SYNTHESIS OF POLY(ETHYLENE GLYCOL) BLOCK COPOLYMERS AS POTENTIAL WATER-SOLUBLE DRUG CARRIERS

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Dedicated to Dr Blahoslav Sedlacek on the occasion of his 70th birthday.

The synthesis of a model water-soluble drug carrier based on poly(ethylene glycol) (PEG) block copolymers is described. In the copolymers, two blocks of PEG are linked by a biodegradable oligopeptide or amino acid linkage containing the glutamic acid residue. 4-Nitroaniline as a drug model is attached to the γ-carboxyl group of glutamic acid of the polymer carrier via an enzymatically degradable oligopeptide spacer. The oligopeptides used were potential substrates for chymotrypsin. The relationship between the structure of oligopeptides linking two PEG blocks and the rate of chymotrypsin-catalyzed polymer chain degradation as well as the relationship between the structure of the spacer and kinetics of drug model release from the carrier after incubation in chymotrypsin solution is discussed in detail. The results showed that by modifying the structure of oligopeptides in the polymer construct, changes in the rates of both polymer degradation and the drug model release can be achieved in a very broad range.

A number of water-soluble polymeric drug carrier systems have been developed and their conjugates, e.g. with antitumour agents have undergone biological and clinical evaluations[1]. According to the nature of the polymer used in the synthesis, polymeric carriers can be divided into two categories. In the first category are biodegradable polymers, mostly of natural origin. Biodegradability of such polymers facilitates metabolic removal from the organism after their administration and prevents their accumulation in the body. Unfortunately, degradable natural polymers are frequently immunogenic[2] and also their modification with a drug reduces their susceptibility to enzymatic attack and hence their use as drug carriers is also limited.

Polymers with a nondegradable polymer backbone, mostly fully synthetic, are in the second category. Their clinical use is restricted to conjugates of molecular weight lower than the renal threshold. Such polymers can be readily excreted from the body.

Many new drug delivery systems based on hydrophilic water-soluble polymer carriers of drugs have been developed. The design of the systems was optimized using the results of model studies verifying the feasibility of their synthesis and their ability to

meet all requirements associated with their optimal function. The synthesis of polymer substrates and enzymatically catalyzed drug model (4-nitroaniline) release from drug carriers based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers[3,4], copolymers of *N*-vinylpyrrolidone with maleic anhydride[5] and PEG derivatives containing oligopeptide 4-nitroanilides[6] incorporated in their structures were studied. In all cases, the carriers were synthetic, nondegradable polymers. The use of such nondegradable carriers in pharmaceutical preparations can be associated with certain problems (no or too slow elimination of a polymer from the body).

Recently, synthetic polymeric carrier systems based on block copolymers of poly(ethylene glycol) were developed[6–8]. PEG already has regulatory approval for the use in pharmaceutical formulations and some of its conjugates passed successfully clinical trials[9]. Development of new drug carrier systems based on block PEG copolymers containing biodegradable linkages in the main chain facilitating its enzymatic degradation can, in principle, solve the problems connected with the drug carrier excretion from the organism. The PEG derivatives[6] described recently, had the oligopeptide spacer linked to either one or both ends of the nondegradable polymer main chain.

The present study deals with derivatives of PEG bearing the oligopeptide sequence with the drug model connected to the γ-carboxylic group of the glutamic acid residue inside the biodegradable linkage. Two types of polymer substrates were synthesized and their enzymatic degradation studied. First group are the substrates containing biodegradable linkage in the main polymer chain. Second group includes substrates bearing a biodegradable spacer in the side chain between the polymer main chain and drug model. For the structures, see the reaction schemes.

In our system, PEG blocks are interconnected with biodegradable oligopeptide or amino acid-containing linkages with the glutamic acid residue in the structure. Drug can be attached to the γ-carboxylic group of the glutamic acid of the polymeric carrier via an enzymatically degradable oligopeptide spacer. All oligopeptide sequences in the conjugate have to be prepared as substrates for specific enzymes present in the living body to ensure controlled degradability of the whole system. Moreover, a suitable targeting moiety, e.g. an antibody, can be incorporated into the conjugate. The general structure of such a drug delivery system is presented in Scheme 1.

This work presents the synthesis and results of biodegradability tests of a model system verifying the feasibility of realization of such PEG carrier system. α-Chymotrypsin and 4-nitroaniline were used as the enzyme and the drug models, respectively.

The study of the relationship between the structure of oligopeptide (amino acid)–PEG conjugates and their hydrolysis catalyzed by α-chymotrypsin was carried out using two types of conjugates: (i) Block copolymers consisting of two methyl-PEG (mPEG) chains connected by an amino acid or oligopeptide linkage with at least one of amino acids being glutamic acid and (ii) block copolymers of methyl-PEGmPEG bearing in

their side chains oligopeptide sequences terminating in the drug model molecule (4-nitroaniline). Oligopeptide is attached to γ-carboxylic group of the glutamic acid residue.

The experimental work included the following synthetic steps and studies:

1. The transformation of hydroxy end group of mPEG into COOH group.

2. The synthesis of the oligopeptide bifunctional reagent containing at least one glutamic acid residue.

3. Coupling of two mPEG molecules using the oligopeptide reagent. Studies of the enzymatic degradation of this block copolymer substrate.

