follows. A solution of the copolymer (200 μL per 1.5-mL Eppendorf test tube, polymer concentration = 10 mg/mL) in PBS (pH = 5.0, 6.5, or 7.4) was incubated at 37°C. At each time (0, 1, 3, 7, 14, and 21 days), methanol (800 μL) was added to the particular Eppendorf test tube, and the precipitated salts were centrifuged out. The free and polymer-bound DOX contents were then determined in supernatant by HPLC with the same system described previously for the determination of the molecular weights of the copolymers, but the absorbance at 488 nm was followed. All release studies were made in triplicate, and all the results were within an experimental error of 5% of the particular measured value.

Radiolabeling of poly(NIPAA-*N*-methacryloyl tyrosinamide) and its formulation with NIPMA–NIPAA–MAGA–DOX

The poly(NIPAA-*co*-*N*-methacryloyl tyrosinamide) copolymer was polymerized in THF as described previously for the degradable polymers. Poly(NIPAA-*co*-*N*-methacryloyl tyrosinamide) (2.2 mg) was dissolved in PBS (200 μL) and chloramine T (2.5 mg), and a solution of $Na^{125}I$ (145 MBq diluted with water to 1000 μL) was added. After 60 min of incubation at room temperature, a solution of ascorbic acid (1.9 mg) in water (10 μL) was added, and the solution was incubated for another 10 min at room temperature. The polymer fraction was separated on a PD-10 desalting column in water, and an aliquot of the polymer fraction equivalent to 0.5 mg of the polymer was added to a freshly prepared solution of NIPMA–NIPAA–MAGA–DOX copolymer (49.5 mg) in cold water. After mixing, the solution was immediately frozen and freeze-dried. The labeling yield was 77%. The radioactivity measurements were performed with a calibrated γ spectrometer with an HPGe detector (Ortec, Oak Ridge, TN) in a defined geometry.

Dissolution study of the radiolabeled NIPMA–NIPAA–MAGA–DOX copolymer

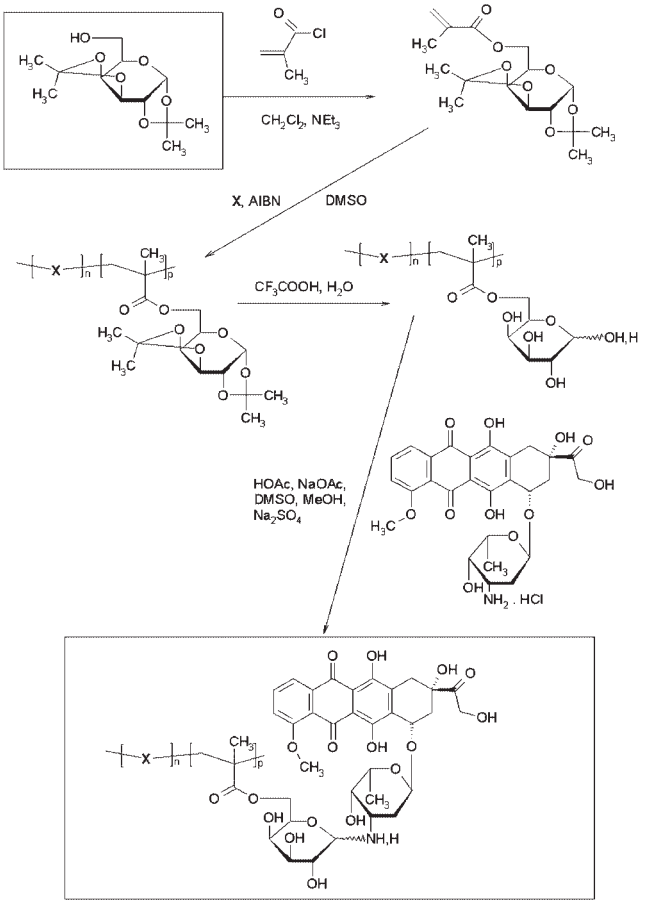
The whole freeze-dried product (see previous discussion) was dissolved in anhydrous DMSO (250 μL). The 1.5-mL Eppendorf vials, each containing 1.00 mL of the incubation solution (PBS; pH = 5.0, 6.5, and 7.4; three repetitions for each incubation solution), were preheated to 37°C in a thermostated bath. The solution of radiolabeled polymer in DMSO (see previous discussion) was then added to the vials (per vial 10 μL). The vials were then incubated at 37°C. At selected time intervals (2, 8, 24, 48, 72, 168, and 336 h), the vial contents were mixed, the polymer precipitate was allowed to settle, and then a 100-μL clear aliquot of supernatant from each vial

was taken. The radioactivity of these aliquots was measured, and the aliquots were heated again to 37°C and returned to the particular Eppendorf vial. The Eppendorf vials were kept at 37°C the whole time. The 100-μL aliquot of the solution of the labeled polymer (10 μL of the stock solution in all the vials diluted to 1 mL with DMSO) was used as the 100% radioactivity value sample. It was proven that the addition of 1% DMSO to water caused a less than 1°C phase separation temperature depression in the studied polymers, and so this effect was neglected in the data evaluation.

