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# Micellar and Antibody-Targeted Polymer Therapeutics

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**Summary:** Synthesis, physicochemical and some biological properties of new actively targeted antibody-containing and passively targeted micellar polymer - doxorubicin conjugates were investigated. Polymer precursors used for the synthesis of the conjugates were based on semitelechelic *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers with reactive groups situated at the polymer chain end or on multivalent copolymer with groups randomly distributed along the polymer backbone. Micellar HPMA-copolymer-based pharmaceuticals were prepared by self-assembly of copolymer–doxorubicin conjugates containing hydrophobic cholesterol ligands attached to the copolymer via hydrolytically degradable spacer. pH-Controlled release of cholesterol derivative is a key-point for disintegration of the micellar drug carrier after delivering the drug to the tumor tissue. Synthesis of star antibody-targeted polymer conjugates takes advantage of reduction of disulfide bridges in antibody with dithiothreitol followed by conjugation with the semitelechelic copolymer thus avoiding modification of the binding site in the antibody for its antigen. Both conjugates differing in their molecular architecture and mechanism of action are promising candidates for in vivo antitumor therapy.

**Keywords:** doxorubicin; drug delivery systems; HPMA copolymers; hydrazone bond; monoclonal anti-CD20 antibody

## Introduction

In the recent few decades many anticancer drugs with remarkable antitumor activity have been developed. Unfortunately, their use in human therapy is often accompanied by undesirable side effects. Conjugation of the drugs with polymers may reduce these side effects, prolong their circulation in blood, improve distribution in the body and increase specificity of the drugs against cancer cells.[1–3]

Recently, we described the synthesis of HPMA copolymers[4] containing the anticancer drug doxorubicin (DOX) bound to the copolymer via the hydrazone linkage susceptible to pH-sensitive hydrolysis. Such polymer–DOX conjugates were fairly stable in aqueous solution at pH 7.4 (pH of bloodstream) and DOX was released in significant amounts at pH 5 – 5.5 (mimicking pH in endosomes). We demonstrated that these conjugates show a high cytostatic activity after incubation with various cancer cell lines and a significant therapeutic effect in the treatment of EL4 lymphoma-bearing mice.[5–8] Later on, it was also published that the antitumor potential of the polymeric prodrugs with hydrazone bound doxorubicin was improved by coupling the prodrugs to human immunoglobulin[9] or by introducing hydrophobic cholesterol substituent, thus forming self-assembling high-molecular-weight supramolecular micellar structures in aqueous solution.[10] The improved anticancer efficacy of the immunoglobulin-containing or micellar conjugates is most likely a result of the high molecular weight (HMW) of the polymer-antibody or supramolecular

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carrier, which is the basis for enhanced accumulation of the prodrug in tumor tissue due to a tumor-related phenomenon described as the enhanced permeability and retention (EPR) effect.[11]

The methods suitable for preparing of antibody-based conjugates can be classified basically in two categories: those involving the random modification of antibody amino acid residues (e.g. acylation of lysine amino groups, alkylation of tyrosines) and those that are highly regioselective. Random modification of antibody in contrast to regioselective modification may impair antigen binding and leads to conjugate heterogeneity.[12] In the past several years, a number of selective methods have been described to introduce reactive groups onto antibody. One of selective modification is based on reduction, using dithiothreitol, tris(2-carboxyethyl)phosphine, aminoethanthiol, of antibody interchain disulfides. Recently, this method was widely used for preparation of antibody-drug conjugates with drugs attached via spacer to free sulfhydril groups on reduced antibody.[13–14]

Here, we describe the synthesis, physicochemical and some biological properties of new micellar and antibody-targeted polymer-DOX conjugates based on HPMA copolymers designed for active or passive tumor targeting. In both conjugates, the anticancer drug DOX is attached to the polymer carrier via pH-sensitive hydrazone bond susceptible to hydrolysis enabling DOX release in mildly acid medium (pH 5 – 5.5). HPMA-based copolymer – doxorubicin conjugates containing the hydrophobic cholesterol moiety attached via a hydrolytically degradable spacer were synthesized to demonstrate the effect of passive targeting. Slow pH-depending release of the cholesterol moiety made possible disintegration of the HMW micellar conjugate to polymer fragments of molecular weight of the original polymers clearable from the body by glomerular filtration. Synthesis of new star-shaped antibody-targeted polymer conjugates takes advantage from the use of reduced antibody (Ab) for conjugation with semitelechelic polymers enabling one-point attachment of the polymer to Ab outside its binding site to antigen. The study of the effect of polymer conjugate architecture onto physicochemical and biological properties of both, passively and actively targeted drug delivery systems is a major goal of this paper.