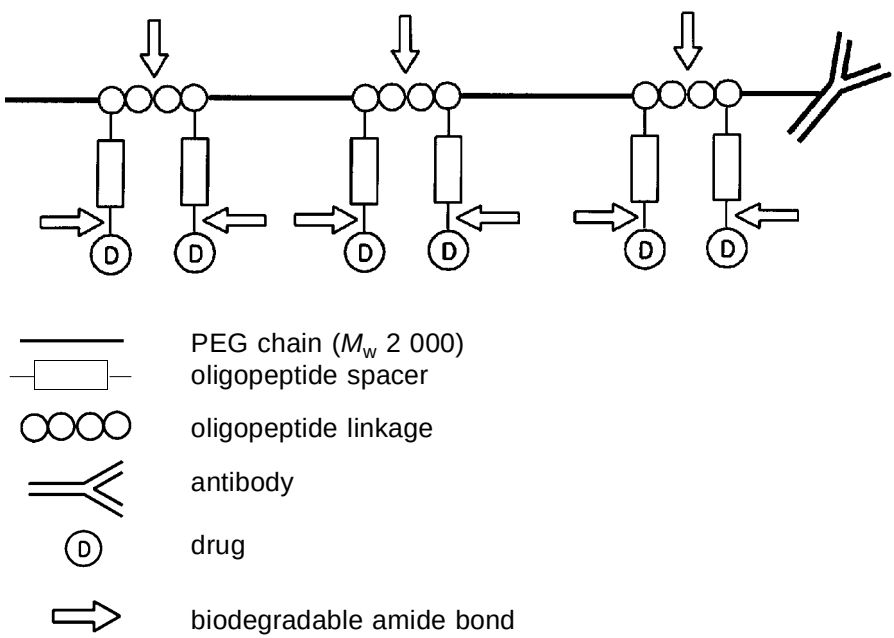
4. The synthesis of the oligopeptide derivatives of 4-nitroaniline and their binding to the side chain of the polymer carrier. Kinetic study of the enzyme-catalyzed 4-nitroaniline release from the carrier.

EXPERIMENTAL

All amino acids were of L-configuration. Their abbreviations are those recommended[10] by IUPAC-IUB.

Materials

Poly(ethylene glycol) monomethyl ether 2000 (mPEG), amino acids, palladium catalyst, α-chymotrypsin and reagents for peptide synthesis were commercial products (Fluka AG, Switzerland). All other chemicals and solvents were of analytical grade. Solvents were purified and dried by usual procedures. The reagents were used without further purification. NMR spectra were recorded in



SCHEME 1

$(CD_3)_2SO$ solutions on a Bruker spectrometer (300 MHz, Switzerland) if not stated otherwise. Chemical shifts (δ-scale) are given in ppm, HMDS was used as an internal standard.

Modification of mPEG Hydroxyl End Group

α-*Methyl*-ω-*(carboxymethoxy)poly(oxyethylene)* (**2**): mPEG 2000 (**1**) (30 g, 15 mmol) was transformed into the sodium salt (mPEG-Na) by reaction with naphthalenesodium[11]. mPEG-Na was then treated with ethyl bromoacetate yielding the ethyl ester derivative which was hydrolyzed[11] with aqueous KOH. The remaining mPEG was removed by the extraction of the alkaline solution (after hydrolysis) by the chloroform–toluene mixture (2 : 1). The aqueous layer was acidified with dilute HCl to pH 3 and the product was extracted into chloroform. The extract was dried over Na_2SO_4, chloroform was distilled off to the volume about 100 ml and the product was precipitated into diethyl ether. The yield of **2** was 17 g (57%). The content of COOH groups was more than 98% as determined both by titration with 0.2 M NaOH and by TAIC derivatization followed by ^{1}H NMR analysis[12].

α-*Methyl*-ω-*(4-nitrophenoxycarbonylmethoxy)poly(oxyethylene)* (**3**): A solution of polymer **2** (2.06 g, 1 mmol), 4-nitrophenol (1.39 g, 10 mmol) and DCC (2.06 g, 10 mmol) reacted in dichloromethane–ethyl acetate (2 : 1) at 0 °C overnight. Acetic acid (0.5 ml) was added to the reaction and the precipitated urea was filtered off after 20 min of stirring. The solvent was evaporated to the final volume about 10 ml and the product was precipitated into 250 ml of dry diethyl ether. The conversion of COOH to COONp groups was higher than 90% as determined by UV spectrophotometry (274 nm, ε 9 500 l mol^{-1} cm^{-1}).

Oligopeptide Bifunctional Reagents

γ-*Benzyl N-tert-butoxycarbonyl glutamate* (**4**): γ-Benzyl glutamate (5 g, 21 mmol), triethylamine (5.9 ml, 42 mmol) and bis(*tert*-butyl) dicarbonate (Boc_2O) (5.5 g, 25 mmol) were suspended in 40 ml of freshly distilled DMF and stirred at 45 °C until the solution became clear (about 3 h). DMF was evaporated under reduced pressure and the oily residue was dissolved in 70 ml of 5% aqueous $NaHCO_3$. The solution was washed with 2 × 50 ml of petrolether to remove the excess of Boc_2O. The solution was then acidified with 50 ml of 5% aqueous $KHSO_4$ and the product was extracted into 2 × 100 ml of ethyl acetate. The collected extracts were dried over Na_2SO_4, filtered and ethyl acetate was distilled off. The yield was 7.3 g (21 mmol, 100%) of an oily, colorless product.

N,N′-Bis(tert-butoxycarbonylphenylalanyl)ethylenediamine (**5**): Boc-Phe-ONp (2 g, 5.18 mmol) was dissolved in DMF (7 ml). Ethylenediamine (0.156 g, 2.59 mmol, 178 μl) was added dropwise under stirring within 5 min. After 30 min of stirring at 20 °C solid product precipitated and the reaction mixture was left at –18 °C overnight. The crude product was filtered off and recrystallized from ethyl acetate–ether yielding 1.24 g (2.02 mmol, 78%) of white crystals melting at 200–203 °C.

N,N′-Bis(phenylalanyl)ethylenediamine bis(trifluoroacetate) (**6**): Compound **5** (0.3 g, 0.49 mmol) was dissolved in TFA (1 ml, 13 mmol). After standing 1 h at 20 °C the excess of TFA was removed under reduced pressure and the viscous residue was triturated with dry ether. Yield 0.31 g (0.48 mmol, 98%) of crystalline **6** was isolated by filtration.

N,N′-Bis(tert-butoxycarbonyl-γ-benzylglutamylphenylalanyl)ethylenediamine (**7**): Acid **4** (169 mg, 0.5 mmol), HOBt (77 mg, 0.5 mmol) and DCC (104 mg, 0.5 mmol) were dissolved in 2 ml of THF at 0 °C. The solution of ammonium salt **6** (160 mg, 0.249 mmol) and triethylamine (70 μl, 0.5 mmol) was added to the reaction mixture in two portions within 30 min under stirring; the temperature was kept at 0 °C for 1 h and then at 20 °C for 5 h. The reaction was completed by addition of a drop of acetic acid and the precipitate was filtered off. The filtrate was evaporated to dryness and a solid residue was dissolved in ethyl acetate. The resulting solution was successively washed with aqueous

$NaHCO_3$, citric acid and $NaHCO_3$. The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. A solid residue was recrystallized from acetone–ether yielding 100 mg (0.1 mmol, 40%) of **7**. For $C_{54}H_{68}N_6O_{12}$ (993.2) calculated: 65.30% C, 6.90% H, 8.46% N; found: 65.10% C, 7.05% H, 8.56% N.

