# Hydrophilic polymers for drug delivery

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SUMMARY: Synthesis and results of biological evaluation of two types of water-soluble polymer drug carrier systems designed for site-specific therapy are described. In the first system, a nondegradable poly[*N*-(2-hydroxypropyl)-methacrylamide] (PHPMA) bears biodegradable oligopeptide side chains, terminated in the targeting antibody and/or anti-cancer drug doxorubicin, randomly distributed along the polymer chain. The other system is based on PEG ($M_w$ 2000) blocks connected with biodegradable $N^2$,$N^6$-bis(glutamyl)-lysine oligopeptide links. This linear water-soluble polymer bears doxorubicin attached to the carboxylic groups of amino acid residues in the oligopeptide links via biodegradable GlyPheLeuGly spacer.
Both systems release doxorubicin *in vitro* after incubation with lysosomal enzyme cathepsin B and exhibit *in vivo* anti-cancer activity in the treatment of selected model mice cancers. PHPMA, PEG and PHPMA-drug carriers, if conjugated with the antibody to form antibody-targeted systems, significantly decrease its immunogenicity (approx. by order of magnitude two).

## Introduction

Water-soluble synthetic polymers conjugated with antibodies or their fragments provide a potential targetable drug delivery system. In recent years, such conjugates based on copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA) have been extensively studied as carriers facilitating site-specific delivery and controlled release of immunosuppressants and anti-cancer drugs[1-3]. In these conjugates, the nondegradable PHPMA backbone is modified by biodegradable oligopeptide side chains (spacers), terminated in the targeting (antibody) and/or drug moieties, randomly distributed along the polymer chain. We have shown that degradation of the spacer and release of the drug from the carrier system is a pre-requisite for the biological activity of some anti-cancer drugs[1-4]. If PHPMA-drug

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conjugates are synthesised with the spacers tailor-made as substrates for lysosomal enzymes (cathepsin B) or their mixtures (tritosomes), the drug can be released at a rate depending on the length and detailed structure of the oligopeptide spacer[5]. In certain cases, nondegradability of the polymer carrier brings about some limitations for therapeutical use of such systems. Recently, a new biodegradable water-soluble drug carrier system based on block copolymers of poly(ethylene glycol) (PEG) has been developed[6,7]. Biodegradation of the conjugate consisting of two low-molecular-weight PEG blocks connected by means of biodegradable oligopeptide linkage with at least one amino acid residue containing carboxylic group have been studied. In the conjugate, a drug model or drug was attached to the carboxylic group of the carrier via an enzymatically degradable oligopeptide spacer.

In this paper, we report on the synthesis and properties of the conjugates based on nondegradable PHPMA or degradable PEG carriers with attached anti-cancer drug doxorubicin (DOX) or model proteins and antibodies, designed for treatment of selected cancers.

## Results and discussion

Schematic structures of polymer-drug conjugates under study are given in Fig. 1. In all polymer-drug conjugates, anti-cancer drug doxorubicin (DOX) was used.

