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International Journal of Pharmaceutics 319 (2006) 29–36
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Controlled release of doxorubicin from thermosensitive poly(organophosphazene) hydrogels

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Received 6 September 2005; received in revised form 25 January 2006; accepted 27 March 2006
Available online 1 April 2006

Abstract

Thermosensitive poly(organophosphazenes) bearing hydrophobic isoleucine ethyl esters group and hydrophilic α-amino-ω-methoxy-poly(ethylene glycol) of the molecular weight 550 along with hydrolysis-sensitive glycyl lactate ethyl esters have been synthesized for sustained delivery of anticancer drug. The aqueous solution of poly(organophosphazenes) containing doxorubicin, that represents chemotherapeutic agent for cancer treatment, was transformed into hydrogel with good gel strength at body temperature via hydrophobic interactions. Solubility of hydrophobic doxorubicin in the aqueous poly(organophosphazene) solution was dramatically improved as compared with that in PBS (0.01 M, pH 7.4). The hydrogel property of poly(organophosphazenes) was affected on incorporation of doxorubicin, resulting in increase of gelation temperature and decrease of gel strength. The release of loaded doxorubicin from the polymer hydrogel was significantly sustained over 20 days and the effect of gel strength, polymer concentration and drug concentration on the release rate were observed. The release of doxorubicin from the polymer gels was effectively controlled by the gel strength. As a result of investigating anticancer efficacy using cancer cell line of mouse lymphoblast of P388D1, the efficacy of doxorubicin was observed to be constant over a prolonged period of times for more than 30 days, indicating that the release of doxorubicin was sustained for a long time without any initial burst release, and that the delivery system was an excellent candidate for locally injectable gel–depot system.

Keywords: Thermosensitive hydrogel; Poly(organophosphazenes); Biodegradable; Doxorubicin; Locally inject able depot-forming system

1. Introduction

Doxorubicin, a representative anthracycline antibiotic and one of the most widely used anticancer drugs, shows high anti-tumor activity (Foye, 1995). However, the chemotherapeutic compound shows non-site specificity as well as strong side effect, including severe immune suppression, myelosuppression, nephrotoxicity, and cardio toxicity (Lowenthal and Eaton, 1996; Klein-Szanto, 1992). In addition, poor solubility in aqueous solution is problematic and surfactants are required for stability in aqueous state. The efforts to minimize its side effect, to advance the enhanced permeability and retention (EPR) effect, and to increase the poor solubility in aqueous solution have resulted in the developments of various drug delivery systems such as micro encapsulation of drug (Gao et al., 2005), conjugation of drug with polymer (Song et al., 2003), and physically loading drug in hydrogel (Zentner et al., 2001; Ruel-Gariepy et al., 2004).

Focusing on hydrogels, the hydrogels formed by chemical cross linking and swelling process (Gehrke et al., 1998; Yang et al., 2002) involved a cytotoxicity of cross linking agents and an inconvenience of surgical implantation. Another class of hydrogel was designed by thermally reversible gelation property (Chen and Hoffman, 1995; Jeong et al., 1997; Lee et al., 2002; Lee and Song, 2004). After locally injected to specific body site, the solution incorporated with drugs by physical mixing can be instantly converted to hydrogel at the injected site via hydrophobic interaction between hydrophobic moieties in the polymers, and the loaded drugs are slowly released through three-dimensional networks of the hydrogel for a long period. Locally injectable and biodegradable hydrogels have attracted a great interest in contemporary drug research because of a simple systemic formulation for drug delivery. The locally injectable hydrogel also have both dose-dependent and time-dependent effects on solid tumor. That is, when higher concentration of the drug (dose-dependence) stay in limited solid tumor tissue