N,N′-Bis(γ-benzylglutamylphenylalanyl)ethylenediamine bis(trifluoroacetate) (**8a**): This compound and all the other ammonium trifluoroacetates were prepared from the corresponding bis(Boc) derivatives according to the procedure described for **6**.

4-Nitrophenyl γ-benzyl-N-tert-butoxycarbonylglutamate (**9**): A solution of compound **4** (7.39 g, 22 mmol), DCC (5 g, 24 mmol) and 4-nitrophenol (3.36 g, 24 mmol) in ethyl acetate (40 ml) was stirred for 1 h at 0 °C and then at 20 °C overnight. After filtering off the precipitate, the solvent was evaporated under reduced pressure and the residue was triturated with dry diethyl ether. The crystalline product was washed with ether. Yield 5.12 g (11.2 mmol, 51%) of **9**; m.p. 117–118 °C. For $C_{23}H_{26}N_2O_8$ (458.5) calculated: 60.26% C, 5.72% H, 6.11% N; found: 60.33% C, 5.67% H, 6.11% N. ^{1}H NMR spectrum: 1.35 s, 9 H (*t*-Bu); 2.05 m, 1 H (H-β); 2.17 m, 1 H (H-β); 2.57 t, 2 H (H-γ); 4.26 dt, 1 H (H-α); 5.07 s, 2 H (CH_2Ph); 7.31 m, 5 H (Ph); 8.22 d, 2 H (ONp).

N,N′-Bis(tert-butoxycarbonyl-γ-benzylglutamyl)ethylenediamine (**10**): A solution of ethylenediamine (0.131 g, 2.18 mmol, 0.147 ml) in THF (5 ml) was added dropwise to a solution of active ester **9** (2 g, 4.37 mmol) in THF (35 ml) with stirring. After 2 h at 20 °C, and 2 h at –18 °C a pale yellow solid was filtered off and recrystallized from THF. The yield was 1.2 g (1.72 mmol, 79%) of white crystals melting at 143–144 °C. For $C_{36}H_{50}N_4O_{10}$ (698.8) calculated: 61.88% C, 7.21% H, 8.02% N; found: 61.95% C, 7.60% H, 8.08% N. ^{1}H NMR spectrum: 1.32 s, 9 H (*t*-Bu); 1.72 m, 1 H (H-β); 1.88 m, 1 H (H-β); 2.30 t, 2 H (H-γ); 3.10 t, 2 H (CH_2N); 3.85 dt, 1 H (H-α); 5.03 s, 2 H (CH_2Ph); 6.50 br, 1 H (NH); 7.28 m, 5 H (Ph); 7.66 s, 1 H (NH).

N,N′-Bis(γ-benzylglutamyl)ethylenediamine bis(trifluoroacetate) (**11a**) was prepared from **10** in the same way as **6** from **5**. For $C_{30}H_{36}F_6N_4O_{10}$ (762.6) calculated: 49.59% C, 4.99% H, 7.71% N; found: 49.17% C, 4.87% H, 7.45% N. ^{1}H NMR spectrum: 2.00 m, 2 H (H-β); 2.42 t, 2 H (H-γ); 3.20 t, 2 H (CH_2N); 3.77 t, 1 H (H-α); 5.03 s, 2 H (CH_2Ph); 7.30 m, 5 H (Ph); 8.49 br, 3 H (NH_3^+).

N,N′-Bis(tert-butoxycarbonylphenylalanyl-γ-benzylglutamyl)ethylenediamine (**12**): A solution of the ammonium salt **11a** (510 mg, 0.702 mmol) and triethylamine (142 mg, 196 μl, 1.404 mmol) in DMF (2.5 ml) was added dropwise to the solution of Boc-Phe-ONp (542 mg, 1.404 mmol) in DMF (2.5 ml, freshly distilled) with stirring. The reaction mixture was kept in a refrigerator (4 °C) overnight. DMF was removed under reduced pressure and a viscous residue was triturated with dry diethyl ether. The crude product was recrystallized from ethyl acetate yielding 616 mg (0.62 mmol, 88%) of a crystalline solid melting at 205–210 °C with decomposition.

N,N′-Bis(phenylalanyl-γ-benzylglutamyl)ethylenediamine bis(trifluoroacetate) (**13a**) was prepared from **12** (see **6**). ^{1}H NMR spectrum: 1.70–2.00 m, 2 H (H-β-Glu); 2.33 m, 2 H (H-γ-Glu); 2.90 m, 1 H (H-β-Phe); 3.07 m, 3 H (CH_2N,H-β-Ph); 4.02 t, 1 H (H-α-Glu); 4.23 dt, 1 H (H-α-Phe); 5.05 s, 2 H (OCH_2Phe); 7.13–7.38 m, 10 H (arom.); 8.06 br, 3 H (NH_3^+); 8.63 d, 1 H (NHCH).

N,N′-Bis(tert-butoxycarbonyl)lysine (**14**): A solution of Boc_2O (25 g, 114 mmol) in dioxane (50 ml) was added to a solution of lysine monohydrochloride (10 g, 54 mmol) and NaOH (4.4 g, 108 mmol) in water (50 ml). The reaction mixture was stirred at 45 °C until it became clear (about 2.5 h). The solvent was evaporated to a final volume of about 20 ml and the resulting solution was diluted with 100 ml of water and washed with petrolether (50 ml). The aqueous layer was acidified with aqueous $KHSO_4$ (8 g in 50 ml) to pH 3 and the product was extracted with ethyl acetate (3 × 50 ml). The collected extracts were washed with aqueous NaCl and dried over Na_2SO_4. Ethyl acetate was taken off under reduced pressure and 18.5 g (53 mmol) of the colorless oily product was obtained. Specific rotation was $[\alpha]_{20}^{D} = +12°$ ($CHCl_3$, *c* 0.01). ^{1}H NMR spectrum (360 MHz): 1.2–1.4 m, 4 H (γ,δ); 1.35 s,