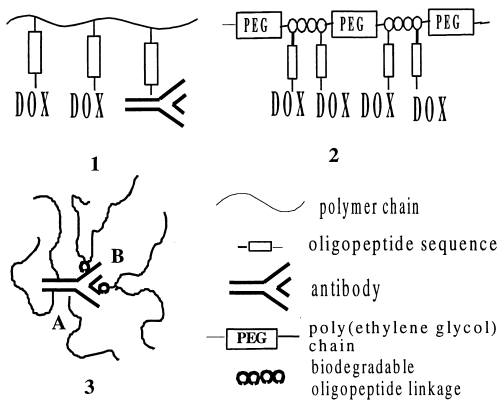


Fig. 1: Schematic structure of polymer conjugates

Three structures of conjugates have been synthesised and studied. Structure **1** represents molecules consisting of PHPMA backbone and biodegradable oligopeptide side chains (spacers), terminated in either the targeting antibody or its $F(ab)_2$ fragment and/or DOX. Conjugate of structure **2** is based on PEG (2000) blocks connected with $N^2$,$N^6$bis(glutamyl)lysine ($Glu_2Lys$) oligopeptide links forming linear water-soluble polymer chain, bearing DOX attached to the carboxylic groups of the oligopeptide links via GlyPheLeuGly spacer. In structure **3**, semitelechelic PHPMA or PEG chains are linked via their terminal functional group to the central antibody molecule. Two types of PEG-antibody conjuga-tes were synthesised; A - antibody modified with single PEG chains and B - antibody modified with two PEG chains doubled by means of lysine molecule *$N^2$,$N^6$*-bis[ω-methyl-poly(oxyethylene)-oxycarbonyl]lysine) ($PEG_2Lys$).

**Synthesis and properties of conjugates with DOX**

In the first step of the synthesis of conjugates of structure **1**, polymer precursors were prepared by radical precipitation copolymerization of HPMA with methacryloylated 4-nitrophenyl ester of oligopeptides and in the second step, DOX or both DOX and antibody were successively attached to the polymer by an aminolytic reaction. In the synthesis, all oligopeptides were tailored as substrates for lysosomal enzymes. For details, see ref.[8,9].

We have shown previously that the *in vitro* rate of DOX release from PHPMA conjugates incubated with lysosomal enzymes can be controlled by changing the detailed structure of the oligopeptide sequences used in the synthesis[10]. Flow cytometry analysis has shown that the intensity of binding of the antibody-targeted conjugates to T-lymphocytes was very high depending on the specificity of antibody, while nontargeted PHPMA-DOX conjugates did not bind to those cells at all[4]. The *in vitro* cytotoxic effect, tested by the inhibition of proliferation of T-lymphocytes ([$^3$H] thymidine uptake), was much higher after application of antibody-targeted conjugates compared with their nontargeted analogues[4,9]. Tests of *in vivo* cytotoxic activity of polymer-DOX conjugates used for the treatment of EL-4 mouse thymoma showed that the use of the free drug was without any significant effect on the mice survival and tumour size. The nontargeted PHPMA-DOX conjugates showed certain but limited effect on the rate of tumour growth and survival of experimental mice. The highest effect was obtained using anti-EL-4 or ATG-targeted conjugates which showed a

significant decrease in the tumour size and extension of the life span in treated mice[11]. The tests confirmed that polymer bound DOX is nontoxic to bone marrow and to reticulocytes in peripheral blood in contrast to the free drug. An example of the activity of PHPMA-DOX conjugates in the treatment of mouse EL-4 lymphoma is given in Fig. 2.

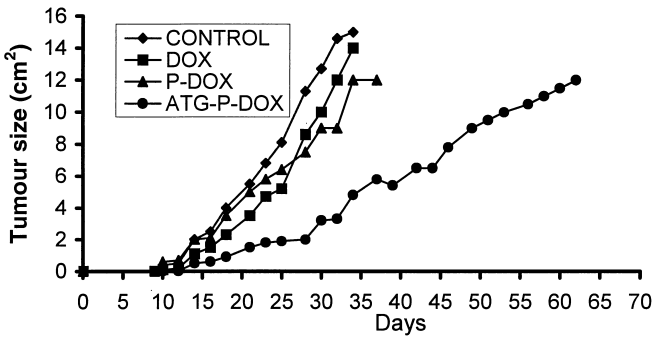


Fig. 2: Effect of DOX, its nontargeted PHPMA (P-DOX) and antibody-targeted (ATG-P-DOX) conjugates on the growth of mouse EL4 T-cell lymphoma

Mice (inbred strain C57BL/10) bearing EL4 T cell lymphoma were treated (i.p.) after tumour inoculation (day 0) on day 3, 5, 7 and 9 with free DOX, nontargeted and antibody-targeted PHPMA-DOX conjugates (dose equivalent to 5 mg/kg DOX). The size of the tumour and survival time were determined. In agreement with our previous studies, there was no significant effect of the free drug (EL-4 lymphoma was selected because its low sensitivity to the treatment with free DOX) on the growth of the tumour and prolongation of survival time. Only a relatively small effect of nontargeted conjugate was observed but, in contrast, significant chemotherapeutic effect (suppressed tumour growth, long survival time) of antibody-targeted conjugate was found. Our results demonstrated that PHPMA-DOX, particularly its antibody-targeted conjugates, are potentially useful systems for treatment of tumours.

