Pharmaceutical Research, Vol. 24, No. 12, December 2007 (© 2007)
DOI: 10.1007/s11095-007-9449-3

Research Paper

Stability in Plasmas of Various Species of HPMA Copolymer–PGE_1 Conjugates

Huaizhong Pan,[1] Pavla Kopečková,[1,2] Jihua Liu,[1] Dong Wang,[4] Scott C. Miller,[3] and Jindřich Kopeček[1,2,5]

Received June 18, 2007; accepted August 28, 2007; published online September 25, 2007

Purpose. To determine the stability of HPMA copolymer–prostaglandin E_1 (PGE_1) conjugates in plasmas of different species and to identify the enzymes responsible for the cleavage of the ester bond.
Methods. The conjugates were incubated in human, rat, and mouse plasma at 37°C in the presence and absence of specific esterase inhibitors. The released PGE_1 was analyzed using an HPLC assay. To evaluate the effect of the conformation of the conjugate on the rate of PGE_1 release, its structure was modified by the attachment of hydrophobic side chains.
Results. The rate of PGE_1 release was strongly species dependent. Whereas the conjugate was stable in human plasma, the PGE_1 release in rat or mouse plasma was substantial. In rat plasma, the ester bond cleavage was mainly catalyzed by butyrylcholinesterase; in mouse plasma, in addition to butyrylcholinesterase, carboxylesterase also contributed to the cleavage. The formation of compact polymer coils stabilized the ester bond.
Conclusions. HPMA copolymer–PGE_1 conjugates are strong candidates as novel therapeutics for the treatment of osteoporosis. The observed species differences in plasma stability of ester bonds are of importance, because the ovariectomized rat model is recommended by the FDA for pre-clinical evaluation.

KEY WORDS: esterase; ester bond; HPMA copolymer conjugates; osteoporosis; plasma.

INTRODUCTION

The design of water-soluble polymer–drug conjugates (macromolecular therapeutics) has been based on advances in polymer chemistry and biomedical sciences. Their unique structural, physicochemical, and biological characteristics result in advantageous properties when compared to low molecular weight drugs (1–3): improved water solubility of hydrophobic low molecular weight drugs with concomitant improvement of bioavailability, protection of unstable drugs from degradation, long-lasting circulation in the bloodstream, decreased non-specific toxicity of the conjugated drug, and increased accumulation of the drug at the tumor site.

Bioconjugate chemistry offers a variety of options for the creation of drug attachment/release points. The stability of the conjugate, the drug release rate, and drug release site often depend on the specific structure of the bond (spacer) between the polymer backbone and the drug moiety. Drugs have been attached to polymer carriers via peptide, saccharide, urethane, ester, disulfide, aromatic azo, and other bonds (4). Combination of enzymatically cleavable sequences with self-elimination groups have been also frequently used in spacer design (5). Usually, the drug-carrier attachment bond (spacer) should have high stability in the bloodstream and the extracellular interstitium, but be cleavable at the target site. Various polymer–drug conjugates contain an ester group either as the major attachment/release point, or as a part of a more complex structure. Due to the instability of the ester bond, the bloodstream stability of conjugates containing ester bonds needs to be evaluated.

Plasma esterases, including paraoxonase (PON1, EC 3.1.8.1), acetylcholinesterase (AChE, EC 3.1.1.7), butyrylcholinesterase (BChE, EC 3.1.1.8), carboxylesterase (CbE, EC 3.1.1.1), and arylesterase (EC 3.1.1.2), play an important role in the stability/efficiency of ester-bond containing conjugates (6). Considerable species differences have been observed in esterase activities. For example, BChE, PON1, albumin esterase, and AChE, but not CbE were detected in human plasma (7). The participation of particular esterases in a cleavage of a substrate can be distinguished using special substrates and inhibitors.

Recently, we designed novel macromolecular therapeutics for the treatment of osteoporosis. Their activity is based on the bone-specific delivery of an anabolic agent, prostaglandin E_1 (PGE_1), mediated by a bone-seeking moiety, aspartic acid octapeptide or alendronate. PGE_1 was linked to the HPMA copolymer via an ester bond connected to 1,6-elimination 4-aminobenzyl alcohol group and a cathepsin K sensitive tetrapeptide (Gly–Gly–Pro–Nle) spacer (8). Ca-

[1] Department of Pharmaceutics and Pharmaceutical Chemistry/CCCD, University of Utah, Salt Lake City, Utah 84112, USA.
[2] Department of Bioengineering, University of Utah, Salt Lake City, Utah 84112, USA.
[3] Department of Radiobiology, University of Utah, Salt Lake City, Utah 84112, USA.
[4] Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, Omaha, Nebraska 68198, USA.
[5] To whom correspondence should be addressed. (e-mail: jindrich.kopecek@utah.edu)

thepsin K is highly expressed in osteoclasts (9); consequently, the spacer will ensure site-specific release at bone resorption locations. The main cleavage mechanism at the target site (bone) is the enzymatic cleavage of the tetrapeptide catalyzed by cathepsin K (rate controlling step) followed by a fast 1,6-elimination reaction resulting in the release of unmodified PGE_1 (8). The stability of the conjugate and the potential mechanism of ester bond cleavage in the blood have been not known.

Conformation of the polymer–drug conjugate may have a strong influence on its biological functions. Associations are the result of inter- and/or intra-molecular interactions, and are typically hydrophobic interactions. The preference for either types of association is intrinsically determined by polymer concentration, and structural parameters such as the size of the polymer chain, or the type, content, and arrangement of hydrophobes in the polymer. The structure of vesicles self-assembled from water-soluble polymers containing hydrophobic side chains depends on the degree of substitution. Low levels of hydrophobicity favor the formation of micelles, higher degrees of substitution produce bilayer vesicles and dense nanoparticles (10). The impact of unimolecular micelle formation on drug release was described by Hörpel *et al*. on cyclophosphamide conjugates with polyethyleneimine containing palmitic acid grafts (11). Similarly, HPMA copolymers with hydrophobic side chains can associate in water forming micelles with hydrophobic side chains inside and hydrophilic polymer chains outside. Micellar shells hindered the penetration of enzyme into the micellar core, and reduced the rate of the enzyme-catalyzed release of *p*-nitroaniline (drug model) (12).

Here, we evaluated the stability in plasma of different species (mice, rat, and human) of HPMA copolymer conjugates containing variable amounts of PGE_1, and of an HPMA copolymer conjugate containing PGE_1, D-Asp_8, and hydrophobic stearyl side chains. Using a set of esterase inhibitors, the esterase(s) responsible for cleavage of the ester bonds in mice and rat plasma have been identified.

MATERIALS AND METHODS

Materials

PGE_1 was obtained from Hawkins (Minneapolis, MN). HPMA (13), *N*-methacryloylglycylglycine 2,3,5-trichlorophenyl ester (MA–Gly–Gly–OTcp) (similarly to 14), *N*-methacryloylglycylglycyl-L-prolyl-L-norleucyl-(4-aminobenzyl alcohol) prostaglandin E_1 ester (MA–Gly–Gly–Pro–Nle–4AB–PGE_1) (8), and HPMA copolymer–PGE_1 conjugates (P-PGE_1) (8) were synthesized as described previously. Prostaglandin A_1 (PGA_1), prostaglandin B_1 (PGB_1), octadecylamine, acetythiocholine chloride, butyrylthiocholine chloride, dithiobis-(2-nitrobenzoic acid), *p*-nitrophenyl acetate, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW 284c51), tetraisopropyl pyrophosphoramide (iso-OMPA), eserine, bis(4-nitrophenyl) phosphate (BNPP), paraoxon, acetylcholinesterase (AChE, from bovine erythrocytes) and butyrylcholinesterase (BChE, from equine serum) were purchased from Sigma (St. Louis, MO). 2-Chlorotrityl chloride resin (100–200 mesh, 1.1 mmole/g), *N*-α-Fmoc protected amino acids, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), and *N*-hydroxybenzotriazole (HOBt) were purchased from EMD Biosciences (San Diego, CA). All other reagents and solvents were purchased from VWR International (West Chester, PA). Human male and female plasmas were obtained from University of Utah Hospital (Blood Bank). Rat plasma was isolated by heart puncture from Sprague Dawley rats. Mouse plasma was obtained through heart puncture from Swiss Webster mice. Heparin was used as an anti-clotting agent for all plasmas.