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doi:10.1016/j.ijpharm.2006.03.032

for a long time (time-dependence), the antitumor activity of anticancer drug is maximized along with minimum side effects. From the viewpoint of this, biodegradable and thermosensitive hydrogel system is superior to other delivery systems such as nano- or micro-encapsulated particles or chemically cross linked hydrogels. Poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) triblock copolymers (Glatter et al., 1994) and poly(*N*-isopropylacrylamide) homopolymers (Chen and Hoffman, 1995) were well known as thermosensitive polymers. However, they have a few inherent defects such as non-biodegradability, initial burst release of drugs loaded from the hydrogels, and cytotoxicity (Desai and Blanchard, 1998; Lim and Sung, 2000). Using poly(L-lactic acid) or poly(lactide-co-glycolide) as a hydrophobic moiety and poly(ethylene glycol) or poly(ethylene oxide) as a hydrophilic moiety, thermosensitive and biodegradable block copolymers have also been reported for drug delivery (Jeong et al., 1997, 1999, 2000; Lee et al., 2001). Insoluble anticancer drugs or protein drugs loaded in the hydrogel were released for a long time (Zentner et al., 2001). Thermoreversible hydrogels like PEG-grafted Spchitosan (Bhattarai et al., 2005a, b) and chitosan/glycerophosphate (Ruel-Gariepy et al., 2000, 2004) were also elucidated.

Recently, the synthesis and characterization of thermosensitive and biodegradable poly(organophosphazene) gels have been reported. The aqueous polymer solutions were reversibly transformed into hydrogels with changes in temperature, and displayed hydrolytic degradation property (Lee et al., 2002; Lee and Song, 2004). Additionally, the most attractive thing is that various hydrophobic, hydrophilic, and other functional substituents can be easily introduced to the polymer backbone, which afford easy controlling of hydrogel characteristics such as gelation temperature, gel strength, and degradability property. Most of poly(organophosphazenes) synthesized exhibited reversible sol–gel transition properties with changes in temperature. The polymer hydrogels showed high gel strength even at relatively low polymer concentration compared with other thermosensitive polymers (Glatter et al., 1994; Chen and Hoffman, 1995; Jeong et al., 1997, 1999, 2000). Besides these good physical properties of poly (organophosphazenes) for drug delivery, thermosensitive polymers play an important role in increasing the solubility of hydrophobic anticancer drugs. The reason is because the hydrophobic moiety in the polymer surrounds drug molecules very effectively as to prevent the attachment of drugs with water molecules, and the hydrophilic moiety in the polymer has good miscibility with water molecules. For example, water-insoluble paclitaxel (4 ug/mL) could dissolve over 10 mg/mL in 23% (w/w) PLGA-PEG-PLGA solution at 4 °C (Zentner et al., 2001). Solubility of hydrophobic anticancer drug in aqueous polymer solution is considered as one of the key factors for the systematic drug delivery formulation. The increased solubility of the hydrophobic drugs in the polymer solutions can avoid the use of additives for increasing the solubility of the drug in an aqueous solution.

In this study, we focused on the release profiles of doxorubicin and anticancer activity of doxorubicin released from poly(organophosphazene) hydrogels. For this, we have synthesized various thermosensitive poly(organophosphazene) gels bearing hydrophobic L-isoleucine ethyl ester (IleOEt) and hydrophilic α-amino-ω-methoxy-PEG with molecular weight of 550 (AMPEG550) along with hydrolysis-sensitive ethyl-2-(*O*-glycyl) lactate (GlyLacOEt). The gelation properties of the aqueous polymer solution with or without doxorubicin were conducted through the viscometric measurement. Release behaviors of doxorubicin from the polymer hydrogels were monitored with respect to the various parameters such as polymer species, polymer concentrations, and drug concentrations. The anticancer activity of released DOX from the hydrogel matrix was measured by counting the dead cell of mouse lymphoblast cell line, P388D1.

2. Materials and methods

2.1. Materials

Hexachlorocyclotriphosphazene was acquired from Aldrich and purified by sublimation at 55 °C under vacuum (about 0.1 mmHg). α-Amino-ω-methoxy-poly(ethylene glycol) with molecular weights of 550 (AMPEG550) was prepared by the published method (Bromberg and Temchenko, 1999). L-Isoleucine ethyl ester (IleOEt) was prepared by the literature method (Greenstein and Winitz, 1961). Ethyl-2-(*O*-glycyl) lactate (GlyLacOEt) was prepared following the method described by the published method (Crommen et al., 1993). Tetrahydrofuran (THF) was dried by reflux over sodium metal and distilled under nitrogen atmosphere. Doxorubicin was purchased from Sigma.

