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Synthesis and characterization of biocompatible poly(organophosphazenes) aiming for local delivery of protein drugs

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Abstract

Biocompatible and thermosensitive poly(organophosphazenes) with a lower critical solution temperature (LCST) below body temperature have been designed with the aim for the local delivery of peptide and protein drugs. These polymers could be synthesized by introducing short chain tri- or tetraethylene glycol as a hydrophilic group and a dipeptide, $GlyGluEt_2$ as a hydrophobic group into the polyphosphazene backbone. The local tolerance tests using rabbits have shown that our polymers are biocompatible. Using the amphiphilic properties of these polymers, in vitro studies were performed for loading and releasing of a human growth hormone (hGH) as a model drug. The entrapment efficiency (%) of hGH by the polymer decreased as its polymer concentration increased, but exhibited high efficiency of more than 95% even at 20% hGH concentration in the polymer. The entrapped hGH has shown to be controlled releasing for 3–4 days.

Keywords: Polyphosphazene; Drug delivery; Thermosensitive polymer; Protein drug

1. Introduction

A variety of thermosensitive polymers have been synthesized for last decades. The thermosensitive polymers usually exhibit in aqueous solution a lower critical solution temperature (LCST), defined as the temperature at which the polymer solution undergoes phase transition from a soluble to an insoluble state when the temperature is raised. These unique properties of the thermosensitive polymers make them useful for applications to many fields such as drug delivery systems (Okano et al., 1990; Mukae et al., 1990; Katono et al., 1991; Yoshida et al., 2003), cell culture (Okano et al., 1995; Yamato et al., 2002), isolation of biomolecules (Monji and Hoffman, 1987; Vaidya et al., 2001), and enzyme activity control (Park and Hoffman, 1988; Ding et al., 1996).

The homopolymer or copolymers of *N*-isopropylacrylamide are representative thermosensitive polymers (Chen and Hoffman, 1995; Gutoska et al., 1995). Their LCST is below body temperature (∼32 °C), and therefore, extensive studies have been performed for applications of these polymers to drug delivery systems, but their utilization is limited because of their non-biodegradability. Most of the thermosensitive polymers including the graft- and block copolymers of poly(ethylene oxide) and poly(vinyl alcohol) derivatives as well as poly(organophosphazenes) bearing alkyl ether side groups are known to be non-biodegradable (Malstom and Lindman, 1992; Allcock and Dudley, 1996; Allcock et al., 1996). Early reported poly(organophosphazenes) with pendent amino acid esters or oligopeptides are hydrolytically degradable (Allcock et al., 1977; Allcock and Chang, 1991) but not thermosensitive, since these polymers are not amphiphilic. Recently, biodegradable and thermosensitive polymers, such as poly(ethylene oxide) (PEO) and poly(L-lactic acid) (PLLA) block copolymers and low-molecular-weight PEG–PLGA–PEG triblock copolymers

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have been reported as injectable drug delivery materials (Jeong et al., 1997; Jeong et al., 2000), but their degradation product (lactic acid) is acidic, which may damage bio-drugs such as protein and DNA drugs.

In particular, the protein drug delivery is attracting a great deal of attention in recent years, since the current active research of proteomics is expected to produce the protein drugs as the major stream of the future drugs. Therefore, many studies have been reported on the protein delivery using biodegradable microspheres (Vila et al., 2002; Sinha and Trehan, 2003; Kim et al., 2004) and hydrogels (Wu et al., 2005). However, unfortunately, no good delivery systems for controlled release of proteins have been found yet because of non-biodegradability of the conventional polymers and/or critical molecular interactions between the polymer substrate and chemically sensitive protein drugs, as above-mentioned.

We have recently reported that amphiphilic poly(organophosphazenes) prepared by stepwise substitutions of the chlorine atoms in poly(dichlorophosphazene) with a hydrophilic poly(ethylene glycols) (PEG) and a hydrophobic amino acid ester are thermosensitive and as such are potential polymeric carriers for biologically active agents (Song et al., 1999; Lee et al., 1999). In most recent years, we have also reported on the poly(organophosphazenes) bearing PEG and oligopeptide esters as side groups and examined their thermosensitive and biodegradable properties (Kim et al., 2004). However, these polymers exhibited a lower critical solution temperature (LCST) in the range of 63–98 °C, which are far higher than body temperature probably due to the high hydrophilic to hydrophobic balance and as such not suitable for local delivery of hydrophobic protein and peptide drugs. Moreover, to our knowledge, there is no report on the biocompatibility of such amphiphilic poly(organophosphazenes) in spite of increasing interests in their potential applications to new drug delivery systems.

