POLY(ETHYLENE GLYCOL)-BASED POLYMER CARRIER OF DOXORUBICIN DEGRADABLE BY BOTH ENZYMATIC AND CHEMICAL HYDROLYSES

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The synthesis and characterization of a water-soluble biodegradable multiblock polyurethane based on poly(ethylene glycol) blocks interconnected by a pentapeptide derivative *N*,*N*′-bis(aspartylprolyl)lysine is described. The pentapeptide linkages are susceptible both to chemical hydrolysis in neutral and mild acid medium (pH 5) and to enzymatic degradation by lysosomal enzyme cathepsin B. Anti-cancer drug doxorubicin was attached to a hydrazide derivative of the polymer via a hydrolytically labile hydrazone bond. Both the pH-triggered hydrolytic release of the drug and the degradation of the polymer carrier by enzymatic and chemical hydrolysis were studied using liquid chromatography.

Keywords: Drug delivery systems; Cancer therapy; Doxorubicin; Poly(ethylene glycol); Peptide synthesis; Polymer-supported drugs; Antineoplastics; Cytostatics; Biodegradable polymers.

Poly(ethylene glycol) (PEG) has been suggested in recent years for a large number of biomedical applications. Because of its good solubility in water and excellent biocompatibility, PEG is often used for modification of biologically active proteins[1–3] (enzymes, antibodies), liposomes[4,5], gene delivery vectors[6–8] (DNA–polycation complexes) or various low-molecular-weight drugs[9–13] (cytostatics). Prolonged blood circulation, improved solubility of hydrophobic drugs, lowered non-specific toxicity or immunogenicity of polymer-modified proteins and peptides, and possible targeting when heterobifunctional PEG is used are the major advantages of "PEGylation". A significant drawback is the non-degradability of PEG limiting the molecular weight (M_w) of the polymer used to values allowing its excretion by glomerular filtration.

We have recently reported[10,14,15] on the synthesis of a biodegradable high-molecular-weight multiblock polymer consisting of blocks of

PEG 2000 and an enzymatically degradable tripeptide H-Glu-Lys(H-Glu-)-OH and investigated its application as a carrier of an anthracycline cytostatic doxorubicin (Dox). A biodegradable high-molecular-weight polymer drug carrier was designed to enable enhanced accumulation of the anti-cancer drug in solid tumors due to EPR (enhanced permeability and retention) effect[16] and elimination of the carrier from a body after anti-cancer drug is released and polymer degraded in target cancer cells. In our current work, we have further improved the structure of the polymer carrier replacing the only enzymatically degradable tripeptide linker with a pentapeptide *N*,*N*′-bis(aspartylprolyl)lysine (**1**), susceptible to much faster intracellular degradation both by lysosomal enzymes and by mild acid hydrolysis (pH 5). The susceptibility of the Asp-Pro peptide bond to hydrolytic degradation in mild acid aqueous solutions is described in peptide chemistry literature[17] as an undesirable property, diminishing the stability of certain peptides and proteins in aqueous medium. We have taken advantage of this fact and designed a new hydrolytically degradable polymer applicable either as a carrier of low-molecular-weight drugs or as a polymer for modification of proteins, liposomes or DNA delivery vectors.

We have converted the pendant carboxylic groups of the linker to the corresponding hydrazide functionalities and covalently attached doxorubicin to the polymer carrier via an acid-labile hydrazone bond. In this study, we present results of both the pH-triggered release of doxorubicin from the multiblock polymer conjugate and the degradation of the polymer carrier at various pH values. Moreover, susceptibility of the polymer carrier to enzyme-catalyzed degradation is demonstrated.

EXPERIMENTAL

Abbreviations. DCM, dichloromethane; Dox, doxorubicin; DMF, *N*,*N*-dimethylformamide; mPEG, α-methoxy-PEG; mPEG-SC, mPEG-succinimidyl carbonate; M_n, number-average molecular weight; M_w, weight-average molecular weight; PEG, poly(ethylene glycol); PEG-BSC, PEG-bis(succinimidyl) carbonate; SEC, size exclusion chromatography; TFA, trifluoroacetic acid; TNBSA, 2,4,6-trinitrobenzene-1-sulfonic acid.

