Hydroxybisphosphonate-Containing Polymeric Drug-Delivery Systems Designed for Targeting Into Bone Tissue

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Received 20 July 2005; accepted 13 October 2005
DOI 10.1002/app.23446
Publication online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: The preparation and characterization of a novel polymeric drug-delivery system designed for bone targeting of antineoplastics is described. The system was based on biocompatible poly[*N*-(2-hydroxypropyl)methacrylamide] carrier containing hydroxybisphosphonate targeting moieties and the model radiotherapeutics ^{125}I or ^{111}In or the anticancer drug doxorubicin. The *in vitro* binding studies with hydroxyapatite as a bone model proved that the system was efficiently adsorbed on this mineral. The systems contained model drugs bound by stable (amide), hydrolytically cleavable (hydrazone) or enzymatically cleavable (Gly-Phe-Leu-Gly tetrapeptide) spacers. It was proven *in vitro* that, in the case of cleavable spacers, the drug could be released from the polymer carrier at a rate depending on the pH or enzymatic stimulus. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 101: 3192–3201, 2006

Key words: copolymerization; drug delivery systems; functionalization of polymers; radical polymerization; water-soluble polymers

INTRODUCTION

The bone, a tissue with a primarily mechanical function, possesses certain differences from other tissues. The main difference is that it is a natural composite containing large quantities of the mineral hydroxyapatite (HA; calcium phosphate), which significantly improves its mechanical properties.[1,2]

The dawn of bisphosphonates (BPs; e.g. alendronate or zolendronate) became a significant contribution to the therapy of diseases that include pathological bone tissue breakdown, such as Paget's disease, osteoporosis, and bone metastases,[3,4] because these agents inhibit the activity of osteoclasts. The mechanism of their action mainly consists of the inhibition of farnesyl transferase activity but also direct interaction with the bone surface. If BPs are applied into the bloodstream, they rapidly accumulate in bone tissue or are eliminated by kidneys. Only small amounts are temporarily retained in the liver and spleen, whereas the accumulation in bones is practically irreversible.[1] The rapid accumulation in bone tissue is caused by the direct chelating retention of BPs with the surface of HA. The selective interaction of BPs with HA is based on the steric analogy of the chemical structures of these agents and diphosphate, chelated in the crystal structure of HA. Diphosphate on the surface of the HA crystals is partly replaced by BPs during the adsorption process.[1] The adsorption of other bone-seeking agents, such as tetracycline, acid poly(amino acids), fluoride, lead, strontium, and complexes of [ethylenedinitrilotetrakis(methylene)] tetrakis(phosphonic acid) with lanthanides,[1,5] is also of chelation origin. This retention is exploited in diagnostic and therapeutic nuclear medicine. The distribution of HA-targeted radiopharmaceuticals is not homogeneous throughout the skeleton, and significantly higher amounts can be found in bone regions with high remodeling activity, such in osteolytic cancer lesions, probably due to the more exposed surface of HA to blood in these tissues.[2,6] Accumulation of these low-molecular-weight agents in metastatic tumor lesions could be more than 10 times higher than that in healthy bones.[1]

It is well known that many cancers develop metastases in bones, and the treatment of such metastases is very difficult.[6] Several low-molecular-weight bone-targeted BP-based prodrugs have been designed for the delivery of antioneoplastic agents, anti-inflammatory agents, radionuclides, and steroid hormones[2,7]

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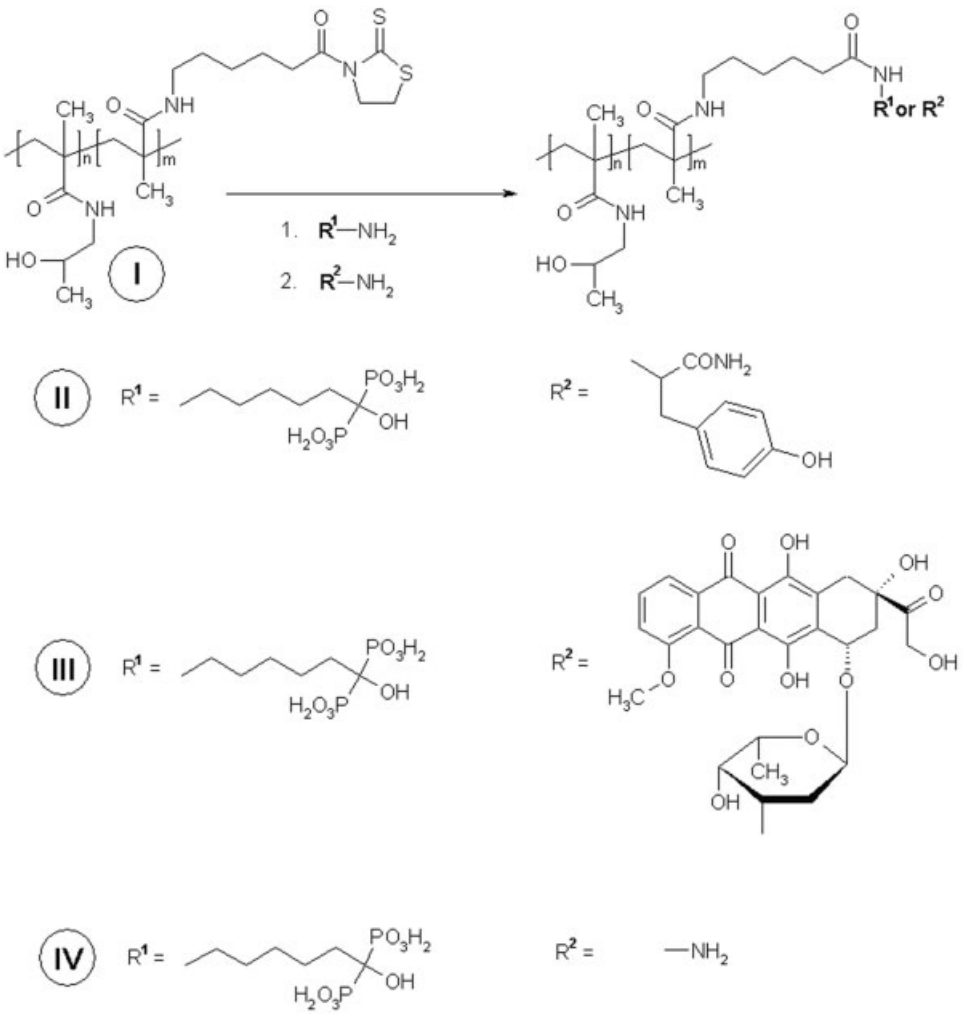
Contract grant sponsor: Grant Agency of the Academy of Sciences of the Czech Republic; contract grant number: B4050408 and A400480616.
Contract grant sponsor: Ministry of Education, Youth and Sports of the Czech Republic; contract grant number: IM 4635608802.



