DEGRADATION BEHAVIOR OF POLY(ETHYLENE GLYCOL) DIBLOCK AND MULTIBLOCK POLYMERS WITH HYDROLYTICALLY DEGRADABLE ESTER LINKAGES

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Diblock and multiblock polymers of poly(ethylene glycol) containing degradable ester bonds between the blocks were synthesized and characterized. Monofunctional poly(ethylene glycol) (PEG 2000) was modified by aliphatic dicarboxylic acids (malonic, succinic, glutaric, maleic) to obtain monocarboxylic polymers PEG-COOH containing ester bonds. Diblock polymers (4000) were prepared by polycondensation of a diamine (ethane-1,2-diamine, L-lysine) and the semitelechelic PEG-COOH. The relationship between the structure of the linkage connecting two PEG blocks and the rate of its hydrolytic degradation was studied at pH 5.5, 7.4 and 8.0. The rate of hydrolysis of all polymers was significant already under mild alkaline conditions (pH 7.4 and 8.0) and increased with increasing pH. The ester bonds of polymers with saturated dicarboxylic acid moieties were stable at pH 5.5. However, the presence of double bond in the acid moiety substantially decreased the stability of the polymer not only in alkaline but also in acid medium. The results of this model study can be utilized in the design of biodegradable high-molecular-weight drug carriers and polymers for preparation of "stealth" systems intended for therapeutic application.

Keywords: PEG; Block copolymers; Degradable bonds; pH sensitivity; Hydrolyses; Protein modification; Drug release; Polymer drugs; Tumour therapy; Drug carriers; Biocompatible polymers.

Biologically active compounds (BAC) like anti-cancer drugs or biologically active proteins are utilized in human therapy of cancer or inborn defects of metabolism and other disorders. Unfortunately, currently used proteins or low-molecular-weight drugs are very quickly entrapped by the reticulo-endothelial system (RES) and metabolized or excreted from organism by glomerular filtration. Moreover, peptides and proteins in native status are immunogenic, antigenic and are rapidly degraded by proteolytic enzymes. In consequence, a variety of polymer-modified BAC were developed to overcome these disadvantages[1,2].

German pathologist and immunologist Paul Ehrlich came already in 1906 with the idea of "magic bullet" aiming directly at the target tissue[3]. This idea was adopted in 1975 by Ringsdorf, who suggested an improved model of a polymeric drug (Fig. 1). According to his concept, a polymer drug consists of polymer backbone (carrier), a biodegradable spacer, a drug and a homing device (targeting moiety)[4].

At present, the most intensively studied polymer drug carriers are based on vinyl polymers (copolymers of *N*-(2-hydroxypropyl)methacrylamide (pHPMA)[5–7], low-molecular-weight styrene-maleic anhydride copolymers[7,8]), synthetic poly(α-amino acid)s (poly-L-lysine, poly(L-glutamic acid), poly[*N*-(hydroxyalkyl)glutamines])[9], polysaccharides (dextran)[7] and proteins (serum albumin)[7] and poly(ethylene glycol) (PEG)[1,6,7,10–13].

PEG is a linear or branched neutral polyether, which is water-soluble, non-toxic, non-immunogenic and non-antigenic. If this polymer is covalently linked to BAC, it can improve solubility of hydrophobic drugs, render proteins non-immunogenic and tolerable, reduce the rate of drug clearance through kidneys, prevent adsorption of proteins on surfaces, alter electro-osmotic flow, facilitate transport of molecules across cell membranes and improve their pharmacokinetics. "PEGylation" prolongs the blood circulation half-life of proteins or peptides and simultaneously limits their renal filtration and alters their biodistribution[1,7].

We have recently reported[14] on the synthesis of a multiblock polymer consisting of PEG 2000 and an enzymatically degradable tripeptide Glu-Lys(Glu)-OH and its application as a carrier of anti-cancer drug doxorubicin.

In our present work, we have designed and synthesized a new polymer carrier of BAC (e.g., proteins or anti-cancer drugs) based on PEG with hydrolytically labile (pH-dependent) ester bonds between PEG blocks. For de-

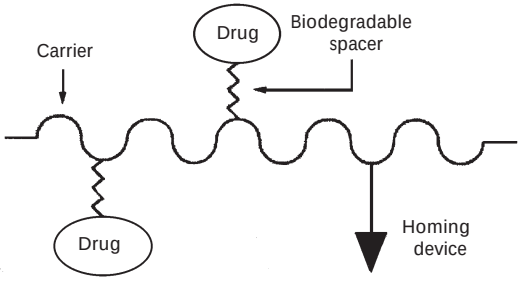


FIG. 1
Ringsdorf's model of polymer drug

tailed study of polymer degradation, we have prepared a series of model diblock polymers with different structure of the linkage susceptible to pH-dependent hydrolysis. The results of degradation studies obtained with these simplified model polymers will serve for future design and preparation of high-molecular-weight polymer drug conjugates. These multiblock polymers, after their conjugation to BAC, are expected to slow down renal excretion and blood plasma clearance of the polymer–BAC conjugates, thus preserving their long-term therapeutic level together with their accumulation in solid tumours by the EPR (enhanced permeability and retention) effect[15] in the case of polymer-modified cytostatic drugs. High polymer–drug conjugate concentrations in tumour could lead to a better treatment efficiency and specificity and, in consequence, to diminished side effects.