Materials and Methods

Doxorubicin hydrochloride, PEG 2000 and cathepsin B were purchased from Fluka (Switzerland). Protected amino acid derivatives, Wang resin and reagents for peptide synthesis were from Novabiochem (Switzerland). All chemicals and solvents were of analytical grade. Solvents were purified and dried by standard procedures. The reagents were used without further purification. NMR spectra were recorded on a Bruker spectrometer (300 MHz, Switzerland). Matrix-assisted laser desorption-ionization time-of-flight mass spectroscopy (MALDI-TOF MS) was carried out using a Bruker Biflex III mass spectrometer. Determination of mo-

lecular weight was carried out by SEC on Äkta Explorer (Amersham Biosciences) equipped with refractive index (RI), UV and multi-angle light scattering DAWN DSP-F (Wyatt Technology Corp., U.S.A.) detectors using TSK columns 3000 and 4000 (50% methanol, 0.1% TFA). The identity of doxorubicin released from polymer was verified by HPLC using a reversed phase column, Nucleosil C18, 250 × 4 mm (Watrex, Czech Republic) and linear gradient methanol–H_2O, 40–90% methanol during 50 min in the presence of 0.1% TFA, UV detector (Shimadzu) at 484 nm, fluorescence detector (Shimadzu) with excitation wavelength at 490 nm, emission at 560 nm. Homogeneity of peptide derivatives was checked by HPLC using the same column and linear gradient water–acetonitrile, 0–100% acetonitrile in presence of 0.1% TFA, UV detector 220 nm. The content of Dox in samples was determined by UV spectrophotometry at 484 nm (ε = 11 500 l mol^{-1} cm^{-1} in H_2O, 13 500 l mol^{-1} cm^{-1} in methanol). The content of hydrazide functionalities was determined by TNBSA assay as described previously[18]. Succinimidyl carbonates of mPEG and PEG (mPEG-SC and PEG-BSC, respectively) were prepared as described in literature[19].

N,*N*′-Bis(aspartylprolyl)lysine Bis(trifluoroacetate) (**1**)

The title pentapeptide was prepared by manual Fmoc/tBu ((9-fluorenylmethoxy)carbonyl/*tert*-butyl) strategy on Wang resin. First, Fmoc-Lys(Fmoc)-OH (3.54 g, 6 mmol) and diisopropylcarbodiimide (DIC) (3.76 ml, 24 mmol) were dissolved in DCM (20 ml) at 20 °C, concentrated to an oily residue 20 min later and re-dissolved in DMF (15 ml) with 1-hydroxybenzotriazole (2.75 g, 18 mmol). The solution was added to Wang resin (2 g, 2.06 mmol of OH groups) followed by diisopropylethylamine (DIEA) (1.07 ml, 6 mmol) and 4-(dimethylamino)pyridine (73 mg, 0.6 mmol) and the suspension was agitated on a rotavapor for 12 h. The resin was isolated by filtration and washed with DMF (3 × 15 ml). The elongation cycle consisted of Fmoc removal with 20% piperidine in DMF (2 × 5 min) and coupling with Fmoc-Pro-OH (2.70 g, 8 mmol) and Fmoc-Asp(OtBu)-OH (3.29 g, 8 mmol), respectively, activated with (benzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate (PyBOP) (4.16 g, 8 mmol) and 1-hydroxybenzotriazole (1.22 g, 8 mmol) in DMF in the presence of DIEA (0.86 ml, 5 mmol) under N_2 (15–45 min). Completion of each coupling step was verified with a ninhydrin test. The final deprotected peptide was cleaved from the resin with a mixture of 95% TFA, 2.5% water, and 2.5% triisopropylsilane (TIS) and the resin beads were removed by filtration. The filtrate was concentrated under vacuum and the residue was triturated with diethyl ether to yield 1.05 g (1.31 mmol, 82%) of peptide **1**. Reversed phase HPLC revealed single peak (UV detection at 220 nm). ^{1}H NMR ($(CD_3)_2SO$): 1.2–1.5 m, 4 H (γ, δ-Lys); 1.5–1.8 m, 2 H (β-Lys); 1.8–2.0 m, 8 H (β-Asp, β-Pro); 2.0–2.4 m, 4 H (δ-Pro); 2.5–2.8 m, 4 H (β-Pro); 2.8–3.2 m, 2 H (ε-Lys); 3.5–3.8 m, 2 H (α-Asp); 4.0–4.2 m, 1 H (α-Lys); 4.5–4.7 m, 2 H (α-Pro); 7.75 t, 1 H (NH-ε-Lys); 8.07 d, 1 H (NH-α-Lys); 8.23 br, 6 H (NH_3^+); 9.50 br, 2 H (COOH). MALDI-TOF MS: 571.6 (M + H).