Scheme 1 Preparation of polymers **II, III,** and **IV**.

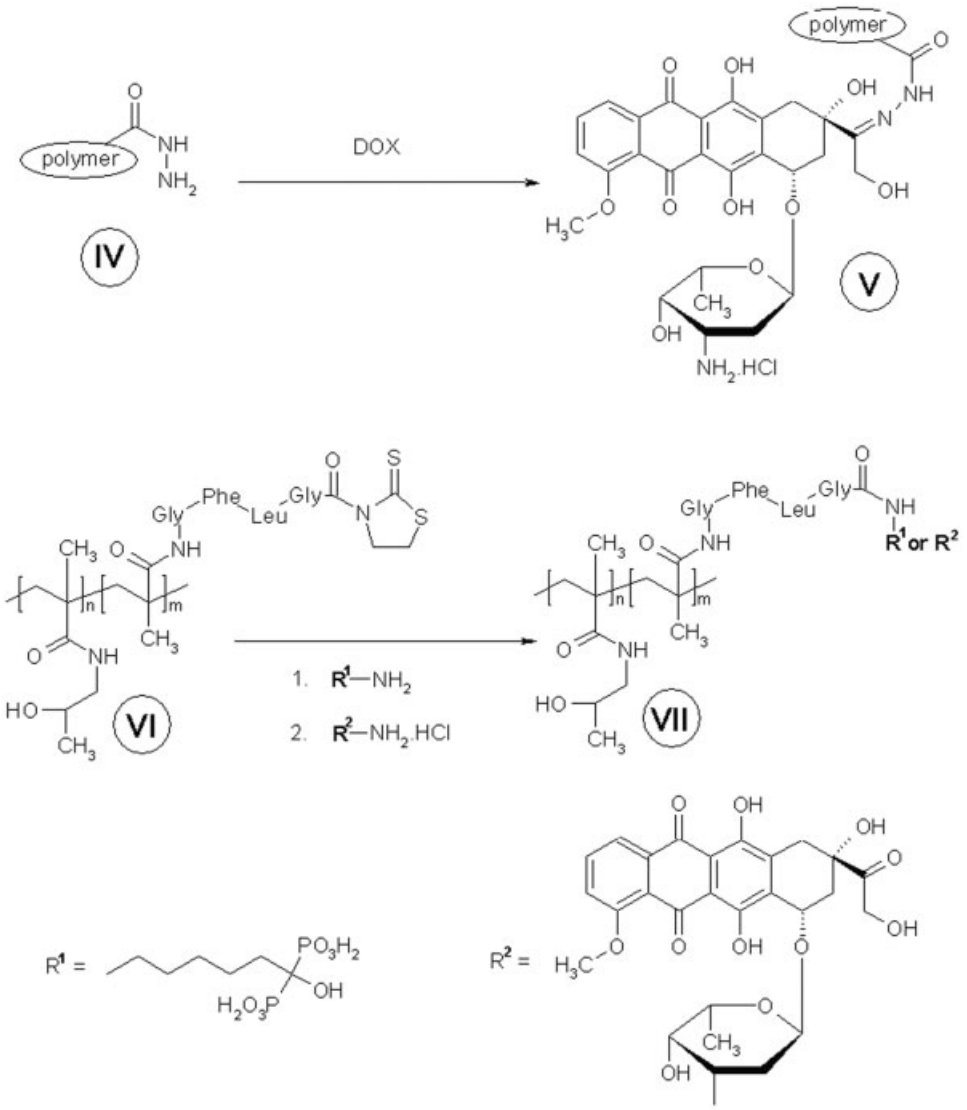
into bone tissue. Of the antineoplastic agents, BP-targeted alkylating cancerostatics, methotrexate, and doxorubicin (DOX) conjugates have been described.[2,7] However, polymeric drug-delivery systems designed for bone targeting have rarely been described; to our knowledge, only a model system for fluorescein[5] and phosphonomethyl polyethyleneimine[8] have been described, although they possess numerous advantages over low-molecular-weight conjugates. Polymeric BP-targeted drug-delivery systems should have longer circulation times due to significantly suppressed renal excretion, and several BP groups per chain could provide a stronger bond to HA. The higher molecular weight of the conjugate should also provide additional selectivity to solid tumors due to the enhanced permeation and retention effect[9] and higher demands for the free HA surface availability.

In this article, we describe a novel drug-delivery system designed for bone targeting of antineoplastics based on biocompatible *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers containing BP-targeting moieties and the radiotherapeutics ^{125}I, ^{111}In, or DOX. The drugs were bound to the polymer chain by a suitable spacer, which enabled drug activation by its release from the carrier controlled by enzymatic or pH-dependent hydrolysis (see Schemes 1 and 2).