Therefore, in the present work, we have designed and synthesized biocompatible and thermosensitive poly(organophosphazenes) with a LCST below body temperature, by employing a shorter PEG such as tri- or tetraethylene glycol along with a dipeptide ethyl ester. Here we report synthesis, characterization and the properties of these novel poly(organophosphazenes).

2. Materials and methods

2.1. Materials

Hexachlorocyclotriphosphazene (Aldrich) was used without further purification. The dipeptide, glycyl-L-glutamic diethyl ester ($GlyGluEt_2$) was prepared by the literature methods (Greenstein and Winitz, 1961; Jones, 1992). Methoxy triethylene glycol (MTriEG) and methoxy tetraethylene glycol (MTetEG) (Fluka) were used without further purification but thoroughly vacuum-dried and stored over molecular sieve 4A before use. Tetrahydrofuran (THF) was dried by boiling at reflux over sodium metal and benzophenone, and then distilled under a nitrogen atmosphere. Chloroform and triethylamine were dried by boiling at reflux over sodium hydride and barium oxide, respectively, and then distilled under the same condition. The growth hormone hGH (Dong-A Pharmacy) was used after confirming by RP-HPLC that the purity was higher than 98%.

2.2. Instruments

Elemental analysis was carried out with a Carlo Erba-EA1108. ^{1}H NMR measurements were made with a Varian Gemini-250 spectrometer operating at 250 MHz in the Fourier transform mode. Proton-decoupled ^{31}P NMR spectra were measured with a Varian Unity INOVA-400 spectrometer operating at 400 MHz using phosphoric acid as an external standard. Gel permeation chromatography was carried out using a Waters Associates HPLC/GPC 150C unit and two styragel columns (Waters styragel HT 4 THF) connected in line at a flow rate of 1.0 ml/min at 40 °C and fitted with a refraction index detector and a computerized data station. Methoxy poly(ethylene glycol) (M_w = 6000, 11,200, 24,800, 42,900, 149,000, 348,000 and 722,000) were used as standards to calibrate the column. The phase transition of the polymer aqueous solutions (10 wt%) was detected using a melting point apparatus: the polymer solution in a capillary tube was immersed in the apparatus and the temperature was slowly raised (Kim et al., 2004). The LCST was identified as the temperature at which the solution became turbid. RP-HPLC was carried out using a styragel column (Waters styragel HT 4 THF) unit and a butyl sillylized silica gel column.

2.3. Synthesis of poly(organophosphazenes)

2.3.1. $[NP(MTriEG)_{0.8}(GlyGluEt_2)_{1.2}]_n$ (1)

Poly(dichlorophosphazene) was prepared as described previously (Sohn et al., 1995). The sodium salt of methoxy triethylene glycol (MTriEG) was prepared by reaction of MTriEG (1.34 g, 8.15 mmol) with 1.05 equivalent of sodium hydride in THF (150 ml) at room temperature for 5 h. The solution was dropped slowly to a solution of poly(dichlorophosphazene) (1.0 g, 8.63 mmol) dissolved in THF (80 ml). The reaction mixture was stirred for 12 h at −78 °C. Meanwhile, glycyl-L-glutamic diethyl ester (2.96 g, 11.39 mmol) was dissolved in dry chloroform (100 ml) containing 3 equivalent of dry triethylamine. The glycyl-L-glutamic diethyl ester solution was added to the polymer solution, which was stirred for 2 days at 50 °C. The reaction mixture was filtered to remove triethylammonium chloride precipitated. After the filtrate was evaporated, the concentrate was precipitated using a solvent pair of THF and *n*-hexane to obtain a yellow precipitate, which was repeated twice in the same solvent system. In order to remove unreacted reactants and inorganic salt, the solution was dialyzed for 1 day against methanol and then 1 day against ultra pure water using cellulose dialysis membranes (molecular weight cutoff: 3.5×10^3, Spectrum Co.). The dialyzed solution was freeze-dried to obtain the polymer **1**.