RESULTS AND DISCUSSION

We decided to use *N*-isopropyl methacrylamide as the main monomer because it had a suitable homopolymer CPT (42.5°C in 0.15*M* aqueous NaCl) and there was a facile possibility to change the CPT both up and down by free-radical copolymerization with other vinylic comonomers. Formerly, we studied hydrazone hydrolytically labile bound to conjugate DOX to a polymer, but because of the strong pH dependence of the DOX release rate in this case, we developed a new type of linker to conjugate DOX to a polymer, the *N*-glycosylamine bond.[14] The MAGA monomeric unit was used to bind DOX by a *N*-glycosylamine bond with the primary amine group of DOX (Scheme 1). This monomeric unit could be readily introduced to the polymer by the copolymerization of MADIGA, a monomer easily available from commercially available bisacetone-protected DIGA[15] by methacroylation and subsequent deprotection of the isopropylidene groups in an acidic environment (Scheme 1). We also studied a similar monomeric unit derived from D-glucose, 3-*O*-methacryloyl-D-glucose, which is analogously readily synthetically available from commercially available 1,2 : 5,6-di-*O*-isopropylidene-α-D-glucofuranose,[16] but such copolymers showed, probably because of steric hindrances, only low to moderate *N*-glycosylamine conjugation yields, so they were no longer studied (data not shown). We decided to copolymerize the MADIGA and deprotect the acetals on the polymer because this provided an advantage over the copolymerization of 6-*O*-methacryloyl galactose in that MADIGA could be easily purified by crystallization, which was not the case for MAGA, which does not crystallize easily.

The polymerization was carried on in tetrahydrofuran at 60°C with AIBN as an initiator, which led to high conversion (typically, ca. 65%) and weight-average molecular weights (M_w = 20–30 kDa) with acceptably low polydispersity values (M_w/number-average molecular weight < 2). The M_w of 20–30 kDa was sufficiently high to suppress intermolecular heterogeneity and, thus, decrease the widening of



Scheme 1 Synthesis of the hydrolytically erodable TRP system. HOAc = acetic acid; NaOAc = sodium acetate.

the CPT and sufficiently low to be below the renal threshold (see previous discussion). MADIGA readily copolymerized with isopropyl methacrylamide. The MADIGA monomeric unit contents in the copolymers closely correlated with the content of MADIGA in the polymerization mixture (R^2 = 0.999):

$$w_{\mathrm{pol}} = 0.9464 w_{\mathrm{mix}}$$

where w_{mix} is the content of MADIGA in the polymerization mixture (mol % of the sum of the monomers) and w_{pol} is content of MADIGA in the resulting copolymer. The copolymer was thus sufficiently chemically homogeneous to avoid the broadening of the CPT.

We used 80% aqueous trifluoroacetic acid to deprotect the acetals from the saccharide moiety in analogy to a low-molecular weight reaction.[17] This method smoothly deprotected all of the isopropylidene groups without crosslinking. The copolymer with a 6-*O*-methacryloyl galactose group content of 10 mol % was used for further studies to offer sufficient DOX binding capacity and cause as low an increase in the CPT of the copolymer as possible.

DOX was conjugated with the saccharide moieties containing polymer in an anhydrous acetic acid–sodium acetate buffer to offer both suitable acidity and ionic strength to shield ionic repulsions between DOX bound to the polymer and free DOX to be bound in solution. The DOX conjugation yields were 66–79%, and the DOX content in conjugate corresponded to the DOX/polymer ratio up to high DOX loadings in such cases. The reaction was done at room temperature in analogy to the synthesis of low-molecular-weight *N*-glycosylamines[18–20] because of DOX stability and also to avoid unwanted Amadori rearrangements, which can take place at elevated temperatures.[19–22] The polymer was purified after conjugation by gel permeation chromatography in methanol. Because most low-molecular-weight glycosylamines are β anomers and α anomers may be isolated only under special conditions (i.e., anhydrous solvents because *N*-glycosylamines mutarotate easily),[18–20,22] we assumed that this *N*-glycosylamine bond formed was also a β anomer.