PEG-based conjugates of DOX (Fig. 3) were prepared by the reaction of polymer precursor with GlyPheLeuGly derivative of DOX differing in configuration of Phe (L-Phe, D-Phe, DL-Phe). Polymer precursor consisting of PEG blocks ($M_w$ 2000) linked via $Glu_2Lys$ links

to form high-molecular-weight polymer was prepared by the reaction of activated PEG with benzyl $N^2,N^6$-bis(γ-benzylglutamyl)lysinate bis(trifluoro acetate)[12] followed by deprotection of benzyl protecting groups. Briefly, PEG 2000 (3.6 g, 1.8 mmol) dried by azeotropic distillation with toluene was dissolved in pyridine (20 $cm^3$) together with 4-(dimethylamino)pyridine (88 mg, 0.72 mmol) and mixed with a solution of disuccinimidyl carbonate (1.8 g, 7.2 mmol) in acetonitrile (15 $cm^3$). The reaction mixture was left in the dark at 25°C overnight. The solvents were evaporated, the residue was dissolved in dry ethyl acetate and the product was isolated by precipitation and filtration after addition of diethyl ether to the cooled ethyl acetate solution. Yield 3.1 g of the active PEG carbonate.

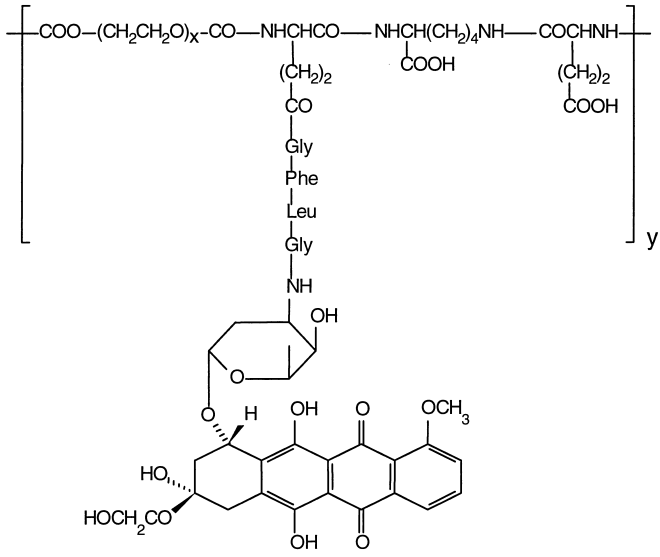


Fig. 3: Structure of PEG-DOX conjugate

The $Glu_2Lys$-linked PEG block polymer was prepared by interfacial methylene chloride - water condensation. A solution of the activated PEG (1.3 g, 0.572 mmol) in methylene chloride (20 $cm^3$) was added to the mixture benzyl $N^2,N^6$-bis(γ-benzylglutamyl)lysinate bis(trifluoro acetate)[12] (0.516 g, 0.572 mmol) and sodium hydrogencarbonate (230 mg, 2.73 mmol) in water (20 $cm^3$) under vigorous stirring at 25°C. The reaction mixture was

acidified with 0.1 M HCl to pH 3 after 5 h stirring. The organic layer was separated, washed with aqueous NaCl and dried over $Na_2SO_4$. The solvent was removed in vacuum, the polymer was suspended in water and freeze-dried. Benzyl protecting groups were removed by 3 h hydrogenation of the polymer (800 mg) in ethanol (10 $cm^3$) using (Pd/C, 10 %) catalyst. The reaction mixture was bubbled with nitrogen, the catalyst was filtered off and ethanol removed in vacuum. The oily residue was dissolved in water and freeze-dried yielding over 700 mg of highly hygroscopic polymer.