EXPERIMENTAL

Materials

(6-Amino-1-hydroxyhexane-1,1-diyl)bis(phosphonic acid) monosodium salt (AHHBP; neridronate) monohydrate was prepared by the condensation of 6-aminohexanoic acid with phosphorous acid and phosphorus trichloride in methanesulfonic acid and subsequent hydrolysis.[10] Hydrazine monohydrate, methacryloyl chloride, 1-aminopropan-2-ol, 6-aminohexanoic acid, glycyl-L-phenylalanine, L-leucylglycine, 2,2′-azobisisobutyronitrile, *N*,*N*-dimethylformamide, thiazolidine-2-thione (TT), *N*,*N*′-dicyclohexylcarbodiimide, ethyl carbazate, L-tyrosinamide,



Scheme 2 Preparation of polymers **V** and **VII.**

dimethyl sulfoxide (DMSO), and DOX hydrochloride were purchased from Fluka Chemie AG (Buchs, Switzerland). 2,4,6-Trinitrobenzene-1-sulfonic acid was purchased from SERVA Feinbiochemica Heidelberg (Heidelberg, Germany).

All other reagents and solvents were analytical grade. Solvents were dried and purified by conventional procedures and distilled before use. Other chemicals purchased from Fluka (Buchs, Switzerland) and Aldrich (Milwaukee, WI) were used without additional purification.

Methods

Synthesis of the monomers

HPMA was synthesized by the reaction of methacryloyl chloride with 1-aminopropan-2-ol in dichloromethane.[11] Schotten–Baumann acylation with methacryloyl chloride in an aqueous alkaline solution was used for the methacryloylation of amino acids and oligopeptides. *N*-Methacryloylglycyl-DL-phenylalanyl-L-leucylglycine[12] and 6-methacrylamidohexanoic acid[13] were prepared as described in the literature. Both monomers bearing TT reactive moieties, 3-[6-(methacrylamido)hexanoyl]thiazolidine-2-thione (Ma-AH-TT) and 3-(*N*-methacryloylglycyl-DL-phenylalanyl-L-leucylglycyl)thiazolidine-2-thione (Ma-GFLG-TT), were synthesized by the reaction of *N*-methacryloylated amino acid or oligopeptide with TT in the presence of *N*,*N*′-dicyclohexylcarbodiimide.[14] Both Ma-AH-TT and Ma-GFLG-TT were pure according to high performance liquid chromatography (HPLC).[14]

Ma-AH-TT: mp = 60°C. ANAL. Calcd for Ma-AH-TT): C, 52.20%; H, 6.41%; N, 9.36%; S, 21.4%. Found: C, 52.51%; H, 6.63%; N, 9.24%; S, 20.83%. ^{1}H-NMR (DMSO-d_6, δ): 1.28 (m, 2H), 1.41 (m, 2H), 1.58 (m, 2H),

TABLE I
Characteristics of Polymers I–VII

Polymer[a]	Side chain[b]	AHHBP (mol %)	DOX (wt %)	M_w	PD[c]
I[d]	AH	—	—	24,600	1.85
II-2[e]	AH	2.53	—	32,000	2.12
II-4[f]	AH	4.02	—	35,000	2.21
III-0	AH	0	3.74[g]	25,200	1.82
III-2	AH	1.37	4.12[g]	27,900	1.76
III-4	AH	3.42	3.68[g]	28,100	1.65
IV-2[h]	AH	1.75	—	34,000	2.10
IV-4[i]	AH	3.32	—	30,400	1.89
V-0	AH	0	8.51[j]	29,000	1.82
V-2	AH	1.75	5.53[j]	32,600	2.11
V-4	AH	3.32	4.23[j]	34,600	1.96
VI[k]	GFLG	—	—	34,000	1.54
VII-0	GFLG	0	5.10[g]	38,200	1.85
VII-4	GFLG	1.60	5.25[g]	43,000	2.11

Contents are in molar percentages of monomeric units.
[a] See Experimental section for structures.
[b] AH = 6-aminohexanoic acid; GFLG = Gly-Phe-Leu-Gly tetrapeptide.
[c] PD = M_w/M_n.
[d] TT groups = 6.85 mol %.
[e] L-tyrosinamide = 1.42 mol %.
[f] L-tyrosinamide = 1.48 mol %.
[g] DOX bound via amide bonds.
[h] Hydrazide groups = 4.30 mol %.
[i] Hydrazide groups = 2.90 mol %.
[j] DOX bound via hydrazone bonds.
[k] TT groups = 7.30 mol %.

1.83 (s, 3H), 3.08 (m, 4H), 3.33 (t, 2H), 4.48 (t, 2H), 5.28 (s, 1H), 5.61 (s, 1H), 7.87 (t, 1H).

Ma-GFLG-TT: ^{1}H-NMR (DMSO-d_6, δ): 0.84 (d, 3H), 0.89 (d, 3H), 1.30–1.70 (m, 3H), 1.84 (s, 3H), 2.70–3.10 (m, 2H), 3.45 (t, 2H), 3.50–3.75 (m, 2H), 4.15–4.35 (m, 1H), 4.45–4.75 (m, 5H), 5.35 (s, 1H), 5.69 (s, 1H), 7.20 (m, 5H), 8.01 (d, 1H), 8.10 (m, 2H), 8.25 (t, 1H).

Preparation of the polymers

Three polymers of each type were prepared and were marked by a Roman number describing the structure type (according to Schemes 1 and 2) and an Arabic number describing theoretical aminobisphosphonate content in molar percentage. The characteristics of the polymers are given in Table I.

Random copolymers of HPMA with Ma-AH-TT (**I**) or Ma-GFLG-TT (**VI**) were prepared[14] by radical polymerization in DMSO [the initiator was 2 wt % 2,2′-azobisisobutyronitrile; monomer concentration = 12.5 wt %; HPMA/Ma-AH-TT (**I**) or Ma-GFLG-TT (**VI**) molar ratio = 11.5 : 1; 60°C; 6 h]. The copolymers were characterized by UV spectrophotometry (the content of TT-terminated side chains; molar extinction coefficient (ε) = 10,300 L mol^{-1} cm^{-1}, λ = 305 nm in methanol) and by size exclusion chromatography [weight-average molecular weight (M_w) and number-average molecular weights (M_n)] after aminolysis of the TT groups with 1-aminopropan-2-ol. Determination of the molecular weights was carried out with a HPLC Äkta Explorer system (Pharmacia) equipped with refraction index (RI), UV, and multiangle light scattering DAWN 8 (Wyatt Technology Corp, Santa Barbara, CA) detectors with 0.3*M* acetate buffer (pH = 6.5) and a Superose 6 column.

