Novel Biodegradable Polyphosphazenes Containing Glycine Ethyl Ester and Benzyl Ester of Amino Acethydroxamic Acid as Cosubstituents: Syntheses, Characterization, and Degradation Properties

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ABSTRACT: Novel biodegradable polyphosphazenes containing glycine ethyl ester and benzyl ester of amino acethydroxamic acid as cosubstituents (PGBP) were synthesized by further modifying poly[bis(glycine ethyl ester)phosphazene] (PGP). The polymers were characterized by IR, ^{1}H-NMR, DSC, and elemental analysis. Degradation experiments were conducted *in vitro* at varied pH conditions. The results indicated that the degradation of PGBP was pH-sensitive. The sample dissoluted after 1.5 days under a physiological condition (pH 7.4) but took more than 20 days under an acidic condition (pH 5–6), which was related to the content of the benzyl ester of amino acethydroxamic acid in the polymer. The "two-stage" degradation mechanism of PGBP was proposed: that the polymer first degraded to a water-soluble polymeric product with the fast break of side groups, followed by the relatively slow scission of the backbone. This property of PGBP may be useful in controlled drug-delivery systems. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 77: 2987–2995, 2000

Key words: polyphosphazene; glycine ethyl ester; benzyl ester of amino acethydroxamic acid; pH-sensitive biodegradation; drug-controlled release

INTRODUCTION

A large variety of synthetic degradable polymers have been developed during the last two decades for drug-controlled release, including polyester, polyorthoester, polyanhydride, and polycarbonate. Recently, polyphosphazene has emerged as a new family of biodegradable polymers with an inorganic backbone consisting of alternating nitrogen and phosphorous atoms and two side groups attached to each phosphorous atom.[1] Their most significant advantage is the ease to link specific side groups to the backbone by efficiently substitutive techniques.[2] Thus, a number of biodegradable polyphosphazenes containing different hydrolytically labile side groups were synthesized. For example, Pucher et al.[3] described a series of degradable polyphosphazenes containing amino acid ester. Allcock and Pucher[4] reported polyphosphazenes with glycolic acid ester and lactic acid ester substituents. Although the controllable degradation rates of polyphosphazenes can be achieved, almost all of them degrade very slowly under physiological conditions.[3–6] This drawback may limit their applications in certain drug-delivery systems, such as a pulsatile drug-delivery system in which fast controlled drug release is required. To overcome this

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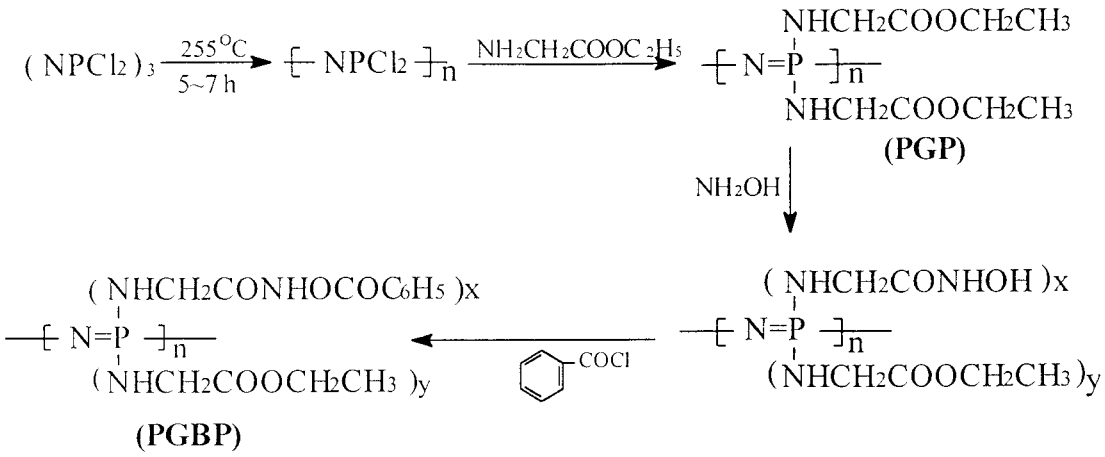


Scheme 1

problem, we recently prepared novel biodegradable polyphosphazenes containing glycine ethyl ester and benzyl ester of amino acethydroxamic acid as cosubstituents (PGBP), which can be hydrolyzed at a higher rate under physiological conditions, but be relatively stable at low pH. Herein, we report some primary results on the preparation, characterization, and degradation properties of PGBP.