It was possible to increase the DOX content even above 15 wt %, but in the case of loadings above 15 wt %, problems with the solubility of the product in aqueous environment and an increase of the apparent molar weight probably due to noncovalent interactions occurred to a more pronounced extent. The apparent molar weight, however, decreased again to the original value during the hydrolytic degradation of the conjugates in an aqueous environment. For instance, the polymer with 11.1 wt % DOX had an apparent M_w of 56 kDa, whereas the polymer containing 17.1 wt % DOX had an apparent M_w of 329 kDa. Both these conjugates were synthesized from the same polymeric precursor with a M_w of 20 kDa, and after incubation in aqueous media, their M_w values dropped to 21 and 23 kDa, respectively.

The CPT of the final copolymer was lowered by the copolymerization of NIPAA (CPT of the homopolymer = 29°C) and by variation with the DOX content to 34°C with DOX and to 40°C without DOX (polymer after degradation). The setting of these temperatures was done on the assumption of additive effects of the increments of the molar contents of NIPAA, MAGA, and DOX on the CPT. A very good agreement between the particular predicted and measured values was achieved (differences in the measured CPTs of the final copolymer from the calculated values were less then 1°C for the particular values before and after degradation). The effect of the MAGA, *N*-isopropyl acrylamide, and DOX monomeric units contents in the copolymers with *N*-isopropyl methacrylamide on the CPT of these copolymers is shown in Figure 1. The increasing content of MAGA monomeric units caused an increase in the CPT consistently with the high hydrophilicity of this monomeric unit, whereas the conjugation of the copolymer with DOX decreased the CPT because of the more hydrophobic nature of DOX. The increasing amount of NIPAA monomeric units caused a linear decrease in the CPT of 0.14°C per mol % (R^2 = 0.988), which was similar to most dependencies described for such systems in the literature.[1] The dependence of the CPT on the monomeric unit content was nonlinear in the case of the DOX and MAGA monomeric unit contents, probably because of steric reasons. The CPTs of the polymers in PBS buffer remained constant (±1°C) in the concentration range 2.0–50 mg/mL coming on force for the parenteral application of these polymers. At lower polymer concentrations, the CPT slightly increased. However, low concentrations would never be used because of the necessity of reaching the effective concentration of conjugated DOX in the target site.

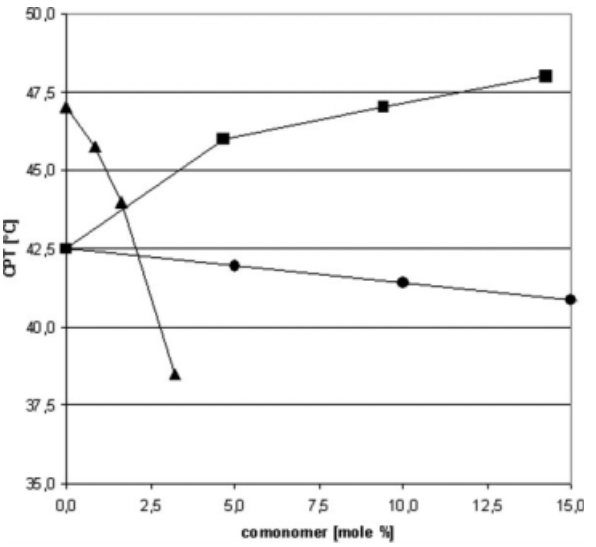


Figure 1 Effect of the copolymer composition on CPT of the copolymers (in PBS buffer; polymer concentration = 25 mg/mL): (●) poly(NIPMA-*co*-NIPAA) (the content of NIPAA monomeric units is the variable), (■) poly(NIPMA-*co*-MAGA) (the content of MAGA monomeric units is the variable), and (▲) the effect of DOX loading on poly(NIPMA-*co*-MAGA) with 10 mol % saccharide monomeric unit (the content of DOX-modified monomeric units is the variable).