*Preparation of the AHHBP-targeted system containing L-tyrosinamide (**II;** see Scheme 1)* Conjugates containing AHHBP and L-tyrosinamide were prepared by the reaction of the polymer precursor containing TT groups (**I**) with AHHBP and tyrosinamide at room temperature in phosphate buffer (0.05*M* NaH_2PO_4/Na_2PO_4; pH = 8.1). The polymer precursor **I** (100 mg; 48 μmol of TT groups) was dissolved in phosphate buffer (1 mL), and then, AHHBP [0 mg (0 μmol, 0 mol %) for polymer **II-0**, 2.7 mg (14 μmol, 2 mol %) for polymer **II-2,** and 5.4 mg (28 μmol, 4 mol %) for polymer **II-4**] was added. The pH of the reaction mixture was kept at 8.1 by the addition of $Na_2B_4O_7$ solution (0.1 mol/L). After 30 min, L-tyrosinamide (2 mg, 11 μmol) was added, and the reaction mixture was stirred for 3 h. The remaining TT groups were aminolyzed by the addition of 1-aminopropan-2-ol (4 μL, 53 μmol). After 10 min, the polymer conjugate was isolated and purified by multiple gel permeation chromatography (Sephadex G-25, Amersham Pharmacia Biotech AB, Uppsala, Sweden), 150-mL bed volume with water as the mobile phase). The polymer fraction was collected and freeze-dried. Polymer conjugates were characterized by size exclusion chromatography as mentioned previously; the AHHBP content was determined from phosphorus analysis, and the L-tyrosinamide content was estimated by amino acid analysis according to ref. 11. Molecular weights were determined as described previously for polymers **I** and **VI**.

*Preparation of HPMA copolymers containing amide-bonded AHHBP and DOX [**iii** (scheme 1) and **vii** (scheme 2)]* Conjugates containing AHHBP and DOX attached via the amide bond were prepared by the reaction of the respective polymer precursor containing TT groups (polymers **I** and **VI**) with AHHBP and DOX · HCl at room temperature in phosphate buffer (0.05*M* NaH_2PO_4/Na_2PO_4) in the dark. The reaction was carried out as described previously for the preparation of polymer **II** with an equimolar amount of DOX · HCl instead of L-tyrosinamide. The polymer conjugate was isolated and purified by multiple gel permeation chromatography (Sephadex LH-20, 200-mL bed volume with methanol as the mobile phase). The polymer fraction was collected, methanol was evaporated, and the conjugate was isolated by precipitation into a mixture of acetone and diethyl ether (3:1 v/v).

The polymer–DOX conjugates were characterized by size exclusion chromatography, amino acid analy-

sis, and phosphorus analysis; the DOX content was determined by UV spectrophotometry in water (ε = 11,500 L mol^{-1} cm^{-1}, λ = 488 nm).

Reference samples (polymers **III-0** and **VII-0**) containing only DOX bound via amide bonds were prepared from polymer precursors **I** and **VI** by the same procedure without the addition of AHHBP.

All the polymer–DOX conjugates were tested for their contents of the free drug with an Äkta Explorer HPLC system (Pharmacia, Uppsala, Sweden) equipped with a Superose 12 column (Amersham Pharmacia Biotech, AB, Uppsala, Sweden), TLC (Kieselgel 60 F_{254}), and a ShimadzuHPLC system (Kyoto, Japan) equipped with a Tessek SGX C18 reverse-phase column (130 × 3 mm; methanol–water gradient = 10–90% methanol, fluorescent detector with excitation wavelength (λ_{ex}) = 488 nm and emission wavelength (λ_{em}) = 560 nm) after chloroform extraction of the free drug from the aqueous polymer solution.

*Preparation of HPMA copolymers with DOX bound with hydrazone bonds (**v**; see schemes 1 and 2)* Conjugates containing amide-bonded AHHBP and hydrazone-bonded DOX (polymers **V-2** and **V-4**) were prepared by a two-step synthesis. In the first step, TT groups of polymer precursor **I** were reacted with AHHBP and hydrazine monohydrate to yield polymers **IV-2** or **IV-4** with side chains terminated in AHHBP molecules and hydrazide groups. In the second step, polymers **IV-2** and **IV-4** were used for the attachment of DOX via hydrazone bonds. The polymer precursor **I** (120 mg, 57.5 μmol) was dissolved in phosphate buffer (see previous discussion; 1.5 mL), and AHHBP [3.4 mg (16.8 μmol; polymer **V-2**) or 6.8 mg (33.6 μmol; polymer **V-4**), respectively] was added under stirring. The pH of the reaction mixture was kept at 8.1 as described previously. After 2 h, the remaining TT groups were hydrazinolyzed by the addition of hydrazine monohydrate (20 μL, 412 μmol). After 10 min of hydrazinolysis, the polymer was isolated and purified by gel permeation chromatography (Sephadex G-25, 150-mL bed volume with water as the mobile phase). The polymer fraction was collected, and polymer **IV** was freeze-dried. The product (**IV**) was characterized by size exclusion chromatography as mentioned previously, the AHHBP content was determined from the phosphorus analysis, and the hydrazide group content of the hydrazide groups was determined by a modified 2,4,6-trinitrobenzene-l-sulfonic acid (TNBSA) assay.[15]

In the second step, the hydrazide group-containing polymers **IV-2** and **IV-4** were used for the attachment of DOX via hydrazone bonds, as described in ref. 15. Polymer conjugates were characterized by size exclusion chromatography, and the DOX and AHHBP contents were characterized as described previously.

The reference sample (polymer **IV-0**) containing only DOX bound via hydrazone bonds was prepared by the same procedure as described previously without the addition of AHHBP in the first step.