Methods

UV–Vis spectra were measured on a Varian Cary 400 Bio UV–Visible Spectrophotometer. Mass spectra of all synthesized compounds were obtained using a mass spectrometer Voyager-DE (STR Biospectrometry Workstation, PerSeptive Biosystems, Framingham, MA). The molecular weight and molecular weight distribution of polymers were measured on the ÄKTA FPLC system (GE Healthcare, formerly Amersham) equipped with UV and RI detectors using a Superose 6 HR10/30 column with PBS (pH 7.3) or PBS/acetonitrile (70/30) as the mobile phase. The average molecular weights were calculated using a calibration with polyHPMA fractions. HPLC analyses of PGE_1, PGA_1, and PGB_1 samples were performed on the Agilent 1100 series HPLC apparatus equipped with a reverse-phase column (ZORBAX 300SB-C18 4.6×250 mm, 5 μm) and a diode-array detector. The mobile phase was a mixture of phosphate buffer (0.02 M, pH 5.0) and acetonitrile, 65:35 at a constant flow rate of 1 ml/min. The PGE_1, PGA_1, and PGB_1 were detected at 205 nm (elution time 9.2 min), 230 nm (elution time 18.1 min) and 280 nm (elution time 18.9 min), respectively using cortisone (elution time 5.3 min) as the internal standard (15,16). Calibration curves for PGE_1, PGA_1 and PGB_1 were obtained using the corresponding peak area *versus* concentration.

N^{α} -[6-Aminohexanoyl-Glycyl-*L*-Prolyl-*L*-Norleucyl-*N*-(6-Aminohexanoyl)]-N^{ε} -[*L*-(Fluorescein-carboxyl)] Lysyl-octa-*D*-Aspartic Acid (FD_8)

FD_8 was synthesized using a solid-phase methodology and manual Fmoc/tBu strategy on 2-chlorotrityl chloride resin as described previously (17). The product was cleaved from the resin by trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H_2O (95/2.5/2.5). Yield 90%. MS (MALDI-TOF): m/z=1919 (M+1).

HPMA Copolymer Precursor Containing PGE_1 and 2,4,5-Trichlorophenyl (OTcp) Active Ester Groups (P-PGE_1-OTcp)

HPMA (40.6 mg), MA–Gly–Gly–Pro–Nle–4AB–PGE_1 (8.5 mg, 3 mol% in the feed), MA–Gly–Gly–OTcp (15.2 mg, 12 mol% in the feed) were dissolved in methanol (0.6 ml) containing 35 mM of 2,2-dimethoxy-2-phenylacetophenone (DMPAP) as the photo-initiator. The solution was bubbled with N_2 for 5 min, then sealed, placed under a fluorescent lamp (1,000 lm/m^2) and polymerized at room temperature for 24 h. The copolymers were isolated by precipitation into

an excess of acetone, purified by re-precipitation from a methanol solution into acetone (three times), and dried under vacuum at room temperature. The PGE_1 content was determined by UV spectroscopy using the molar extinction coefficient of macromonomer 21,000 M^{-1} cm^{-1} (λ_{max} 247 nm, methanol).

HPMA Copolymer Containing PGE_1, Stearyl Side-chains, and Fluorescein Labeled D-Aspartic Acid Octapeptide Targeting Moieties (P-PGE_1-C_{18}-FD_8)

P-PGE_1-OTcp (28 mg), FD_8 (5.6 mg), and octadecylamine (4 mg) were dissolved in 0.5 ml of DMSO, and then DIPEA (10 μl) was added slowly. The reaction mixture was stirred for 6 h, then 1-amino-2-propanol (100 μl) was added and stirring continued for 30 min. Next, the reaction mixture was diluted with 1 ml of methanol and the conjugate was purified by chromatography on a LH-20 column, using methanol as eluent. Yield 35 mg. The content of octadecylamine was determined by HPLC, after the sample was hydrolyzed with 6 N HCl (18). The mobile phase was a gradient of methanol and water at a constant flow rate of 1 ml/min. The content of fluorescein-D-aspartic acid octapeptide (FD_8) in the conjugate was determined by UV–Vis at 500 nm.

Plasma Characterization

The protein content of the plasma was measured by Lowry method calibrated by BSA. The activity of the plasma was measured by acetylthiocholine chloride, butyrylthiocholine chloride (19), and *p*-nitrophenyl acetate (20). The activity to acetylthiocholine chloride (or butyrylthiocholine chloride) was measured in pyrophosphate buffer (pH=9.0) containing acetylthiocholine chloride (or butyrylthiocholine chloride) (3.5×10^{-3} M), dithiobis-(2-nitrobenzoic acid) (1.25×10^{-4} M) and plasma (0.2 vol.%). The release kinetics of 5-thio-2-nitrobenzoic acid was detected at 405 nm ($\varepsilon=1.36\times10^4$ M^{-1} cm^{-1}) at room temperature (r.t.). The activity to *p*-nitrophenyl acetate was measured in PBS buffer (pH=7.3) containing *p*-nitrophenyl acetate (1.0×10^{-3} M) and plasma (0.2 vol.%). The release kinetics of *p*-nitrophenolate was detected at 400 nm at r.t. ($\varepsilon=1.80\times10^4$ M^{-1} cm^{-1}).

Stability Study

PGE_1 stock solution was prepared by dissolving PGE_1 in a small amount of ethanol, then diluting with PBS. The final concentration of PGE_1 was 400 μg/ml and the content of ethanol was less than 10 vol.% (we found that a small amount of DMSO had some effect on stability of plasma and PGE_1 but a small amount of ethanol did not). HPMA copolymer–PGE_1 conjugate stock solution was prepared by dissolving the conjugate in PBS to a final concentration of 400 μg/ml of PGE_1 equivalent. In a typical experiment, 0.5 ml of stock solution of PGE_1 (or the conjugate) was added to 0.5 ml of plasma at 37°C, stirred and incubated. At predetermined time intervals, 80 μl of sample was withdrawn and diluted with 240 μl of methanol. The sample was vortexed and centrifuged, the clear solution was filtered, and the prostaglandins (PGs) content (including PGE_1, PGA_1 and PGB_1) in the filtrate was analyzed by HPLC.

Inhibition Study

Inhibitor stock solutions were freshly prepared by dissolving inhibitors in PBS (if the inhibitor could not dissolve in PBS, then it was dissolved in a drop of ethanol, and then diluted with PBS). HPMA copolymer–PGE_1 conjugate stock solutions (800 μg/ml of PGE_1 equivalent) were prepared by dissolving the conjugate in PBS. In a typical experiment, 0.05 ml of inhibitor stock solution was added to 0.1 ml of plasma at 37°C. After incubation for 1 h, 0.05 ml of stock solution of the conjugate was added. The mixture was incubated for an additional 1 h, then 80 μl of sample was withdrawn and diluted with 240 μl of methanol. The sample was vortexed and centrifuged; the clear solution was filtered and the content of PGs in the filtrate was determined by an HPLC assay.