Yield: 50%. ^{31}P NMR (DMSO), δ (ppm): 2.25(b). ^{1}H NMR (DMSO), δ (ppm): 1.1–1.2 (m, 7.0H), 1.8–2.1 (m, 2.0H), 2.3–2.5 (t, 1.8H), 3.2–3.3 (s, 3.0H), 3.3–3.7 (b, 13.8H), 3.9–4.2 (b, 5.4H), 4.2–4.4 (b, 1.0H). Elem. Anal. (%) calcd for

$[NP(C_7H_{15}O_4)_{0.8}(C_{11}H_{19}O_5N_2)_{1.2}\cdot H_2O]$: C, 44.74; H, 7.35; N, 9.44. Found: C, 43.98; H, 6.90; N, 10.03.

2.3.2. $[NP(MTetEG)_{0.9}(GlyGlu(Et_2)_{1.1})]_n$ (2)

This polymer was prepared by the same procedure using methoxy tetraethylene glycol. Yield: 36%. ^{31}P NMR (DMSO), δ (ppm): 0.349(b). ^{1}H NMR (DMSO), δ (ppm): 1.1–1.3 (m, 5.7H), 1.8–2.1 (m, 1.8H), 2.2–2.4 (t, 1.6H), 3.2–3.3 (s, 3.0H), 3.3–3 (b, 13.7H), 3.8–4.2 (b, 4.4H), 4.2–4.4 (b, 1.01H). Elem. Anal. (%) calcd for $[NP(C_9H_{19}O_5)_{0.9}(C_{11}H_{19}O_5N_2)_{1.1}\cdot H_2O]$: C, 45.37; H, 7.54; N, 8.38. Found: C, 45.09; H, 7.96; N, 8.60.

2.4. Biocompatibility test

Assay for the biocompatibility of the present polymers was performed using male New Zealand White rabbits (3.0–3.5 kg) according to the standard procedure (Ogawa et al., 1996). Fifteen rabbits were kept under controlled conditions of light/dark cycles of 12/12 h, humidity of 55 ± 10% and temperature of 22 ± 3 °C for 1 week in separate cages and provided with free access to water and food. After the adaptation period 12 healthy rabbits were selected and divided into four groups to examine biocompatibility of the polymer solution with and without drug at 3rd and 7th day post injection. The polymer samples used for the biocompatibility test were prepared by the following method. Polymer **1** was dissolved in the PBS solution to make a 15% (w/v) solution, which was stirred in cold room for 1 day, and then hGH was added to the solution, which was once again diluted by PBS solution to make a 12.5% (w/v) polymer solution containing 1 mg of hGH per millilitre, and filtered through a 0.45 μm filter (MILLEX-GV$_4$). After shaving the back of the rabbits, 1 ml of the sample solution was injected subcutaneously to the left back of the rabbits and on the other side 1 ml of the saline solution as reference was injected in the same way.

2.5. Entrapment efficiency and in vitro release of hGH

The human growth hormone (hGH) with an average molecular weight of 22,000 was employed as a model drug to examine the entrapment efficiency and in vitro releasing profile of the drug. The entrapment efficiency of hGH by polymer **1** was studied using 12.5% (w/v) polymer solutions, in which hGH was added to make 0, 2, 5, 10 and 20% (w/w) hGH/polymer in aqueous solutions. The mixtures were precipitated by raising the polymer solution temperature to 37 °C, and then the residual free hGH concentration in each solution was measured by SEC-HPLC.