Isotopic labeling studies

*Isotopic labeling of polymer **II** with ^{125}i*
A solution of polymer **II** (3 mg) in phosphate-buffered saline (PBS; pH = 7.4, 200 μL), aqueous $Na^{125}I$ solution (9 MBq/mL, 20 μL), and aqueous chloramine T solution (10 mg/mL, 50 μL) were placed in this order in an 1.5-mL Eppendorf test tube and incubated for 30 min at 23°C. Iodination was then quenched by the addition of aqueous L-ascorbic acid (10 mg/mL, 80 μL). The high-molecular-weight fraction was then separated with a PD-10 desalting column with aqueous NaCl (0.15 mol/L) as the mobile phase, 1.5 mL fractions were taken, and their radioactivity was measured with a NaI(Tl) scintillation counter (74038 type, RFT, Dresden, Germany). The relative errors of the radioactivity counts were within 5%. The total volume of the high-molecular-weight fraction was then adjusted to 3.0 mL.

*Isotopic labeling of polymer **II** with ^{111}in* Polymer **II** (3 mg) was dissolved in an Eppendorf test tube in aqueous ammonium acetate (0.5 mol/L, 300 μL), aqueous $^{111}InCl_3$ solution (9 MBq/mL, 20 μL) was added, and the mixture was incubated for 30 min at 23°C. The polymer fraction was separated with a PD-10 column with aqueous ammonium acetate (0.5 mol/L) as the mobile phase. Radioactivity was measured as described for ^{125}I.

HA binding studies

The weights and volumes are specified in the figure legends for the particular cases. HA was shaken in a 1.5-mL Eppendorf test tube in a buffered solution of the polymer at room temperature (23°C). The suspension was then centrifuged, and polymer sorption was assayed from the decrease in absorbance (DOX-containing polymers **III, V,** and **VII;** λ = 488 nm, 1-cm cuvette, Unicam Helios α UV–visible spectrophotometer, ChromSpec, Ltd., Prague, Czech Republic) and radioactivity (radiolabeled polymer **II**) of the supernatant aliquots.

pH-activated release of DOX from polymers V-0, V-2, and V-4

HA (600 mg) was shaken with the solution of polymer **V-4** in PBS (0.5 mg/mL polymer **V**, pH = 7.4, 12 mL) for 30 min at 23°C. The suspension was then centrifuged, and the absorbance of the supernatant was measured (λ = 488 nm, 1-cm cuvette) to calculate the amount of adsorbed polymer. The sediment was re-

suspended in water (20 mL) and centrifuged. The sediment washing procedure was repeated once more, and the sediment was resuspended in PBS buffer of a particular pH (5.0 or 7.4) up to a total volume of 12 mL. The suspension was then incubated at 37°C. Aliquot samples (1.2 mL) were taken after the suspension was shaken at particular time points and centrifuged, and the absorbance of the supernatant was measured (λ = 488 nm, 1-cm cuvette) to calculate the amount of released DOX.

To compare the rate of DOX release from the conjugates adsorbed on HA with those incubated in solution, the stability and rate of release of DOX from the polymer–DOX conjugates **V-0, V-2,** and **V-4** were investigated after the incubation of polymer samples in phosphate buffers (0.1*M* phosphate buffer with 0.05*M* NaCl, pH = 5 or 7.4) at 37°C. In all cases, the final DOX concentration was 0.5 m*M*. At predetermined time intervals, solutions of the polymers were tested for the free DOX content in solution after its extraction from the incubation media into chloroform.[15] Briefly, a mixture of a polymer solution (0.1 mL) and buffer (0.2*M* $Na_2CO_3/NaHCO_3$, pH = 9.8, 0.3 mL) was extracted with chloroform (0.8 mL). DOX was determined with the HPLC Shimadzu system as described previously. The calibration (yield of extraction) was carried out with DOX · HCl. All drug-release data are expressed as the amount of free DOX relative to the total DOX content in the conjugate.

Enzymatic release of DOX from conjugates VII-0 and VII-4 by cathepsin B

HA (600 mg) was shaken with a solution of polymer **VII-4** in PBS (0.5 mg/mL polymer **V**, pH = 7.4, 12 mL) for 30 min at 23°C. The suspension was then centrifuged, and the supernatant absorbance was measured (λ = 488 nm, 1-cm cuvette) to calculate the adsorbed amount of polymer. The sediment was resuspended in water (20 mL) and centrifuged. The sediment washing procedure was repeated once more, and the sediment was resuspended in phosphate buffer (pH = 6, 0.1*M* NaH_2PO_4/Na_2PO_4, 0.05*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid, 5 m*M* reduced glutathione) up to a total volume of 12 mL. Cathepsin B was added to a final concentration of 0.5 μmol/L, and the suspension was then incubated at 37°C. The control experiment with enzymatic release of DOX from polymer **VII-4** in solution was made with the same concentration of the polymer and enzyme as the release of DOX from HA-bound polymer **VII-4**. The activity of a cysteine proteinase–cathepsin B was determined with N_a-benzoyl-L-arginine-4-nitroanilide hydrochloride (Bz-Arg-NAp) as a substrate.[16] Samples (1.2 mL) were taken after the suspension was shaken at particular time intervals and centrifuged, and the supernatant absorbance was measured (λ = 488 nm, 1 cm) to calculate the amount of released DOX.

RESULTS AND DISCUSSION

Preparation of the polymers

All polymers were prepared from precursors containing reactive *N*-acyl thiazolidine-2-thione groups, which are known to undergo rapid aminolysis, although they are relatively stable to hydrolysis.[14] The selectivity of TT groups in aminolysis in aqueous environment is much higher than, for example, the selectivity of 4-nitrophenyl ester groups, which are usually used for the synthesis of HPMA copolymer/DOX conjugates.[12] Moreover, the TT groups do not interfere with the free-radical polymerization as much as 4-nitrophenyl esters do,[12] which enables better control over the polymerization process. L-Tyrosinamide, hydrazine, targeting AHHBP, and amide-bonded DOX were thus attached to the polymer with the reaction of the corresponding amines with the particular polymeric precursors, **I** or **VI,** respectively. DOX was attached to the hydrazide-containing polymer **IV** by condensation. The separation of DOX from polymers containing BP moieties must be done in saline environment to prevent ion interaction from worsening the separation.