EXPERIMENTAL

Materials

Hexachlorocyclotriphosphazene $(NPCl_2)_3$ (mp 112–114°C) was obtained from Fluka Chemie AG (Buchs, Switzerland). Myoglobin (M_w 18,800) and FITC–dextran (M_w 71,200) were purchased from Sigma Chemical Co. (St Louis, MO). Benzoyl chloride, benzene, triethylamine, and light petrol (bp 30–60°C) were completely dried and distilled before use. Glycine ethyl ester hydrochloride was synthesized in our lab (mp 144°C).[7] All other chemicals were used as received.

Synthesis (Scheme 1)

Synthesis of Poly[bis(glycine ethyl ester)phosphazene] (PGP)

The monomor $(NPCl_2)_3$ (4 g) was sealed in a well-dried and vacuumed vessel (20 mL). The vessel was heated in the oven at 255°C for 5–7 h until the reactant almost solidified. The product was dissolved in benzene and precipitated from light petrol under a dry N_2 atmosphere. The substitution reaction on the polymer backbone was carried out immediately by a similar method as described by Ruiz et al.[8] Glycine ethyl ester hydrochloride (10 g, 0.07 mol) was mixed with benzene (100 mL) and triethylamine (10 mL, 0.07 mol), stirred, and refluxed for 6 h, then cooled and filtered. Another 3.6 mL of triethylamine was added to the filtrate. Then, the mixture was slowly dropped into the solution of $[NPCl_2]n$ (1.4 g, 0.024 mol P—Cl) in benzene (50 mL) which had been cooled to 0°C. After completing the additions, the reaction was maintained at 0°C for 8 h, followed at 25°C for 12 h. The insoluble hydrochloride salt was removed by filtration and the filtrate was concentrated by evaporation in a vacuum to about 10 mL. PGP was obtained by precipitating from petrol. The yield was 30%.

Synthesis of PGBP

Hydroxylamine hydrochloride was dissolved in methanol and converted into free hydroxylamine by the addition of an equivalent amount of sodium methylate. The sodium chloride was collected on a filter, and the solution of free hydroxylamine was added to a methanol solution containing PGP with stirring at room temperature. At the same time, additional sodium methylate was dissolved in the reactant solution to modulate the pH to about 9. The mixture was allowed to stand overnight. Then, the solvent was removed from the vacuum. The dry resultant was dissolved in 20 mL distilled water and treated with potassium hydroxide. Then, excess benzoyl chloride was added slowly dropwise, while the solution was vigorously shaken. After several

minutes, a white solid, PGBP, precipitated, which was separated by centrifugation, dried in a vacuum, and extracted several times with ethyl ether to remove benzoic acid. The yield was about 72%.

Characterization

Infrared Analysis

IR analysis was performed on a Nicolet 5DX FTIR spectrophotometer. The samples were film-cast in methylene chloride onto KBr plates.

^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR)

^{1}H-NMR measurement was carried out at 90°C by an Avance DMX500 instrument using deuterated DMSO as a solvent and internal Me_4Si as a shift reference.

Elemental Analysis

The carbon, hydrogen, and nitrogen content in the polymer was obtained by elemental analysis using an EMA EA-1106 elemental analyzer.

Differential Scanning Calorimetry (DSC)

The glass transition temperature (T_g) was determined by a Perkin–Elmer DSC-7 thermal analyzer at a heating rate of 10°C min^{-1} from −70 to 200°C.