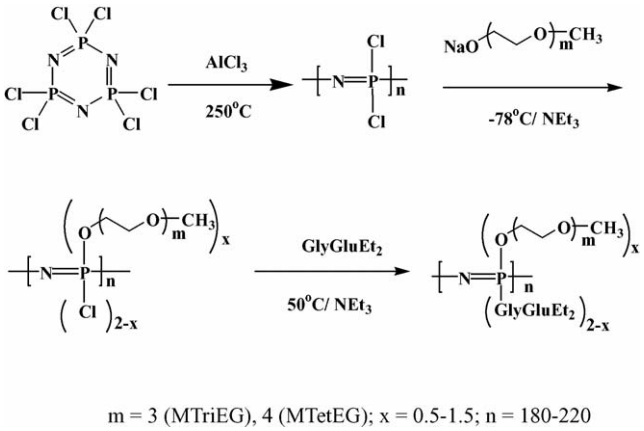
The samples used to study the drug releasing profile were prepared by the following method. The polymer was dissolved in 10 ml of PBS solution to make a 15% (w/v) polymer solution, which was stirred in a cold room for 1 day. The drug was added to the solution and once again diluted by PBS solution to make a 12.5% (w/v) polymer solution containing 1 mg of hGH per millilitre, and then filtered through a 0.45 μm filter (MILLEX-GV$_4$). From the filtrate, an aliquot (1 ml) was taken into each of seven vials, and warmed up to 37 °C to precipitate the hGH-entrapped polymer, which was immediately separated by centrifugation and dispersed in the same amount of the fresh PBS solution. The amount of hGH released to the buffer solution from the drug loaded polymer **1** was measured on 0.25, 1, 2, 3, 4, 6 and 7th day, and the released hGH was confirmed and analyzed by SEC-HPLC.

3. Results and discussion

3.1. Synthesis and characterization

The present polymers were synthesized according to the reaction route depicted in Scheme 1. The chloropolymer prepared by the author's procedure (Sohn et al., 1995) from hexachlorocyclotriphosphazene was subjected to stepwise nucleophilic substitution reactions by hydrophilic methoxy triethylene glycol (MTriEG) or methoxy tetraethylene glycol (MTetEG) and then by a hydrophobic dipeptide, glycyl-L-glutamic diethyl ester ($GlyGluEt_2$).

The resultant copolymers were obtained as yellow viscoelastic solids, which are fairly soluble in most polar organic solvents and very soluble in water below their LCST. The polymers prepared were characterized by means of multinuclear (^{1}H, ^{31}P) NMR spectroscopies, GPC and elemental analysis. The ^{1}H and ^{31}P NMR spectra of polymer **2** are shown in Fig. 1.



Scheme 1. The synthetic scheme for poly(organophosphazenes) with methoxy tri- and tetraethylene glycol and dipeptide ethyl ester as side groups.

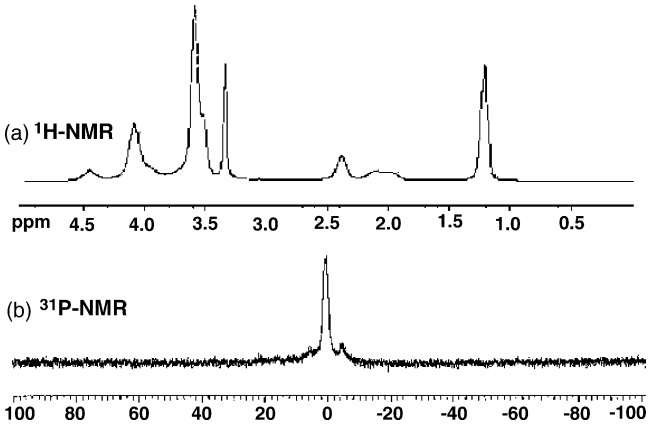


Fig. 1. ^{1}H NMR (a) and ^{31}P NMR (b) spectra of polymer **2** in DMSO.

Table 1
Characteristics of poly(organophosphazenes)

Polymer	Formula	LCST (°C)	$M_w \times 10^{-4}$
1	$[NP(MTriEG)_{0.8}(GlyGluEt_2)_{1.2}]_n$	25	9.2
2	$[NP(MTetEG)_{0.9}(GlyGluEt_2)_{1.1}]_n$	27	18
3[a]	$[NP(MPEG350)_{1.05}(GlyGluEt_2)_{0.95}]_n$	81	18

[a] Kim et al. (2004).

The mole ratio of MTetEG and the dipeptide diethyl ester of polymer **2** was calculated from the integration ratio of the ethyl protons of the dipeptide diethyl ester and the methoxy protons of MTetEG appearing at 1.2 and 3.3 ppm, respectively, in Fig. 1(a). The ^{1}H NMR spectrum of polymer **2** measured in D_2O has shown exactly the same pattern as that measured in DMSO in the figure, which indicates that the present polymer does not form micelles in aqueous solution, probably due to the random orientation of both hydrophilic and hydrophobic groups of the polymer. The ^{31}P NMR spectrum in Fig. 1(b) shows small amounts of –O–P–O– and –N–P–N– in addition to the major peak of –O–P–N– in the backbone. The mole ratio, LCST and molecular weight of the synthesized polymers are listed in Table 1.