The characteristics of all of the polymers and polymer conjugates are summarized in Table I. The molecular weight of all of the polymer conjugates slowly increased after the attachment of AHHBP, L-tyrosinamide, hydrazine, or DOX, but no significant branching or grafting was found. In all cases, the molecular weight of polymer conjugates remained under the limit of renal threshold for HPMA-based polymers.[17] Because of this, all of the polymers fulfilled basic requirements for elimination from the organism by glomerular filtration and prevention of accumulation in the body. In conjugation reactions, four different moieties were attached to the side chains of the polymer precursors **I** and **VI**. In the case of AHHBP, L-tyrosinamide, and hydrazine, the total conversion of TT groups was between 70 and 100%, depending on the combination of amines used for attachment. On the other hand, only 40–60% of DOX was attached to the polymer, independently of the type of conjugation, amide, or hydrazone bonds. The lower conversion of DOX attachment was probably affected by the bulkiness of the DOX molecules.

The spacers between the drug or the targeting moiety and a polymer were selected to be either stable (polymers **II** and **III**) or to be able to undergo controlled release of the drug by pH-dependent (polymer **V**) or chemical or enzymatic (polymer **VII**, by lysosomal cathepsin B) hydrolysis.

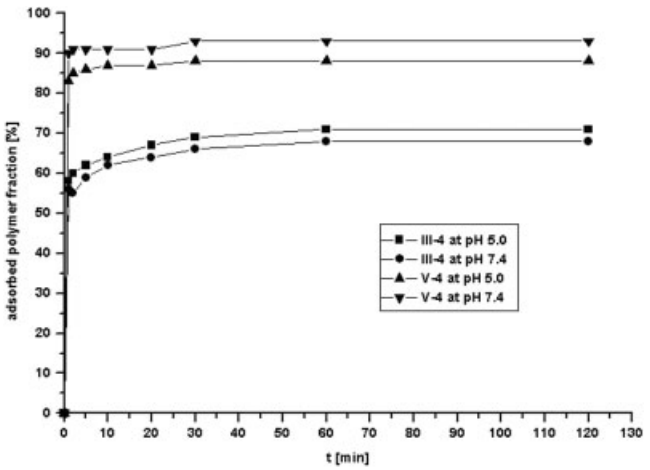


Figure 1 Rate of adsorption of polymers **III-4** and **V-4** on HA at pH 5.0 and 7.4. Experimental conditions: polymer solution (0.5 mg/mL, 15 mL) in the particular buffer and HA (400 mg) and 1 mL of sample.

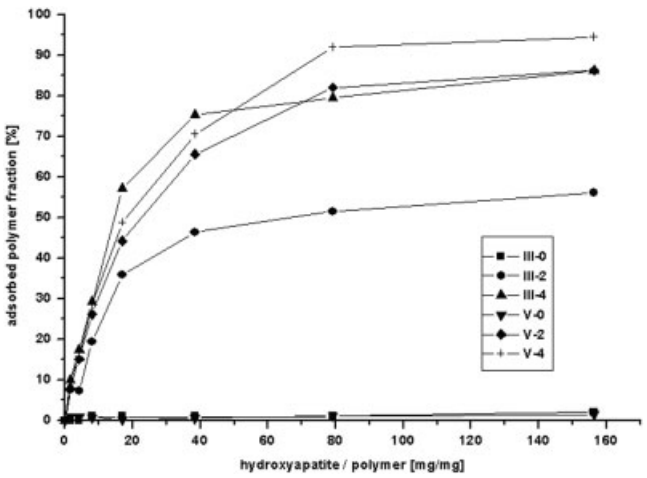


Figure 2 Dependence of adsorption on HA on the polymer/HA weight ratio for polymers **III-0, III-2, III-4, V-0, V-2,** and **V-4.** Experimental conditions: polymer solution (0.5 mg/mL, 2.5 mL) in PBS (pH 7.4) was added to HA; incubation time = 60 min.

Sorption studies on HA

HA was chosen as a model of bone tissue.[18] Polymers **II, III, V,** and **VII,** containing BP moieties, rapidly adsorbed on the surface of HA. The rate of adsorption was very fast and was independent of pH in the biologically relevant pH range between 5.0 and 7.4, with most of the polymer being adsorbed within 1 min (see Fig. 1). Clearly, the amount of the polymer adsorbed on HA was highly dependent on the content of targeting BP moieties. The polymers without targeting BP moieties (**III-0** and **V-0**) did not adsorb on HA, whereas with increasing content of BPs, the degree of sorption significantly increased (the extent of sorption was 1, 86, and 94% for polymers **V-0, V-2,** and **V-4,** respectively; see Fig. 2 for the dependence of adsorption on HA on the polymer/HA weight ratio). Incomplete adsorption could have been due to both the inherent chemical and molecular polydispersity (PD) of the polymers, which caused there to always be some untargeted but drug-containing polymer chains in the conjugate. It was also clear that several targeting moieties attached to each polymer chain were needed to have at least one targeting moiety exposed on the surface of the polymer globule and to achieve optimal interaction with the receptor of the substrate (HA). The latter effect was described for albumin, which requires at least five aminobisphosphonate moieties to be effectively targeted into bones.[18]

This effect was also proven by the sorption experiments conducted with the $^{111}In^{3+}$–polymer **II-4** complex and HA. Although $^{111}InCl_3$ alone was quickly and quantitatively adsorbed on HA, the $^{111}In^{3+}$–polymer **II-4** complex was adsorbed to a lower extent (e.g., 85% for the $^{111}In^{3+}$–polymer **II-4** complex, see Table II). Because low-molecular-weight trivalent ion–BP complexes and BPs were shown[19] to possess the same affinity to bone tissue and HA and $^{111}In^{3+}$ interacts with BP polymer or HA only, there must have been some polymer chains in solution with small content of BPs insufficient for adsorption.