RESULTS AND DISCUSSION

Structure of the Conjugates

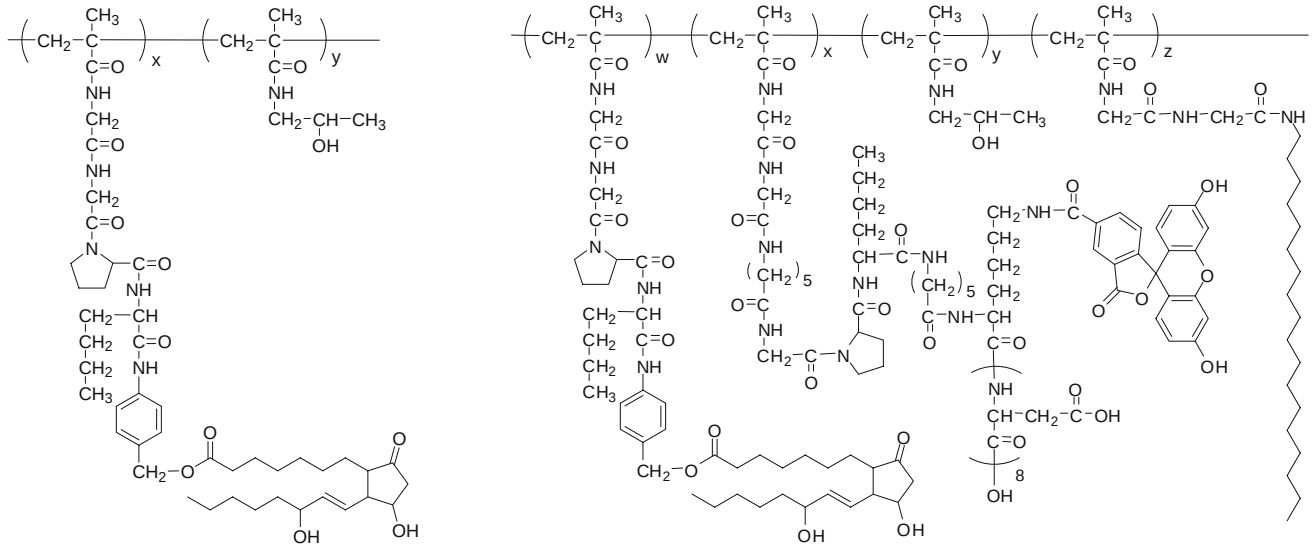
A novel bone targeting, HPMA copolymer based, PGE_1 delivery system was recently designed (8). PGE_1 was bound to the HPMA copolymer backbone via a spacer, composed of a cathepsin K sensitive tetrapeptide (Gly–Gly–Pro–Nle) and a self-eliminating 4-aminobenzyl alcohol structure. The spacer can be cleaved by cathepsin K, followed by 1,6-elimination reaction to release free PGE_1. In the chemical structure of the conjugate, there is an ester bond connecting PGE_1 and the 4-aminobenzyl alcohol moiety. Three HPMA copolymer–PGE_1 conjugates, C1, C2, and C3, containing different amounts of PGE_1 (Scheme 1a) were evaluated in this study. Another conjugate (C4) contained, in addition to PGE_1, fluorescently labeled lysyl-D-aspartic acid octapeptide, and hydrophobic stearyl side chains (Scheme 1b). In all conjugates, PGE_1 was bound to the HPMA copolymer carrier via its C-1 COOH group (see Table I for the characterization of the conjugates). Modification at this position contributes to the stabilization of the conjugate against metabolism (21). On the other hand, this attachment point, being an ester bond, may be susceptible to enzymatically catalyzed hydrolysis in the blood stream.

Stability of PGE_1 and its HPMA Copolymer Conjugates in Plasma of Different Species

Plasma Characterization

The plasmas were characterized by their activity to selected substrates and by protein content. The protein content of the human, rat and mouse plasma were 5.7±0.5, 5.8±0.5, and 5.4±0.5 mg/ml, respectively.

The activity of the plasmas to acetylthiocholine chloride, butyrylthiocholine chloride, and *p*-nitrophenyl acetate were determined. Typical results are shown on Fig. 1. The variation of activities when different batches of plasma have been used was less than 5%.



a Conjugates C1 − C3 (P-PGE_1) **b** Conjugate C4 (P-PGE_1-C_{18}-FD_8)

Scheme 1. Chemical structure of **a** HPMA copolymer–PGE_1 conjugates C1, C2, and C3 and **b** HPMA copolymer–PGE_1–FD_8 (fluorescein labeled D-aspartic acid octapeptide) conjugate C4, containing stearyl side-chains. See Table I for the composition of the conjugates

Stability of PGE_1

PGE_1 has a β-hydroxycyclopentanone structure. It can undergo dehydration in aqueous solution, under acidic or basic conditions, to give PGA_1, which can isomerize to PGB_1 in alkaline solution (Scheme 2). Paul *et al* found the degradation of PGE_1 in aqueous solutions followed apparent first-order kinetics, was pH dependent, and occurred at r.t. (16). We have compared the stability of the PGE_1 in human, rat, and mouse plasmas (Fig. 2). The PGE_1 degraded fast in all plasmas with similar degradation rates. The PGE_1 dehydrated to PGA_1 and quickly isomerized to PGB_1 in all plasmas. The content of PGA_1 in the incubation solution was negligible. We cannot exclude the presence of PGX_1 and PGX_2 in the incubation mixtures (16). However, the PGB_1 was by far the main peak and its structure is the most stable one. Consequently, PGX_1 and PGX_2 could be present only in minute amounts.

Stability of HPMA Copolymer–PGE_1 Conjugates

The stability of HPMA copolymer–PGE_1 conjugates was measured in human (male and female), rat, and mouse plasmas (Fig. 3). The rate of PGE_1 release was strongly species dependent. Whereas the conjugate appeared to be stable in human plasma and in PBS (control), the PGE_1 release in rat or mouse plasma was substantial, with mouse plasma producing the fastest rate of release. These data suggest that the release of the PGE_1 in rat or mouse plasma was due to enzymatically catalyzed hydrolysis of the ester bonds in the HPMA copolymer–PGE_1 conjugate.

The shape of the PGE_1 release curves was altered due to the conversion of free PGE_1 into PGB_1. Curves of total prostaglandins (PGs) release were also provided. Their shape is consistent with profiles observed with other enzymatically cleavable polymer–drug delivery systems (see, e.g. ref. 22).

PGE_1 release experiments in mixed plasmas were performed to determine if the stability of the conjugate in human plasma was due to the presence of inhibitors or absence of a particular enzyme. Rat (or mouse) plasma was co-incubated with human plasma for 1 h at 37°C, then HPMA copolymer–PGE_1 conjugate C2 was added to the incubation mixture. After additional 1 h and 4 h, the released PGE_1 and total PGs were measured (Table II). Mixtures of rat (or mouse) plasma and PBS were used as controls. The total released PGs (PGE_1+PGB_1) at both time intervals in rat/human (or mouse/human) plasma mixture were similar to results obtained when the conjugate was incubated with the mixtures of rat plasma/PBS (or mouse plasma/PBS). These results demonstrated that human plasma had no significant effect on the activity of rat and mouse plasma. Apparently, there is no inhibitor present in human plasma, which could influence the activity of relevant enzymes in rat or mouse plasma.

The stability of the PGE_1 moiety when attached to HPMA copolymer was estimated by spiking experiments.