18 H (*t*-Bu); 1.4–1.7 m, 2 H (β); 2.9 m, 2 H (ε); 3.8 m, 1 H (α); 6.75 t, 1 H (NH-ε); 7.0 d, 1 H (NH-α).

N,N′-Bis(tert-butoxycarbonyl)lysine benzyl ester (**15**): Compound **14** (18.5 g, 53 mmol), benzyl alcohol (5.5 ml, 53 mmol), 4-(dimethylamino)pyridine (DMAP) (1.2 g, 10 mmol) and DCC (12 g, 58 mmol), were dissolved in ethyl acetate (100 ml) at 0 °C. The reaction mixture was stirred for 20 min at 0 °C and then left for 5 h at 20 °C. The progress of the reaction was checked by TLC (silica gel/ethyl acetate). The precipitated were was filtered off and the filtrate was washed with aqueous $CuSO_4$ solution (3 × 50 ml). A bluish precipitate (probably Cu(II) complex with DMAP) was removed with the aqueous layer. The organic layer was evaporated to dryness and an oily product was used for the next reaction. ^{1}H NMR spectrum ($CDCl_3$, 360 MHz): 1.2–1.4 m, 4 H (γ,δ); 1.4 s, 18 H (*t*-Bu); 1.5–2.0 m, 2 H (β); 3.05 m, 2 H (ε); 4.3 m, 1 H (α); 4.5 br, 1 H (NH); 5.10–5.25 m, 3 H (CH_2Ph,NH); 7.35 m, 5 H (Ph).

Lysine benzyl ester bis(trifluoroacetate) (**16**): Compound **15** (about 50 mmol) was dissolved in TFA (20 ml, 260 mmol). The reaction mixture was kept 1 h at 20 °C in a flask equipped with $CaCl_2$ tube. TFA was removed under reduced pressure, the residue was dissolved in water (100 ml) and washed with diethyl ether (2 × 50 ml) to remove the unreacted benzyl alcohol. The water layer was filtered and freeze-dried. An amorphous hygroscopic product was used in the next reaction. ^{1}H NMR spectrum (360 MHz): 1.2–1.4 m, 2 H (γ); 1.5 m, 2 H (δ); 1.6–1.8 m, 2 H (β); 2.75 t, 2 H (ε); 4.1 t, 1 H (α); 5.25 s, 2 H (CH_2Ph); 7.4 m, 5 H (Ph); 7.6–8.2 br, 3 H (NH_3^+); 8.2–8.6, 3 H (NH_3^+).

N,N′-Bis(tert-butoxycarbonyl-γ-benzylglutamyl)lysine benzyl ester (**17**): To an ice-cooled solution of acid **4** (8 g, 21 mmol), ester **16** (4.87 g, 10.5 mmol), triethylamine (2.93 ml, 21 mmol) and HOBt (2.84 g, 21 mmol) in THF (100 ml, freshly distilled) a solution of DCC (4.77 g, 23 mmol) in THF (30 ml) was added. The reaction was carried out for 1 h at 0 °C and for 5 h at 20 °C with stirring. The precipitate was removed by filtration, THF was evaporated to dryness and the viscous residue was dissolved in ethyl acetate (200 ml). The solution was successively washed with aqueous 5% $NaHCO_3$ (50 ml), 5% citric acid (100 ml) and aqueous 5% $NaHCO_3$ (50 ml). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The residue was dissolved in diethyl ether and evaporated again. The amorphous solid was recrystallized from ether–hexane (3 : 1). The yield of **17** melting at 89–92 °C was 6.0 g (6.86 mmol, 65%). ^{1}H NMR spectrum (360 MHz): 1.35 s, 18 H (Boc); 1.2–1.4 m, 4 H (Lys-γ,Lys-δ); 1.6–2.0 m, 6 H (Glu-β,Lys-β); 2.35 m, 4 H (Glu-γ); 3.0 m, 2 H (Lys-ε); 3.9 m, 1 H (Glu-α); 4.0 m, 1 H (Glu-α); 4.25 m, 1 H (Lys-α); 5.04 s, 2 H (CH_2Ph); 5.05 s, 2 H (CH_2Ph); 5.06 s, 2 H (CH_2Ph); 7.35 m, 15 H (arom); 6.85 d, 1 H (NH-Glu); 6.95 d, 1 H (NH-Glu); 7.8 t, 1 H (NH-Lys-ε); 8.25 d, 1 H (NH-Lys-α).

N,N′-Bis(γ-benzylglutamyl)lysine benzyl ester bis(trifluoroacetate) (**18**): Compound **17** (0.5 g, 0.57 mmol) was dissolved in TFA (3 ml, 0.39 mmol) and the mixture was left for 1 h at 20 °C. TFA was removed in vacuo and the residue was covered with dry diethyl ether. After 24 h, the non-crystalline solid was dissolved in 10 ml water. The aqueous layer was separated and the traces of ether were removed under reduced pressure. The water solution was freeze-dried yielding 0.6 g (98%) of a hygroscopic product. ^{1}H NMR spectrum (360 MHz): 1.2–1.5 m, 4 H (Lys-γ, Lys-δ); 1.5–1.85 m, 2 H (Lys-β); 2.0 m, 4 H (Glu-γ); 2.45 m, 4 H (Glu-γ); 3.05 m, 2 H (Lys-ε); 3.8 br, 1 H (Glu-α); 3.9 br, 1 H (Glu-α); 4.3 m, 1 H (Lys-α); 5.1 m, 6 H (CH_2Ph); 7.35 m, 15 H (Ph); 8.2 br, 6 H (NH_3^+); 8.5 t, 1 H (NH-ε); 8.9 d, 1 H (NH-α).

N,N′-Bis(γ-benzylglutamylphenylalanyl)hexamethylenediamine bis(trifluoroacetate) (**8b**), *N,N′-bis-(γ-benzylglutamyl)hexamethylenediamine bis(trifluoroacetate)* (**11b**) and *N,N′-bis(phenylalanyl-γ-benzylglutamyl)hexamethylenediamine bis(trifluoroacetate)* (**13b**) were prepared similarly to **8a**, **11a** and **13a** starting with hexamethylenediamine instead of ethylenediamine.