Taking into account the literature data[1,6,7,16], it can be expected that the multiblock polymers based on PEG conjugated with biologically active proteins would protect the proteins to degradation during their transport in organism and, at the same time, preserve their natural biological activity. The proteins would be covered with the high-molecular-weight polymer, which is water-soluble, non-toxic, non-immunogenic and non-antigenic. Simultaneously, the polymer biodegradability would ensure its excretion from organism by glomerular filtration after delivery of the protein–polymer conjugate to the target tissue. We believe that the polymers described in this paper fulfil, at least partly, these requirements.

EXPERIMENTAL

Material and Method

Poly(ethylene glycol) monomethyl ether 2000 (mPEG), poly(ethylene glycol) 2000 (PEG), malonyl chloride, succinic anhydride, glutaric anhydride, maleic anhydride, L-lysine (Lys), ethane-1,2-diamine (ED), *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*,*N*-diisopropylethylamine (DIEA), 4-(dimethylamino)pyridine (DMAP), dimethylformamide (DMF) were commercial products (Fluka AG, Switzerland).

All other chemicals and solvents were of analytical grade. mPEG and PEG were dried by azeotropic distillation with toluene. Solvents were purified and dried by usual procedures. The reagents were used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker spectrometer (300 MHz). TLC of PEG derivatives: silica gel 60 F_{254} (Merck), chloroform–methanol 5:1, detection with a solution of NH_4SCN (3.40 g), $Co(NO_3)_2$ (0.56 g), H_2O (20 ml). Molecular weight of the polymers was determined by SEC on TSK columns 3000 SW and 4000 SW (50% methanol, 0.05% TFA, 0.5 ml/min, RI detector) on FPLC system (Pharmacia, Sweden) calibrated with PEG standards. Titration of terminal COOH groups of carboxylic polymers (**3**, **6**, **9**, **13**) was performed with 0.05 M NaOH using a TIM 900 titrator (Radiometer Copenhagen). Polymers **2**, **5**, **8** and **11** were purified by SEC on a preparative column TSK 3000 SW in 40% methanol with 0.03% trifluoroacetic acid.

Succinimidyl mPEG Malonate (**1**)

mPEG (2.28 g, 1.14 mmol) was dissolved in dichloromethane (DCM) (10 ml) and the solution was bubbled with argon. Malonyl chloride (0.7 ml, 1.43 mmol) was dissolved in DCM (6 ml) under argon. The polymer solution was added to the malonyl chloride solution followed by DIEA (0.195 ml, 1.14 mmol) under vigorous stirring. After 3 h standing, DCM and excess of malonyl chloride were removed on a rotary evaporator. The brown residue was dissolved in DCM (12 ml), and *N*-hydroxysuccinimide (0.276 g, 1.14 mmol) and DIEA (0.2 ml, 1.14 mmol) were added. The mixture was stirred overnight, the brown precipitate was filtered off and the filtrate was concentrated on a rotary evaporator. The residue was dissolved in methanol (5 ml) and polymer **1** was isolated by precipitation into diethyl ether followed by filtration.

Diblock Polymer **2**

Polymer **1** (0.5 g, 0.229 mmol) was dissolved in dry tetrahydrofuran (THF) (5 ml) and a solution of ED (7.64 μl, 0.115 mmol) in THF was gradually dropped to the dark-brown polymer solution under vigorous stirring. The reaction mixture turned orange. The course of reaction was followed by analytical SEC. The solvent was removed after 20 h standing and polymer **2** was precipitated into diethyl ether and purified by preparative SEC. ^{13}C NMR ($CDCl_3$): 59.04 (CH_3O); 41.25 (CO-CH_2-CO); 29.67 (NH-CH_2CH_2-NH); 70.83 ($CH_2CH_2O)_{45}$. ^{1}H NMR ($CDCl_3$): 4.29 m, 4 H (CH_2-O-CO); 3.52–3.74 m, 360 H (CH_2-CH_2-O, CH_2-NH); 3.44 s, 4 H (CO-CH_2-CO); 3.37 s, 6 H (CH_3-O). Yield 67% (475 mg).