Table I. Characteristics of the Conjugates

Conjugate	Structure	Mw (kD)	Mw/Mn	PGE_1 (mol%)	FD_8 (mol%)	C_{18} (mol%)
C1	P-PGE_1-3	21.0	1.4	3.1	–	–
C2	P-PGE_1-5	28.0	1.3	4.8	–	–
C3	P-PGE_1-10	N.D.	N.D.	10.1	–	–
C4	P-PGE_1-C_{18}-FD_8	N.D.	N.D.	2.5	1.2	4.6

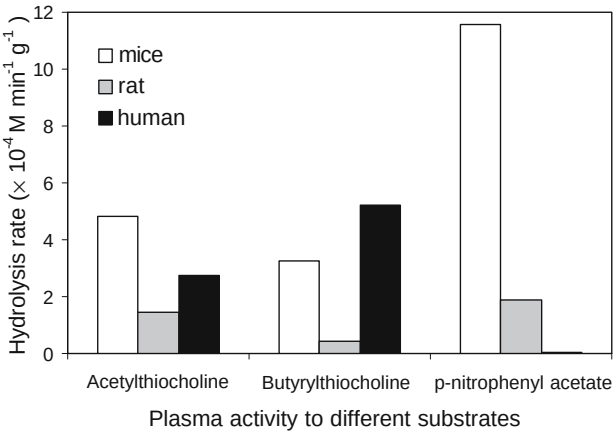
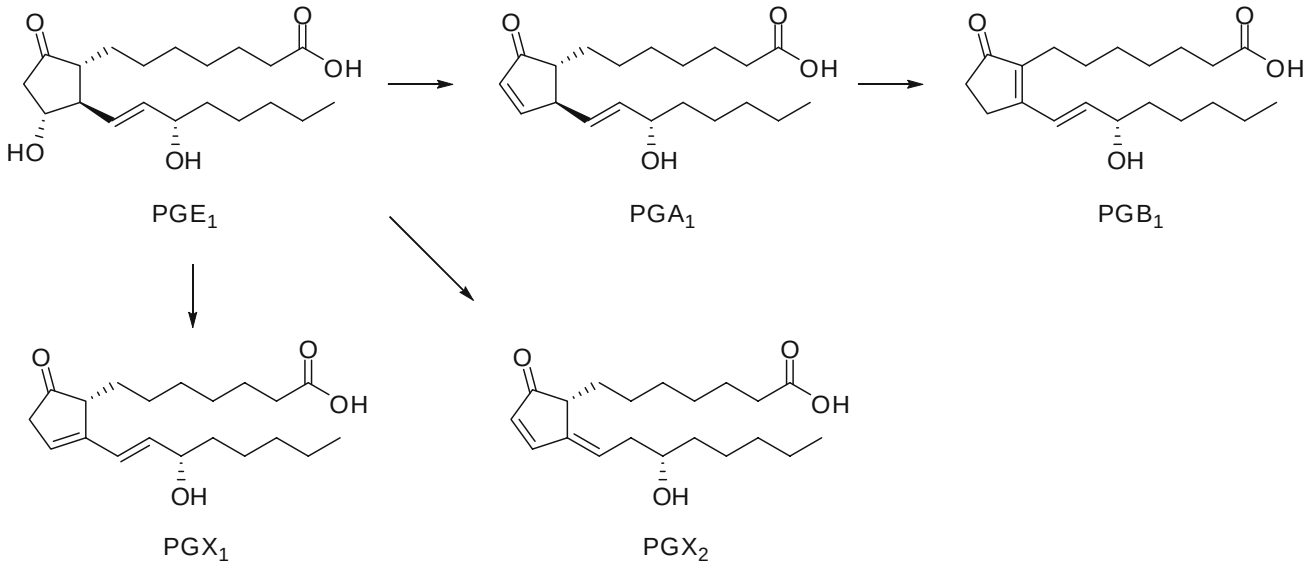


Fig. 1. The activity of human, rat, and mouse plasma to selected substrates

After 12 h incubation of HPMA copolymer–PGE_1 conjugate C2 in human plasma, rat (or mouse) plasma was added to the incubation mixture. As expected, the cleavage rate of PGE_1 from the conjugate immediately increased (Table III). There was no difference in the total PGs released when pre-incubation was performed in human plasma or in PBS. However, the amount of PGE_1 present in the mixture was different. The main reason was the fact that in the mixture of plasmas the protein content was twice as high as in the mixture of PBS with rat (or mouse) plasma. Based on the protein content of human (5.7±0.5 mg/ml), rat (5.8±0.5 mg/ml), and mouse (5.4±0.5 mg/ml) plasma, the protein content of human–rat mixed plasma was about 5.8 mg/ml, and human–mouse mixed plasma was about 5.6 mg/ml. On the other hand, the protein content of the PBS–rat plasma mixture would have been about 2.9 mg/ml, and of the PBS–mouse plasma mixture about 2.4 mg/ml. Since the rate of conversion of PGE_1 to PGB_1 by plasma proteins was similar (Fig. 2), the higher protein content apparently corresponded to a faster conversion of PGE_1 to PGB_1. In addition, the small amount of released PGE_1 during incubation in human plasma also contributed to the observed phenomena.

Identification of Mouse and Rat Plasma Enzymes Involved in the Cleavage of the Ester Bonds of the Conjugate

An excellent overview of esterases involved in the cleavage of prodrugs was recently published (6). In addition to Enzyme Commission (EC) numbers, esterases have been divided into three groups, A, B, and C, based on their specificities (23–25). Type A esterases are not inhibited by organophosphates, they hydrolyze them. Type B esterases are inhibited by organophosphates, whereas type C esterase are not. PON1 belongs to the esterase-A group, it requires Ca^{2+} for activity and stability. PON1 can be inhibited by EDTA, metals, and mercurial compounds, but not by organophosphates. On the other hand, AChE, BChE and CbE belong to the esterase-B group. The active site of these enzymes contains a catalytic triad composed of serine, glutamate, and histidine residues. AChE and BChE can be distinguished by special substrates and inhibitors. AChE is selective for acetylcholine and BChE is selective for butyrylcholine (6). CbE has wide substrate specificities. AChE, BChE and CbE can be inhibited by organophosphate compounds, such as paraoxon. AChE and BChE can be very specifically inhibited by 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51) and tetraisopropyl pyrophosphoramide (iso-OMPA), respectively. Eserine inhibits both AChE and BChE; it can be used to distinguish AChE and BChE from CbE. CbE can be inhibited by bis(4-nitrophenyl) phosphate (BNPP) (6,26). The structure of selected inhibitors and the corresponding esterases are summarized in Table IV. In addition to esterases, some proteins exhibit activity in the cleavage of ester bonds. Human serum albumin (HSA) is known for its activity in the activation of ester containing prodrugs (27). However, HAS produces low cleavage rates and contributes minimally to the overall enzymatic activity (6).



Scheme 2. Transformation of PGE_1 to PGA_1 and PGB_1. Adapted from (16)

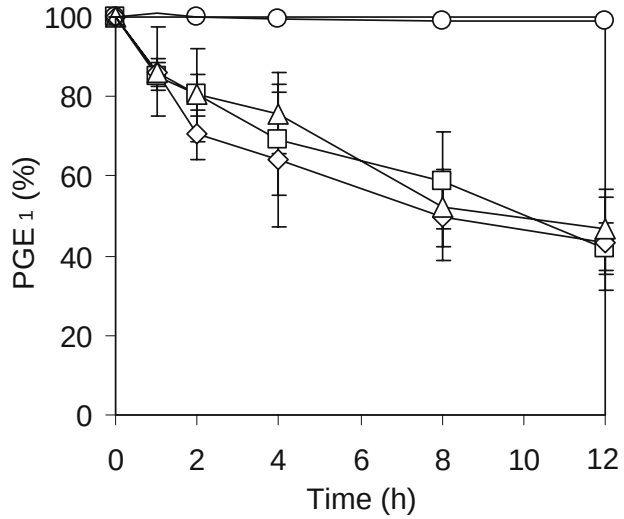


Fig. 2. Stability of PGE_1 in different plasmas at 37°C. Incubation system: *open circles* PBS; *open squares* human plasma; *open diamonds* rat plasma; *open triangles* mouse plasma

Based on published data (26), we selected BW284c51 for AChE, iso-OMPA for BChE, eserine for both AChE and BChE (to distinguish them from CbE), and BNPP for detecting CbE. In addition, we used paraoxon for AChE, BChE, and CbE, and EDTA for PON1 (Table IV) (6,7).