## Materials and Methods

### Chemicals

1-Aminopropan-2-ol, methacryloyl chloride, 2,2́-azobisisobutyronitrile (AIBN), 6-aminohexanoic acid (AH), 2-iminothiolane, cholesterol, dimethylformamide (DMF), *N*,*N*́-dicyclohexylcarbodiimide (DCC), phthalaldehyde (OPA), *N*-ethyldiisopropylamine, *N*-(2-aminoethyl)maleimide trifluoroacetate, succinimidyl 3-maleimidopropanoate (SMP), 5,5́-disulfanylbis(2-nitrobenzoic acid) (Ellmańs reagent), cysteine, dithiothreitol, dimethyl sulfoxide (DMSO), 4-oxopentanoic acid, *tert*-butyl carbazate, trifluoroacetic acid (TFA) and doxorubicin hydrochloride (DOX.HCl) were purchased from Fluka. 2,4,6-Trinitrobenzene-1-sulfonic acid (TNBSA) was purchased from Serva. Anti-CD20 Ab (clone MEM97) was a kind gift of EXBIO co. (Czech Republic).

### Synthesis of Monomers and Derivatives

*N*-(2-Hydroxypropyl)methacrylamide (HPMA),[15] m.p. 69 - 70 °C (calc. 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),[16] m.p. 110-114 °C (calc. C 57.70%, H 8.33%, N 13.46%; found C 57.96%, H 8.64%, N 13.25%) and 6-methacrylamidohexanohydrazide (Ma-ah- $NHNH_2$),[17] m.p. 79–81 °C (calc. C 56.32, H 8.98, N 19.70; found C 56.49, H 8.63, N 19.83), were prepared as described previously.

Cholest-5-en-3β-yl 4-oxopentanoate (LEV-CHOL) was prepared by the reaction of 4-oxopentanoic acid with cholesterol. 2.0 mmol of cholesterol and 2.2 mmol of 4-oxopentanoic acid were dissolved in 6 mL of dichloromethane (DCM). 2.4 mmol of

 

dicyclohexylcarbodiimide (DCC) were dissolved in 0. 5 mL of DCM and a few crystals of 4-(dimethylamino)pyridine was added. Both solutions were cooled to -18 °C. The reaction mixture was left at −18 °C for 1 h, then at 4 °C for 16 h and at laboratory temperature for 2 h. Unreacted DCC was removed by reaction with 100 μL of acetic acid. After 30-min incubation, the precipitated *N*,*N*́-dicyclohexylurea was filtered off. The product was purified by column chromatography (silica gel 60) in a mixture DCM/methanol (30: 1 by vol.). The product was crystallized from DCM. Yield: 62%, m.p. 66–67 °C (calc. C 79.29%, H 10.81%; Found C 78.69%, H 10.83%), TLC: DCM/methanol (30: 1), $Rf = 0.8$; selected peaks of $^1$H-NMR (300 MHz, $CDCl_3$): δ 5.42 t, 1H (C = CH-$CH_2$); δ 4.63 m, 1H (CO-O-CH-$(CH_2)_2$); δ 2.78 t,