mPEG Succinate (**3**)

mPEG (6.186 g, 3.1 mmol), succinic anhydride (1.535 g, 15.5 mmol) and DMAP (0.376 g, 3.1 mmol) were reacted in distilled THF at 25 °C for 55 h. The solvent was evaporated under vacuum. The crude product was dissolved in DCM and the remaining DMAP was removed by extraction with cold aqueous solution of $CuSO_4$ and NaCl. The organic layer was dried over anhydrous Na_2SO_4, filtered and the polymer was precipitated with diethyl ether and purified by SEC (Sephadex LH20, CH_3OH). TLC has shown a single spot with R_F 0.35. Titration of the polymer with 0.05 M NaOH showed the theoretical amount of COOH groups. ^{1}H NMR ($CDCl_3$, 300 K) with addition of 10 μl of trichloroacetyl isocyanate before measurement[17] did not prove the presence of free mPEG 2000. ^{1}H NMR ($CDCl_3$): 3.36 s, 3 H (CH_3-O); 3.52–4.26 m, 180 H (CH_2-CH_2-O)$_{45}$; 2.62–2.64 m, 4 H (CO-CH_2CH_2-CO). Yield 84% (5.46 g).

Succinimidyl mPEG Succinate (**4**)

Polymer **3** (614 mg, 0.3 mmol), *N*-hydroxysuccinimide (67 mg, 0.58 mmol) and DCC (121 mg, 0.58 mmol) were reacted in THF solution overnight at 4 °C and at 25 °C for 8 h. The precipitated DCU was filtered off and the active ester was isolated by precipitation with diethyl ether followed by filtration. Yield 84% (541 mg).

Diblock Polymer **5**

Activated polymer **4** (200 mg, 0.09 mmol) was dissolved in THF (4 ml) and ED (3 μl, 0.04 mmol) was added to the polymer solution under vigorous stirring. The mixture was

stirred overnight. The white precipitate was removed by filtration, the filtrate was concentrated under reduced pressure and the title product was precipitated with diethyl ether and purified by preparative SEC. ^{1}H NMR ($CDCl_3$): 4.21 m, 4 H (CH_2-O-CO); 3.52–3.74 m, 360 H (CH_2-CH_2-O, CH_2-NH); 3.37 s, 6 H (CH_3-O); 2.70 t, 4 H (O-CO-CH_2); 2.46 t, 4 H (CH_2-CO-NH). Yield 68% (148 mg).

mPEG Glutarate (**6**)

mPEG (5.779 g, 2.9 mmol), glutaric anhydride (1.439 g, 12.6 mmol) and DMAP (0.353 g, 2.9 mmol) were dissolved in distilled THF (15–20 ml) and the mixture was heated to reflux for 19 h. The product was isolated and purified as described for polymer **3**. Titration of COOH groups on the polymer with 0.05 M NaOH showed 91 mole % of theory. Yield 84% (5.62 g).

Succinimidyl mPEG Glutarate (**7**)

Polymer **7** was prepared starting from polymer **6** according to the procedure described for polymer **4**. Yield 97% (569 mg).

Diblock Polymer **8**

Diblock polymer **8** was synthesized as described for diblock polymer **5**. ^{1}H NMR ($CDCl_3$): 4.23 m, 4 H (CH_2-O-CO); 3.52–3.74 m, 360 H (CH_2-CH_2-O, CH_2-NH); 3.37 s, 6 H (CH_3-O); 2.38 t, 4 H (O-CO-CH_2); 2.23 t, 4 H (CH_2-CO-NH); 1.98 t, 4 H (CH_2-CH_2-CH_2). Yield 54.5% (266 mg).

mPEG Maleate (**9**)

mPEG (3.82 g, 1.91 mmol), maleic anhydride (0.797 g, 8.13 mmol) and 4-octylcatechol were dissolved in distilled DCM (10–15 ml). The reaction mixture was heated to reflux and stirred for 32 h. The solution gradually turned yellow. The solvent was removed under reduced pressure, the residue was dissolved in methanol and the crude product was purified by SEC (Sephadex LH20, CH_3OH). The content of COOH group in the polymer was 89 mole % of theory (by titration). Yield 70% (2.782 g).

Succinimidyl mPEG Maleate (**10**)

Polymer **10** was prepared from polymer **9** in an analogous way as described for polymer **4**. Yield 92% (290 mg).

Diblock Polymer **11**

Diblock polymer **11** was prepared from polymer **10** as described for polymer **5**. Yield 20% (50 mg).

Diblock Polymer **12**

mPEG succinimidyl succinate (**4**; 121 mg, 0.055 mmol), L-lysine (4.04 mg, 0.028 mmol) and DIEA (47 μl, 0.277 mmol) were dissolved in distilled DMF (1.3 ml) and stirred at 25 °C for

2 days. The precipitate of NHS was filtered off, the solvent was removed under reduced pressure and the product was isolated by precipitation with diethyl ether followed by filtration. Yield 75.4% (89 mg).