Table V shows the results of cleavage of Conjugate C2 (P-PGE_1-5) in rat and mouse plasmas in the presence of inhibitors. Paraoxon blocked the PGE_1 release, demonstrating that cleavage was caused by esterase activity. EDTA did not inhibit the cleavage, so the cleavage was not caused by PON1. For rat plasma, iso-OMPA, eserine, and BNPP almost stopped the cleavage, but BW284c51 only slightly lowered the cleavage rate. It appears that BChE predominantly contributed to the cleavage of the conjugate in rat plasma. For mouse plasma, BNPP decreased the cleavage rate at both inhibitor concentrations used, whereas iso-OMPA lowered the cleavage rate only at the higher concentration (1 mM). Thus, it appears that in mouse plasma two enzymes, BChE and CbE, contributed to the cleavage of the conjugate.

For confirmation of the esterase-mediated hydrolysis, HPMA copolymer-PGE_1 conjugate was incubated with two commercial cholinesterase esterases, AChE (bovine) and BChE (equine), which have similar structures as their corresponding rodent enzymes. At high enzyme concentrations (>10 U/ml), BChE released PGE_1; whereas, the release of PGE_1 by AChE was negligible (data not shown). These results confirmed that certain esterases have the potential to cleave the ester bond in the conjugate. Due to its lower specificity, BChE possesses a higher activity toward cleavage of the ester bond in HPMA copolymer-PGE_1 conjugate than AChE (6).

It is interesting to note that human plasma contains BChE. However, the release of PGE_1 from the HPMA copolymer–PGE_1 conjugate in human plasma was minimal (Fig. 3). This suggests that the activity of BChE in human plasma to PGE_1–benzyl ester bond in HPMA copolymer–PGE_1 conjugate is considerably lower than in rat and mouse plasmas.

Impact of the Conformation of HPMA Copolymer–PGE_1 Conjugates on the Rate of PGE_1 Release

In aqueous solutions, polyHPMA assumes a random coil conformation (28). Incorporation of hydrophobic side-chains into HPMA copolymers may result in intra- and/or intermolecular association between the hydrophobic moieties. The critical role of side-chain terminal moieties on the association of macromolecules was demonstrated in experiments with HPMA copolymers containing azobenzene side-chains. The association of the copolymer was reversibly controlled by photoirradiation. Photoinduced change of the azobenzene configuration from *trans* (apolar) to *cis* (high dipole moment) resulted in decreased polymer interactions (29,30).

The distinction between the inter- and intra-molecular association of side-chains can be made using a combination of physicochemical methods. HPMA copolymers containing side-chains terminated in chlorin e_6 (a photosensitizer) were evaluated by determination of the quantum yield of singlet oxygen formation and by light scattering. The decrease of the quantum yield indicated polymer association, while minimal changes in the hydrodynamic volumes of the conjugates (as observed by dynamic light scattering) indicated that the association was intra-molecular (31,32).

Conformation can also influence the biological properties of polymer conjugates, and may affect the rate of enzymatic release of drugs or ligands (12,33). Higher amounts of the hydrophobic drug PGE_1 bound to one HPMA macromolecule resulted in a lower rate of drug release (8). Association of heptapeptide (YILIHRN, ligand of the CD21 receptor) side-chains in HPMA copolymer conjugates resulted in a decrease of the rate of enzymatically catalyzed release of doxorubicin (34). Micellization/aggregation of water-soluble polymer carriers also impairs pure hydrolysis (11).

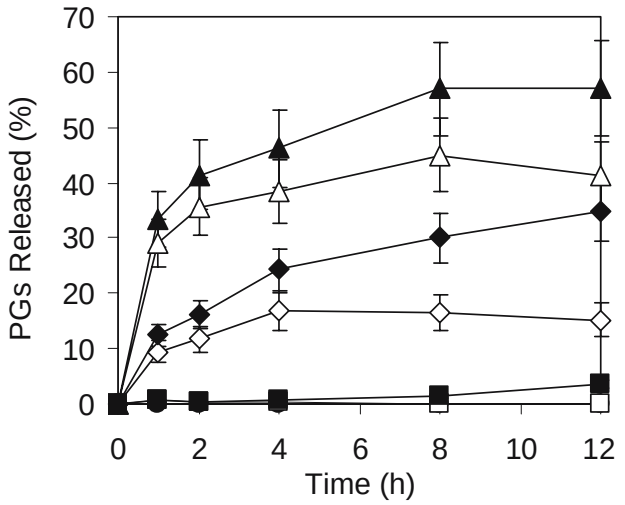


Fig. 3. Release of PGE_1 from conjugate C2 (P-PGE_1-5) in human, rat, and mouse plasma at 37°C. Incubation system: *open circles* PBS (control); *open squares* PGE_1 released in human plasma; *closed squares* total PGs released in human plasma; *open triangles* PGE_1 released in mouse plasma; *closed triangles* total PGs released in mouse plasma; *open diamonds* PGE_1 released in rat plasma; *closed diamonds* total PGs released in rat plasma

Table II. Release of PGs from Conjugate C2 (P-PGE_1-5) in Plasma Mixtures

Incubation System	Released PGs (%)[a]			
	1 h		4 h	
	PGE_1	Total	PGE_1	Total
Rat+human plasma[b]	17.4	24.1	22.8	46.7
Rat plasma+PBS	18.1	22.5	28.2	45.7
Mouse+human plasma	35.7	46.3	41.0	63.6
Mouse plasma+PBS	41.6	48.3	49.5	63.5

[a] Measured three times, the maximum standard deviation was less than ±5.0.
[b] 0.1 ml of rat plasma with 0.1 ml of human plasma were pre-incubated at 37°C for 1 h. Then 0.2 ml of conjugate C2 (P-PGE_1-5) stock solution (400 μg/ml of PGE_1 equivalent) was added. All incubations were done accordingly. $Total = PGE_1 + PGB_1$.

The structure of the resulting vesicles (micelles, bilayer vesicles, or dense nanoparticles) depends on the content of hydrophobic moieties (10,35). It appears that the biological properties of a water-soluble polymer conjugate with a hydrophobic drug need to be optimized to produce release profiles suitable for a particular application. Two approaches have been chosen to investigate the impact of conformation change on the stability of the ester bond in HPMA copolymer conjugates: manipulation of the PGE_1 content and introduction of hydrophobic side-chains (stearyl grafts). The micellization-induced incorporation of the PGE_1 containing side-chains into the hydrophobic core would impede the formation of the enzyme–substrate complex and thus permit to control the non-specific hydrolysis of the ester bond.

Manipulation of the Content of PGE_1 Moieties in the Conjugate

In our previous cathepsin K cleavage study, it was found that the rate of PGE_1 release from HPMA copolymer–PGE_1 conjugates depended on the composition of the conjugate (8). The higher the PGE_1 content in the conjugate, the slower the cathepsin K mediated PGE_1 release. This was a result of association of hydrophobic side-chains in aqueous media, which rendered the formation of the enzyme–substrate complex more difficult. Similar phenomena were observed in rat and mouse plasmas. Figure 4 shows the release of PGE_1 from two HPMA copolymer–PGE_1 conjugates, Conjugate C1, containing 3.1 mol% of PGE_1, and Conjugate C3, containing 10.1 mol% of PGE_1, during incubation in rat and mouse plasma. Comparing data of Fig. 3 (cleavage of Conjugate C2) and Fig. 4 indicates that the higher the content PGE_1 in the conjugate the higher the stability of the ester bond in plasma.