The polymer precursor was obtained after activation carboxylic groups of the product by the reaction with 4-nitrophenol. The polymer acid (200 mg), 4-nitrophenol (100 mg) and *N,N'*-dicyclohexylcarbodiimide (DCC) (140 mg) were dissolved in methylene chloride (2 $cm^3$) at 0°C. The reaction mixture was kept at 4 °C overnight, dicyclohexylurea (DCU) was removed by filtration and the polymer was purified by GPC (Sephadex LH-20, $CH_2Cl_2$). The polymer was isolated by freeze-drying from benzene resulting in a yellowish powder which, however, "melts" when not kept under vacuum. Yield 200 mg (90 %) of the polymer precursor.

Preparation of H-GlyPheLeuGly derivative of DOX starting from methyl esters of dipeptides H-GlyPhe-OMe and H-LeuGly-OMe was carried out using methods common in peptide chemistry. H-Gly-Phe-OMe.HCl (3.0 g, 11 mmol) was tritylated in suspension in dry $CHCl_3$ (25 $cm^3$) with trityl chloride (3.1 g, 11.1 mmol) in the presence of triethylamine (2.3g, 22.7 mmol) at 25°C (2 h). Chloroform was evaporated and the oily residue was diluted with ethyl acetate (60 $cm^3$). Triethylamine hydrochloride was filtered off, the filtrate was washed with water (2x20 $cm^3$) and dried over $MgSO_4$. The product was crystallised from propan-2-ol-hexane. Yield 4.5 g (9.4 mmol, 85 %) of white crystalline product, m.p. 154-156°C, TLC: $R_f$ = 0.92 (ethyl acetate). Methyl ester protecting group was removed by alkaline hydrolysis of Tr-Gly-Phe-OMe (3.0 g, 6.27 mmol) with aqueous KOH (6.3 $cm^3$, 1 $mol \cdot dm^{-3}$) in dioxane (25 $cm^3$). The reaction mixture was stirred at 45°C for 1 h and then evaporated to dryness under reduced pressure. The product was obtained after acidification of aqueous solution with acetic acid, its extraction to ethyl acetate and tritration with hexane. Yield 96 %, TLC: $R_f$ = 0.75 (ethyl acetate). Tetrapeptide was prepared by the reaction of Tr-Gly-Phe-OH (0.89 g, 1.92 mmol), HCl.Leu-Gly-OMe (0.46 g, 1.93 mmol) and HOBt (0.30 g, 1.93 mmol) in DMF (8 $cm^3$) using the DCC method (0.40 g, 1.93 mmol DCC

in 1 $cm^3$ DMF) in the presence of triethylamine (0.27 $cm^3$, 1.93 mmol). After 48 h at 4°C, the precipitated DCU was filtered off and the solution was concentrated under vacuum to an oily viscous residue. The crude product was redissolved in ethyl acetate (30 $cm^3$), the solution obtained was washed with aqueous solutions of $NaHCO_3$ and NaCl, dried over $Na_2SO_4$ and the final product was obtained by crystallisation from ethyl acetate-hexane. Yield 33 % of pure L-Phe isomer, m.p. 172-175°C. TLC: $R_f = 0.55$ (ethyl acetate). Tr-Gly-L-Phe-Leu-Gly-OH was prepared from its methyl ester by alkaline hydrolysis as described above for Tr-GlyPhe-OH. Tr-Gly-L-Phe-Leu-Gly-ONSu was prepared from Tr-Gly-L-Phe-Leu-Gly-OH (0.5 g, 0.788 mmol) and HONSu (0.1 g, 0.869 mmol) using DCC (0.2 g, 0.969 mmol) in THF (8 $cm^3$) at 0°C. DCU was filtered off, THF was removed under reduced pressure and the crude product was crystallised from propan-2-ol - ether yielding 0.32 g (0.437 mmol, 55 %) of the active ester, m.p. 109-113°C.