Molecular Weight Determination

The molecular weights of the polymers were determined by a Knauer vapor-pressure osmometer (VPO) in DMSO at 90°C using sucrose octaacetate (MW = 678.58) as a reference.

Polymer Degradation

Considering the interference with assays of the hydrolysis product of polyphosphazene, a phosphorus-free buffer was required. Therefore, the buffers used here were citric acid/sodium citriate for pH 5.0 and 6.0 and boronic acid/borax for pH 7.4 and 8.0, respectively.

Samples of the disc shape (2.5 mm in diameter, 2 mm in length) were fabricated by compression in a mold under a pressure of 500 kg/cm^2 at 40°C for 2 min. Hydrolysis of the polymers *in vitro* was examined by immersing samples in each buffer solution at 37°C and the samples were recovered periodically. The weight loss was measured gravimetrically. The molecular weight changes were determined by VPO. The hydrolysis products of PGBP, phosphate and benzoic acid, were assayed by the ascorbic acid method[8] and high-performance liquid chromatography (HPLC) [Shimadzu C-R4A chromatopac system with C_{18} as the stationary phase and methanol/water/acetic acid (60/40/2 v/v) as mobile phase at flow rate of 0.8 mL/min: λ = 254nm as a detection wavelength using a UV detector, benzoic acid as reference], respectively.

Drug-Release Studies

Myoglobin (MG) and fluorescein isothiocyanate dextran (FITC–dextran) were used as model drugs. Prior to making drug-loaded samples by the same method stated in the degradation studies, the micronized polymers were sieved into a particle size range of 90–150 μm and mixed with the same size range of model drugs. Ten percent drug-loaded samples were placed in 5 mL of the buffer at 37°C. The buffer solutions were frequently replaced to maintain perfect sink conditions. MG release was monitored by visible absorbance at 420 nm using a UV-vis spectrophotometer (Shimatz-1201). FITC–dextran release was measured by fluorescence intensity (Hitachi F4000; the excitation wavelength was 496 nm and the emission wavelength was 520 nm).

RESULTS AND DISCUSSION

Polymer Synthesis

Several methods have been developed to prepare hydroxamic acid derivatives.[9] Among them, the most common one is the reaction between an ester and hydroxylamine. This generality of the reaction allowed it to be applied to prepare novel polyphosphazenes containing hydroxamic acid as a side group. Thus, PGBP was synthesized by modifying the fully glycine ethyl ester-substituted polyphosphazene with varying amounts of free hydroxylamine to convert partial glycine ethyl ester to amino acethydroxamic acid, followed by further esterification with benzoyl chloride. During the reaction between PGP and hydroxylamine, sodium methlate caused the reaction to take place rapidly at room temperature. Only after 1 h, the reactant gave a red coloration with ferric chloride, indicating the existence of hydroxamic acid. Two polymers designated as

Table I Some Data of PGBP

Polymer	Feed Ratio of PGP/ NH_2OH (by mol)[a]	M_n	T_g (°C)		Elemental Analyses (%) C	N	H	Conversion Ratio (%)[d]
PGBP-1	2:1	6430	34	f[b]	44.01	15.96	5.12	40
				c[c]	44.74	16.53	5.22	
PGBP-2	1:5	7400	55	f	48.14	16.01	4.61	80
				c	48.66	16.32	4.46	

[a]The amount of PGP was calculated based on —$NHCH_2COOCH_2CH_3$ group.
[b]Experimental values.
[c]Theoretical values.
[d]Based on the percent of glycine ethyl ester converted to benzyl ester of amino acethydroxamic acid.

PGBP-1 and PGBP-2 in Table I were prepared in different feed ratios of PGP/NH_2OH. They were both white power, but PGBP-1 was soluble in alcohol, CH_2Cl_2, THF, and DMSO, whereas PGBP-2 was insoluble in alcohol and THF.