Attachment of Hydrophobic Grafts into the HPMA Copolymer Conjugate

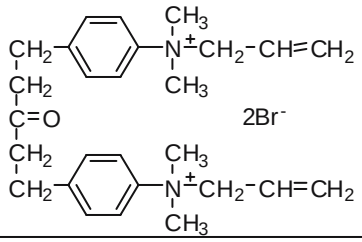
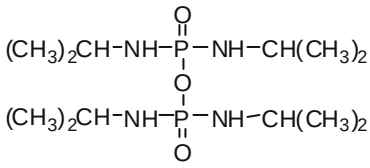
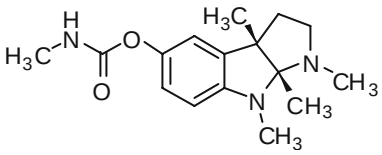
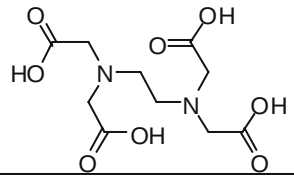
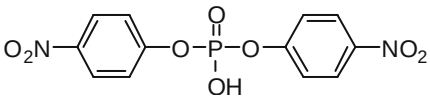
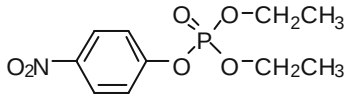
To demonstrate the possibility to control the hydrophobicity and micellization of the conjugates, we introduced stearyl grafts into their structure. To this end, an HPMA copolymer precursor containing *N*-methacryloylglycylglycine 2,4,5-trichlorophenyl ester (MA–GG–OTcp) monomer units was synthesized. Hydrophobic side-chains (grafts) were incorporated by polymer-analogous aminolysis of OTcp groups with octadecylamine. The structure of the conjugate C4 is shown in Scheme 1b. The content of hydrophobic PGE_1 moieties and stearyl grafts are variables, which provide a tool to control the rate of hydrolysis. Figure 5 shows the release of PGE_1 from conjugate C4 (P-PGE_1-C_{18}-FD_8) containing PGE_1 (2.5 mol%), stearyl grafts (4.6 mol%), and D-aspartic acid octapeptide (FD_8, 1.2 mol%; bone-targeting moiety; it impacts also the solubility of the conjugate) by incubation in rat and mouse plasmas. Compared with the HPMA copolymer–PGE_1 conjugate C1 (similar PGE_1 content), the

Table III. Release of PGs from Conjugate C2 (P-PGE_1-5), Pre-incubated 12 h in Human Plasma, by Rat or Mouse Plasma

Incubation System	Released PGs After Addition of Rat or Mouse Plasma (%)[a]			
	1 h		4 h	
	PGE_1	Total	PGE_1	Total
Human plasma/rat plasma[b]	13.2	33.6	16.2	47.4
PBS/rat plasma	24.6	32.6	26.6	43.0
Human plasma/mouse plasma	32.5	54.8	33.2	67.8
PBS/mouse plasma	46.0	54.3	50.5	65.9

[a] Measured three times, the maximum standard deviation is less than ±5.0.
[b] 0.5 ml of conjugate C2 (P-PGE_1-5) stock solution (400 μg/ml of PGE_1 equivalent) was mixed with 0.5 ml of human plasma and pre-incubated for 12 h at 37°C. Then 0.5 ml of rat plasma was added. All incubations were done accordingly. $Total = PGE_1 + PGB_1$. At 12 h, PGs released by cleavage in human plasma were 0.1% (PGE_1) and 3.3% (total); in PBS the PGE_1 released was minimal.

Table IV. The Structure of the Selected Inhibitors and their Specific Enzymes

Inhibitor	Specific enzyme	Inhibitor	Specific enzyme
BW284c51 	AChE	Iso-OMPA 	BChE
Eserine 	AChE BChE	EDTA 	PON1
BNPP 	CbE AChE BChE	Paraoxon 	CbE AChE BChE

introduction of stearyl side-chains resulted in a reduced PGE_1 release rate in both rat and mouse plasma. In addition, the conversion of PGE_1 to PGB_1 proceeded at a slower rate during the incubation of Conjugate C4. Apparently, the stability of the PGE_1 moiety in the conjugate also improved. One may speculate that the hydrophobic side-chain association reduced the accessibility of the plasma proteins to the PGE_1 moiety.

Similar observations were made with other polymer systems. In poly(amino acid) polymers bearing hydrophobic grafts, lower levels of hydrophobic grafts and a reduced degree of polymerization resulted in the formation of bilayer membranes (35), whereas higher levels of hydrophobic grafting resulted in the production of solid nanoparticles (36). Introduction of hydrophobic alkyl grafts into poly(L-lysine citramide imide) resulted in the formation of nanosized multimolecular aggregates in aqueous media. The size of these aggregates was influenced by concentration, ionic strength, degree of ionization, alkyl substitution, and alkyl group length (37).

It is interesting to note, that the rate of PGE_1 release could be also manipulated (decreased) by the addition of a phospholipid, 1-stearoyl-2-oleoyl-3-phosphatidylcholine, into the rat plasma incubation mixture (Fig. 6). It appears that the

Table V. Effect of Inhibitors on the Cleavage of Conjugate C2 (P-PGE_1-5) in Rat and Mouse Plasma

Inhibitor Concentration (mM)	Relative PGs Release (%)	
	Rat	Mouse
BW284C51		
0.2	74.7	82.4
1.0	42.9	78.9
iso-OMPA		
0.2	3.5	74.5
1.0	2.0	14.3
Eserine		
0.2	42.8	79.4
1.0	8.3	6.3
BNPP		
0.2	10.9	34.7
1.0	2.4	19.5
Paraoxon		
0.2	3.2	1.2
1.0	2.3	2.1
EDTA (10 mM)	92.1	93.1

PGs released related to no inhibitor present.

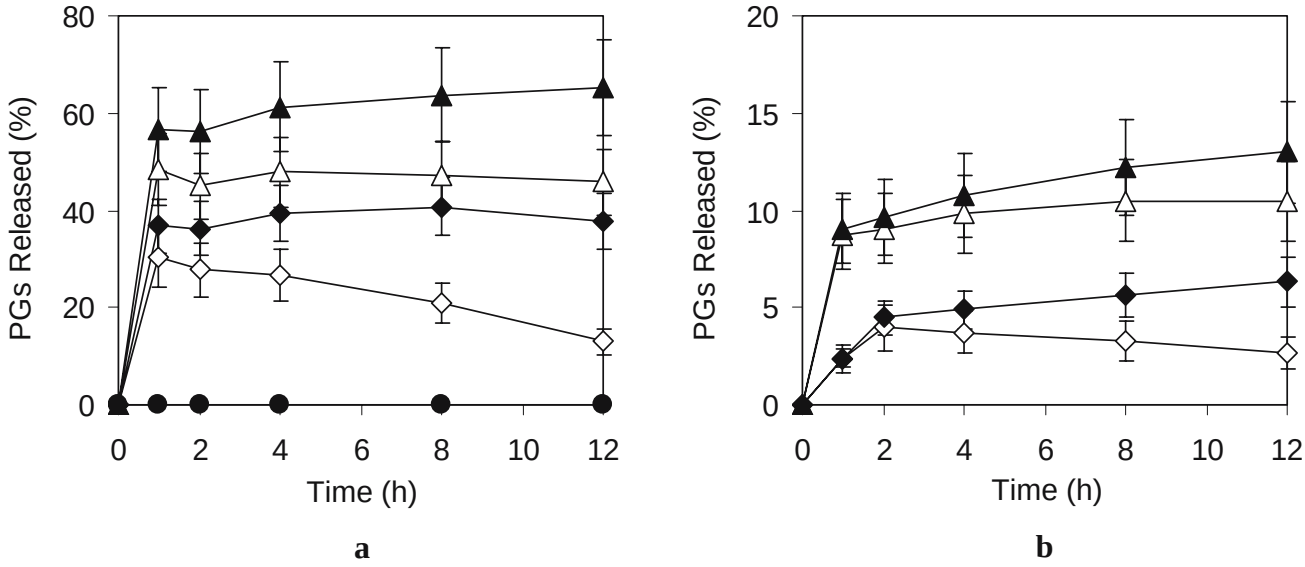


Fig. 4. Release of PGE_1 from **a** Conjugate C1 (P-PGE_1-3) and **b** conjugate C3 (P-PGE_1-10) in rat and mouse plasma at 37°C. Incubation system: *closed circles* total PGs released in PBS; *open triangles* PGE_1 released in mouse plasma; *closed triangles* total PGs released in mouse plasma; *open diamonds* PGE_1 released in rat plasma; *closed diamonds* total PGs released in rat plasma. See Table I for the composition of the conjugates

hydrophobicity of the phospholipid improved the hydrophobic attraction between the hydrophobic side-chains of the HPMA copolymer–PGE_1 conjugate. The association of the lipid molecules with the hydrophobic chains of the HPMA copolymer–PGE_1 conjugate apparently resulted in a change of the microenvironment and reduced access of enzymes to the ester bond. Similarly, Uchegbu and coworkers found that cholesterol improved the attraction between hydrophobic chains of cetyl linear polyethylenimine (38).