$(mPEG\text{-}AspPro)_2Lys$ (**2**)

Pentapeptide **1** (50 mg, 0.063 mmol) reacted with mPEG-SC (268 mg, 0.125 mmol) in DMF (2 ml) with DIEA (0.045 ml, 0.25 mmol) overnight. Analytical SEC of the reaction mixture revealed about 70 wt.% of a fraction with M_w >4000 corresponding to the title product. The title diblock polymer was isolated by precipitation into diethyl ether followed by preparative SEC (TSK 3000 SW column, 50% methanol–water, 0.1% TFA), evaporation of the corre-

sponding fraction and lyophilization to yield 180 mg (70%) of white polymer **2**. For MALDI-TOF spectrum of the polymer, see Fig. 1.

[mPEG-AspPro)$_2$Lys]-(NHNH$_2$)$_3$ (**3**)

Polymer **2** (50 mg, 0.03 mmol of COOH) and diisopropylcarbodiimide (15 µl, 0.1 mmol) were dissolved in DCM (0.5 ml) at 0 °C and hydrazine hydrate (5 µl, 0.1 mmol) was added to the reaction mixture under stirring and cooling. After 5 h at 25 °C, the solvent was evaporated under reduced pressure and the residue was suspended in water (1 ml). An insoluble solid (1,3-diisopropylurea) was removed by centrifugation and the filtrate was chromatographed on Sephadex G-25 in water. The polymer fraction was lyophilized to yield 33 mg (66%) of the title polymer. TNBSA assay of hydrazide groups showed quantitative conversion.

[PEG-(AspPro)$_2$Lys]$_n$ (**4**)

Multiblock polymer **4** was prepared similarly to diblock polymer **2** starting with PEG-BSC (560 mg, 0.25 mmol), pentapeptide **1** (200 mg, 0.25 mmol) and excess of DIEA (0.45 ml, 2.5 mmol). The product was isolated by precipitation into diethyl ether followed by SEC (Sephadex LH 20, methanol), evaporation of the high-molecular-weight fraction and lyophilization yielding 430 mg (77%) of white polymer **4**. Analytical SEC (TSK 4000 SW column, 50% methanol–water, 0.1% TFA, light scattering detector) showed M_w = 43 000, polydispersity M_w/M_n = 3.2.

{PEG-[(AspPro)$_2$Lys](NHNH$_2$)$_3$}$_n$ (**5**)

Polymer **4** (150 mg, 0.18 mmol of COOH) and diisopropylcarbodiimide (0.137 ml, 0.88 mmol) were dissolved in DCM (1.5 ml) at 0 °C. Within 1 min, a gel formed, which gradually re-dissolved after addition of hydrazine hydrate (0.043 ml, 0.88 mmol). The sol-