Synthesis of Substrates **19**–**24**

General procedure: A solution of DCC (103 mg, 0.5 mmol) in THF (2 ml) was added to a solution of bisammonium salt **8a**, **8b**, **11a**, **11b**, **13a** or **13b** (0.121 mmol), polymer **2** (500 mg, 0.243 mmol), HOBt (77 mg, 0.5 mmol) and triethylamine (70 μl, 0.5 mmol) in THF (6 ml) at 0 °C. The reaction was kept at –18 °C for 1 h and then at 4 °C overnight. A drop of acetic acid was added and the urea was filtered off after 30 min. THF was removed under reduced pressure and a solid residue was dissolved in methanol (3 ml); the solution was filtered and purified by GPC (Sephadex LH60/methanol). A polymer fraction of the molecular weight above 4 000 was collected, methanol was evaporated and the residue was freeze-dried from water. Thus the polymer benzyl esters **19a**, **19b**, **20a**, **20b**, **21a** and **21b** were obtained in 60–90% yields. The catalytic hydrogenation of **19a**, **20a** and **21a** with Pd/C (10%) in methanol with 1% of acetic acid led to polymers **22**, **23** and **24**, respectively, with two carboxylic groups in the side chain.

Synthesis of Substrates **25** and **26**

Nitrophenyl ester **3** (1 g, 0.41 mmol) dissolved in THF (10 ml) reacted with ammonium salt **18** (186 mg, 0.205 mmol) and triethylamine (63 μl, 0.454 mmol) in THF (5 ml) at 20 °C for 12 h. THF was evaporated, the residue was dissolved in chloroform (8 ml) and the polymer was precipitated into dry diethyl ether (200 ml). The crude product (0.9 g) was purified by GPC (Sephadex G50/H_2O, refractive index detector). The fraction of molecular weight above 4 000 was collected and polymeric benzyl ester **25** was isolated by freeze-drying from the water solution. The hydrogenation (see the previous paragraph) led to polymer **26** containing three carboxylic groups. ^{1}H NMR spectrum (360 MHz): 1.20–1.40 m, 4 H (Lys-γ,δ); 1.50–2.05 m, 6 H (Lys-β,Glu-β); 2.35 t, 4 H (Glu-γ); 3.01 m, 2 H (Lys-ε); 3.25 s, 6 H (OCH_3); 3.52 m, 360 H (OCH_2CH_2); 3.91 s, 4 H (OCH_2CO); 4.18–4.48 m, 3 H (Glu-α,Lys-α); 5.07 s, 2 H (CH_2Ph); 5.08 s, 2 H (CH_2Ph); 5.11 s, 2 H (CH_2Ph); 7.33 m, 15 H (arom); 7.65 dd, 2 H (NH-Glu); 8.02 t, 1 H (NH-Lys-ε); 8.47 d, 1 H (NH-Lys-α).

Oligopeptide 4-Nitroanilides **27**–**32**

These compounds were prepared by standard methods of peptide synthesis[13], using the carbodiimide method for linking COOH and NH_2 groups and Boc group for the protection of α-amino groups. The following derivatives were synthesized[6,14]: H-Phe-NAp (**27**), H-Gly-NAp . HCl (**28**), H-Gly-Gly-Phe-NAp . HCl (**29**), H-Gly-Val-Phe-NAp . HCl (**30**), H-Ala-Gly-Val-Phe-NAp . HCl (**31**), H-Ala-Gly-Gly-Phe-NAp . HCl (**32**).

Synthesis of Substrates **33**–**38**

General procedure: A solution of polymer **22** (50 mg, 1.14 . 10^{-5} mol), the respective oligopeptide 4-nitroanilide (**27**–**32**) (1.14 . 10^{-5} mol), triethylamine (5 μl, 35 mmol, when working with hydrochlorides), HOBt (7.66 mg, 0.05 mmol) and DCC (10 mg, 0.05 mmol) in acetonitrile (2 ml) was kept at 4 °C overnight and, after addition of a drop of acetic acid, in urea was filtered off after 1 h. The solvent was evaporated to dryness and the residue was dissolved in methanol (0.5 ml). The pure product was isolated by GPC (Sephadex LH 20, methanol, UV detector 315 nm) followed by freeze-drying from water. The content of the 4-nitroanilide groups bound to the polymer was determined spectrophotometrically (λ 315 nm, ε 12 800 l mol^{-1} cm^{-1}). Yield 20–45% (based on the starting 4-nitroanilide).

Enzymatic Cleavage of Polymeric Substrates

The concentration of the active sites in α-chymotrypsin was determined by the active site titration with Z-tyrosine 4-nitrophenyl ester[15] (60% of active protein).

Kinetic measurements: The release of 4-nitroaniline from polymer substrates **33**–**38** incubated in α-chymotrypsin solution ($1 . 10^{-8}$–$7 . 10^{-7}$ mol l^{-1} in buffer 0.08 mol l^{-1} Tris and 0.1 mol l^{-1} $CaCl_2$, pH 8.0 adjusted with HCl) was monitored with an LKB Ultrospec II spectrophotometer (25 °C, 410 nm, ε 8 440 l mol^{-1} cm^{-1}). Kinetic constants K_M, k_{cat} and k_{cat}/K_M were determined using Lineweaver–Burke plots[16]. The enzyme concentrations differed according to the substrate, the substrate concentrations were in the range $1 . 10^{-5}$–$1 . 10^{-4}$ mol l^{-1}.

Chymotrypsin-catalyzed hydrolysis of substrates **19**–**26**: The reaction was carried out at the polymer concentration of 5 mg ml^{-1}, i.e. $2 . 10^{-3}$ mol l^{-1} of potential sites of cleavage, and at the enzyme concentration $2.6 . 10^{-6}$ mol l^{-1} in the same buffer at 37 °C. The extent of hydrolysis and the rate of formation of a polymer with M_w ca 2 000 were determined using a GPC column TSK 3000 (50% methanol, 0.1% TFA, 0.7 ml min^{-1}) from the area of the corresponding peaks. The rate of formation was considered a measure of the cleavage rate.