2.2. Preparation of polymers

2.2.1. Synthesis of $[NP(IleOEt)_{1.20}(AMPEG550)_{0.80}]_n$ (1)

Polymer **1** was synthesized similarly by the procedure stated in the previous report (Lee et al., 2002). Poly(dichlorophosphazene) was prepared as described previously (Sohn et al., 1995). In brief the polymer synthesis procedure is as follows. L-Isoleucine ethyl ester hydrochloride (8.11 g, 42.1 mmol) suspended in dry THF (100 mL) containing triethylamine (15.51 g, 153.25 mmol) was added slowly to poly(dichlorophosphazene) (4.0 g, 34.52 mmol) dissolved in dry THF (100 mL). The reaction mixture was stirred for 4 h at 4 °C and then for 48 h at room temperature. After AMPEG550 (26.58 g, 48.33 mmol) dissolved in dry THF (100 mL) containing triethylamine (5.6 g, 55.2 mmol) was added to the polymer solution, the reaction mixture was stirred for 1 day at room temperature and for 1 day at 40–50 °C. The reaction mixture was filtered; the filtrate was concentrated and poured into *n*-hexane to obtain a precipitate, which was reprecipitated twice in the same solvent system. The polymer product was further purified by dialysis in methanol for 2 days and then in distilled water for 2 days at 4 °C. The dialyzed solution was freeze-dried to obtain polymer **1**. Yield: 70%. ^{31}P NMR($CDCl_3$), δ (ppm): 19.6. ^{1}H NMR ($CDCl_3$, spectra not shown), single broad peak appeared at 0.8–1.0 δ (ppm) corresponds to the 6H of two methyl groups in the isoleucine and single broad from 1.1 to

1.3 δ (ppm) corresponds to the 5H of methyl groups of ester and CH_2 group in isoleucine. Single broad peak appeared at 1.3–1.6 δ (ppm) corresponds to 1H of CH group attached to methyl group in the isoleucine. Single broad peak appeared at 1.6–1.9 δ (ppm) correspond to 1H of NH group resulted due to amide linkage of isoleucine with poly(organophosphazene) and shift was observed due to the linkage. Broad single peak appeared at 2.8–3.1 δ (ppm) corresponds to 2H of CH_2 group attached to NH_2 group in the AMPEG. The shifts in the position of the above protons were observed due to amide bond formation between PEG and polyphosphazene back-bone. Doublet was appeared at 3.4 δ (ppm) corresponds to 3H of methoxy group ($-OCH_3$) in AMPEG. A single broad peak at 3.5–3.9 δ (ppm) corresponds to 40H of ethylene groups in AMPEG. A broad peak at 3.9–4.1 δ (ppm) corresponds to 1H of CH group attached to amine group in the isoleucine and broad peak at 4.1–4.3 δ (ppm) corresponds to 2H of CH_2 group in isoleucine ethyl ester ($-CH_2CH_3$).