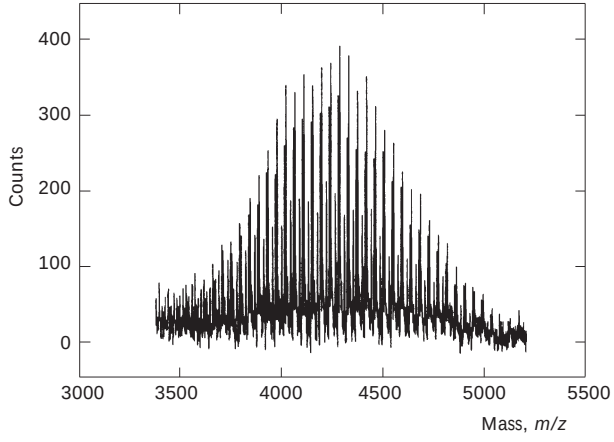


FIG. 1
MALDI-TOF mass spectrum of diblock polymer **2**. The molecular weight difference (44 units) between neighboring peaks corresponds to the CH_2CH_2O repeating unit in PEG

vent was evaporated 5 h later and the residue was suspended in water (3 ml). An insoluble solid (1,3-diisopropylurea) was removed by filtration and the filtrate was chromatographed on Sephadex G-25 in water. The polymer fraction was lyophilized to yield 140 mg (93%) of polymeric hydrazide **5**. Molecular weight distribution (checked by SEC) was identical with that of polymer **4**. TNBSA assay of hydrazide groups has shown quantitative conversion.

{PEG-[$(AspPro)_2$Lys](NHN=Dox)}$_n$ (**6**)

Polymer **5** (80 mg, 0.09 mmol of $NHNH_2$) and doxorubicin hydrochloride (18 mg, 0.03 mmol) were reacted in dry methanol (1 ml) in the presence of acetic acid (10 μl) and anhydrous sodium sulfate (10 mg) under stirring overnight. The solution was filtered and chromatographed on Sephadex LH-60 in methanol. A red polymer fraction was evaporated to dryness, dissolved in water and lyophilized to yield 71 mg (89%) of the polymer-Dox conjugate **6**. The content of Dox in the conjugate was 10.3 wt.%.

Hydrolytic Degradation of Diblock Polymers **2** and **3**

Enzymatic hydrolysis with cathepsin B (final concentration 8×10^{-7} mol l^{-1}, determined as described[20], pH 6.0) and non-enzymatic hydrolyses were performed with diblock polymers **2** (in phosphate buffers of pH 3.0, 5.0, 6.0, and 7.4) and **3** (at pH 5.0) as substrates (10^{-3} mol l^{-1}) at 37 °C. The progress of the polymer degradation was monitored by SEC (TSK 3000 SW column, 50% methanol–water, 0.1% TFA, RI detector) following the amount of mPEG 2000 released.

Hydrolytic Release of Doxorubicin from Polymer Carrier

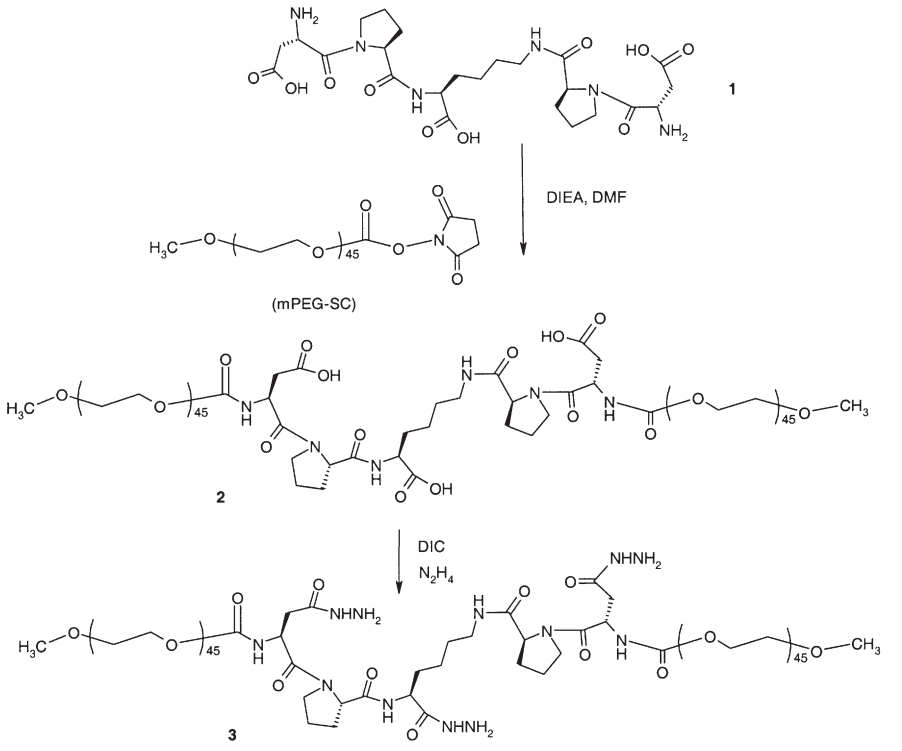
Polymer–Dox conjugate **6** (15 mg ml^{-1}) was incubated in 0.66 M phosphate buffers pH 5.0 and 7.4 at 37 °C. The amount of free Dox was determined by SEC of the reaction mixture from the relative area of the corresponding peak on column TSK 3000, 80% methanol, 20% of 0.3 M sodium acetate buffer, pH 6.7, UV detector 484 nm.