From the infrared spectra of PGBP-1 and PGBP-2, specific peaks assignments are as follows: 3350 (—NH—), 3100–2800 [CH(ϕ), —CH_2—,—CH_3,], 1750 [—O—C(=O)—], 1650 [—C(=O)NH—], 1600, 1580 (—C_6H_5), 1220 [—C(=O)—O—CH_2—], and 1150 cm^{-1} (—N=P—). Furthermore, the absorbance at 1650 cm^{-1} of PGBP-2 was obviously stronger than that of PGBP-1, which indicated that PGBP-2 contained more benzyl ester of amino acethydroxamic acid as a side group than did PGBP-1.

In the ^{1}H-NMR spectra of PGBP-1 and PGBP-2, similar distributions of the peaks were obtained (Fig. 1). The peaks at 1.2 ppm can be assigned to —CH_3; 3.78 ppm, to —CH_2CH_3; 4.15

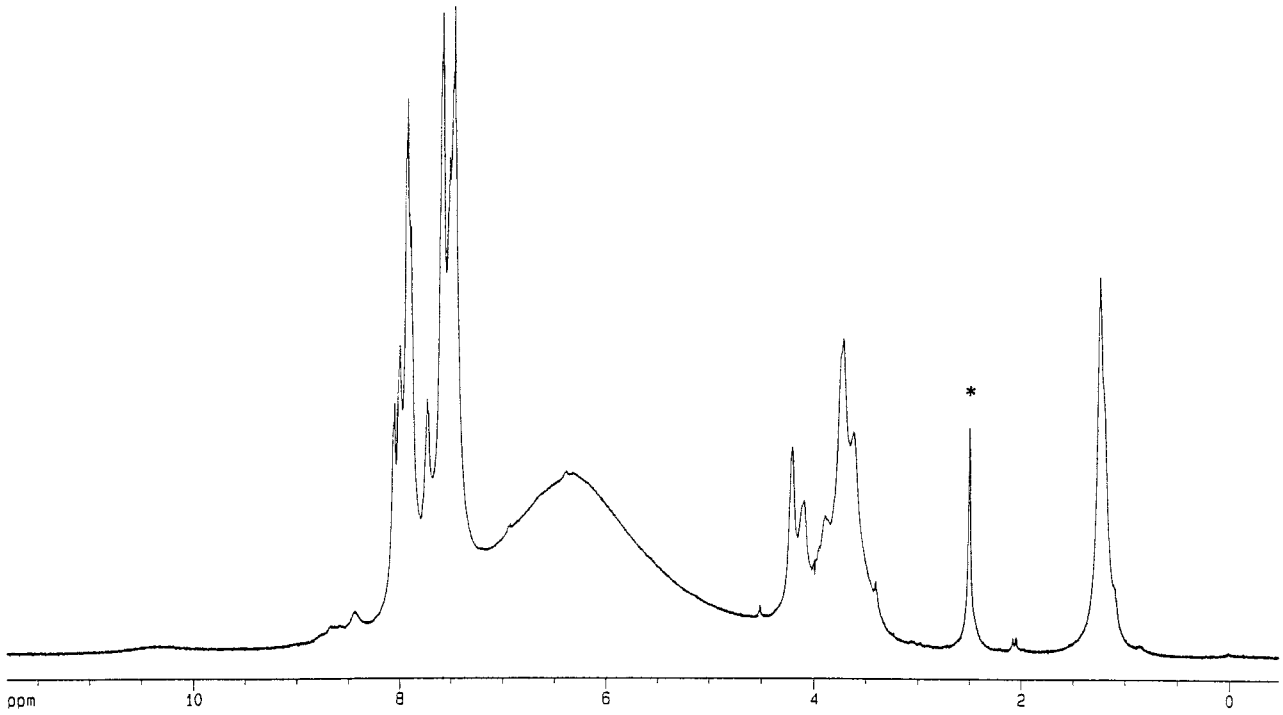


Figure 1 ^{1}H-NMR spectrum for PGBP-2 measured at 90°C by an Avance DMX500 instrument using 6*d*-DMSO as a solvent and internal Me_4Si as a shift reference. *Solvent peak.