2.2.2. Synthesis of [NP (IleOEt)$_{1.11}$(GlyLacOEt)$_{0.05}$ (AMPEG550)$_{0.84}$]$_n$ (**2**)

Polymer **2** was synthesized similarly by the procedure stated in the previous report (Lee and Song, 2004). L-Isoleucine ethyl ester hydrochloride (7.50 g, 38.31 mmol) suspended in anhydrous THF (100 mL) containing triethylamine (15.51 g, 153.25 mmol) was added slowly to poly(dichlorophosphazene) (4.0 g, 34.52 mmol) dissolved in dry THF (100 mL). The reaction mixture was stirred for 4 h at 4 °C and then for 20 h at room temperature. To this mixture, triethylamine (0.70 g, 6.90 mmol) and ethyl-2-(*O*-glycyl) lactate ammonium oxalate (0.46 g, 1.73 mmol) dissolved in acetonitrile (50 mL) were added and the reaction mixture was stirred for 19 h in an ice-water bath. AMPEG550 (31.89 g, 58.00 mmol) dissolved in THF (100 mL) containing triethylamine (23.47 g, 231.94 mmol) was added to the polymer solution and the reaction mixture was stirred for 2 days at 40–50 °C. The reaction mixture was filtered; the filtrate was concentrated and poured into *n*-hexane to obtain a precipitate, which was reprecipitated twice in the same solvent system. The polymer product was further purified by dialysis in methanol for 2 days and then in distilled water for 2 days at 4 °C. The dialyzed solution was freeze-dried to obtain pure polymer **2**. Yield: 80%. ^{31}P NMR ($CDCl_3$), δ (ppm): 19.6. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.0 (s, 6H), 1.1–1.3 (b, 8H), 1.3–1.6 (b, 4H), 1.6–1.9 (b, 1H), 2.8–3.1 (b, 2H), 3.4 (s, 3H), 3.5–3.9 (b, 40H), 3.9–4.1 (b, 3H), 4.1–4.3 (b, 4H) and single broad peak appeared at 5.0–5.1 corresponds to 1H of CH group in the GlyLacOEt.

2.2.3. [NP(IleOEt)$_{1.13}$(GlyLacOEt)$_{0.05}$(AMPEG550)$_{0.82}$]$_n$ (**3**)

IleOEt (42.11 mmol), GlyLacOEt (1.73 mmol), and AMPEG550 (50.39 mmol) were used. Yield: 83%. ^{31}P NMR ($CDCl_3$), δ (ppm): 19.6. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.0 (s, 6H), 1.1–1.3 (b, 8H), 1.3–1.6 (b, 4H), 1.6–1.9 (b, 1H), 2.8–3.1 (b, 2H), 3.4 (s, 3H), 3.5–3.9 (b, 40H), 3.9–4.1 (b, 3H), 4.1–4.3 (b, 4H), 5.0–5.1 (b, 1H).

2.2.4. [NP(IleOEt)$_{1.13}$(GlyLacOEt)$_{0.01}$(AMPEG550)$_{0.86}$]$_n$ (4)

IleOEt (41.42 mmol), GlyLacOEt (1.12 mmol), and AMPEG550 (51.77 mmol) were used. Yield: 80%. ^{31}P NMR ($CDCl_3$), δ (ppm): 19.6. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.0 (s, 6H), 1.1–1.3 (b, 8H), 1.3–1.6 (b, 4H), 1.6–1.9 (b, 1H), 2.8–3.1 (b, 2H), 3.4 (s, 3H), 3.5–3.9 (b, 40H), 3.9–4.1 (b, 3H), 4.1–4.3 (b, 4H), 5.0–5.1 (b, 1H).

2.3. Instruments and measurements

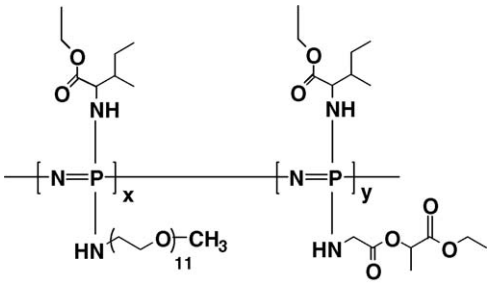
Proton-decoupled ^{31}P NMR spectra were measured with a Varian Gemini-300 spectrometer operating at 121.4 MHz using triphenyl phosphate as an external standard. ^{1}H NMR measurements were made with the same spectrometer operating at 300 MHz in the Fourier transform mode. Aqueous polymer solutions were prepared, dissolving the polymer in phosphate buffered solution (PBS) by continuous stirring at low temperature (4 °C) for 3 days. The viscosity measurements on the aqueous polymer solutions were performed on a Brookfield RVDV-III + viscometer between 5 and 60 °C. The measurements were carried out with a set spindle speed of 0.05 rpm and 1 °C raise in temperature in 3 min. Thermal analysis of the polymers was carried out using Dupont DSC 2100 TA Instruments. The sample was heated at a rate of 5 °C/min in the range of −100 to 40 °C. Gel permeation chromatography was carried out using a GPC system (Waters 1515) with a refractive index detector (Waters 2410). Chromatographic conditions includes the connection of two styragel columns (Waters styragel HR 5E) in line at a flow rate of 0.8 mL/min at a column temperature of 35 °C. THF containing 0.1 wt.% of tetrabutylammonium bromide was used as an eluent. Polystyrenes (M_w: 1140, 3570, 14 100, 28 700, 65 300, 181 000, 613 000, 1 010 000, 2 660 000) were used as standards to calibrate the column.