RESULTS AND DISCUSSION

Synthesis of Polymers

The synthesis of pentapeptide diamine linker **1** started by attachment of Fmoc-Lys(Fmoc)-OH to the resin and, after removal of both protecting groups, the Pro and Asp residues were attached to both NH_2 groups of lysine. The crude peptide **1** cleaved from the solid support was sufficiently homogeneous (>98% by HPLC) to be used for the reaction with mPEG-SC or PEG-BSC without further purification.

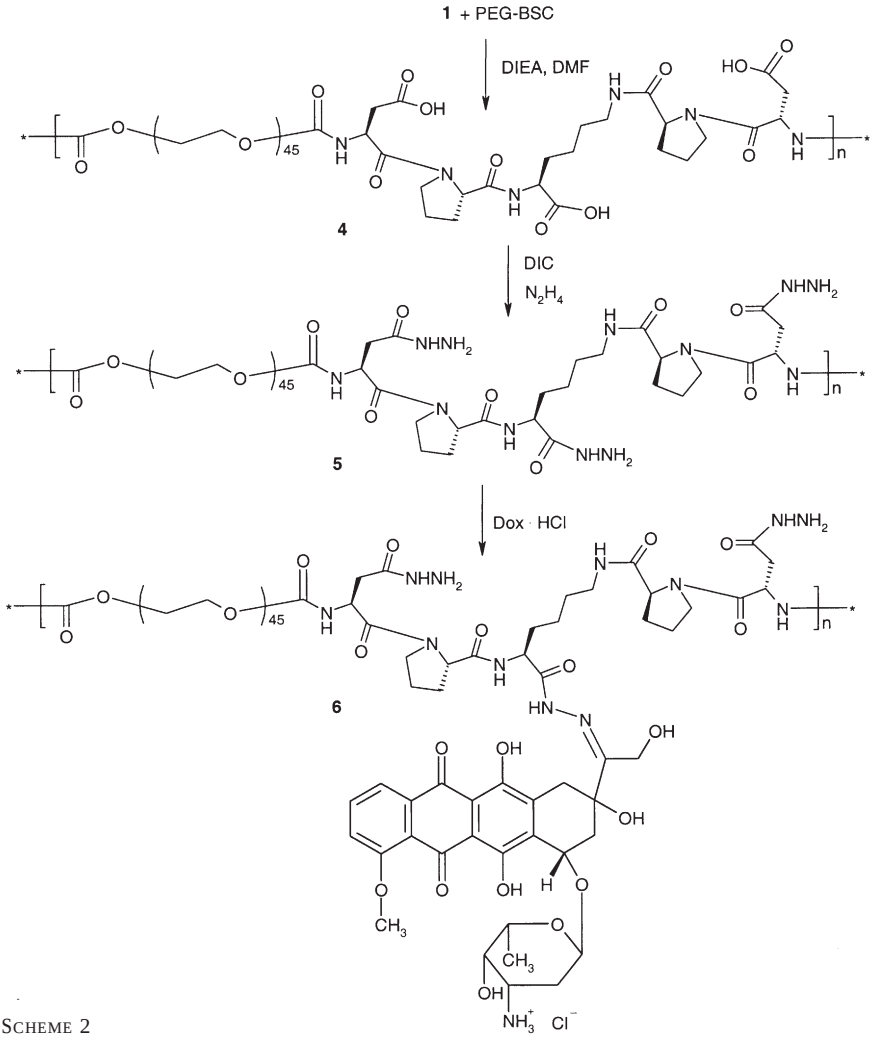
The reaction of diamine linker **1** with mPEG-SC in DMF (Scheme 1) gave model diblock polymer **2** characterized by SEC and MALDI-TOF MS (Fig. 1). In the next step, polymer **2** reacted with hydrazine hydrate in the presence of diisopropylcarbodiimide to model diblock hydrazide polymer **3**. The