The dependence of adsorption on HA, the polymer-to-HA weight ratio, and the adsorption rate (see previous discussion) were practically independent of pH in the biologically relevant pH range between 5 and 7.4. We also checked whether Ca^{2+} ions, present in bloodstream and being strongly chelated by BPs, interfered with the sorption of polymers on HA. There was no influence of various concentrations of Ca^{2+} ions on the sorption of the polymers on HA when the Ca^{2+}/polymer AHHBP molar ratio in incubation solution was 0, 0.5, 1, and 10. This finding was in accordance with the adsorption mechanism model, which includes direct chelation of BPs to calcium in the crystal structure of HA due to steric analogy of BPs with diphosphate (see previous discussion[1]). The adsorption of the prepared polymeric BPs on HA was not

TABLE II
Sorption of Radiolabelled Polymers II-0, II-2, and II-4 on Hydroxyapatite

Polymer	Isotope	Adsorbed polymer fraction (%)
II-0	^{125}I	3
II-2	^{125}I	57
II-4	^{125}I	77
II-2	^{111}In	92
II-4	^{111}In	85
$InCl_3$	^{111}In	93

Experimental conditions: polymer solution (0.5 mg/mL, 0.5 mL) in the PBS (pH = 7.4), HA = 12.5 mg, and incubation time = 60 min.

purely electrostatically driven because the polymeric BPs were negatively charged and the ζ potential of HA in the buffers used was -19.2 mV at pH 5.0 and -33.2 mV at pH 7.4, as measured with a Zetasizer ZS3600 (Malvern Instruments, Worcestershire, UK).

Polymer **V,** containing hydrazide and DOX moieties, was more quickly and more completely adsorbed on the surface of HA than the polymers with amide bond-bound DOX and L-tyrosinamide (**II, III,** and **VII**). We assumed that this effect was caused by protonizable amino groups of bound DOX present in polymer **V** but not in polymers **II, III,** and **VII,** which decreased the net total negative charge of the polymer caused by BP groups. This reduced the electrostatic repulsive forces between polymer **V** and the HA surface. It also reduced the electrostatic repulsion among the adsorbed molecules of polymer **V,** so the achievable surface density of the polymers **V** on the surface of HA was higher (see Fig. 2), although multilayer adsorption was still unlikely to occur.

Isotope labeling studies

The first system designed for bone metastases diagnostics and therapy was a radionuclide-delivery system based on the electrophilic iodination of the phenol ring of L-tyrosinamide with ^{131}I (β^- emitter, for therapy) and the chelation of $^{111}In^{3+}$ (an orbital electron capture emitter for scintigraphic imaging) ions into the BP moiety of polymer **II** respectively. The two approaches used the BP targeting moiety in its free (^{131}I) or complexed (^{111}In) form, respectively. ^{125}I was used as model isotope for ^{131}I because of the easier measurement of radioactivity and a longer decay halftime and, thus, significantly easier handling ($^{131}I - \beta^-$, decay halftime ($t_{1/2}$) = 8.04 days; ^{125}I − EC, $t_{1/2}$ = 59.4 days). Polymer **II** was labeled with $Na^{125}I$ in 67% yield and with $^{111}InCl_3$ in greater than 99% yield. The stability of both isotope labels was sufficient (>99% for 24-h incubation at room temperature in 0.15*M* NaCl). The sorption of polymer **II** labeled with $Na^{125}I$ on HA was lower than that of polymer **II** labeled with $^{111}InCl_3$ (see Table II). This could have been caused by partial instability of the trivalent cation–BP complexes in the environment containing competing ions, which was reported for ethidronate–radionuclide complexes used for diagnostics and therapy of skeleton.[6] Iodine isotopes could be of special advantage in such cases because the tyrosinamide moiety is stable in iodation and leaves the BP moiety free for interaction with HA. Iodine also has isotopes suitable for all main types of radiodiagnostics and radiotherapy (^{125}I for scintigraphy, ^{124}I for positron emission tomography, ^{131}I for therapeutic β^-).

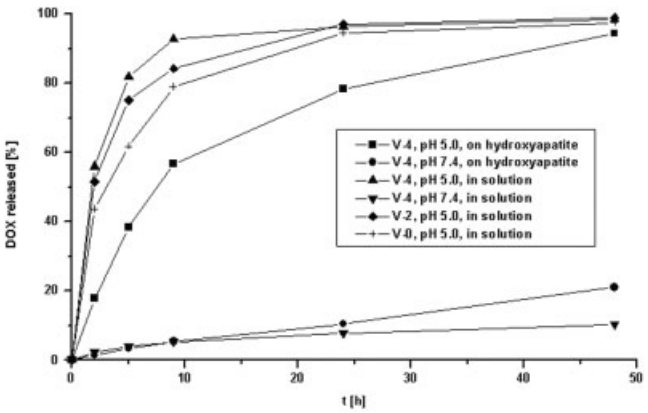


Figure 3 pH-dependent DOX release from polymer **V**.

pH-activated release of DOX from polymers V-0, V-2, and V-4

The interstitial space of numerous solid tumors and endosome content has a lower pH than the bloodstream (pH = 5–6 in comparison to pH = 7.4 in bloodstream).[15,20] It is thus advantageous to use polymeric prodrugs with the drug bound by a bond that is stable at pH 7.4 during transport but that hydrolyzes under mildly acidic conditions. One of the bonds suitable for attachment of the commonly used anticancer anthracycline antibiotic DOX is a hydrazone bond.[15,20] Polymer **V,** with hydrazone bonds between DOX and the polymer carrier containing BP units as targeting moieties, was used as a model case. Polymer **V** was adsorbed on HA, a buffer was added, and DOX cleavage was assayed spectrophotometrically in the supernatant. Unlike the polymeric precursor, which was tightly bound to HA, free DOX did not interact with HA. DOX was released according to first-order kinetics (R^2 = 0.9933 at pH 5.0 and R^2 = 0.9956 at pH 7.4) and 12 times faster at pH 5.0 than at 7.4 (see Fig. 3); the corresponding halftimes were 12.3 h at pH 5.0 and 147.5 h at pH 7.4. Seventy eight percent of DOX was released at pH 5.0 within 24 h, whereas at pH 7.4, it was only 10%.