These results indicate that several options are available for the manipulation of the structure and properties of the HPMA copolymer-based PGE_1 delivery system. The remaining option to be pursued is the modification of the design of the spacer and avoidance of the ester bond or an increase of the steric hindrance around it.

CONCLUSIONS

The stability of the ester bond in novel HPMA copolymer–PGE_1 conjugates containing an anabolic agent PGE_1 linked to the polymer backbone via an ester bond connected to 1,6-elimination 4-aminobenzyl alcohol group and cathepsin K sensitive tetrapeptide spacer were studied. The stability of the ester bond in human plasma bodes well for the potential intravenous administration of HPMA copolymer–PGE_1 conjugates. Interestingly, this ester bond

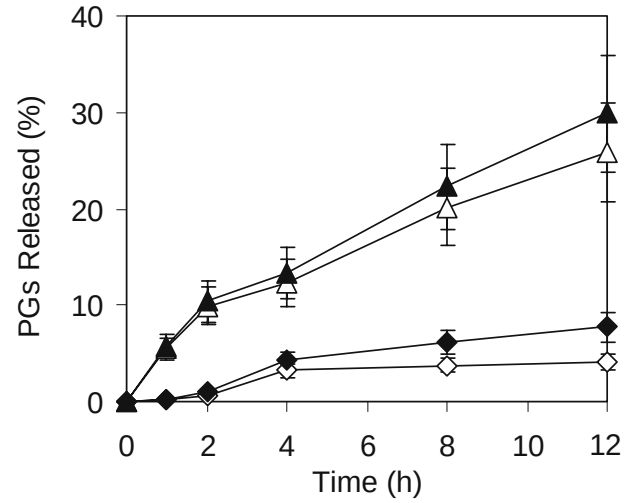


Fig. 5. Release of PGE_1 from Conjugate C4 (P-PGE_1-C_{18}-FD_8) in rat and mouse plasma at 37°C. Incubation system: *open triangles* PGE_1 released in mouse plasma; *closed triangles* total PGs released in mouse plasma; *open diamonds* PGE_1 released in rat plasma; *closed diamonds* total PGs released in rat plasma

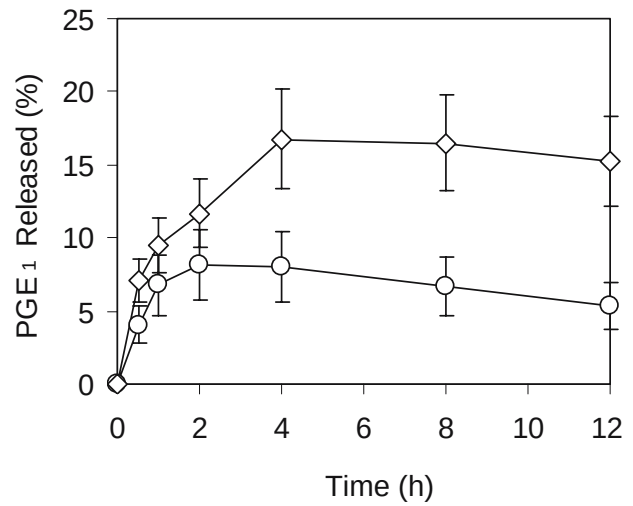


Fig. 6. Release of PGE_1 from conjugate C2 (P-PGE_1-5) in rat plasma containing 1-stearoyl-2-oleoyl-3-phosphatidylcholine at 37°C. *Open diamonds* rat plasma; *open circles* rat plasma containing 0.2 mg/ml phospholipid.

was cleaved relatively fast in mouse and rat plasma. Enzyme inhibitor studies revealed that in rat plasma BChE was the esterase responsible for cleavage. In mouse plasma, in addition to BChE, CbE also participated in the hydrolysis of the ester bond. The observed species differences in plasma stability of ester bonds are of importance for the pre-clinical evaluation of new conjugates. The ovariectomized rat and a non-rodent species are models recommended by the FDA for pre-clinical evaluation of drugs for the treatment of osteoporosis (39). A better understanding of the differences in the enzymatic activities in different species may enhance the translation of pre-clinical data into well-designed clinical trials.

The impact of the conformation of the macromolecular conjugate on the stability of ester bond in rat and mouse plasma was evaluated. The increase of content of hydrophobic drug per macromolecule, or the modification of the carrier with hydrophobic stearyl side chains (grafts) resulted in the aggregation of the conjugates, probably to unimolecular micelles, with concomitant increase of their stability. These results, as well as data on bone-binding (40), cathepsin K cleavage (8), and pharmacokinetics and biodistribution (41), suggest that HPMA copolymer–PGE_1 conjugates are strong candidates as novel therapeutics for the treatment of osteoporosis and other musculoskeletal diseases (42,43).

ACKNOWLEDGMENT

We thank Monika Sima for the outstanding technical assistance and Songqi Gao for kindly providing part of the rat and mouse plasmas. The research was supported in part by NIH grant GM069847.