As stated previously, the copolymer with a CPT of 34°C before and 40°C after degradation was chosen for further degradation, labeling, and dissolution studies. This polymer was prepared with 45.5 mol % *N*-isopropyl methacrylamide, 44 mol % *N*-isopropyl acrylamide, 10 mol % MADIGA, and 0.5 mol % *N*-methacryloyl tyrosinamide (a radioiodinable monomer) in the polymerization mixture. The content of *N*-methacryloyl tyrosinamide was sufficiently low not to significantly influence the CPT of the copolymer. After deprotection, a 1 : 4 DOX·HCl polymer

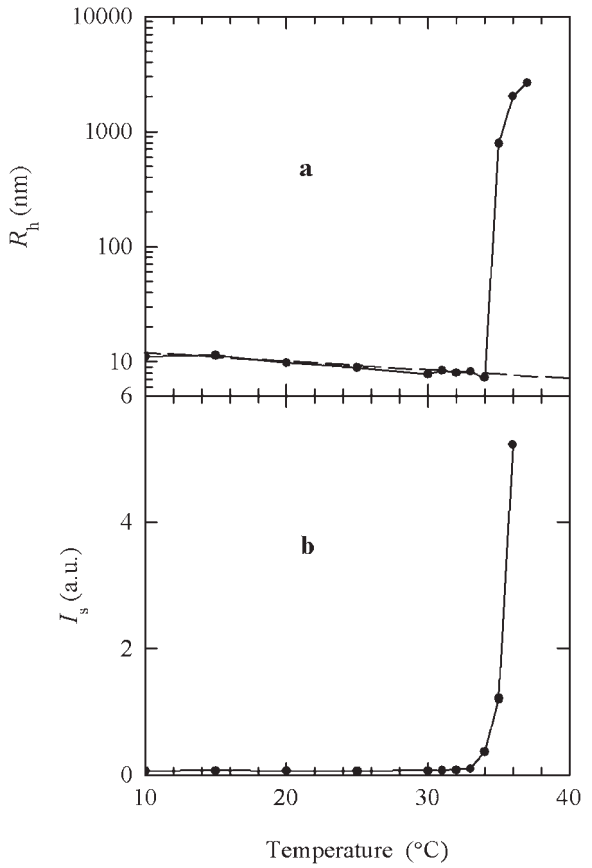


Figure 2 Temperature dependences of (a) R_H and (b) I_S for a fresh solution of the thermoresponsive hydrolytically degradable polymer before degradation (in PBS buffer; polymer concentration = 2.0 mg/mL).

ratio in the conjugation mixture was used, which led to a 13.2 wt % DOX content in the final product.

We also studied whether the phase separation behavior of the TRP was classic phase separation with coil-to-globule transition in a narrow temperature range, which was followed by phase separation to macroscopic precipitate, or also whether micelle-like behavior could be observed at temperatures below the apparent CPT.[6] The latter case occurred when a relatively large hydrophobic moiety was attached to the polymer, so blocklike behavior prevailed. Because the DOX moiety was relatively bulky, we tested whether such behavior took place here. As shown by the temperature dependence of the hydrodynamic radii (R_H's) and I_S (Fig. 2), no formation of micelles occurred, and the decrease in R_H due to chain desolvation and collapse was rapidly followed by macroscopic aggregation in temperatures above the CPT.

The *in vitro* *N*-glycosylamine bond degradation study was carried on in a milieu mimicking the biological environment, in PBS at pH 7.4 at body temperature (37°C). The hydrolysis of *N*-glycosylamine bonds (measured as free DOX release) showed a relatively fast onset during the first 3 days and then slowed down but continued for the following 3 weeks (Fig. 3). The most plausible explanation for this was that DOX released by hydrolysis was in apparent equilibrium with the polymer in a microenvironment of phase-separated polymer, where the diffusion of DOX to the incubation buffer was significantly slowed down by steric hindrance, as is seen, for example, in polymeric micelles.[7] It is known that TRPs of poly(*N*-isopropyl acrylamide) type contain large amounts of water in a phase-separated state shortly after phase transition, but gradually, within the course of time, lose this water, which makes the separated phase more condensed.[3,4] We thus assumed that the first burst period corresponded to the state where the phase-separated polymer still contained large amounts of water and the diffusion of DOX outside the polymer was not so restricted. This was a difference between this system and our previous system with hydrazone bonds,[6] where the dissolution-controlling hydrophobic moiety was an aliphatic ketone. In that case, no such effect was observed, probably because of the lower steric demands of aliphatic ketones in comparison to DOX.