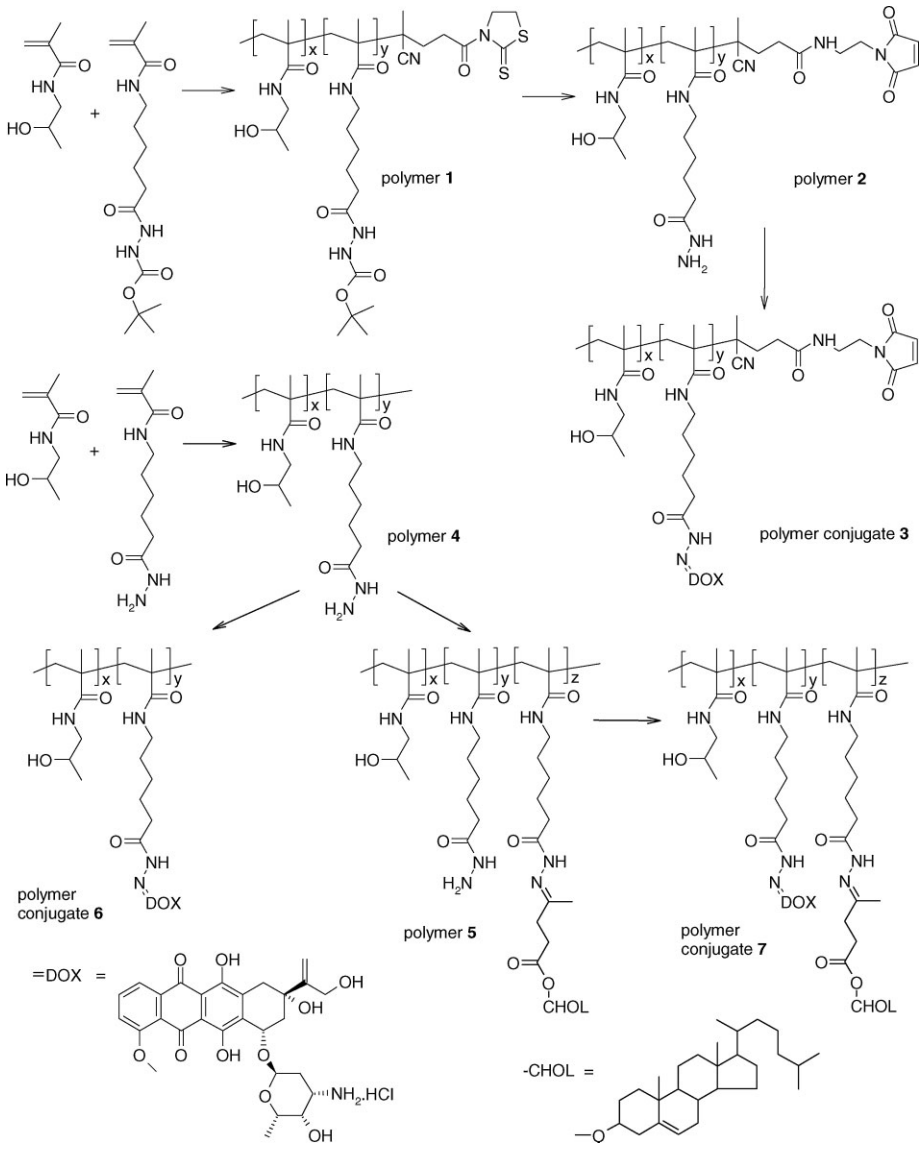


***Figure 1.***
Scheme of the synthesis of HPMA polymers **1**, **2**, **4** and **5** and polymer conjugates **3**, **6** and **7**

 

2H (CO-$CH_2$-$CH_2$-CO-O); δ 2.61 t, 2H (CO-$CH_2$-$CH_2$-CO-O); δ 2.36 d, 2H (CO-O-CH-$CH_2$-C = CH); δ 2.24 s, 3H ($CH_3$-CO); δ 0.72 s, 3H (C(18)$H_3$).

### Synthesis of Polymer Precursors and Polymer-Drug Conjugates

Semitelechelic copolymer precursor (**1**, Table 1) containing Boc-protected hydrazide groups was prepared by radical copolymerization of HPMA and Ma-ah-NHNH-Boc in DMSO using the initiator 3,3́-[azobis(4-cyano-4-methyl-1-oxobutane-4,1-diyl)]bis(thiazolidine-2-thione) (ABIC-TT) as described in ref.[9] The end-chain reactive maleimide (MI) group was introduced into polymer **1** by the reaction of thiazolidine-2-thione (TT) group with *N*-(2-aminoethyl)maleimide in DMF as previously reported.[9] Semitelechelic polymer **2** bearing hydrazide groups was obtained after removal of Boc protecting groups in concentrated TFA .

A random copolymer of HPMA with Ma-ah-$NHNH_2$ (polymer **4**) was prepared by radical copolymerization in methanol (AIBN, 1 wt.%; monomers concentration 18 wt.-%; mol ratio HPMA - Ma-ah-$NHNH_2$ 93: 7; 60 °C; 17 h) as described in.[17]

Random terpolymer bearing cholesterol ligands (polymer **5**) was prepared by reaction of hydrazide groups of polymer **4** with LEV-CHOL: 10 wt.% methanolic solution of polymer **4** (100 mg) was added to LEV-CHOL (4.9 mg) and stirred. Immediately, 40 μL of acetic acid was added under stirring into the reaction mixture for 24 h at room temperature. Polymer **5** was isolated by precipitation into an excess of ethyl acetate and purified by precipitation from methanol into ethyl acetate. The polymer precursor was filtered off and dried in vacuum to constant weight .

Semitelechelic polymer–DOX precursor **3**, nontargeted polymer - DOX conjugate **6** and micellar polymer - DOX conjugate **7** were prepared by the reaction of the respective polymer hydrazide precursors with DOX.HCl in methanol in the dark as described previously.[5]

### Synthesis of Antibody – Polymer – DOX Conjugates

Anti-CD20 antibody-containing polymer–DOX conjugates were prepared from DOX-containing polymer precursors **2** and **3** by the reaction with sulfanyl groups-containing anti-CD20 Ab. Ab-containing conjugates **8 - 10** were prepared by the reaction of the MI end group of polymers **2** and **3** with the sulfanyl groups of the modified Ab. The sulfanyl groups were introduced into the Ab either by modification of a part of 6-amino groups of lysine residues of the Ab with 2-iminothiolane (ITH), as described in [7] or by mild reduction of disulfides in the IgG-type Ab with dithiothreitol (DTT) (Figure 2). The amount of sulfanyl groups in modified Ab was determined by the reaction with Ellman's reagent.[18]