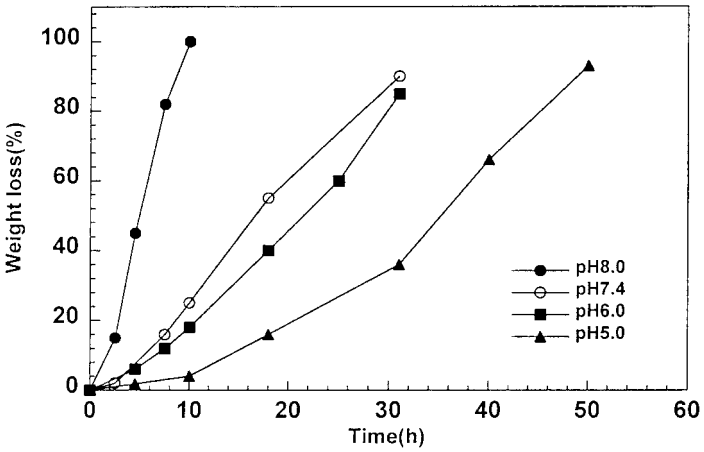


Figure 2 Cumulative weight loss of PGBP-1 in pH 8.0, 7.4, 6.0, and 5.0 buffer at 37°C.

ppm, to —$NHCH_2$—; and 7.3–8.2 ppm, to —C_6H_5. The centered broad peak at 6.4 ppm at 90°C, but moved to 5.2 ppm at 120°C, corresponded to —NH—. The conversion ratio of glycine ethyl ester to benzyl ester of amino acethydroxamic acid of PGBP-1 and PGBP-2 was calculated from the peak integration data. The conversion ratio of PGBP-1 and PGBP-2 was about 40 and 80%, respectively, indicating that it could be well modulated by changing the feed ratio of PGP to hydroxylamine. These results were also confirmed by the carbon, hydrogen, and nitrogen content of PGBP-1 and PGBP-2 measured by elemental analysis (Table I).

DSC analysis was employed to measure the T_g's of PGBP-1 and PGBP-2. Due to the introduction of stiff benzene groups, the higher conversion ratio of the reaction, the higher the T_g of the polymer. As shown in Table I, the T_g of PGBP-1 and PGBP-2 was 34 and 55°C, respectively, and no T_m was observed for either of them, which is important in some cases. These relatively low T_g's facilitated the fabrication of drug formulations at mild conditions, especially for delicate bioactive substances.

Degradation

The degradation of polymers in varied pH buffers was examined to evaluate the influence of pH on their degradation behavior. Figure 2 shows that the weight loss of PGBP-1 gradually increased with increasing pH of the buffer and all samples dissoluted within 50 h in the range of pH 5.0–8.0. However, a great dependence of the hydrolysis rate of PGBP-2 on the pH was observed in Figure 3. It took only 12 h for the sample to dissolute completely in a pH 8.0 buffer, 1.5 days in pH 7.4,

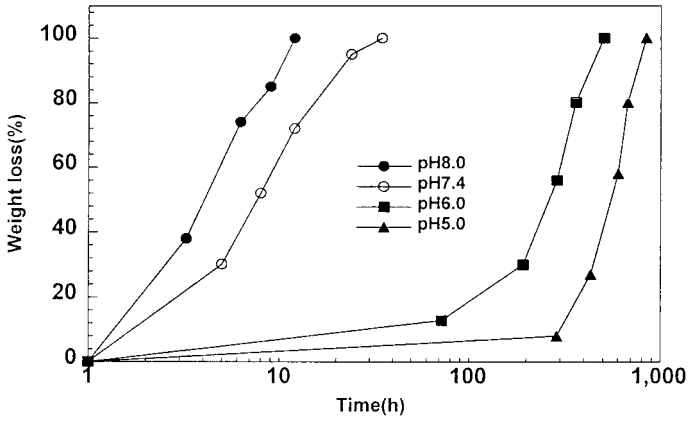


Figure 3 Cumulative weight loss of PGBP-2 in pH 8.0, 7.4, 6.0, and 5.0 buffer at 37°C.