2.4. In vitro release of doxorubicin

Poly(organophosphazenes) were dissolved in PBS solution (0.01 M, pH 7.4) containing 5 wt.% of lactose at 4 °C and known amount of doxorubicin was added to the polymer solutions. A 0.5 mL of the polymer solution was transferred to millicell (Ø: 12 mm, Millipore) and millicells were incubated at 37 °C for 30 min, in order to transform the solutions into hydrogels. The millicells containing hydrogels were soaked in 10 mL PBS solution pre-heated to 37 °C and incubated in water bath (KMC-1205SW1, Vision, Korea) at 37 °C under mild shaking motion (50 rpm). PBS solutions were periodically renewed with fresh buffer. The amount of DOX released into the buffer was determined using UV–vis spectroscopy at a wave length of 495 nm (Optizen 2120UV, Mecasys, Korea) and the total amount released was calculated from the established standard curve. All processes were carried out under light-hindered conditions.

2.5. Antitumor activity of released doxorubicin

Mouse lymphoblast cell line, P388D1 was grown in RPMI 1640 (Gibco BRL, Life Technologies Inc., USA) supplemented



Scheme 1. Structure of thermosensitive poly(organophosphazene).

with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL, Life Technologies Inc.) and 5% mixture of penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL) (Antibiotic-Antimycotic, Gibco BRL, Life Technologies Inc.). After being sub cultured for 24 h, exponentially growing cell suspensions were distributed into 24 well culture plates at a density of 100 000 cells/1.5 mL. Millicell containing 10 wt.% polymer and 0.2% (w/v) doxorubicin was soaked in the 24 well culture plates filled with pre-cultured cancer cell. The plates were incubated in CO_2 incubator (Sanyo) adjusted to 37 °C in a humidified atmosphere consisting of 50 mL/L CO_2 in air. After 12 and 24 days, the old cultured cells were replaced with the newly cultured cells (density of 100 000 cells/1.5 mL) in order to monitor the release of doxorubicin from the hydrogels. The proliferation and the cell death of P388D1 cells during the period of experiments were monitored by counting cell number at every second day interval. Cell number counting was carried out by following method. Cell suspension was mixed with equal volume of 0.08% trypan blue solution (Sigma Chemical Co., USA). The mixture was transferred to the hemacytometer and viable cells (unstained cells) were counted.

3. Results and discussion

3.1. Characterization of poly (organophosphazenes)

The synthesized thermosensitive poly(organophosphazenes) contain hydrophobic isoleucine ethyl esters (IleOEt), hydrophilic α-amino-ω-methoxy-PEG (AMPEG) and hydrolysis-sensitive glycyl lactate ethyl ester (GlyLacOEt) as substituents as shown in Scheme 1. Four copolymers were prepared with different molar ratios of substituents and its groups by varying the types and molar ratios of substituents. The polymer