PEG Bis-succinate (**13**)

PEG (8.153 g, 4 mmol), succinic anhydride (4 g, 40 mmol) and DMAP (0.977 g, 8 mmol) were dissolved in distilled THF (60 ml) and reacted at 25 °C under stirring for 50 h. The product was isolated, purified and characterized according to the procedure described for mPEG succinate (**3**). TLC: R_F 0.32. Titration of COOH groups on the polymer with 0.05 M NaOH showed 100% of the theoretical amount. Yield 76% (6.855 g).

PEG Bis(succinimidyl succinate) (**14**)

Active diester **14** was synthesized from polymer **13** (102 mg, 0.046 mmol), *N*-hydroxysuccinimide (21 mg, 0.19 mmol) and DCC (38 mg, 0.19 mmol) as described for polymer **4**. Yield 56% (62 mg).

Multiblock Polymer **15**

Multiblock polymer **15** was prepared from polymer **14** (62 mg, 0.026 mmol) and L-lysine (3.79 mg, 0.026 mmol) in the presence of DIEA (22 μl, 0.132 mmol) according to the procedure for diblock polymer **12**. M_w = 6060, M_w/M_n = 1.92. Yield 53% (35 mg).

Hydrolysis of Polymers

Diblock polymers **2**, **5**, **8**, **11**, **12** and multiblock polymer **15** (1 mg/ml) were incubated in phosphate buffers (0.07 mol/l) adjusted to pH 5.5, 7.4 and 8.0 at 37 °C. The extent of hydrolysis was determined by SEC comparing the area under the corresponding peaks.

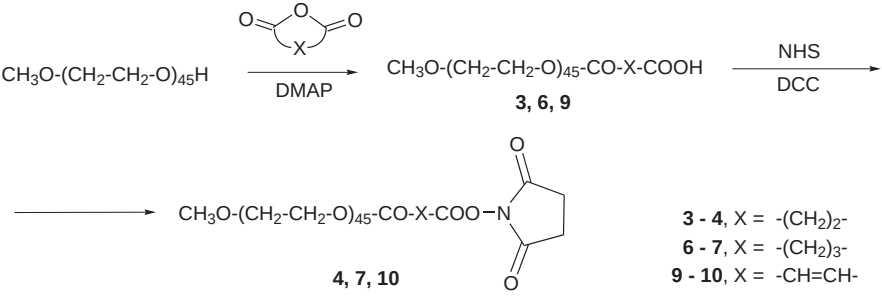
RESULTS AND DISCUSSION

The aim of this work was to design and to synthesize new PEG-based block polymers susceptible to pH-dependent hydrolysis for modification of either protein therapeutics (e.g., enzymes, proteins and antibodies) or low-molecular-weight drugs (e.g., cytostatics). This study was performed with model diblock polymers, consisting of only two blocks of PEG linked via an ester linkage to facilitate evaluation of the degradation experiments. The relationship between the detailed structure of the linkage and the rate of the polymer hydrolysis at various pH was studied.

*Synthesis of Polymers **2**, **5**, **8** and **11***

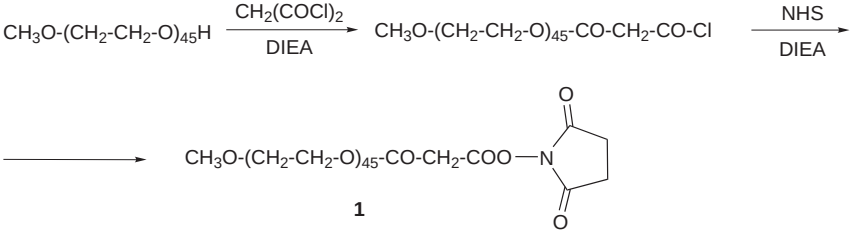
Conversion of a terminal hydroxy group of PEG to carboxylic group can be achieved by various methods leading either to relatively stable derivatives or to polymers with a degradable bond adjacent to the COOH group[18]. The

hydrolytically stable PEG-carboxylic acids can be prepared, for instance, by reaction of sodium salt of PEG with alkyl bromoacetate or 3-bromopropanoate followed by alkaline hydrolysis of the alkyl ester. On the other hand, analogous PEG-carboxylic acids prepared by reaction of terminal PEG hydroxy groups with anhydrides of dicarboxylic acids are hydrolyzed in aqueous solutions at different rates depending on the structure of the anhydride used for their synthesis. In consequence, any BAC modified by reaction of such PEG-carboxy derivatives can be finally released from the polymer under physiological conditions. If these PEG derivatives are further utilized for the synthesis of high-molecular-weight block polymers for drug delivery, the excretion of their degradation products (original PEG blocks) is guaranteed. The synthesis started with the modification of mPEG with excess anhydrides of both saturated and unsaturated dicarboxylic acids to yield polymer acids **3**, **6** and **9**. The corresponding succinimidyl esters **4**, **7** and **10** were prepared using the carbodiimide method[19] (Scheme 1).