We also tested the pH dependence of the DOX release rate because the pH in solid tumors and inflamed tissues, for which this polymer was intended, is generally lower than the pH of blood plasma. We used buffers of pH 7.4 (the pH of blood plasma), pH 6.5 (the typical pH of the interstitial space in solid tumors), and pH 5.0 (the pH in late endosomes, where the polymer may get after endocytosis of the partially degraded polymer). The DOX release (Fig. 3) was only slightly pH dependent in the studied pH range, which is an advantage for local application because the release of DOX and, thus, the degradation of the polymer should be

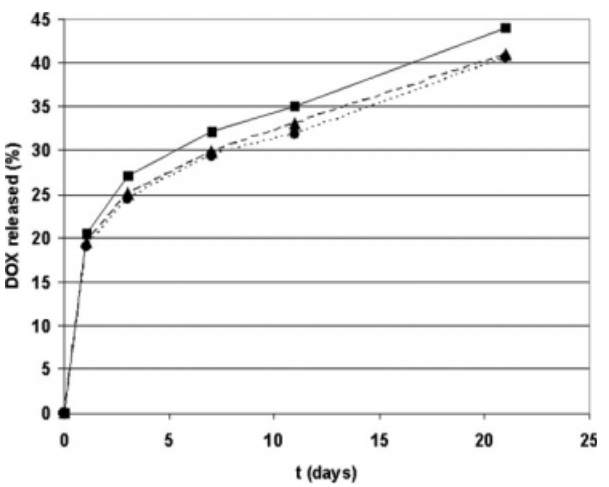


Figure 3 *In vitro* release of DOX from the *N*-glycosylamine conjugate into the PBS buffer: pH (▲) 5.0, (●) 6.5, and (■) 7.4.

reliably case-to-case independent of the application site, which is important for the possible therapeutic effects caused by both DOX and radioactivity (see previous discussion). This was in good agreement with the kinetics of hydrolysis of low-molecular-weight *N*-glycosylamines.[20]

Iodine 125 was used as a model of the therapeutic isotope ^{131}I because of its longer $T_{1/2}$ ($T_{1/2}$ = 59.4 days for ^{125}I and $T_{1/2}$ = 8.040 days for ^{131}I), which enabled us to conduct longer degradation studies compared to ^{131}I with the same iodine radiochemistry. Direct radiolabeling of the polymer with or without DOX via standard electrophilic iodination with sodium iodide with chloramine T or iodogen as an oxidizer resulted in medium labeling yields (ca. 60%), however, the label was not stable in an aqueous environment (ca. 30% of the radiolabel was cleaved out to solution within 24 h). Because radioiodination of the monomer or early polymer precursors would cause manufacturing problems (especially large volumes of liquid radioactive waste), we decided to radiolabel a small amount of thermoresponsive poly(NIPAA-*co*-*N*-methacryloyl tyrosinamide), which has a CPT in isotonic PBS buffer of about 29°C, and formulate it to 100× excess of the biodegradable system. According to our previous study,[23] poly(NIPAA-*co*-*N*-methacryloyl tyrosinamide) is biocompatible and forms long-lasting depoes after injection to the target tissue, and dissoluted fractions are immediately excreted from the body without specific organ distribution, and its radioiodination is completely stable. Formulation with the bioerodable polymer should increase the rate of elimination and contribute to the therapeutic effect with the synergic cytostatic influence of DOX. Such a system is also easier to manufacture, and the radioactivity-to-DOX ratio may be more operatively customized. The release of radioactivity from the separated phase to solution during bioerodable polymer degradation was significant but not quantitative (2 h after beginning, 88.9 ± 5.4, 84.6 ± 5.5, and 87.1 ± 1.8% of radioactivity were in the phase-separated polymer at pH values of 7.4, 6.5, and 5.0, respectively; after 336 h, 71.6 ± 3.0, 68.6 ± 3.2, and 74.1 ± 2.9% of radioactivity were in the phase-separated polymer at pH values of 7.4, 6.5, and 5.0, respectively; between, the trend was linear; other data are not shown), even after 2 weeks, when nearly all of the bioerodable polymer was dissolved. The radioactivity retained in the separated phase was lower than in the case of poly(NIPAA-*co*-*N*-methacryloyl tyrosinamide) alone (where there was 95.0 ± 3.0% radioactivity in the phase-separated polymer, which was not dependent on time). This was in agreement with the assumption of interpolymer interactions between the degradable and nondegradable polymers. Because the studied model system was closed, in organisms, where the dissolved components are continuously washed out, one may expect significantly enhanced radiolabeled polymer elimination from the target site after the system fulfills its task.