SCHEME 1

polycondensation of PEG-BSC with peptide **1** (Scheme 2) yielded high-molecular-weight multiblock polyurethane **4** of M_w = 43 000. A large excess of tertiary amine (diisopropylethylamine) in the reaction mixture was found to be advantageous to neutralize TFA which was non-covalently bound to peptide linker **1** in higher than the stoichiometric ratio.

Surprisingly, the polycondensation completely failed when an interfacial reaction was attempted in DCM and aqueous sodium hydrogencarbonate solution. This system was successfully used[10] for preparation of analogous multiblock polymers containing GluLys(-Glu) linkages with benzyl ester protecting groups on the carboxyls. However, the polycondensation in DMF yielded the target polymer of sufficient M_w and already with free COOH groups of Lys and Asp residues, hence alternative reaction conditions were not further investigated. The pendant carboxylic groups were subsequently modified with hydrazine hydrate and finally doxorubicin was attached to some of the hydrazide functionalities of the polymer carrier by hydrazone bond to yield polymer–drug conjugate **6**.



SCHEME 2

Hydrolytic Degradation of Polymers

The rate of hydrolysis of model diblock polymer **2** in buffers of various pH was studied. The rate (evaluated by the amount of mPEG released) increased with decreasing pH of the solution. When polymer **2** was incubated with a lysosomal enzyme cathepsin B, its degradation rate was significantly higher than in the control buffer (pH 6.0) without enzyme (Fig. 2), indicating the susceptibility of the Asp-Pro-Lys(Asp-Pro)-OH sequence to hydrolysis by intracellular proteases. Cathepsin B was chosen as a representative of

lysosomal enzymes responsible for intracellular degradation of polymer carriers after their uptake by target cells *in vivo*.

It should be noted that the degradation of the high-molecular-weight multiblock polymer **4** produces not only PEG 2000 but also longer polymer fragments. Therefore the rate of release of PEG 2000 would be lower than that from model diblock polymer **2**. However, the initial concentration of the hydrolyzable peptide bonds is nearly twice higher in the case of the multiblock polymer than in the case of the diblock model (if the same weight concentration is used). In consequence, the amount of PEG 2000 released after 48 h of incubation in a buffer at pH 5 of the two polymers was not much different – 24% of PEG 2000 was released from diblock polymer **2** (see Fig. 2) while 18% of PEG 2000 was released from multiblock polymer **4**.

The presence of biodegradable peptide linkages connecting PEG blocks into high-molecular-weight polymer renders polymer **4** a good candidate for a lysosomotropic drug carrier or a polymer suitable for preparation of "stealth" biologically active proteins or particles for extended systemic circulation. The degradability of the polymer under *in vivo* conditions enables elimination of its metabolites by glomerular filtration even if a high-molecular-weight polymer (i.e., suitable for passive tumor targeting[16]) is used. It can be assumed that, due to its acid-sensitive peptide bonds, the