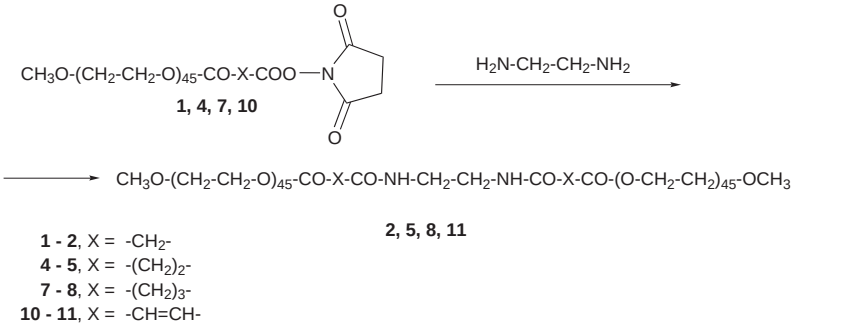
SCHEME 1

Succinimidyl mPEG malonate (**1**) was obtained by acylation of the end hydroxy group of mPEG with malonyl chloride followed by reaction of the intermediate chloride with NHS (Scheme 2).



SCHEME 2

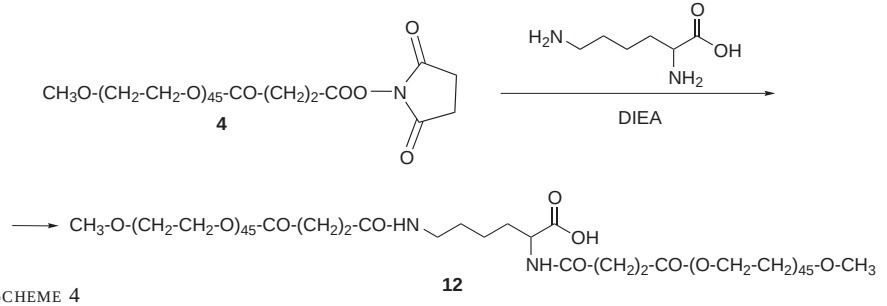
Diblock polymers **2**, **5**, **8** and **11** were prepared by condensation of active esters **1**, **4**, **7** and **10** with ethane-1,2-diamine (Scheme 3).



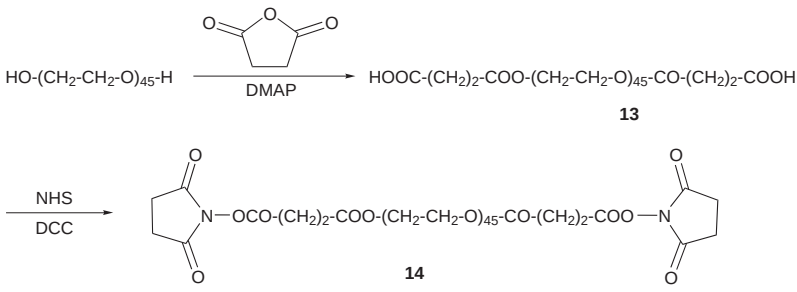
SCHEME 3

*Synthesis of L-Lysine Polymers **12** and **15***

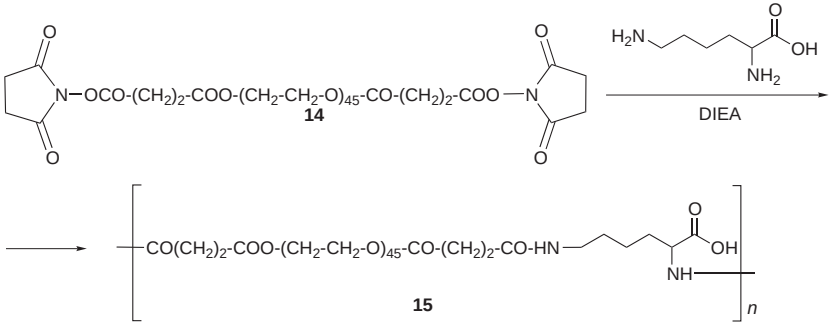
PEG bis-succinate **13** was prepared from bifunctional PEG 2000 and succinic anhydride in the same way as carboxylic polymer **3**. The terminal COOH groups were converted to active succinimidyl esters through the reaction with NHS (Scheme 5). Polymers **12** and **15** were obtained by condensation and polycondensation of active esters **4** and **14**, respectively, with L-lysine as a diamine linker in the presence of DIEA in DMF (Schemes 4 and 6). Zalipsky et al.[20] reported on polymers with molecular weight about 100 000 obtained by polycondensation of lysine ethyl ester and PEG-bis(succinimidyl carbonate). Pechar et al.[14] described analogous polymers with molecular weight about 30 000 using tripeptide Glu-Lys(Glu)-OH as a spacer. In this work, using herein described dicarboxylic acids and diamine linkers we achieved M_w only 6060 with polydispersity 1.92. These results demonstrate that the polymerisation degree of the PEG multiblock polymers prepared by polycondensation of activated PEG with different diamine linkers significantly depends on their structure.