3.2. Thermosensitivity

It is generally known that the phase transition from a soluble to an insoluble state of thermosensitive polymers in aqueous solution is attributed to a change mainly in the hydrogen bonding interaction between the polymer and the solvent water molecules. The polymers with a LCST are soluble in aqueous solution below their LCST through the strong hydrogen bonding interaction but precipitate at temperatures higher than their LCST due to weakening of the interaction. Therefore, decrease in the hydrophilicity of the polymer causes decrease in the LCST of the polymer.

We have shown in our previous works that the LCST of organophosphazenes can be controlled by appropriate combination of the hydrophilic and hydrophobic groups (Sohn et al., 2004; Kim et al., 2004). In particular, the poly(organophosphazenes) bearing a long chain poly(ethylene glycol) with an average molecular weight larger than 350 and a dipeptide ethyl ester all exhibited LCSTs above 60 °C far higher than body temperature. Therefore, in the present study, shorter chain tri- and tetraethylene glycols were employed to reduce the LCST of the polymer dramatically. As shown in Table 1, polymers **1** and **2** exhibit their LCSTs at 25 and 27 °C, respectively, which are below body temperature and seem to be appropriate for local drug delivery.

3.3. Biocompatibility and hydrolytic degradability

In order to be used as a drug carrier, the polymer itself and the drug-loaded polymer should not show any acute inflammation, and abnormal histological symptoms should be insignificant or reversible. The results of the local tolerance tests of polymer **1** and hGH-loaded polymer **1** are shown in Fig. 2 and Tables 2–4. The photographs of necropsy of the injected area of the rabbit

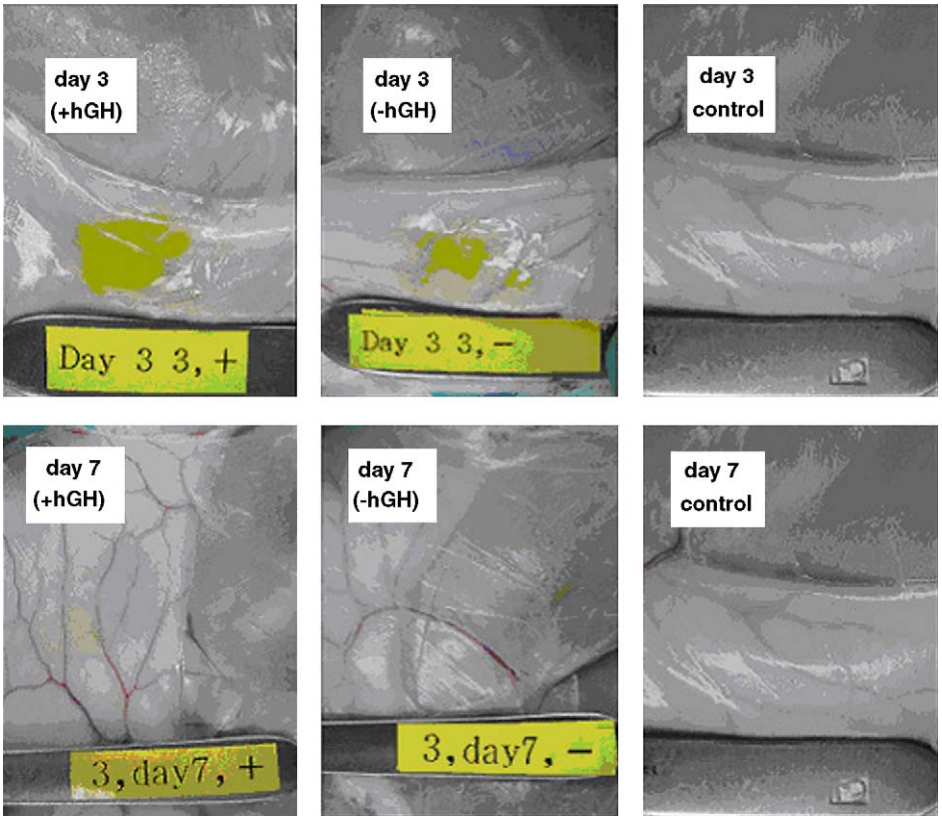


Fig. 2. Photographs of injection areas at 3rd and 7th day after injecting polymer **1** solution loaded (+hGH) and unloaded with hGH (−hGH), and saline (control).