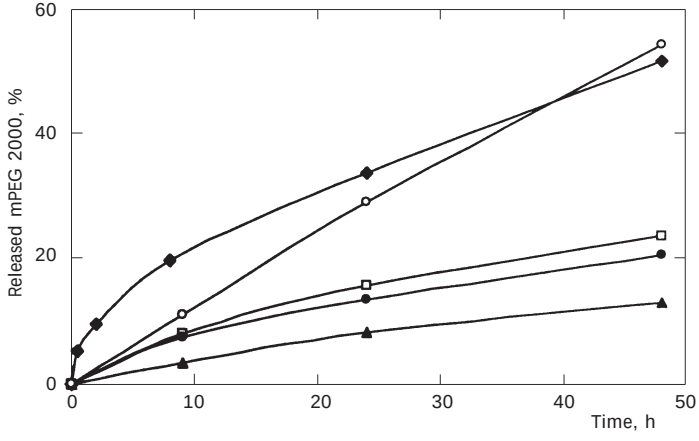


FIG. 2

Hydrolytic degradation of diblock copolymer **2**. Enzymatic hydrolysis was performed with cathepsin B (8×10^{-7} mol l^{-1}, pH 6.0 (◆), 37 °C) and non-enzymatic hydrolysis in buffers of various pH (10^{-3} mol l^{-1}; pH 3.0 (○), 5.0 (□), 6.0 (●), and 7.4 (▲); 37 °C). The progress of polymer degradation was monitored by SEC (TSK 3000 SW column, 50% methanol–water, 0.1% TFA) following the amount of mPEG 2000 released. Standard deviation did not exceed 5%

polymer would be finally degraded even in endosomal compartments of cells with a limited or no activity of lysosomal enzymes. Moreover, the multivalency of the highly hydrophilic polymer **4** offers possibilities not only for attachment of anti-cancer drugs but also for modification of therapeutically important proteins (enzymes, cytokines, antibodies), for surface coating of gene delivery vectors based on DNA–polycation complexes, liposomes or viruses and, after attachment of cell receptor-specific molecules, it enables their targeting[7,21,22] on specific cells and tissues *in vivo*.

The unique ease of mild acid hydrolysis of polymer **4** is directly related to the presence of the unmodified Asp-Pro peptide bond. Thus, a question arises whether the hydrolysis rate would be influenced by the modification of the β-carboxylic group of Asp residue with hydrazine, which is necessary, e.g., for preparation of polymer–Dox conjugate **6**. Therefore, model diblock hydrazide polymer **3** was prepared and its degradability by chemical and cathepsin B-catalyzed enzymatic hydrolysis was investigated. The results of the study shown in Fig. 3 demonstrate that rates of both the enzymatic and non-enzymatic hydrolyses (pH 5.0) were significantly slowed down compared with those of corresponding polymer **2** (Fig. 2) with free COOH groups. Nevertheless, the hydrolysis was not eliminated completely and, consequently, we may assume that also the metabolic products arising from possible *in vivo* application of polymer–Dox conjugate **6** (and, conse-

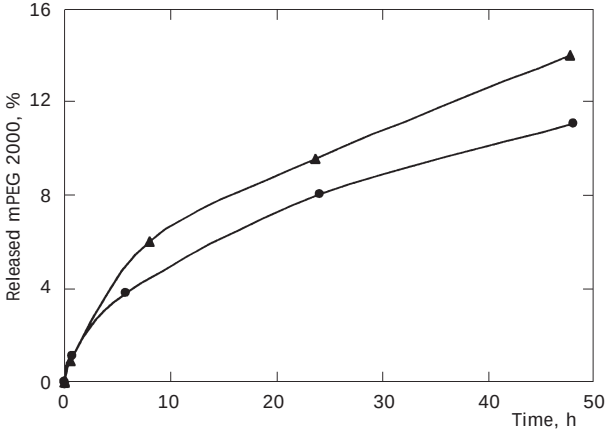


FIG. 3

Hydrolytic degradation of diblock copolymer **3**. Enzymatic hydrolysis was performed with cathepsin B (▲; 8×10^{-7} mol l^{-1}, pH 6.0, 37 °C) and non-enzymatic hydrolysis in phosphate buffer pH 5.0 (●; 10^{-3} mol l^{-1}, 37 °C). The progress of the polymer degradation was monitored by SEC (TSK 3000 SW column, 50% aqueous methanol, 0.1% TFA) following the amount of mPEG 2000 released. Standard deviation did not exceed 5%

quently, also those of polymer hydrazide **5**) would be finally excreted from organism by glomerular filtration.