SCHEME 4



SCHEME 5



SCHEME 6

Hydrolytic Degradation of the Ester Bonds Between PEG Blocks

Diblock polymers containing degradable ester bonds were incubated in phosphate buffer (0.07 mol/l) of pH 5.5, 7.4 and 8.0. Every pH value represented a definite environment in organism – pH 5.5 was found[21] inside the endosomal compartment of cells, pH 7.4 (physiological) in blood and pH 8.0 in intestinal tract. Hydrolysis rate of ester bonds was expressed as the amount of released PEG 2000.

We studied the influence of pH on the rate of hydrolysis of polymer diblock models (Fig. 2), hypothesizing that the rate of hydrolysis of ester bond would increase with increasing pH. Polymers containing saturated dicarboxylic acid residues **2**, **5** and **8** were readily hydrolyzed in mildly alkaline medium (pH 7.4 and 8.0); however, they proved to be completely stable under acid conditions (pH 5.5). On the other hand, the presence of double bonds conjugated with the ester carbonyl (polymer **11**) significantly promoted degradation of the polymer in acid medium (pH 5.5) while re-

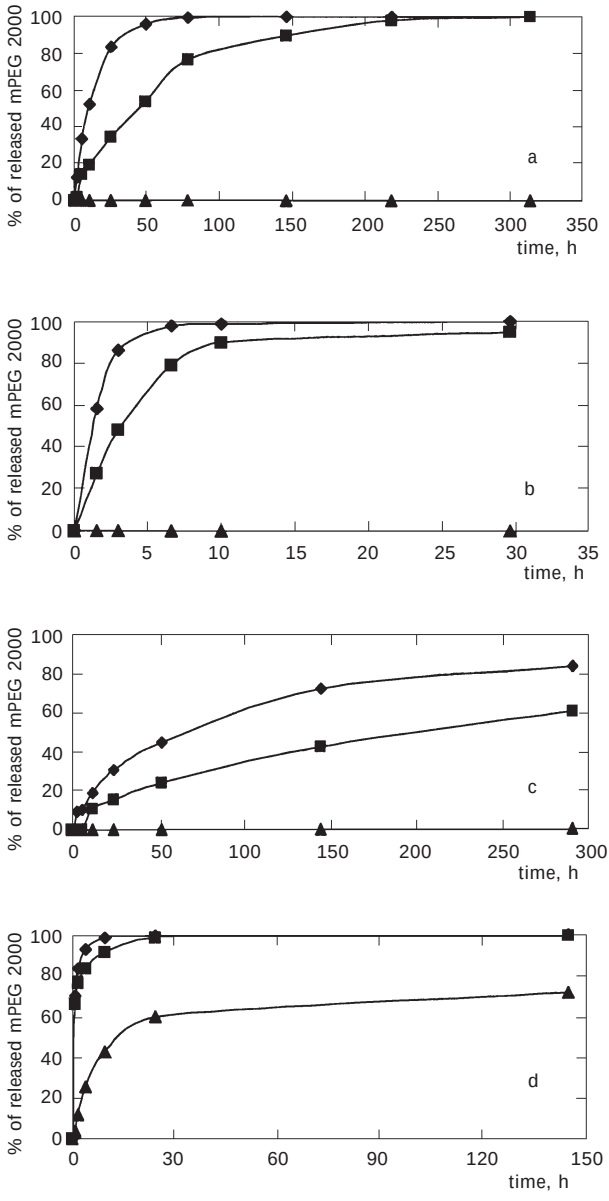


FIG. 2

Conversion curves of the hydrolysis of diblock polymers **2** (a), **5** (b), **8** (c) and **11** (d) at various pH: 8.0 (◆), 7.4 (■), 5.5 (▲). Concentration of polymers 1 mg/ml, phosphate buffer 0.07 mol/l; 37 °C

taining its hydrolysis rate in alkaline medium when compared with the analogous saturated polymer **5**.

Comparison of stability of various polymer esters at the same pH (Fig. 3) indicates that the increasing number of methylene groups in dicarboxylic acid structure prolongs the degradation time. The malonic acid derivative **2** is an exception to this trend, probably due to its special structure with extremely acid methylene hydrogen atoms. We may speculate that this acidity shifts the equilibrium between keto and enol tautomeric form of the malonate in favour of the latter and thus hinders the carbonyl from nucleophilic attack during hydrolysis. In consequence, it results in even lower hydrolysis rate compared with the succinate derivative **5**.