RESULTS AND DISCUSSION

The aim of this work was to verify the idea of synthesis of a drug polymer conjugate, capable of releasing at a controlled rate a drug or its model in the biological environment. In such a system the drug release should be accompanied or followed by a controlled degradation of polymer carrier to yield biocompatible low-molecular-weight products excretable from the body, e.g. by glomerular filtration. Two investigations were carried out:

– the study of the relationship between the structure of the oligopeptide-based linkage connecting two PEG blocks and the rate of the degradation of the conjugate and

– the study of the relationship between the structure of the oligopeptide spacer connecting the polymer chain with a drug model molecule and the rate of a drug model release from such carrier.

Synthesis of Polymeric Substrates **19**–**26**

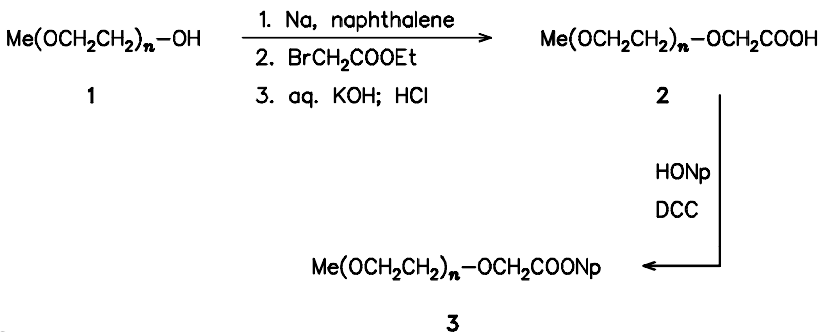
The synthesis of the COOH derivative of mPEG (**2**) was performed as follows. The active 4-nitrophenyl ester of **2** (**3**) was prepared using the carbodiimide method (Scheme 2).

Substrates **19a**, **19b**, **20a**, **20b**, **21a**, **21b** and **25** were prepared by the reaction of **2** or **3** with bifunctional reagents **8a**, **8b**, **11a**, **11b**, **13a**, **13b** and **18**, respectively. Catalytic hydrogenation of **19a**, **20a**, **21a** and **25** yielded polymers **22**, **23**, **24** and **26**, respectively (Scheme 3).

Substrates **33**–**38** were synthesized by the reactions of **22** with amino acid or oligopeptide 4-nitroanilides **27**–**32** (Scheme 4).

Chymotrypsin-Catalyzed Degradation of the Main Chain of the PEG Block Copolymers

By the reaction of mPEG derivatives **2** or **3** with diamines **8a**, **11a**, or **13a** and after removal of benzyl protecting group (see Scheme 3) polymer carriers containing two carboxylic groups in the molecule were obtained. Two molecules of drug, drug model or oligopeptide drug model derivative can be attached to the carriers. When diamine **18** containing the lysine residue was used in the synthesis, a polymer carrier containing three carboxylic groups was obtained. In this case carboxylic groups of different reactivity were introduced into the polymer molecule, facilitating different modification of each type of carboxyls (γ-carboxylic group of glutamic acid, carboxylic group of lysine), e.g. binding both drug and targeting moiety to the same polymer chain. Both systems were tested for their susceptibility to chymotrypsin-catalyzed hydrolysis.



SCHEME 2

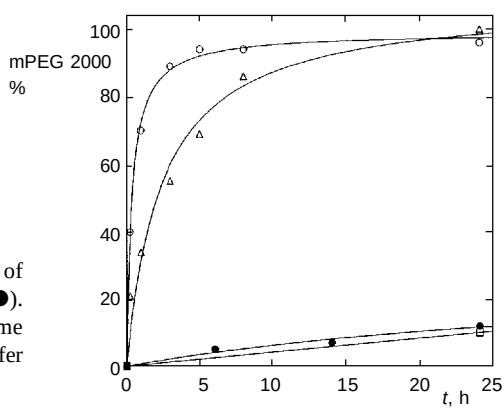
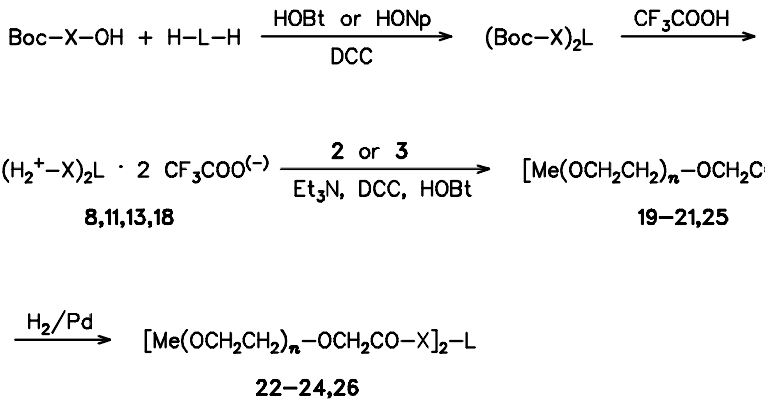


FIG. 1
mPEG 2000 released by chymotrypsin cleavage of substrates **19a** (❐), **20a** (Δ), **21a** (○) and **25** (●). Substrate concentration 2 . 10^{-3} mol l^{-1}, enzyme concentration 2.6 . 10^{-6} mol l^{-1}, pH 8.0, buffer 0.08 mol l^{-1} Tris and 0.1 mol l^{-1} $CaCl_2$, 37 °C

Polymer substrates **19**–**26** (Scheme 3) were incubated in chymotrypsin solutions and the rate of their degradation (release of an mPEG derivative of molecular weight approximately 2 000) was determined by GPC. The results presented in Figs 1–3 show that all polymers under study were susceptible to enzymatic cleavage. Polymers containing phenylalanine residue in the structure were degraded much faster than those containing only glutamic acid residue. This finding is in agreement with the view of the substrate specificity of chymotrypsin[17]. Chymotrypsin cleaves specifically the amide bond of amino acids containing bulky hydrophobic substituents (Phe, Tyr), capable of hydrophobic interactions with the active site of the enzyme. The finding that the polymers containing only γ-benzyl glutamate or even glutamic acid in the linkage were