Tr-Gly-L-Phe-Leu-Gly-DOX was prepared by the reaction of the active ester (117 mg, 0.16 mmol) with DOX.HCl (95 mg, 0.164 mmol) in dry DMF (2.8 $cm^3$) in the presence of triethylamine (23 µl, 0.164 mmol) at 25°C. After 24 h, the solvent was evaporated to dryness and the crude product was chromatographed on silica gel using chloroform-methanol (95:5) as an eluent. The yield of 76% was obtained. The trityl-protecting group was removed by the reaction with 75% acetic acid (2.8 $cm^3$, 35 mmol) under stirring at room temperature for 1 h. Excess of acetic acid was removed by evaporation, the product was redissolved in water and freeze-dried. The yield was 57 % of the acetate salt. The Gly-D-PheLeuGly-DOX was prepared accordingly. Acetate of Gly-L-PheLeuGly derivative of DOX (39 mg) was conjugated with PEG precursor (100 mg) in DMF (2 $cm^3$) in the presence of triethylamine (50 µl) at room temperature. After filtration, DMF was evaporated, the oily product dissolved in methanol and purified by GPC on a column packed with Sephadex LH-20. The final product (Fig. 2) was obtained by freeze-drying of its aqueous solution and characterised by HPLC (TSK 4000 column, mobile phase 50 % aqueous methanol containing 0.1 % TFA). $M_w$ 29 200, polydispersity 3.3. The content of DOX was 5.6 wt % (UV spectrophotometry). Detailed synthesis and characterisation of PEG-DOX conjugates will be described in a separate paper. *In vitro* degradation of conjugates was estimated after their incubation with lysosomal enzyme cathepsin B. A

comparison of the rates of DOX release from PHPMA and PEG carriers is shown in Fig. 4. There was only a small difference in the rate of DOX release from both conjugates, the release from PEG conjugate being slower probably due to higher steric hindrance to the formation of the polymer-enzyme complex. The results of HPLC analysis (column TSK PW 3000, methanol-water) of products of cathepsin B-catalysed hydrolysis confirmed degradation of $(Glu)_2Lys$ links in the main chain, resulting in formation of PEG, $M_w = 2000$ (data not shown).

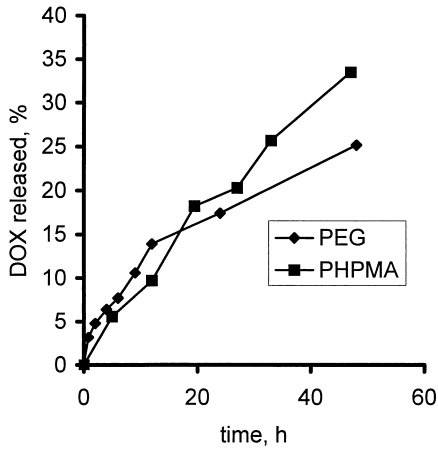


Fig. 4: Release of doxorubicin from polymer conjugates incubated with cathepsin B in phosphate buffer, pH 5.5, 37°C

Preliminary results of *in vivo* tests of anti-tumour activity of PEG conjugates against mice colorectal C 26 carcinoma in BALB/C mice are shown in Fig. 5.

Mice were treated i.v. on day 8, 9 and 10 after i.p. inoculation of $10^5$ cancer cells (on day 0) by 5mg/kg of DOX or an equivalent of its conjugates. Regression of the tumour growth after treatment with free DOX was not significant. In contrast, both PEG-DOX conjugates were more efficient and the effect (regression of tumour growth and prolongation of life span) of the nontargeted PEG-DOX conjugates was more pronounced than in the case of treatment of EL4 lymphoma with PHPMA-DOX (Fig. 2). These preliminary results justify

us to assume that antibody-targeted forms of PEG conjugates will be even more effective than their nontargeted forms. Detailed studies are under way.