Table 2
Results of histopathological analysis of rabbit skin at 3rd and 7th day post injection of polymer **1** solution with (+hGH) and without (−hGH) human growth hormone

Skin	3 day +hGH		3 day −hGH		7 day +hGH		7 day −hGH		3 day saline		7 day saline	
Findings	1	2	1	2	1	2	1	2	1	2	1	2
Hemorrhage	−	−	−	−	−	−	−	−	−	−	−	−
Hyperemia	−	−	−	−	−	−	−	−	−	−	−	−
Cell infiltration	+	+	−	±	−	±	−	−	−	−	−	−
Edema	−	−	−	−	−	−	−	−	−	−	−	−
Degeneration	−	±	−	−	−	−	±	−	−	−	−	−
Necrosis	−	−	−	−	−	−	−	−	−	−	−	−
Regeneration	−	−	−	−	−	−	−	−	−	−	−	−
Fibrosis	−	−	−	−	−	−	−	−	−	−	−	−
Individual score	1	1	0	0	0	0	1	1	0	0	0	0
Average score	1.0		0.0		0.0		0.0		0.0		0.0	

−: No significant change; ±: very slight; +: slight; ++: moderate; +++: marked.

Table 3
Results of histopathological analysis of rabbit muscle at 3rd and 7th day post injection of polymer **1** solution with (+hGH) and without (−hGH) human growth hormone

Muscle	3 day +hGH		3 day −hGH		7 day +hGH		7 day −hGH		3 day saline		7 day saline	
Findings	1	2	1	2	1	2	1	2	1	2	1	2
Hemorrhage	−	−	−	−	−	−	−	−	−	−	−	−
Hyperemia	−	−	−	−	−	−	−	−	−	−	−	−
Cell infiltration	±	±	−	±	+	−	±	−	−	−	−	−
Edema	−	−	−	−	−	−	−	−	−	−	−	−
Degeneration	−	−	−	−	−	−	−	−	−	−	−	−
Necrosis	−	−	−	−	−	−	−	−	−	−	−	−
Regeneration	−	−	−	−	−	−	−	−	−	−	−	−
Fibrosis	−	−	−	−	−	−	−	−	−	−	−	−
Individual score	0	0	0	0	1	0	0	0	0	0	0	0
Average score	0.0		0.0		0.5		0.0		0.0		0.0	

−: No significant change; ±: very slight; +: slight; ++: moderate; +++: marked.

back in the figure do not show any symptoms on the skin and muscle. A small amount of polymer **1** remained is seen at the injected area in the size of 0.8 cm in diameter at the 3rd day post injection, but finally almost disappeared at the 7th day. The histopathological analysis data of the rabbit skin and muscle listed in Tables 2 and 3, respectively, show a few cases of very slight or slight cell infilteration in monocyte and eosinophil, but in overall exhibit excellent biocompatibility of polymer **1** comparable to the saline solution. In conclusion, as shown in Table 4, there was neither abnormal clinical nor any other particular necropsy findings for both cases of polymer **1** drug-loaded and unloaded, compared with that of the control saline. Therefore, the polymer and the polymer loaded with hGH were proved to be biocompatible.

The hydrolytic degradability of the present polymer **1** was examined in the same way as in our previous work (Kim et al., 2004). Its degradation profile was found to be almost the same as that of poly(organophosphazenes) with pendent groups of PEG350 and glycyl glutamate, and its half life in the neutral buffer solution was approximately 30 days, which may be reduced by introducing a small amount of depsipeptide (Crommen et al., 1992). However, it is more important from the viewpoint of protein drug delivery that complete hydrolytic degradation of the polyphosphazene backbone results in ammo-

Table 4
Summary of the local tolerance test results

Days after injection	Injected sample	Clinical and skin symptoms	Necropsy findings		Remarks
			Skin	Muscles	
3	Polymer **1** (−hGH)	–	Sample remained (0.8 cm)	–	All samples are biocompatible: no difference was found between the test polymer solutions and saline
	Polymer **1** (+hGH)	–	Sample remained (0.8 cm)	–	
7	Polymer **1** (−hGH)	–	Sample faintly remained	–	
	Polymer **1** (+hGH)	–	Sample faintly remained	–	