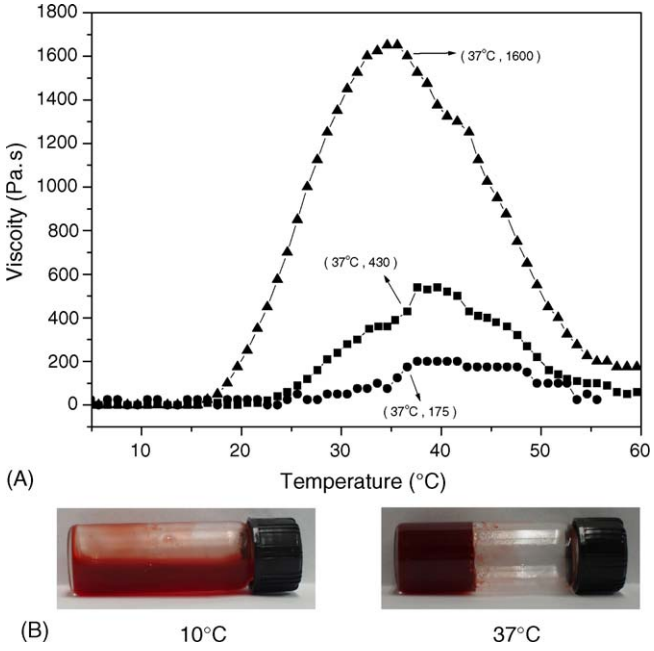


Fig. 1. Change of viscosity of polymer **1** with the various concentrations of the polymer (A). The concentrations were adjusted as 7 wt.% (●), 10 wt.% (■), and 15 wt.% (▲), respectively. The polymer solution with doxorubicin showed reversible gelation behavior at the temperature between 10 and 37 °C (B).

products obtained were characterized by means of multinuclear NMR spectroscopy, DSC, GPC, and rheometer. The polymers are listed in Table 1. The mole ratio of isoleucine ethyl ester, glycyl lactate ethyl ester, and α-amino-ω-methoxy-PEG of the polymer were calculated from the integration ratios of ^{1}H NMR spectra between methyl protons (6H) of the isoleucine ethyl ester, CH (1H) of glycyl lactate ethyl ester, and ethylene protons (40H) of α-amino-ω-methoxy-PEG appearing at 0.8–1.0, 5.0–5.1, and 3.9–4.1 ppm, respectively (spectra not shown). The polymers had almost same glass-transition temperatures (T_g) of −68 °C. The polymers were found to have average molecular weights in the range (1.5–5.7) × 10^4. The polymers were soluble in cold water as well as in several organic solvents such as THF, methanol, and acetone.

The gelation behavior of polymer **1** in pH 7.4 phosphate buffered solution was examined by measuring the viscosity as a function of temperature and polymer concentration was shown in Fig. 1A. The aqueous polymer solution (10 wt.%) which was at low temperature started to become viscous as the tempera-

Table 1
Characteristics of thermosensitive poly(organophosphazenes)[a]

Polymer	Structure	M_w (×10^4)	T_g (°C)	T_{ass} (°C)[b]	T_{max} (°C)[c]	$V_{37\,°C}$ (Pa s)[d]	V_{max} (Pa s)[e]
1	$[NP(IleOEt)_{1.20}(AMPEG550)_{0.80}]_n$	5.7	−68	20	40	430	540
2	$[NP(IleOEt)_{1.11}(GlyLacOEt)_{0.05}(AMPEG550)_{0.84}]_n$	4.4	−68	23	41	65	240
3	$[NP(IleOEt)_{1.13}(GlyLacOEt)_{0.05}(AMPEG550)_{0.82}]_n$	2.1	−67	20	38	80	120
4	$[NP(IleOEt)_{1.13}(GlyLacOEt)_{0.01}(AMPEG550)_{0.86}]_n$	1.5	–	15	33	–	388

[a] Viscosity was measured at 10 wt.% of polymer concentration in the phosphate buffered solution (pH 7.4).
[b] The association temperature at which the viscosity starts to increase sharply.
[c] The temperature at which viscosity reaches the maximum value.
[d] Viscosity at 37 °C.
[e] Viscosity at T_{max}.