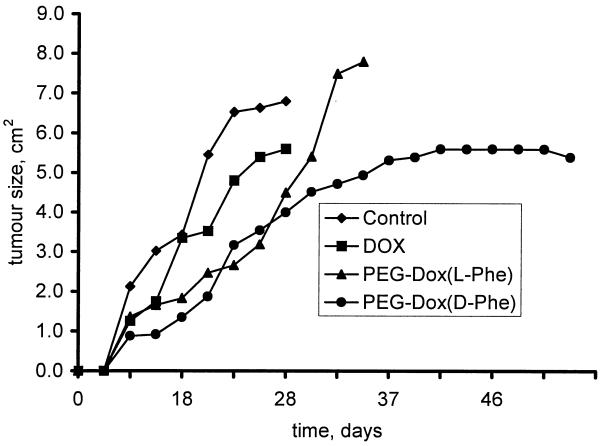


Fig. 5: Growth of the colorectal C 26 carcinoma in BALB/C mice

The use of antibody-polymer conjugates as drug carriers may be accompanied by several problems, immunogenicity being the most important. This is why we synthesised and studied immunogenicity of polymer-antibody conjugates of structure **3** (Fig.1.)

**Polymer conjugates with antibodies and their immunogenicity**

Conjugates of structure **3** were prepared by the reaction of anti-Thy 1,2 antibody with semitelechelic polymer precursors with the aim to study immunogenicity of polymer-antibody conjugates designed as targetable drug carriers. PHPMA precursor was prepared by radical chain-transfer copolymerization of HPMA using 3-sulfanylpropanoic acid as a chain transfer agent followed by activation of terminal carboxylic group with *N*-hydroxysuccinimide[13,14]. The molecular weight ($M_w$) of the PHPMA 11 000 was obtained. The precursor α-methyl-ω-[(succinimidyloxycarbonyl)oxy]-poly(oxyethylene) (MeO-PEG-COOSu), $M_w$ 2000, was prepared as described earlier[12]. It was directly used for antibody modification and also for preparation of the precursor consisting of two PEG chains linked via lysine moiety ($N^2$,$N^6$-bis(MeO-PEG-CO)$_2$Lys-OSu). MeO-PEG-COOSu (1g) was

dissolved in 4 $cm^3$ DMF and added to a solution of H-Lys(OBzl)·2TFA (116 mg) and triethylamine (51 mg) at 15°C and the mixture was stirred for 3h. DMF was evaporated in vacuum, the product was dissolved in methanol and, after filtration, purified by GPC (Sephadex LH-60, methanol). The benzyl ester-protecting group was removed by 3 h hydrogenation in methanol using (Pd/C, 10 %) as a catalyst. The reactive succinimidyl ester was prepared in DMF by the reaction with *N*-hydroxysuccinimide using the DCC coupling method similarly to PHPMA[14].

Conjugates of polymer precursors with anti-Thy 1,2 antibody (ATS) were prepared by mixing in various ratios antibody and the respective polymer precursor in phosphate buffer pH 7.2 at 15°C using pH stat. The reaction mixture was stirred at pH 8.0 (sodium tetraborate) for 4 h and kept in refrigerator overnight. The resulting conjugates were separated from the unreacted polymer by GPC using Sephadex G-25 and phosphate buffer, pH 7.2 as an eluent. The amount of the protein in the conjugate was estimated using amino acid analysis and the degree of substitution of amino groups in the original antibody by polymer was determined using the trinitrobenzenesulfonate (TNBS) method[15]. The immunogenicity of conjugates was estimated as described[14]. The results of immunogenicity tests are given in Table 1.

Tab. 1. Immunogenicity od polymer-glycoprotein conjugates. (Anti-Thy 1,2 (a-Thy 1,2), IgG and polymer conjugates were used as antigens for detection and determination of antibody titres)

| Structure | Polymer content in conjugate, wt % | Degree of $NH_2$ substitution, % | Antibody titre x $10^{-6}$ |
|---|---|---|---|
| PEG-a-Thy 1,2 | 23 | 29 | 1.60 |
| PEG-a-Thy 1,2 | 28 | 39 | 0.82 |
| $(PEG)_2$-Lys-a-Thy 1,2 | 16 | 20 | 2.1 |
| $(PEG)_2$-Lys-a-Thy 1,2 | 36 | 26 | 1.6 |
| PHPMA -a-Thy 1,2 | 61 | 38 | 1.0 |
| -a-Thy 1,2 (Control) | 0 | 0 | 130 |
| PHPMA-DOX-IgG | 75 | n.d. | 1.0 |
| IgG (Control) | 0 | 0 | 260 |