***Table 1.***
Characteristics of polymer precursors

| Polymer precursor | $M_w$ | $M_w$/ $M_n$ | Hydrazide (mol.%) | End-group | $M_{n,T}$[c] | $M_n$/ $M_{n,T}$[d] | $R_H$[e] (nm) |
|---|---|---|---|---|---|---|---|
| **1**[a] | 29000 | 1.89 | 5.9 | TT | 11800 | 1.31 | – |
| **2**[a] | 37000 | 1.94 | 5.9 | MI | 15800 | 1.18 | 4.5 |
| **3**[a,b] | 41200 | 1.90 | – | MI | 18900 | 1.14 | 4.7 |
| **4** | 27000 | 1.80 | 5.7 | – | – | – | 4.3 |
| **5** | 28500/230000[g] | 1.90 | 4.4/1.3[f] | – | – | – | 14.3 |

[a]semitelechelic copolymer.
[b]polymer precursor with 12.6 wt.% of DOX attached via hydrazone bond.
[c]number-average molecular weight calculated from the TT or MI group content.
[d]functionality of the polymer.
[e]hydrodynamic radius in aqueous solution.
[f]LEV-CHOL content in mol.%.
[g]apparent molecular weight in aqueous solution.

 

***Table 2.***
Characteristics of polymer carriers and conjugates

| Polymer conjugate | Polymer | $M_w$ | $M_w/M_n$ | DOX (wt.%) | Ab (wt.%) | $R_H$[a] (nm) |
|---|---|---|---|---|---|---|
| **6** | **4** | 32500 | 1.86 | 9.8 | – | 4.7 |
| **7** | **5** | 26500/240000[b] | 1.88 | 8.5 | – | 14.2 |
| **8** | **3** | 620 000 | 1.91 | 6.05 | 39.5[c] | 20.5 |
| **9** | **2** | 416 000 | 1.58 | – | 47.1[d] | 15.2 |
| **10** | **3** | 423 000 | 1.52 | 5.41 | 46.8[d] | 17.1 |

[a]Hydrodynamic radius in aqueous solution.
[b]apparent molecular weight in aqueous solution.
[c]Ab with thiol groups introduced by modification with ITH.
[d]Ab with thiol groups generated by DTT reduction.

*Preparation of Ab Reduced with DTT*

105 mg of Ab dissolved in 10 mL of phosphate buffer (0.05 M $NaH_2PO_4$/$Na_2PO_4$, 0.1 M NaCl, 0.01 EDTA, pH 7.4) was added under gentle stirring to 388 μL of 1.7 M dithiothreitol solution in water. The reaction mixture was stirred for another 1 h at room temperature. Low-molecular-weight impurities were removed by gel filtration on a column packed with Sephadex G-25 (Pharmacia) with phosphate buffer eluent (pH 7.4).

*Conjugation of Polymers with Modified Ab*

110 mg of polymer **3** dissolved in 6 mL of phosphate buffer (0.05 M $NaH_2PO_4$/$Na_2HPO_4$, 0.1 M NaCl, pH 7.4) was added to a stirred solution of 100 mg of reduced Ab (6 μmol SH) in 10.6 mL of phosphate buffer. After 1 h the conjugate was separated from low-molecular-weight impurities by gel filtration (Sephadex G25, 0.02 M phosphate buffer with 0.15 M NaCl, pH 7.4). The solution containing Ab - polymer conjugate **10** was desalted by ultrafiltration using a PM 100 membrane (Amicon), lyophilized and kept at 4 °C.

## Purification and Characterization of Conjugates

The Ab content in the conjugate was estimated by amino acid analysis (Shimadzu, Japan, precolumn OPA derivatization) and the DOX content by UV spectrophotometry. Free DOX was estimated by HPLC after extraction with chloroform from aqueous solution of the conjugate. Determination of molecular weights was carried out with an HPLC Shimadzu system equipped with UV, RI Optilab®-rEX and multiangle light scattering DAWN EOS (Wyatt Technology Co., USA) detectors using 0.3 M acetate buffer (pH 6.5) and Super-

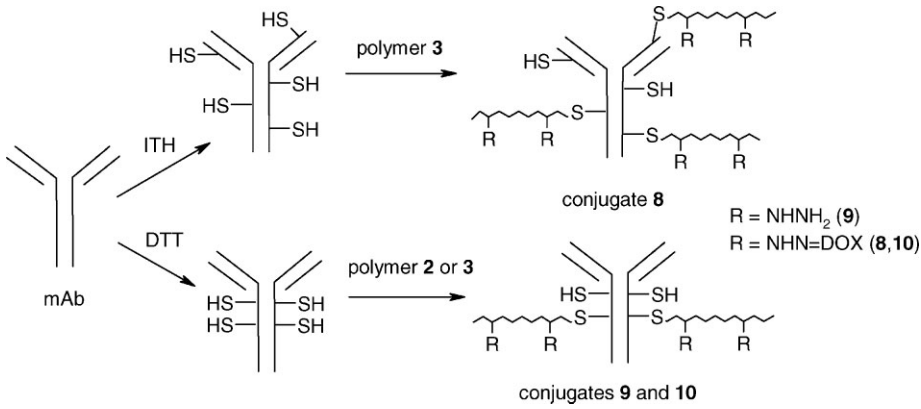


***Figure 2.***
Scheme of the synthesis of monoclonal antibody containing polymer conjugates **8**, **9** and **10**

 