We also studied the influence of the structure of diamine linker on the hydrolysis rate of diblock polymer prepared from mPEG succinate. The hydrolysis of polymer **12** (Fig. 4) follows the hydrolysis trend observed for polymers with ED – the highest hydrolysis rate is achieved at pH 8.0. The hydrolysis is slightly slower at physiological pH (7.4) than in alkaline medium (pH 8.0). It does not proceed at all in mild acid medium (pH 5.5). The presence of L-lysine in the structure of polymer backbone significantly slowed down the rate of hydrolysis of polymer **12** compared with the diblock polymer **5**, containing ED as a linker (Fig. 5). We can hypothesize that the carboxylic group of lysine residue contributes to the higher stability as it locally acidifies the vicinity of the hydrolyzed ester bond. Moreover, the four methylene groups of the lysine residue side chain increase the hydrophobicity of the polymer compared with the analogous ED deriv-

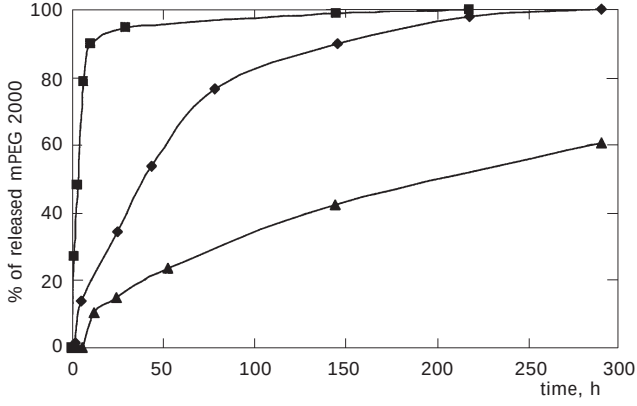


FIG. 3

Comparison of conversion curves of the hydrolysis of conjugates **2**, **5** and **8** at pH 7.4. Malonyl (diblock **2**) (◆), succinyl (diblock **5**) (■), glutaryl (diblock **8**) (▲)

ative **5**. The use of L-lysine incorporated in the polymer backbone is an easy way of introduction of the carboxylic group available for attachment of BAC.

The multiblock polymer **15** is an example of polymer precursor which could be utilized as a drug carrier for therapeutic application or for modification of biologically active proteins. Such polymer should protect protein to undesirable degradation during the transport in the organism, prolong its circulation in blood, decrease protein immunogenicity and improve

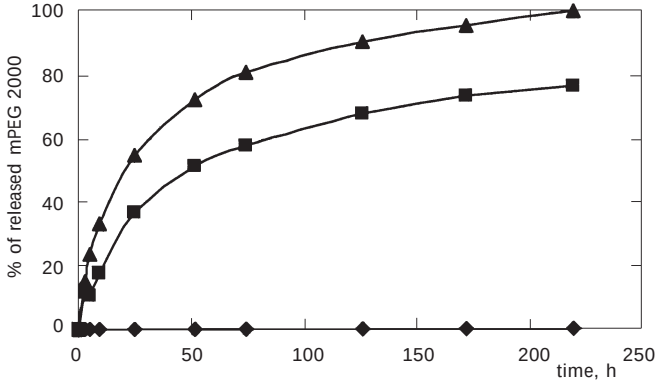


FIG. 4
Conversion curves of the hydrolysis of lysine diblock derivative of mPEG succinate (polymer **12**) at various pH: 8.0 (▲), 7.4 (■), 5.5 (◆). For hydrolysis conditions, see Fig. 2

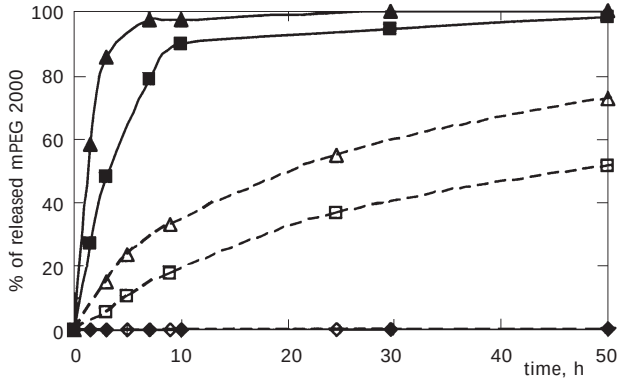


FIG. 5
Comparison of conversion curves of the hydrolysis of diblock mPEG succinates with different diamine linkers Lys (polymer **12**) at pH 5.5 (◇), 7.4 (□), 8.0 (△) and ED (polymer **5**) at pH 5.5 (◆), 7.4 (■), 8.0 (▲). For hydrolysis conditions, see Fig. 2

pharmacological properties of the protein. Hydrolysis of polymer **15** in phosphate buffers at various pH is shown in Fig. 6. It should be noted that the degradation product of the multiblock polymer **15** is not only PEG 2000 but also longer fragments. Therefore the rate of release of PEG 2000 would be lower than in the case of diblock polymer **5**. However, the initial concentration of the hydrolysable ester bonds is twice higher for the multiblock than for the diblock if the same weight concentration (1 mg/ml) is used. In consequence, the measured rate of PEG 2000 release of the two polymers is very similar.