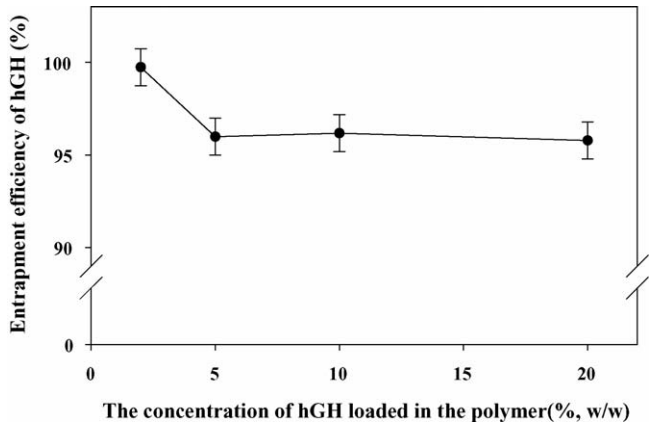


Fig. 3. The entrapment efficiency (%) of hGH depending on its polymer concentration in the aqueous solution of polymer **2** (12.5%, w/v).

nium phosphate (Lee et al., 1999), which is neutral and compatible with protein drugs, in contrast to organic acid residues resultant from degradation of organic acid polymers.

3.4. Entrapment efficiency and in vitro release of hGH

The drug entrapment efficiency by the present polymer **1** depending on its polymer concentration is shown in Fig. 3. The entrapment efficiency (%) of hGH decreases as its polymer concentration increases but exhibited high efficiency of more than 95% even at 20% hGH concentration of the polymer, which is promising for practical application. The mechanism of entrapment of hGH in the present polymer is not clear but seems to be simply due to a hydrophobic interaction between the hydrophobic part of the polymer and the protein drug molecules, since the present polymer does not form micelles, as above-mentioned.

Our target releasing profile of hGH loaded in the polymer carrier was a zero-order release for 1 week with a minimum burst effect and an cumulative release over 90%. Fig. 4 shows the cumulative releasing profile of hGH loaded in 12.5% (w/w) polymer solution. The initial release post injection was 30.8%, which is not serious and acceptable, but most of the drug seems to be released in 3 or 4 days, which is a little faster than we expected. Therefore, along with in vivo study of the present polymer, further study is underway for structural modification.

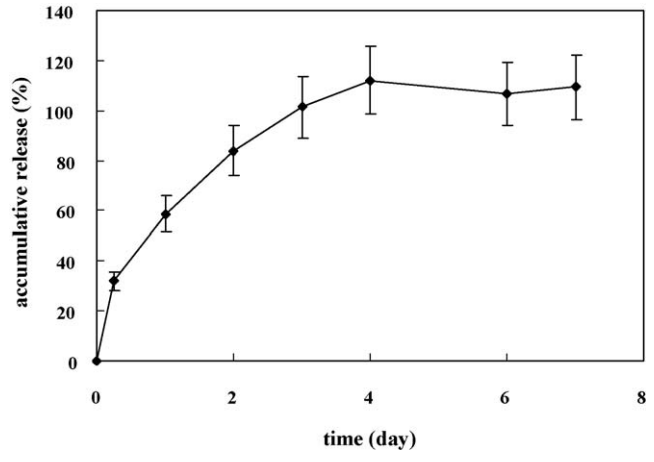


Fig. 4. The cumulative releasing profile (%) of hGH loaded in the polymer **2** solution (12.5%, w/v).

In conclusion, new biocompatible and thermosensitive poly(organophosphazenes) with a LCST below body temperature could be synthesized by introducing short chain tri- or tetraethylene glycol as a hydrophilic group and a dipeptide ethyl ester, $GlyGluEt_2$ as a hydrophobic group into the polyphosphazene backbone. The local tolerance tests using rabbits have shown that the present polymers are biocompatible, and the amphiphilic properties of these polymers seem to be useful for local delivery of hydrophobic drugs such as peptide and protein drugs. In vitro studies performed for loading and releasing of hGH as a model drug using the present polymers showed reasonably good results, and in vivo study is underway.

Acknowledgement

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