ose™ 6 column or using methanol - sodium acetate buffer (0.3 M; pH 6.5) mixture (80: 20 vol.%; flow-rate 0.5 mL.min$^{-1}$) and TSKgel G3000SWxl column for polymers **5** and **7**. For calculation of molecular weights dn/dc values 0.175 for HPMA-based polymers in acetate buffers and 0.184 for HPMA-based polymers in methanol-acetate buffer mixture were used. The thiazolidine-2-thione (TT) group content was determined spectrophotometrically on a Helios α (Thermochrom) spectrophotometer ($\varepsilon_{305}$ = 10 700 L.mol$^{-1}$.cm$^{-1}$ (methanol) [19]).

The hydrazide groups content was determined by a modified TNBSA assay as described previously.[4] The MI groups content in polymer precursors was determined by a modified Ellman's assay as a difference between concentration of cysteine in solution before and after reaction with MI groups of the polymer.[16]

The LEV-CHOL content was determined by $^1$H-NMR (Bruker, 300 MHz). Integral intensities from $^1$H-NMR spectrum in $(CD_3)_2SO$ were compared: δ 5.32 t, 1H (C = CH); δ 4.70 br, 1H (CH-OH).

Hydrodynamic radii ($R_H$) in PBS buffer (pH 7.4; 0.15 M NaCl; polymer concentrations: 10 mg/mL for conjugate bearing hydrophobic substituent, 20 mg/mL for conjugate without hydrophobic substituent) were measured with a Nano-ZS instrument (ZEN3600, Malvern, UK). The intensity of scattered light was detected at angle $\theta = 173^\circ$. The wavelength of laser was 632.8 nm. For evaluation of dynamic light scattering data, the DTS(Nano) program was used. The values were a mean of at least five independent measurements. Apparent molecular weight ($M_{app}$) of conjugates was analysed by the Zimm procedure at single angle $\theta = 173^\circ$.

The values of critical micellar concentration (CMC) of polymer 5 in water was estimated using the pyrene fluoroprobe.[20]

### In Vitro Release of Doxorubicin from Polymer Drug Conjugates

The rate of DOX release from conjugates containing the drug attached via hydrazone bonds was investigated in the course of incubation of the conjugate in phosphate buffer at pH 5 or 7.4 (0.1 M phosphate buffer with 0.05 M NaCl) at 37 °C using extraction of released DOX followed by HPLC analysis as described previously.[5] The final concentration of the conjugates in incubation media was equivalent to 0.5 mM DOX.

### In Vitro Degradation of the Micellar Polymer Conjugates

The micellar polymer conjugate **7** was incubated in saline-sodium phosphate buffers (0.1 M sodium phosphate; 0.05 M NaCl; 1 mM EDTA; pH 5 or 7.4) at 37 °C at final polymer concentration 10 mg/mL. In the fixed time intervals, the amount of released LEV-CHOL was determined by HPLC after extraction into organic solvent from incubation aqueous solution as follows: 150 μL of polymer solution was mixed with 0.8 mL of $CHCl_3$ and gently shaken for 10 min. Organic phase was removed, dried and the released LEV-CHOL was determined by reverse-phase HPLC (Chromolith Performance RP-18e, 100 × 4.6 mm; 5 μm) with UV detection at 240 nm and isocratic elution acetonitrile: propan-2-ol (85:15 vol.%, flow-rate 1 mL.min$^{-1}$].

### In Vitro Study of Efficacy of Binding of Anti-CD20 Antibody to its Antigen

Conjugates or MEM97 antibodies were biotinylated using N-Hydroxysuccinimide activated biotins that react with primary amino groups of lysine residues (Sulfo-NHS-LC-Biotin, Pierce). Binding of biotinylated conjugates 8 - 10 or MEM97 Ab to Raji, EL4 or EL4-CD20 cells was tested using a standard flow cytometry. In all incubations the PBS buffer (2% FTS, 2 mM EDTA, 0.05% $NaN_3$) was used. 1x10$^5$ Raji, EL4 or EL4-CD20 cells were incubated for 10 min with 0.5 μg/mL of Fc block (anti CD16 and anti CD32 Ab, eBiosciences) and then 10 μg/mL of Ab equivalent in biotinylated Ab-polymer conjugates or native Ab was added. After 40-min incubation, the cells were washed three times with buffer and incubated with streptavidin-allophycocyanin (APC) (1:1000, Exbio

 

Czech Republic) for 20 min. After washing, mean the fluorescence intensity (MFI) of APC was analysed in live (Hoechst-negative) cells using a LSRII flow cytometer. All the presented data are based on at least three independent experiments.