The immunogenicity of immunoglobulins (anti-Thy 1,2 or IgG) dramatically decreased (two order of magnitude) after their modification with both hydrophilic polymers (PEG,

PHPMA).The decrease in immunogenicity of polymer conjugates depends on the degree of protein substitution. The higher the degree of substitution the lower immunogenicity. Unfortunately, the higher degree of substitution of amino groups in the antibody molecule, the higher is the probability of damaging of the binding site of the antibody for its antigen and hence the efficiency of targeting can be decreased. This problem can be overcome using polymer structures facilitating attachment of two polymer chains via one binding site to the antibody (in our case ($N^2$,$N^6$-(MeO-PEG-CO)$_2$Lys-OSu in (PEG)$_2$-Lys-a-Thy 1,2 conjugates). Our results showed that there is no significant difference between the effects of PHPMA and PEG modification. A significant and even more pronounced decrease in immunogenicity than in the case of conjugates with single polymer chains was observed in tests with targeted anti-cancer drugs - PHPMA-DOX conjugates with antibodies. In this case, immunogenicity of the polymer-modified immunoglobulin decreased more than 200 times).

The results presented in this paper demonstrate high potential of hydrophilic polymers in the development of new site-specific drug delivery systems designed for human therapy.

## Acknowledgement

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## References

[1] K. Ulbrich, *J. Bioact. Compat. Polym.* **6**, 348 (1991)
[2] R. Duncan, *Anti-Cancer Drugs* **3**, 175 (1992)
[3] B. Říhová, J. Strohalm, D. Plocová, V. Šubr, J. Šrogl, M. Jelínková, M. Šírová, K. Ulbrich, *J. Controlled Release* **40**, 303 (1996)
[4] M. Jelínková, J. Strohalm, D. Plocová, V. Šubr, M. Šťastný, K. Ulbrich, B. Říhová, *J. Controlled Release* **52**, 253 (1998)
[5] J. Strohalm, V. Šubr, T. Hirano, Y. Ito, K. Ulbrich, *J. Controlled Release* **49**, 123 (1997)
[6] M. Pechar, J. Strohalm, K. Ulbrich, E. Schacht, *Macromol. Chem. Phys.* **198**, 1009 (1997)
[7] K. Ulbrich, M. Pechar, J. Strohalm, V. Šubr, B. Říhová, *Macromol. Symp.* **118**, 577 (1997)
[8] B. Říhová, J. Strohalm, D. Plocová, K. Ulbrich, *J. Bioact. Compat. Polym.* **5**, 249 (1990)
[9] K. Ulbrich, V. Šubr, J. Strohalm, D. Plocová, M. Jelínková, B. Říhová, *J. Controlled Release,* in press
[10] K. Ulbrich, J. Strohalm, V. Šubr, D. Plocová, R.Duncan, B.Říhová, *Macromol. Symp.* **103**, 177 (1996)

[11] B. Říhová, J. Šrogl, M. Jelínková, M. Burešová, V. Šubr, K.Ulbrich, *Ann. N.Y. Acad. Sci*. **831**, 57 (1997)
[12] M. Pechar, J. Strohalm, K. Ulbrich, *Collect. Czech. Chem. Commun*. **60**, 1765 (1995)
[13] Č. Koňák, L. Mrkvičková, O. Nazarova, K. Ulbrich, L.W. Seymour, *Supramol. Sci*. **5**, 67 (1998)
[14] D. Oupický, K. Ulbrich, B. Říhová, *J. Bioact. Compat. Polymers*, in press
[15] S.L. Snyder, P.Z. Sobocinski, *Anal. Biochem*. **64**, 284 (1975)