ture is raises to about 23 °C (T_{ass}), and its viscosity reaches to maximum at 40 °C (T_{max}). The gel formed at 40 °C was transparent but gradually becomes opaque as the temperature was further raised and then gradually started to shrink by expelling water, leading to a shrunken gel. Beyond this temperature, its viscosity gets lower and finally, a turbid solution was obtained over 52 °C. It has been proposed that gelation of the thermosensitive poly(organophosphazenes) bearing hydrophobic amino acid esters and AMPEG occurs via a physical cross-linking mechanism (Lee et al., 2002; Lee and Song, 2004). Similarly, the gelation of present polymer seems to be attributed to the hydrophobic interaction between the side chain fragments (–CH–(CH_3)–CH_2CH_3) of isoleucine ethyl ester which acts as a physical junction in the polymer solution while hydrogen-bonding interaction between hydrophilic parts of the polymer and water molecules is preserved. The gelation properties of the polymers were dependent on their concentration. The sharpness of viscosity curves and the magnitude of their viscosity increased with increasing concentration of polymer **1** solutions. The viscosity of 15 wt.% polymer solution was sharply increased at 17 °C and attained the maximum viscosity (V_{max}) of 1600 Pa s but the viscosity of the 7 wt.% polymer solutions was slightly increased above 23 °C and its V_{max} was 200 Pa s. Fig. 1B photographically demonstrates the sol–gel transition of polymer **1** solution containing doxorubicin. The polymer solution containing doxorubicin, which was at low temperature (below 15 °C), was observed to change rapidly into gel state at body temperature. The gelation properties of different polymers in PBS solution (10 wt.%) were listed in Table 1. As the molar ratio of hydrophobic IleOEts in the polymer increased, the maximum viscosity (V_{max}) increased but T_{max}, temperature at which viscosity reaches the maximum decreased. These results indicated that the molar ratio of the substituents is major factor to determine discordant gel properties such as T_{max} and V_{max} against the molar ratios, and that fine control of the molar ratio should be required for satisfying ideal V_{max} and T_{max}. Polymer **4** has a V_{max} of 388 Pa s at 33 °C, but no viscosity at 37 °C, can be explained that the hydrogel was rapidly collapsed to form turbid solution on raising the temperature due to breakage of the bonds between hydrophilic AMPEG and water molecules in the hydrogel. Polymers **3** and **4** were shown different T_{max} and V_{max} even though they contain same molar amount IleOEt. This was due to the difference in the molar ratios of GlyLacOEt between the polymers and can be explained that GlyLacOEts have semi-hydrophobic and hydrolysis sensitive properties, so they can be easily located in hydrophobic region. In aqueous polymer solutions the hydrolysis sensitive character of GlyLacOEt dominates the hydrophobic character resulting in the ionization of lactate moiety, which further results in the weakening of rigid inner network structure of hydrogel. Polymer **3** possess high molar ratio of GlyLacOEt compared to polymer **4** and because of the above mentioned reason polymer **3** exhibits high T_{max} and low V_{max} on comparison.

The gelation property of the poly(organophosphazene) was also affected by the amount of doxorubicin loaded in the polymer solution as shown in Fig. 2. As the amount of doxorubicin in the polymer solution increased, increase in T_{max} and the decrease in V_{max} were observed. Because of the above reason, the V_{max} was sharply decreased to half in the case of doxorubicin adjusted to 0.2% in 10 wt.% polymer solution when compared with hydrogel without doxorubicin. It has been confirmed that the solubility of doxorubicin in the polymer solution (PBS, pH 7.4, 10 wt.%) was about 2 mg/mL while that in PBS (pH 7.4) was 0.046 mg/mL. The addition of doxorubicin to the polymer solution resulted in the decrease of V_{max} and increase of T_{max} on increasing the drug concentration and whereas other hydrophobic anticancer drug paclitaxel showed increase in V_{max} (data not shown). The difference in the gelation behavior can be explained that doxorubicin is a hydrophobic chemotherapeutic agent but contains a hydrophilic glucosamine unit in the molecule, so it can be assumed that the hydrophobic part of doxorubicin interacted with hydrophobic region of the polymers but the hydrophilic glucosamine unit of doxorubicin disturbed the hydrophobic interaction between the polymer chains resulted in alteration in the gelation behavior, i.e. increase of T_{max} and decrease of V_{max}.