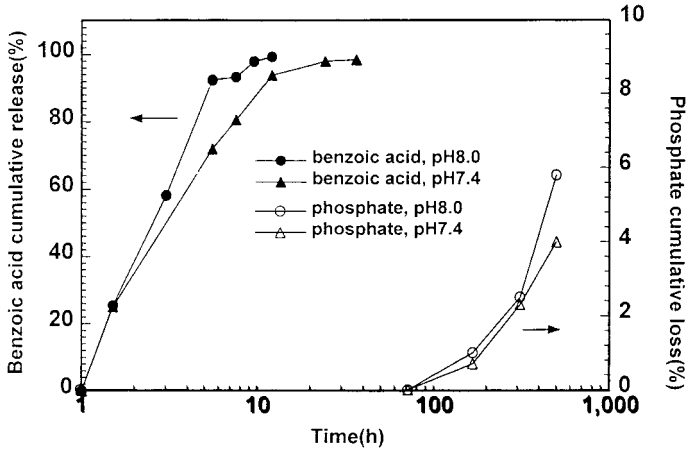


Figure 4 Benzoic acid and phosphate cumulative release of PGBP-2 in pH 8.0 and 7.4 buffer at 37°C.

but more than 20 days in weak acidic media (pH 5.0 and 6.0). This is in apparent contrast to the behavior reported by Ruiz et al.[8] for the fully glycine ethyl ester-substituted polyphosphazene (PGP). In their case, hydrolysis was slower in pH 7.0 than in basic or acidic pH.

To understand this phenomenon, the degradation mechanism of PGBP was further studied. It was known that the benzyl ester of hydroxamic acid decomposed to benzoic acid by a Lossen rearrangment.[10] Therefore, benzoic acid was used to represent the hydrolysis of the side group of the polymer. Also, the corresponding cumulative phosphate loss represented the backbone cleavage of polyphosphazene. Figure 4 shows that the benzoic acid release increased against the time until the polymer sample dissoluted completely at pH 8.0 and 7.4. But the release of phosphate occurred after the sample dissoluted completely, which lagged seriously behind that of benzoic acid, indicating that this side group was more readily cleaved than was the backbone. So, it was reasonable to assume that the whole degradation proceeding of PGBP was composed of two phases: In the first phase, the break of side groups played a prominent role in that the benzyl ester of amino acethydroxamic acid was hydrolyzed to produce amino groups, probably accompanied with the hydrolysis of glycine ethyl ester to glycine.[1] As a result, the polymer degraded to a water-soluble polymeric product. In the second phase, a soluble polymeric product continued thorough degrada-

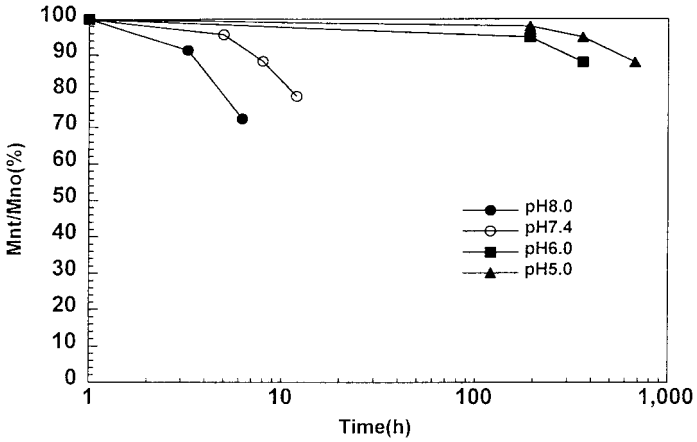
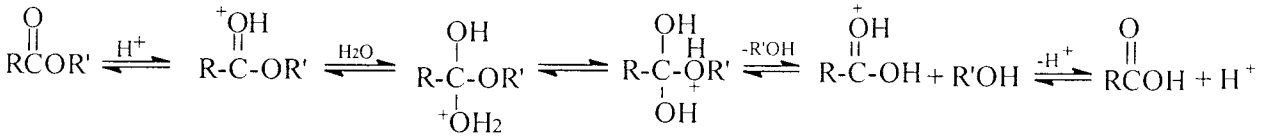