*Hydrolytic Release of Doxorubicin from Polymer–Drug Conjugate **6***

It is now generally accepted that the release of a low-molecular-weight cytostatic compound doxorubicin from its conjugate with a synthetic polymer carrier is a prerequisite for successful therapeutic use of the conjugate. The release mechanism is usually based either on enzymatic or chemical hydrolysis. While the systems based on enzymatic hydrolysis require time-consuming and costly synthesis of a suitable oligopeptide substrate as a spacer between the drug molecule and polymer carrier, formation of acid-labile hydrazone bond between Dox and PEG multiblock carrier described in this work is quite a straightforward single-step reaction.

It has been shown previously[13] that doxorubicin (or another hydrophobic drug) covalently linked to a hydrophilic polymer can physically entrap free drug molecules to the resulting conjugate. In consequence, it is quite difficult to separate the unbound drug (always remaining after the conjugation reaction) from the polymer conjugate by chromatography in aqueous buffers. We have found that quantitative chromatographic separation can be achieved by chromatography in methanol (preparative scale) or in a

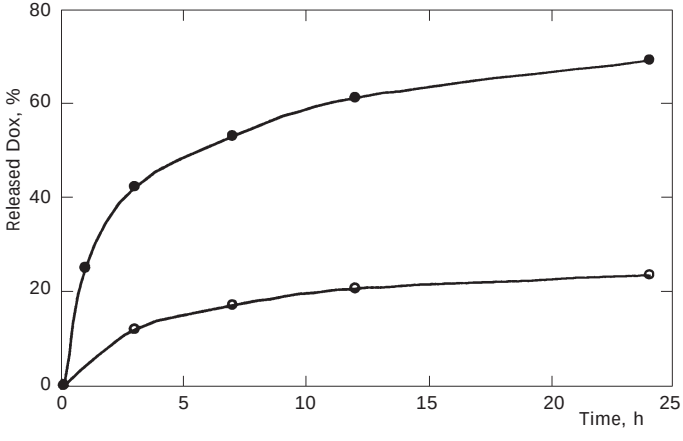


FIG. 4

Dox released by hydrolysis of polymer **6** incubated in phosphate buffers at pH 5.0 (●) and 7.4 (○) at 37 °C. Concentration of Dox was 3×10^{-3} mol l^{-1}. The amount of free Dox was determined by SEC of the reaction mixture from the area of the corresponding peak (column TSK 3000 SW, 80% MeOH, 20% of 0.3 M sodium acetate buffer, pH 6.7, UV detector set at 480 nm). Standard deviation did not exceed 5%

mixture of methanol with an aqueous buffer (analytical scale). The latter method was used for determination of the amount of the drug released at different pH.

The difference between the rate of hydrolysis of the polymer–Dox conjugate **6** at pH 7.4 and 5.0 (Fig. 4) demonstrates relative stability of the polymer drug in an environment typical of the bloodstream (pH 7.4) and rapid release of the active compound (doxorubicin) in a buffer modelling endosomal compartments (pH 5.0) of tumor cells[23]. The resulting polymer hydrazide **5** would be finally (albeit slowly) degraded to polymer fragments of sufficiently low M_w that could be excreted from organism by glomerular filtration as shown in the preceding section.

CONCLUSION

A new multivalent multiblock PEG-based polymer suitable for modification of biologically active compounds was synthesized and characterized. The rate of its *in vitro* hydrolytic degradation increased with decreasing pH of incubation media. The polymer was found to be degradable also by a lysosomal enzyme cathepsin B. Covalent attachment of anti-cancer drug doxorubicin to the polymer via acid-labile hydrazone bond was described and its hydrolysis and rate of drug release at pH 5.0 (mimicking pH inside endosomes) and at pH 7.4 (pH of blood) was determined.

Biological evaluation of this new biodegradable polymer as a carrier of cytostatics and as a polymer for modification of biologically active proteins (e.g., enzymes or antibodies) and viral and non-viral gene delivery vectors is under way.

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