## Results and Discussions

The synthesis and study of physicochemical and preliminary biological properties of new types of polymer cancerostatics designed for passive or active tumor targeting are described. A new micellar polymer carrier was obtained by attachment of a hydrophobic substituent, a cholesterol derivate, to side chains of the linear HPMA-based copolymer. As recently reported, self-assembling of cholesterol-substituted polymers in aqueous media leads to the formation of micellar structures[10] with the size exceeding the limit for glomerular filtration, and hence passively accumulated in solid tumors due to the EPR effect. The novelty of the micellar system described here consists in disintegration of the HMW carrier in target cells after hydrolysis of the pH-sensitive hydrazone spacer between the hydrophobic substituent and polymer backbone resulting in polymer fragments excretable from the body by glomerular filtration.

A second system, based on star-shaped Ab-containing polymer - drug conjugate, was intended for active tumor-targeting. Synthesis of the system was based on the reaction of HPMA copolymer precursor with thiol groups introduced into Ab by mild reduction of a part of cystine units with DTT. This reduction does not influence the Ab binding site and enables the consecutive reaction of Ab with the MI groups of semitelechelic HPMA-based polymers forming the star structure of the conjugate. Both drug carrier systems are bearing hydrazide groups enabling subsequent attachment of DOX via hydrazone bond susceptible to pH-controlled hydrolysis.

### Synthesis of Semitelechelic and Random Copolymers

Semitelechelic polymer precursor **1** was prepared by radical copolymerization of HPMA with a protected methacrylohydrazide initiated with ABIC-TT, an initiator containing two reactive TT groups. The use of ABIC-TT enables preparation of HPMA copolymers with more or less one chain-terminating reactive TT group if polymerization is performed under specific conditions. Physicochemical characteristics (molecular weight, polydispersity and functionality) can be controlled by proper selection of solvent, concentration of initiator and other reactants, and polymerization temperature. For the synthesis of conjugates with modified Ab, the TT end-group was transformed to maleimidyl group, suitable for selective reaction with the Ab sulfanyl groups. Conversion of TT to MI end-groups does not significantly change the molecular weight and polydispersity of polymer precursors while their functionality slightly decreases close to unity indicating that, on average, every polymer chain contained one MI end-group.

The random polymer precursor **4** containing hydrazide groups was prepared by radical copolymerization of HPMA with a monomer bearing hydrazide groups (Ma-ah-$NHNH_2$) initiated with AIBN. Molecular weights, polydispersity and the content of hydrazide groups in copolymer **4** are given in Table 1. The amount of hydrazide groups in polymer **4** was sufficient for attachment of both, derivative and the drug. Polymer precursor **5** was prepared by reaction of hydrazide groups of polymer **4** with carbonyl groups of the CHOL-LEV derivative in the presence of acetic acid. The carbonyl group was introduced into cholesterol by its acylation with 4-oxopentanoic acid using the DCC method. Attachment of CHOL-LEV to polymer **4** did not significantly change molecular weight and polydispersity of the polymer precursor, when measured by HPLC in an organic solvent. On the other hand, polymer **5** in aqueous solution forms supramolecular

 

structures of high molecular weight $M_w$ and hydrodynamic radius $R_H$. The apparent molecular weight increased from 28 000 to 230 000. Likewise $R_H$ increased from 4.3 to 14.3 nm. Such increase in $M_w$ and $R_H$ is important for an efficient EPR effect and accumulation of the micellar drug delivery system in the solid tumors.

Attachment of DOX to polymer precursors was performed by a procedure described earlier.[5] Attachment of DOX had no significant influence on the molecular weight, polydispersity, and aqueous solution properties of the polymer conjugates.

### Synthesis of Anti CD20-Antibody - Polymer Conjugates

The reaction of Ab containing thiol groups (introduced by the reaction with ITH) with polymer precursor **3** containing MI end-group enables attachment of 10 - 20 polymer chains to one Ab molecule. After conjugation we have observed only small amounts of the unbound polymer in the crude product (up to 12 wt.%). A high yield of conjugation could be ascribed to high reactivity of MI groups with sulfanyl groups in Ab.

Introduction of sulfanyl groups into antibody molecule via modification of amino groups with ITH is a common method. Its major drawback consists in partial loss of the binding activity of a modified antibody to its antigen because the amino groups of the active site are also involved in the modification reaction. This was why we used for introduction of sulfanyl groups into Ab a more specific method - reduction of disulfide bonds located outside the binding site for antigen with DTT.[12] As mentioned in the literature,[14] reducing agents such as DTT caused preferential reduction of heavy-light chain disulfides. After reduction of anti-CD20 Ab no significant changes in molecular weight, hydrodynamic radius or binding affinity of the Ab to antigen were observed. Approximately 8-10 sulfanyl groups were formed per one Ab molecule. This number of SH groups enabled the synthesis of a polymer conjugate with the antibody/polymer ratio similar to that prepared from ITH-modified Ab. Two polymers (**2** and **3**) containing MI end-group were used for the reaction with SH groups of modified Ab with the aim to prepare antibody-targeted conjugates with the drug attached via a pH-labile spacer or drug-free polymer conjugate as a control. Molecular weight of conjugates **9** and **10** were slightly lower than that of the conjugate synthesized from ITH-modified Ab (conjugate **8**) because of a lower number of polymer chains attached to the Ab. GPC analysis proved narrow distribution of molecular weight confirming star structure. Only a small content of unbound polymer was found in the crude product (less than 10 wt.%).