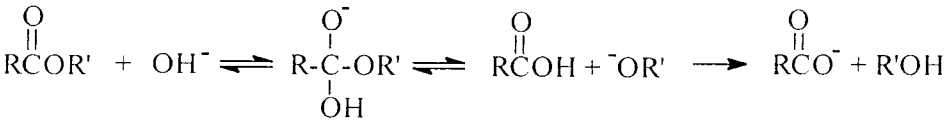
Figure 5 M_n change of PGBP-2 in pH 8.0, 7.4, 6.0, and 5.0 buffer at 37°C. Mno and Mnt are the M_n of PGBP-2 at initial and specific times, respectively.

tion by the scission of the backbone to phosphates and ammonia. This assumption can also be confirmed by the MW change plotted against the time in Figure 5. At pH 8.0 and 7.4, the MW of PGBP-2 reduced only 28 and 20%, until the sample dissoluted completely. Also, at pH 5.0 and 6.0, a small MW change could be found after 100 h, indicating that the samples degraded in the same manner but at a low rate under an acid condition.

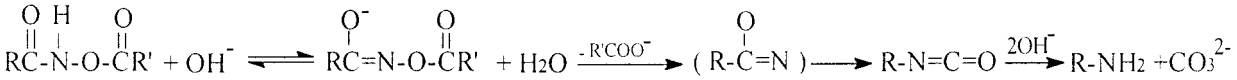
On the other hand, the different hydrolysis mechanism of amino acid ester and hydroxamic acid ester should be taken into account. Their respective hydrolysis mechanisms are illustrated as follows:

Ester hydrolysis process:





Hydroxamic acid ester hydrolysis process:[11,12]



Due to the acid- or base-catalyzed effect on glycine ethyl ester, the hydrolysis rate of PGP was enhanced under an acidic or basic condition. But the actual concentration of the hydroxide ion is an important factor to make the hydroxamic acid ester moiety quite sensitive toward hydrolysis. So, introducing an amount of benzyl ester of amino acethydroxamic acid as cosubstituents resulted in a drastic increase of the degradation at pH 7.4. In addition, the degradation characteristic of PGBP was determined to a great extent by the content ratio of hydroxamic acid ester to amino acid ester. PGBP-2, carrying 80% benzyl ester of amino acethydroxamic acid, apparently displayed the hydrolysis property of a hydroxamic acid ester, being stable at pH 5.0 and 6.0 but very

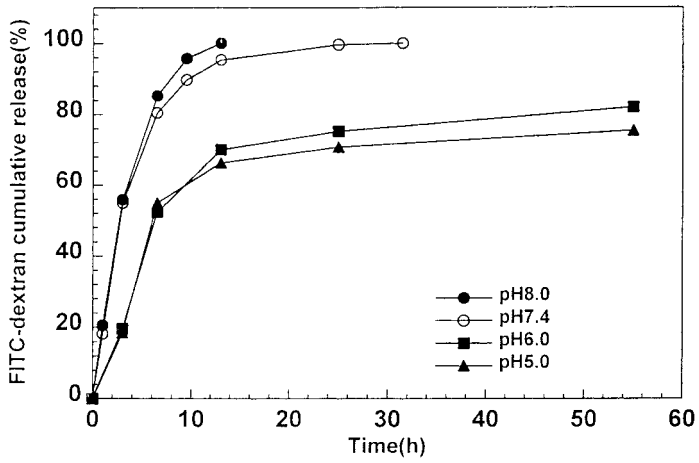


Figure 6 Cumulative release of FITC–dextran from 10% drug-loaded PGBP-2 discs in pH 8.0, 7.4, 6.0, and 5.0 buffer at 37°C.