	X	L
19a	Glu(OBz)	NH-$(CH_2)_2$-NH
19b	Glu(OBz)	NH-$(CH_2)_6$-NH
20a	Glu(OBz)-Phe	NH-$(CH_2)_2$-NH
20b	Glu(OBz)-Phe	NH-$(CH_2)_6$-NH
21a	Phe-Glu(OBz)	NH-$(CH_2)_2$-NH
21b	Phe-Glu(OBz)	NH-$(CH_2)_6$-NH
22	Glu(OH)	NH-$(CH_2)_2$-NH
23	Glu(OH)-Phe	NH-$(CH_2)_2$-NH
24	Phe-Glu(OH)	NH-$(CH_2)_2$-NH
25	Glu(OBz)	$N^{\alpha},N^{\varepsilon}$-Lys-OBz
26	Glu(OH)	$N^{\alpha},N^{\varepsilon}$-Lys-OH

SCHEME 3

also susceptible to degradation, was surprising. Clearly, in this case the rate of degradation was more than one order of magnitude lower than in the case of dipeptide linkages. No degradation was observed if the polymers were incubated in buffers in the absence of enzyme. The polymers in which benzyl protecting group was removed from glutamic acid residue, were degraded more slowly (see Figs 1 and 2) when compared with the protected analogs. The presence of free carboxylic group in the vicinity of the cleavable amide bond probably decreases the ability of phenylalanine to interact and form a complex with a hydrophobic pocket of the active site of the enzyme.

Polymers **19b**, **20b** and **21b** containing a hexamethylenediamine instead of ethylenediamine unit were used for the study of the enzymatic degradation by chymotrypsin under the same conditions as their ethylenediamine analogs. The courses of the hydrolysis are shown in Fig. 3. The presence of the more hydrophobic hexamethylenediamine residue in the linkage results in an increase of the rate of degradation of the substrate containing only glutamic acid, while the rate of degradation of the dipeptide-containing substrates is slightly lower. Incorporation of lysine molecule into the linkage does not bring any dramatic change in polymer degradability.

In all cases under study the dipeptide-containing linkages were degraded with the highest rates, the substrates with -PheGlu- being degraded faster than those with -GluPhe- sequence.

Model studies of the chymotrypsin-catalyzed degradation of mPEG block copolymers confirmed that a biodegradable polymer drug carrier can be prepared by incorporation oligopeptide-containing linkages into the main PEG chain. These linkages must be tailor-made as substrates for respective enzymes. The rate of polymer main

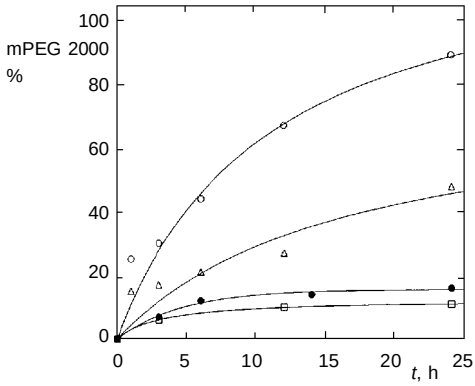


FIG. 2
mPEG 2000 released by chymotrypsin cleavage of substrates **22** (❐), **23** (Δ), **24** (○) and **26** (●). For conditions, see Fig. 1

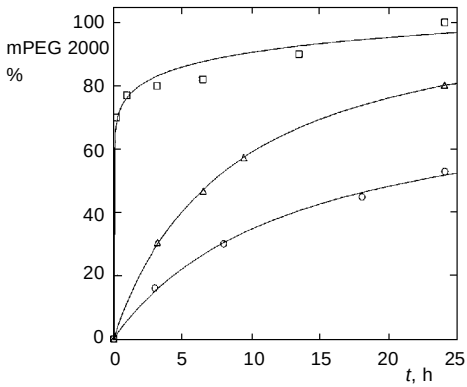


FIG. 3
mPEG 2000 released by chymotrypsin cleavage of substrates **19b** (○), **20b** (Δ) and **21b** (❐). For conditions, see Fig. 1

chain degradation can be significantly influenced by changing the length and detailed structure of the linkage connecting two PEG chains.

Chymotrypsin-Catalyzed Release of 4-Nitroaniline from mPEG Block Copolymers

The Effect of the Composition of the Spacer

Substrates **33**–**38** (Scheme 4) contain an oligopeptide (amino acid) spacer which is terminated in a drug model (4-nitroaniline). Except for substrate **34**, the ultimate unit in

TABLE I
Kinetics of chymotrypsin-catalyzed hydrolysis[a] of polymeric substrates **33**–**38**

Side chain	k_{cat}, s^{-1}	K_M . 10^4, mol l^{-1}	k_{cat}/K_M, l mol^{-1} s^{-1}
-Phe-NAp	0.091	3.959	231
-Gly-NAp	0.000	–	0
-Gly-Gly-Phe-NAp	0.510	9.138	558
-Gly-Val-Phe-NAp	12.475	9.023	14 125
-Ala-Gly-Val-Phe-NAp	63.960	1.658	386 000
-Ala-Gly-Gly-Phe-NAp	0.431	7.468	577

[a] In a buffer of pH 8.0 containing 0.08 mol l^{-1} Tris and 0.1 mol l^{-1} $CaCl_2$ at 25 °C.

TABLE II
Effect of the type of the polymer main chain on k_{cat}/K_M (l mol^{-1} s^{-1}) on the chymotrypsin-catalyzed hydrolysis of oligopeptide side chains[a]

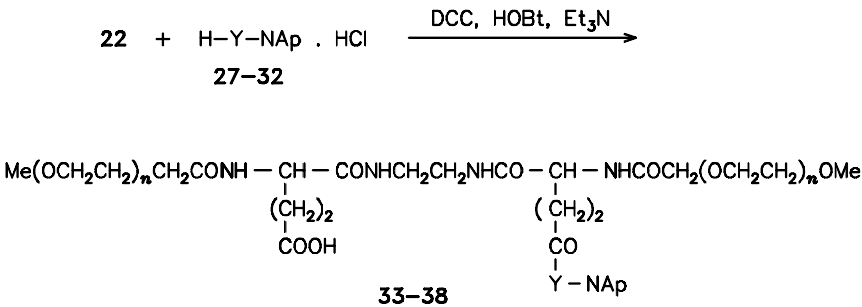
Side chain	HPMA[b]	MA-VP[c]	PEG[d]	PEG[e]
-Phe-NAp	0	0	80	231
-Gly-NAp	–	–	–	0
-Gly-Gly-Phe-NAp	110	–	481	558
-Gly-Val-Phe-NAp	245	1 280	9 550	14 125
-Ala-Gly-Val-Phe-NAp	14 200	–	123 000	386 000
-Ala-Gly-Gly-Phe-NAp	–	–	995	577
-Gly-Gly-Val-Phe-NAp	6 300	15 000	–	–