### In Vitro Degradation of Micellar Polymer Carriers

Our previous studies showed that passive accumulation of the HPMA copolymers in solid tumors (EPR effect)[21,22] strongly depends on molecular weight of the polymer, the HMW polymers being accumulated most efficiently. On the other hand, the HMW carriers must contain biodegradable linkages susceptible to intracellular or extracellular degradation to the products excretable from the organism. An increase in molecular weight could be obtained using various strategies, such as branching, grafting or self-assembling to supramolecular structures. The self-assembling micellar HMW polymer–drug conjugates describe previously[10] contained a polymer carrier with hydrophobic cholesterol substituents attached via stable ester bonds. Disintegration of these micellar conjugates, after delivery of the drug, is controlled by the critical micellar concentration (CMC). Below CMC, micelles dissolve to single polymer chains which could be removed from the body by glomerulal filtration. In case of cholesterol this concentration is very low, CMC = 10.5 mg/L, so the micelles will be stable for long time of circulation after administration into animals.

New micellar conjugates described here contain hydrophobic cholesterol moieties attached via hydrolytically degradable

 

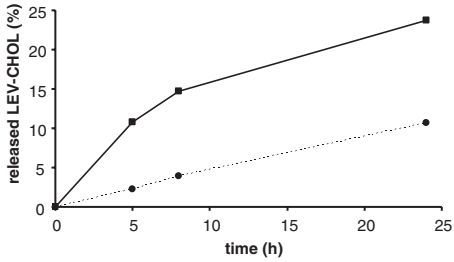


*Figure 3.*
Release of LEV-CHOL from micellar polymer conjugate **7** incubated in phosphate buffers at 37 °C. (■ —) pH 5; (●- - -) pH 7.4. Polymer concentration was 10 mg/mL.

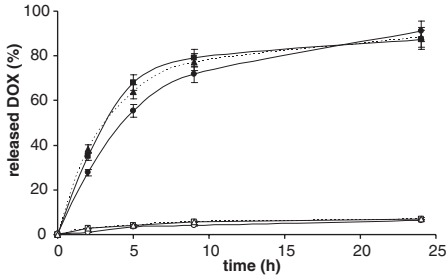


*Figure 4.*
Release of DOX from linear polymer conjugate **6**, micellar polymer conjugate **7** and star Ab-containing polymer conjugate **10** incubated in phosphate buffers at 37 °C. (■ —) conjugate **6**, pH 5; (● —) conjugate **7**, pH 5; (▲ - - -) conjugate **10**, pH 5; (△ - - -) conjugate **6**, pH 7.4; (□ —) conjugate **7,** pH 7.4; (○ —) conjugate **10**, pH 7.4. Polymer concentration was equivalent to 0.5 mM DOX.

spacers. In this case disintegration of the system is controlled not only by CMC, but preferably by pH-dependent hydrolysis of the hydrazone bonds linking hydrophobic cholesterol moieties. Study of the in vitro LEV-CHOL release showed that the hydrazone bond is fairly stable in buffer solutions at pH 7.4 and 37 °C (Figure 3) modeling the blood medium. Only a minor release (10%) of LEV-CHOL was observed within 24 h of incubation. On the other hand, ca. 25% of LEV-CHOL was released within 24 h after incubation in a buffer of pH 5 (37 °C) simulating conditions in endosomes of target cells. These results allow us to hypothesize that the new micellar conjugate is quite stable in blood circulation being disintegrated after the drug and LEV-CHOL release in the tumor tissue due to pH-controlled hydrolysis.

### Release of DOX from Polymer Conjugates

Study of the in vitro DOX release showed that the hydrazone bond used for DOX attachment in all linear, micellar and Ab-containing polymer conjugates are fairly stable in buffer solutions at pH 7.4 at 37 °C (Figure 4) modeling the blood medium. Only a minor release of DOX was observed within the 24-h incubation. On the other hand, ca. 90% of DOX was released within 24 h after incubation in a buffer of pH 5 (37 °C) simulating conditions in endosomes of target cells. The rate of DOX release is only slightly dependent on the detailed structure and size of the polymer-DOX conjugates and the effect of steric hindrance hydrolysis taken into consideration in case of micellar or Ab-containing HMW conjugates is negligible. To resume drug release experiments, all the new micellar and Ab-containing polymer-DOX conjugates fulfill the basic requirement for an efficient anticancer prodrug - stability in blood circulation and fast release of the active drug after entering tumor cells or tissue.