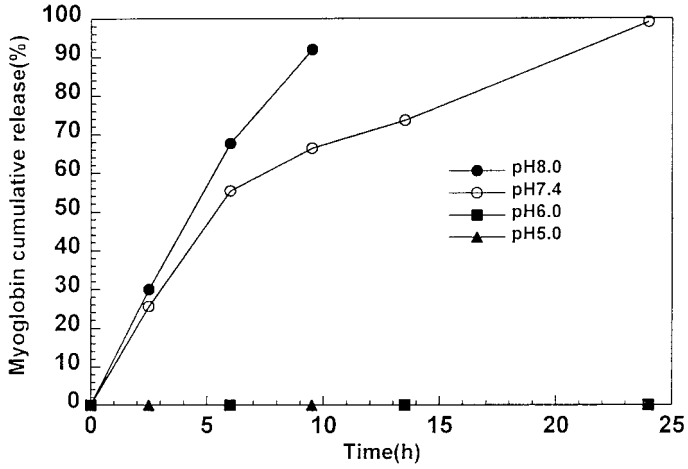


Figure 7 Cumulative release of myoglobin from 10% drug-loaded PGBP-2 discs in pH 8.0, 7.4, 6.0, and 5.0 buffer at 37°C.

liable at pH 7.4 and 8.0. Since PGBP-1 had almost an equal content of the benzyl ester of amino acethydroxamic acid and glycine ethyl ester, it degraded slower at pH 7.4 than did PGBP-2, but much faster at pH 6.0 and 5.0 than did PGBP-2. Thus, PGBP-2 exhibited a stronger pH-dependent degradation characteristic than that of PGBP-1. This finding was consistent with a recent study carried out by Ulbrich and coworkers.[13,14]

Drug Release

With the aim to investigate the suitability of PGBP-2 as an erodible support for controlled drug release, FITC–dextran (polysaccharide) and myoglobin (protein) were used as model drugs. In Figure 6, the release of FITC–dextran at pH 8.0 and 7.4 accorded with the degradation of the polymer, and the process of drug release was somewhat extended at pH 6.0 or 5.0, but the release pattern was not significantly different. It was revealed that FITC–dextran was released from the PGBP-2 matrix at a rate controlled by diffusion, by the rate of degradation of the polymer, or by the combination of both processes depending on the pH of the release media and the polymer degradation stage. However, it should be noted that the release of MG coincided with the corresponding degradation of the polymer at pH 7.4 or 8.0, but the polymer maintained MG quite well at pH 6.0 and 5.0 (Fig. 7). The strong pH-dependence of the MG release may be interpreted as follows: PGBP-2 bore negative charges after one of its side groups, glycine ethyl ester, hydrolyzed to glycine. Since MG (isoelectric point is 6.9) is an amphoteric polymer, it carried positive charges at pH 5.0 or 6.0, which formed salt bridges with negative charges of PGBP-2. The formed water-insoluble polyelectrolyte complexes retained MG in the matrix. When pH was above the IEP of MG, such as pH 7.4 or 8.0, MG carried negative charges, and the interactions between MG and the polymer turned to electrostatic repulsion, MG was released as the polymer degraded.

CONCLUSIONS

Novel polyphosphazenes containing 40 and 80% benzyl ester of amino acethydroxamic acid side groups (PGBP-1, PGBP-2) were synthesized by further modifying PGP with different amounts of hydroxylamine. The degradation mode of PGBP involves fast hydrolysis of the side groups followed by slow cleavage of the backbone. The dependence of the polymer degradation on pH was observed. Moreover, it became more important with increasing the conversion ratio of glycine ethyl ester to benzyl ester of amino acethydroxamic acid. PGBP-2 samples dissoluted after more than 20 days at pH 6.0, but much more quickly at pH 7.4 (1.5 days). MG release from PGBP-2 also exhibited similar pH-dependence. Owning these properties, this novel biodegradable polyphosphazene may be useful for drug-controlled release.

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