[a] For experimental conditions, see Table I. [b] Copolymers of HPMA and methacroylated oligopeptides[3,5]. [c] Copolymers of maleic anhydride and *N*-vinylpyrrolidone[3,5]. [d] PEG with carboxyl end groups[6]. [e] PEG block copolymer (this study).

the spacer is the phenylalanine residue. It was shown before[3,5,6] that such structures are susceptible to the chymotrypsin-catalyzed hydrolysis. The rate of hydrolysis and 4-nitroaniline release was characterized by the value k_{cat}/K_M. The results summarized in Table I are in accordance with those obtained with substrates containing 4-nitroaniline moiety situated at the end of the main polymer chain[6].

Polymer substrates **33**–**38** differing in the composition of oligopeptide spacers were incubated in buffer solutions containing chymotrypsin and the kinetics of 4-nitroaniline release was studied. In addition to the rate constant k_{cat} and Michaelis–Menten constant K_M, the ratio k_{cat}/K_M was calculated and used as a value characterizing the rate of drug model release from mPEG block copolymer carrier. The results of kinetic measurements are shown in Table I. The rate of 4-nitroaniline release strongly depended on the structure of the oligopeptide spacer, the prerequisite being structure susceptible to chymotrypsin degradation, i.e., that with phenylalanine as the terminal amino acid residue. When a glycine spacer was used (in **34**), no release was observed.

The lowest rate of 4-nitroaniline release was observed for the substrate containing only phenylalanine in the spacer. Nevertheless, even in this case the rate of drug model release is relatively high which can be explained by participation of the glutamic acid residue on the formation of complex between the substrate and the active site of the enzyme. The longer the spacer is, the higher is the rate of drug model release. The



	Y
33	Phe
34	Gly
35	Gly–Gly–Phe
36	Gly–Val–Phe
37	Ala–Gly–Val–Phe
38	Ala–Gly–Gly–Phe

SCHEME 4

impact of the detailed structure of the oligopeptide sequence on the rate of release was dramatic. When a hydrophobic amino acid was placed in the penultimate position (Val in **37**) the rate of 4-nitroaniline release increased by three orders of magnitude compared to a similar spacer containing in the same position the glycine residue (tetrapeptide sequence)[17]. In tripeptide spacers, the effect of the presence of Val residue was also remarkable (cf. **35** and **36**), but not as pronounced as in the case of tetrapeptide spacer. Serious explanation of this effect would require more detailed study.

The Effect of the Polymer Chain

Table II summarizes the results of kinetic studies of 4-nitroaniline release from polymer substrates based on copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA), copolymers of maleic anhydride with *N*-vinylpyrrolidone (MA–VP) and poly(ethylene glycol)s (PEG) with different end groups. Even though the molecular weights of the polymers were not equal, some common dependences are evident. The presence of phenylalanine in the vicinity of the 4-nitroaniline residue in subsite 1 is a prerequisite[17] for the chymotrypsin-catalyzed 4-nitroaniline release from all the polymer substrates. Glycine-containing polymer conjugates did not release any 4-nitroaniline when incubated in chymotrypsin solutions. The length and structure of the oligopeptide spacer influenced the rate of 4-nitroaniline release from all types of polymer carriers in a similar way and relative rates of 4-nitroaniline release from various carriers were in a correspondence. Lower rates of release observed for HPMA and MA–VP copolymers can be explained by the effect of higher molecular weights of the polymer carrier (20 000–30 000, PEG conjugates 2 000 and 4 000) and by the sterically unfavourable location of the oligopeptide sequence in side chains of the carriers. Both these effects result in higher steric hindrance to the access of the enzyme to the oligopeptide substrate and hence decrease the ability of the enzyme to hydrolyze the substrate.

Surprisingly enough, a comparison of the results obtained for both PEG carrier structures shows that polymer substrates based on more complex block mPEG copolymers released 4-nitroaniline with higher rate than the substrates based on PEG with modified end groups. This increase in the rate of degradation could be explained by the participation of glutamic acid fragment ($-CH_2CH_2CO-$) in the formation of the complex between amino acids of the substrate and the active site of the enzyme in block copolymer substrates. The effect is more pronounced for substrates containing only Phe in the spacer. If -PheNAp is bound directly to the polymer chain (HPMA, MA–VP), the influence of steric hindrance was significant and no degradation was observed. In the case of mPEG block copolymer, PheNAp was attached to the polymer chain via glutamic acid residue and such an extension of the spacer was sufficient to promote the release of 4-nitroaniline. A simple PEG substrate bearing PheNAp bound directly at

polymer chain ends also released the drug model molecule under similar conditions, but with a lower rate.

Model studies of chymotrypsin-catalyzed 4-nitroaniline release from mPEG block copolymer carriers demonstrated the feasibility of the synthesis of polymer drug carriers, which are biodegradable in the environment containing enzyme and which can release a drug or a drug model from the carrier at a wide range of rates controlled by the length and detailed structure of the oligopeptide linkage between the drug model and polymer chain. The prerequisite for such a release is the optimal structure of the oligopeptide, tailor-made as a substrate for the respective enzyme.

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ABBREVIATIONS

Boc	*tert*-butoxycarbonyl
Boc_2O	bis(*tert*-butyl) dicarbonate
Bz	benzyl
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DCU	*N*,*N*′-dicyclohexylurea
DMAP	4-(dimethylamino)pyridine
DMF	dimethylformamide
HOBt	1-hydroxybenzotriazol
HPMA	*N*-(2-hydroxypropyl)methacrylamide
mPEG	α-methyl-ω-hydroxypoly(oxyethylene)
NAp	4-nitroanilide
Np	4-nitrophenyl
PEG	α-hydro-ω-hydroxypoly(oxyethylene)
TAIC	trichloroacetyl isocyanate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Z	benzyloxycarbonyl

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