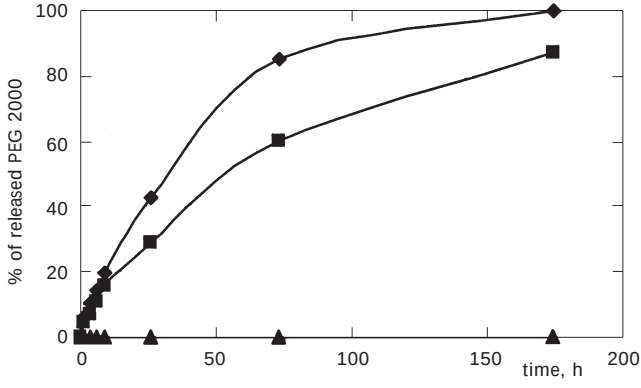


FIG. 6
Conversion curves of the hydrolysis of multiblock derivative of PEG bis-succinate (polymer **15**) with lysine as a diamine linker at various pH: 5.5 (▲), 7.4 (■), 8.0 (◆). For hydrolysis conditions, see Fig. 2

CONCLUSIONS

Diblock and multiblock PEG-based polymers containing degradable ester bonds were synthesized and characterized. We found that the detailed structure of the ester linkage affects the hydrolysis rate of the diblock polymers. The results of this model study can be utilized in the design of a biodegradable high-molecular-weight polymer for therapeutic applications.

LIST OF ABBREVIATIONS

BAC	biologically active compound
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DCM	dichloromethane
DCU	*N*,*N*′-dicyclohexylurea
DIEA	*N*,*N*-diisopropylethylamine
DMAP	4-(dimethylamino)pyridine

DMF	dimethylformamide
ED	ethane-1,2-diamine
FPLC	fast protein liquid chromatography
M_n	number-average molecular weight
M_w	weight-average molecular weight
mPEG	α-methyl-ω-hydroxypoly(oxyethylene)
NHS	*N*-hydroxysuccinimide
PEG	α-hydro-ω-hydroxypoly(oxyethylene)
RI	refractive index
SEC	size exclusion chromatography
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography

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REFERENCES

1. Harris J. M.: *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications*. Plenum Press, New York 1992.
2. Roberts M. J., Bentley M. D., Harris J. M.: *Adv. Drug. Delivery Rev.* **2002**, *54*, 459.
3. Ehrlich P.: *Studies in Immunity*. Plenum Press, New York 1906.
4. Ringsdorf H.: *J. Polym. Sci., Polym. Symp.* **1975**, *51*, 135.
5. Etrych T., Chytil P., Jelínková M., Říhová B., Ulbrich K.: *Macromol. Biosci.* **2002**, *2*, 43.
6. Putnam D., Kopeček J.: *Adv. Polym. Sci.* **1995**, *122*, 55.
7. Veronese F. M., Morpurgo M.: *Il Farmaco* **1999**, *54*, 497.
8. Maeda H.: *Proc. AFMC Int. Med. Chem. Symp.* **1995**, 197.
9. Hoste K., Schacht E., Seymour L.: *J. Controlled Release* **2000**, *64*, 53.
10. Veronese F. M.: *Biomaterials* **2001**, *22*, 405.
11. Greenwald R. B., Conover C. D., Choe Y. H.: *Crit. Rev. Ther. Drug Carrier Syst.* **2000**, *17*, 101.
12. Greenwald R. B.: *J. Controlled Release* **2001**, *74*, 159.
13. Greenwald R. B., Choe Y. H., McGuire J., Conover C. D.: *Adv. Drug. Delivery Rev.* **2003**, *55*, 217.
14. Pechar M., Ulbrich K., Šubr V., Seymour L. W., Schacht E. H.: *Bioconjugate Chem.* **2000**, *11*, 131.
15. Maeda H., Wu J., Sawa T., Matsumura Y., Hori K.: *J. Controlled Release* **2000**, *65*, 271.
16. Martinez A., Pendri A., Xia J., Greenwald R. B.: *Macromol. Chem. Phys.* **1997**, *198*, 2489.
17. Vandoorne F., Loccufier J., Schacht E.: *Makromol. Chem. Rapid. Commun.* **1989**, *10*, 271.
18. Geckeler K., Bayer E.: *Polym. Bull.* **1980**, *3*, 347.
19. Bodanszky M., Bodanszky A.: *The Practice of Peptide Synthesis*. Springer-Verlag, Berlin, Heidelberg 1994.
20. Zalipsky S., Gilon C., Zilkha A.: *J. Macromol. Sci., Part A* **1984**, *21*, 839.
21. Diwu Z., Chen C. S., Stass S. A., Mixon A. G.: *Chem. Biol.* **1999**, *6*, 411.