REFERENCES

1. J. Kopeček. Soluble biomedical polymers. *Polim. Med.* **7**:191–221 (1977).
2. J. Kopeček, P. Kopečková, T. Minko, and Z.-R. Lu HPMA copolymer–anticancer drug conjugates: design, activity, and mechanism of action. *Eur. J. Pharm. Biopharm.* **50**:61–81 (2000).
3. V. Cuchelkar and J. Kopeček. Polymer–drug conjugates. In I. F. Uchegbu and A. G. Schätzlein (eds.), *Polymer–Drug Conjugates*, CRC Press, Boca Raton, Florida, 2006, pp. 155–182.
4. H. Pan and J. Kopeček. Multifunctional water-soluble polymers for drug delivery. In V. P. Torchilin (ed.), *Multifunctional Pharmaceutical Nanocarriers*, Springer, New York, 2007, in press.
5. P. L. Carl, P. K. Chakravarty, and J. A. Katzenellenbogen. A novel connector linkage applicable in prodrug design. *J. Med. Chem.* **24**:479–480 (1981).
6. B. M. Liederer and R. T. Borchardt. Enzymes involved in the bioconversion of ester-based prodrugs. *J. Pharmaceutical Sci.* **95**:1177–1195 (2006).
7. B. Li, M. Sedlacek, I. Manoharan, R. Boopathy, E.G. Duysen, P. Masson, and O. Lockridge. Butyrylcholinesterase, paraoxonase, and albumin esterase, but not carboxylesterase, are present in human plasma. *Biochem. Pharmacol.* **70**:1673–1684 (2005).
8. H. Z. Pan, P. Kopečková, D. Wang, J. Y. Yang, S. Miller, and J. Kopeček. Water-soluble HPMA copolymer–prostaglandin E-1 conjugates containing a cathepsin K sensitive spacer. *J. Drug Targeting* **14**:425–435 (2006).
9. F. H. Drake, R. A. Dodds, I. E. James, J. R. Connor, C. Debouck, S. Richardson, E. Lee-Rykaczewski, L. Coleman, D. Rieman, R. Barthlow, G. Hastings, and M. Gowen. Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. *J. Biol. Chem.* **271**:12511–12516 (1996).
10. I. F. Uchegbu, S. Anderson, and A. Brownlie. Polymeric vesicles. In I. F. Uchegbu and A. G. Schätzlein (eds.), *Polymer–Drug Conjugates*, CRC Press, Boca Raton, Florida, 2006, pp. 131–153.
11. G. Hörpel, W. Klesse, H. Ringsdorf, and B. Schmidt. Micellforming co- and block copolymers for sustained drug release. 28th International Symposium on Macromolecules IUPAC, Amherst, MA, 1982, Proceedings.
12. K. Ulbrich, Č. Koňák, Z. Tuzar, and J. Kopeček. Solution properties of drug carriers based on poly[*N*-(2-hydroxypropyl)-methacrylamide] containing biodegradable bonds. *Makromol. Chem.* **188**:1261–1272 (1987).
13. J. Kopeček and H. Bažilová. Poly[*N*-(2-hydroxypropyl)methacrylamide]-1, radical polymerization and copolymerization. *Eur. Polym. J.* **9**:7–14 (1973).
14. P. Rejmanová, J. Labský, and J. Kopeček. Aminolyses of monomeric and polymeric 4-nitrophenyl ester of *N*-methacrylamino acid. *Makromol. Chem.* **178**:2159–2168 (1977).
15. R. Gatti, R. Gotti, V. Cavrini, M. Soli, A. Bertaccini, and F. Carparelli. Stability study of prostaglandin E1 (PGE1) in physiological solutions by liquid chromatography (HPLC). *Int. J. Pharm.* **115**:113–117 (1995).
16. M. Paul, N. Razzouq, G. Tixier, and A. Astier. Stability of prostaglandin E_1 (PGE_1) in aqueous solutions. *Eur. J. Hospital Pharmacy Sci.* **11**:31–36 (2005).
17. M. Pechar, P. Kopečková, L. Joss, and J. Kopeček. Associative diblock copolymers of poly(ethylene glycol) and coiled coil peptides. *Macromol. Biosci.* **2**:199–206 (2002).
18. D. Wang, M. Pechar, W. Li, P. Kopečková, D. Brömme, and J. Kopeček. Inhibition of cathepsin K with lysosomotropic macromolecular inhibitors. *Biochemistry* **41**:8849–8859 (2002).
19. G. L. Ellman, K. D. Courtney, V. Andres, and R. M. Featherstone. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**:88–95 (1961).
20. Y. Sakurai, S. F. Ma, H. Watanabe, N. Yamaotsu, S. Hirono, Y. Kurono, U. Kragh-Hansen, and M. Otagiri. Esterase-like activity of serum albumin: characterization of its structural chemistry using *p*-nitrophenyl esters as substrates. *Pharm. Res.* **21**:285–292 (2004).
21. K. R. Kozak, B. C. Crews, J. L. Ray, H. H. Tai, J. D. Morrow, and L. J. Marnett. Metabolism of prostaglandin glycerol esters and prostaglandin ethanolamides *in vitro* and *in vivo*. *J. Biol. Chem.* **276**:36993–36998 (2001).
22. P. Rejmanová, J. Pohl, M. Baudyš, V. Kostka, and J. Kopeček. Polymers containing enzymatically degradable bonds. 8. Degradation of oligopeptide sequences in *N*-(2-hydroxypropyl)methacrylamide copolymers by bovine spleen cathepsin B. *Makromol. Chem.* **184**:2009–2020 (1983).
23. W. N. Aldridge. Serum esterases 1. Two types of esterases (A and B) hydrolysing *p*-nitrophenylacetate, propionate and butyrate, and a method for their determination. *Biochem. J.* **53**:110–117 (1953).
24. W. N. Aldridge. Serum esterases 2. An enzyme hydrolysing diethyl *p*-nitrophenyl phosphate (E 600) and its identity with the A-esterase of mammalian sera. *Biochem. J.* **53**:117–124 (1953).
25. F. Bergmann, R. Segal, and S. Rimon. A new type of esterase in hog-kidney extract. *Biochem. J.* **67**:481–486 (1957).
26. V. Simeon, E. Reiner, M. Skrinjaric-Spoljar, and B. Krauthacker. Cholinesterases in rabbit serum. *Gen. Pharmacol.* **19**:849–853 (1988).
27. A. Salvi, P. A. Carrupt, J. M. Mayer, and B. Testa. Esterase-like activity of human serum albumin toward prodrug esters of nicotinic acid. *Drug Metab. Dispos.* **25**:395–398 (1997).
28. M. Bohdanecký, H. Bažilová, and J. Kopeček. Poly[*N*-(2-hydroxypropyl)methacrylamide]. II. Hydrodynamic properties of dilute solutions. *Europ. Polym. J.* **10**:405–410 (1973).
29. Č. Koňák, P. Kopečková, and J. Kopeček. Photoregulated association of *N*-(2-hydroxypropyl)methacrylamide copolymers with azobenzene containing side-chains. *Macromolecules* **25**:5451–5456 (1992).

30. J. Kopeček, P. Kopečková, and Č. Koňák. Biorecognizable polymers: Design, structure, and bioactivity. *J. Macromol. Sci. Pure Appl. Chem.* **A34**:2103–2117 (1997).
31. J. G. Shiah, Č. Koňák, J. D. Spikes, and J. Kopeček. Solution and photoproperties of *N*-(2-hydroxypropyl)methacrylamide copolymer–meso-chlorin e_6 conjugates. *J. Phys. Chem.* **B101**:6803–6809 (1997).
32. J. G. Shiah, Č. Koňák, J. D. Spikes, and J. Kopeček. Influence of pH on aggregation and photoproperties of *N*-(2-hydroxypropyl) methacrylamide copolymer–meso-chlorin e_6 conjugates. *Drug Deliv.* **5**:119–126 (1998).
33. D. Putnam and J. Kopeček. Polymer conjugates with anticancer activity. *Adv. Polym. Sci.* **122**:55–123 (1995).
34. H. Ding, P. Kopečková, and J. Kopeček. Self-association properties of HPMA copolymers containing an amphipatic heptapeptide. *J. Drug Targeting* **15**:465–474 (2007).
35. W. Wang, L. Tetley, and I. F. Uchegbu. The level of hydrophobic substitution and the molecular weight of amphiphilic poly-L-lysine-based polymers strongly affects their assembly into polymeric bilayer vesicles. *J. Colloid Interface Sci.* **237**:200–207 (2001).
36. W. Wang, L. Tetley, and I. F. Uchegbu. A new class of amphiphilic poly-L-lysine-based polymers forms nanoparticles on probe sonication in aqueous media. *Langmuir* **16**:7859–7866 (2000).
37. S. Gautier, M. Boustta, and M. Vert. Poly(L-lysine citramide), a water-soluble bioresorbable carrier for drug delivery: Aqueous solution properties of hydrophobized derivatives. *J. Bioact. Compatible Polym.* **12**:77–98 (1997).
38. W. Wang, X. Qu, A. I. Gray, L. Tetley, and I. F. Uchegbu. Self assembly of cetyl linear polyethylenimine to give micelles, vesicles, and dense nanoparticles. *Macromolecules* **37**:9114–9122 (2004).
39. D. D. Thompson, H. A. Simmons, C. M. Pirire, and H. Z. Ke. FDA guidelines and animal models for osteoporosis. *Bone* **17**:125S–133S (1995).
40. D. Wang, S. C. Miller, M. Sima, P. Kopečková, and J. Kopeček. Synthesis and evaluation of water-soluble polymeric bone-targeted drug delivery systems. *Bioconjugate Chem.* **14**:853–859 (2003).
41. D. Wang, M. Sima, R. L. Mosley, J. P. Davda, N. Tietze, S. C. Miller, P. R. Gwilt, P. Kopečková, and J. Kopeček. Pharmacokinetic and biodistribution studies of a bone-targeting drug delivery system based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers. *Mol. Pharmaceutics* **3**:717–725 (2006).
42. D. Wang, S. Miller, P. Kopečková, and J. Kopeček. Bone-targeting macromolecular therapeutics. *Adv. Drug Delivery Rev.* **57**:1049–1076 (2005).
43. The Osteoporosis Methodology Group and The Osteoporosis Research Advisory GroupMeta-analysis of therapies for postmenopausal osteoporosis. *Endocrine Rev.* **23**:496–507 (2002).