Similar experiments were made with polymer **V-4** in solution to compare the rate of DOX release from polymers adsorbed on HA and from polymers in solution. We observed that the rate of DOX release at pH 5 decreased after adsorption of polymer **V-4** on HA, whereas at pH 7.4, we found the opposite trend: the rate of DOX release was increased by adsorption on HA (see Fig. 3). This phenomenon was probably associated with the different behavior of HA in solutions of different pH and should be studied further.

In addition, we determined the influence of the number of BP units on the rate of DOX release from polymers **V.** Although it increased the number of BP moieties bound to the polymer, the rate of DOX release from this polymer increased in the same order

(**V-0** < **V-2** < **V-4**; see Fig. 3), independently of the pH of the incubation media in the biologically relevant pH range between 5 and 7.4. We assumed that the increase in the rate of DOX release was associated with a decrease in pH localized near the polymer chain (in the vicinity of hydrolyzed hydrazone bonds) due to the increasing number of acidic BP units bound to the same polymer chain. A similar effect was observed in HPMA copolymers also bearing, except hydrazone bond-bound DOX, free carboxylic groups (unpublished results). The rate of DOX release was in all cases much faster in buffers at pH 5 than in buffers at pH 7.4, which enabled us to hypothesize that these types of conjugates will not release significant amounts of DOX during transport in the blood stream but will release it only after they have entered the target tumor tissue with the lower pH.

Enzymatic release of DOX from conjugates VII-0 and VII-4 by cathepsin B

Enzyme activation, for example, lysosomotropic activation, has been used in (polymeric) prodrug design because it can be used for selective release of the drug in the target tissue (cells) from an inactive precursor by a tissue- or cell-specific enzyme. The tetrapeptide Gly-Phe-Leu-Gly is an example of such a spacer susceptible to enzymatic degradation that has been used in water-soluble polymer drug-delivery systems for anticancer drugs.[11,21] Polymer **VII-4** with DOX bound to this tetrapeptide spacer was adsorbed on HA, cathepsin B was added, and DOX cleavage was assayed spectrophotometrically in the supernatant. To determine the effect of polymer **VII-4** adsorption on HA and the content of BP moieties in polymers **VII** on the rate of enzymatic release of DOX, we carried out release experiments with polymers **VII-0** and **VII-4** in solution containing cathepsin B in the same concentration. We observed (see Fig. 4) that the introduction of BP units into the polymer resulted in a small decrease in the rate of DOX release due to cathepsin B. On the contrary, after the adsorption of polymer **V-4** on HA, the rate of DOX release by cathepsin B decreased significantly to approximately one-half that obtained in solution. This decrease in the rate of enzymatic drug release could have come from increased steric hindrance of the access of the enzyme to the polymer tetrapeptide substrate after its adsorption on solid HA surface. Nevertheless, even if the polymer carrier is adsorbed on the solid surface, it can release a significant amount of the active free DOX to ensure its therapeutic effect.

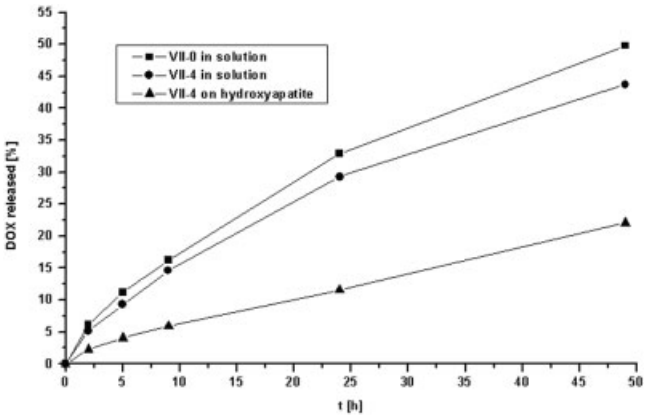


Figure 4 Cathepsin B dependent enzymatic release of DOX from polymers **VII-0** and **VII-4**.

CONCLUSIONS

Preparation and characterization of novel polymeric drug-delivery systems designed for bone targeting of antineoplastics based on biocompatible HPMA copolymers containing hydroxybisphosphonate targeting moieties and the model drugs radiotherapeutics ^{125}I, imaging agent ^{111}In, or anticancer drug DOX were described, and the basic characteristics were given. The *in vitro* binding studies on HA as a bone model proved that the copolymers of various structures were very quickly and efficiently adsorbed on this mineral. Adsorption was not dependent on pH, but it was influenced by the BP content in the copolymers. In the polymers, DOX was bound through stable (amide), pH cleavable (hydrazone), or enzymatically cleavable (Gly-Phe-Leu-Gly) spacers. The efficient *in vitro* release of DOX was demonstrated as the result of both pH and enzymatic stimuli. Interaction with and attachment of the drug carrier system to its target; the bone model HA, resulted in a slight decrease of the rate of DOX release from the pH-sensitive hydrazone bond-containing carrier system, whereas the decrease in the rate of DOX release from the enzymatically degradable system was more pronounced.

The pH-dependent release of DOX from hydrazone bonds was only slightly dependent on the bond to HA in comparison to solution, whereas in the case of enzymatically degradable system, the release rate was considerably lower after binding to HA.

The authors thank Čestmír Koňák, Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague, for the ζ potential measurements and Jana Zimová, Nuclear Research Institute, Řež near Prague, for the kind gift of $^{111}InCl_3$ solution and valuable comments regarding labeling with ^{111}In.

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