The depo formed should also be as small as possible to suppress the radiation burden of normal tissues surrounding the tumor; however, the emitter must have sufficient range to affect the whole tumor tissue (but this is the case in most the therapeutically considerable radionuclides). Multiple injections may solve the problem with tumors of nonspherical shape. Phase separation should thus be as fast as possible to prevent the broadening of the depo formed. In the case of the polymers described in this article, phase separation was practically immediate. According to our results with a nonbioerodable polymer on mice,[23] the depo formed was very small, covering even a smaller part of the murine muscle.

CONCLUSIONS

A new polymeric drug-delivery system designed for possible local chemoradiotherapy with an injectable TRP with a radionuclide and DOX, which will serve as an antiproliferative agent and hydrophobic moiety controlling bioerosion at the same time, was synthesized and characterized. DOX was bound to the polymer carrier by an *N*-glycosylamine bond. DOX was slowly released during incubation in aqueous milieu at 37°C, which caused nearly complete dissolution of the bioerodable polymer within about 2 weeks. The model radionuclide iodine 125, bound to a small amount of poly(NIPAA) with copolymerized *N*-methacryloyl tyrosinamide, was retained in the separated phase and slowly dissolved during the incubation.

References

1. Gil, E. S.; Hudson, S. A. Prog Polym Sci 2004, 29, 1173.
2. Alarcon, C. D. H.; Pennadam, S.; Alexander, C. Chem Soc Rev 2005, 34, 276.
3. Starovoytova, L.; Spevacek, J.; Trchova, M. Eur Polym J 2007, 43, 5001.
4. Starovoytova, L.; Spevacek, J. Polymer 2006, 47, 7329.
5. Hruby, M.; Kucka, J.; Mackova, H.; Lebeda, O.; Ulbrich, K. Chem Listy 2008, 102, 21.
6. Hruby, M.; Kucka, J.; Lebeda, O.; Mackova, H.; Babic, M.; Konak, C.; Studenovsky, M.; Sikora, A.; Kozempel, J.; Ulbrich, K. J Controlled Release 2007, 119, 25.
7. Neradovic, D.; van Nostrum, C. F.; Hennink, W. E. Macromolecules 2001, 34, 7589.
8. Chytry, V.; Ulbrich, K. J Bioactiv Compat Polym 2001, 16, 427.
9. Butler, W. M.; Merrick, G. S. Int J Radiat Oncol Biol Phys 2008, 71, S142.
10. Aronowitz, J. N. Brachytherapy 2008, 7, 55.
11. Kissel, M.; Peschke, P.; Subr, V.; Ulbrich, K.; Schuhmacher, J.; Debus, J.; Friedrich, E. PDA J Pharm Sci Technol 2001, 55, 191.
12. Chenoufi, N.; Raoul, J. L.; Lescoat, G.; Brissot, P.; Bourguet, P. J Nucl Med 1998, 39, 900.

13. Brahimi-Horn, M. C.; Pouyssegur, J. Oxygen Sensing Hypoxia-Induced Responses 2007, 43, 165.
14. Hruby, M.; Kucka, J.; Konak, C.; Vetrik, M.; Lebeda, O.; Ulbrich, K. Proceedings of the 10th European Symposium on Controlled Drug Delivery, Noordwijk aan Zee, Amsterdam, The Netherlands, April 2008; p 174.
15. Kondo, S.; Sasai, Y.; Kuzuya, M.; Furukawa, S. Chem Pharm Bull 2002, 50, 1434.
16. Al-Bagoury, M.; Buchholz, K.; Yaacoub, E. J. Polym Adv Technol 2007, 18, 313.
17. May, J. A.; Sartorelli, A. C. J Med Chem 1979, 22, 971.
18. Metlitskikh, S. V.; Koroteev, A. M.; Koroteev, M. P.; Shashkov, A. S.; Korlyukov, A. A.; Antipin, M. Y.; Stash, A. I.; Nifantiev, E. E. Russ Chem Bull 2005, 54, 2890.
19. Capon, B. Chem Rev 1969, 69, 407.
20. Monsigny, M.; Quetard, C.; Bourgerie, S.; Delay, D.; Pichon, C.; Midoux, P.; Mayer, R.; Roche, A. C. Biochimie 1998, 80, 99.
21. Roscic, M.; Horvat, S. Bioorg Med Chem 2006, 14, 4933.
22. Bridiau, N.; Benmansour, M.; Legoy, M. D.; Maugard, T. Tetrahedron 2007, 63, 4178.
23. Kucka, J.; Hruby, M.; Kozempel, J.; Lebeda, O. J Labelled Compd Radiopharm 2007, 50(S1), S459.