### Binding Efficacy of Anti-CD20 Antibody - modified Conjugates

The in vitro binding efficacy of Ab-containing conjugates (after biotinylation) or of native Ab to EL4 or EL4-CD20 cells was tested using a standard flow cytometry procedure by measuring mean fluorescence intensity (MFI) of APC in living (Hoechst-negative) cells. Two cell lines were used representing CD20-positive (EL4 transfected with CD20) or CD20-negative (EL4) experimental tumors. The binding efficacy of native Ab and DTT-reduced Ab was compared first. As can be seen in Figure 5, no significant difference in binding activity to EL4-CD20 cells was observed for both Ab samples. Native Ab and DTT-reduced Ab exhibited almost the same MFI demonstrating that the reduction of disulfides in Ab with DTT did not influence the binding affinity of

 

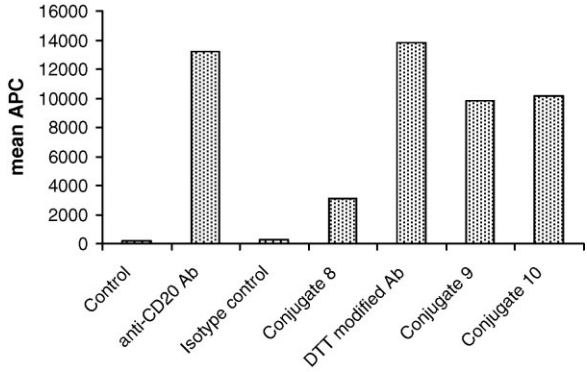


***Figure 5.***
Efficacy of binding of native anti-CD20 Ab, DTT-modified anti-CD20 Ab and its Ab-containing polymer conjugates **8-10** to EL4 CD20 cells, expressed as MFI of APC.

Ab to its CD20 receptor. This shows that this method is suitable for Ab modification enabling its subsequent conjugation with a polymer. When EL4 T-cell leukemia cells (without CD20 antigen) were used as a control, no Ab or Ab conjugate binding to the cells was observed (data not shown).

Tests of the binding efficacy of Ab-polymer conjugates showed that the method of Ab conjugation to polymer strongly influenced its binding efficacy to the antigen when CD20-positive line EL4-CD20 was used (Figure 5). Conjugate **8,** where 6-amino groups of lysine residues in Ab were involved in conjugation (reaction of MI-containing precursor with ITH-modified Ab), showed a very low binding activity, much lower than that of the free Ab. On the other hand, polymer-Ab conjugates **9** and **10**, prepared from DTT-reduced Ab, showed a significantly retained binding activity, three times higher than that of conjugate **8**. No significant divergence was found between conjugates of DTT-reduced Ab without drug and those containing DOX attached via pH-labile hydrazone bond. We can conclude that conjugation of anti-CD20 Ab with polymer carriers via ITH-modified Ab dramatically decreased the binding of conjugated Ab to the antigen present on antigen-positive cells. Modification of the amino groups near or at the binding site almost tired out the efficacy of binding to the CD20 antigen. The new method of conjugation using the reaction with DTT-reduced Ab is a highly efficient method suitable for preparation of Ab-polymer conjugates with well-defined structures, preserving the efficacy of binding to the antigen. A small drop-off in binding activity can be ascribed to steric hindrance of polymer chains, which is present in any modification of antibody with a polymer.

## Conclusion

Two drug delivery systems based on HPMA copolymers were designed for tumor targeting. Their synthesis, physicochemical and some biological characteristics were described. anti-CD20-targeted water-soluble conjugate of a star structure bearing doxorubicin was designed for active targeting on tumor cells expressing CD20 antigen. In the conjugate a defined number of semitelechelic DOX-bearing polymer chains are coupled to the sulfanyl groups of targeting Ab by one-point attachment to form a star structure. The sulfanyl groups were introduced into Ab by mild reduction of cystine disulfides in Ab. After conjugation with a polymer, the conjugate showed a well-defined star structure with a rather narrow distribution of molecular weights and preserved efficacy of

 

binding of Ab to its specific cell membrane antigen.

A micellar HPMA-based polymer conjugate containing hydrophobic cholesterol moiety attached via pH-sensitive hydrazone bond was designed for passive targeting of DOX to solid tumors. The conjugate formed supramolecular structures in aqueous solutions, polymer micelles, which are expected to show enhanced accumulation in solid tumors due to the EPR effect. After their intracellular disintegration, allow elimination of the polymer from body by glomerular filtration. Both types of conjugates bear DOX attached via hydrazone bonds enabling specific intracellular drug release.

The new strategy in the synthesis of antibody-targeted and micellar polymer-drug conjugates opens new perspectives for polymer drug conjugates in targeted drug delivery.

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