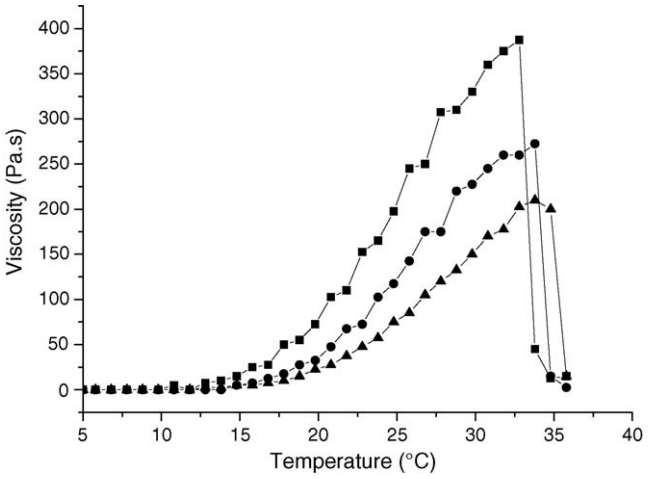


Fig. 2. Change of viscosity of polymer **4** with the various concentrations of doxorubicin adjusted as 0% (■), 0.1% (●), and 0.2% (▲), respectively.

3.2. *In vitro release profiles of doxorubicin*

Fig. 3 shows the release behaviors of doxorubicin loaded in various hydrogels of poly(organophosphazenes) and poloxamer at 37 °C. Significant initial burst release of doxorubicin was observed in the poloxamer hydrogel system despite the use of high polymer concentration 23 wt.%. On the other hand, release of doxorubicin was sustained in all cases of poly(organophosphazene) hydrogels. The release of doxorubicin from the hydrogels of polymers **2** and **3** exhibited similar profiles and it was continued for 20 days. Among the polymer hydrogels, polymer **1** provided excellent control of the release of doxorubicin over 30 days without initial burst release.

This result indicated that the release of doxorubicin from the polymer hydrogels was clearly influenced by their hydrogel strength. As listed in Table 1, the hydrogel viscosities of polymers **2** and **3** were 65 and 80 Pa s at 37 °C, respectively,

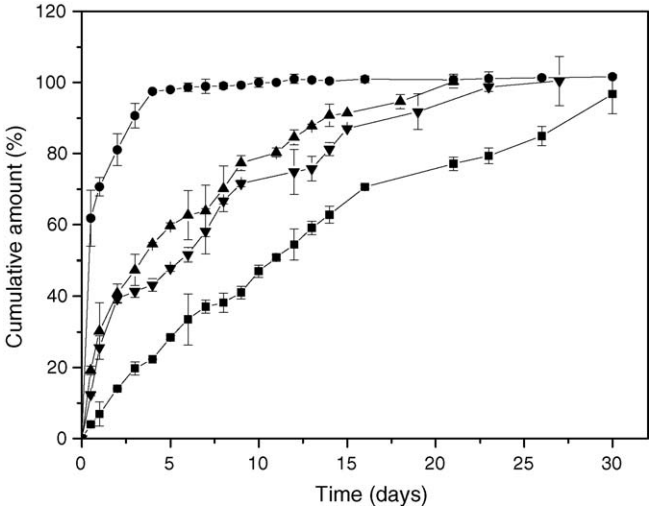


Fig. 3. Cumulative release of doxorubicin from various polymer hydrogels at 37 °C. The concentrations of poloxamer (●) and synthesized poly(organophosphazenes) (polymer **1**: ■; polymer **2**: ▲; polymer **3**: ▼) were 25 and 10 wt.%, respectively. Doxorubicin was dissolved in each polymer solution by 1% (w/v).

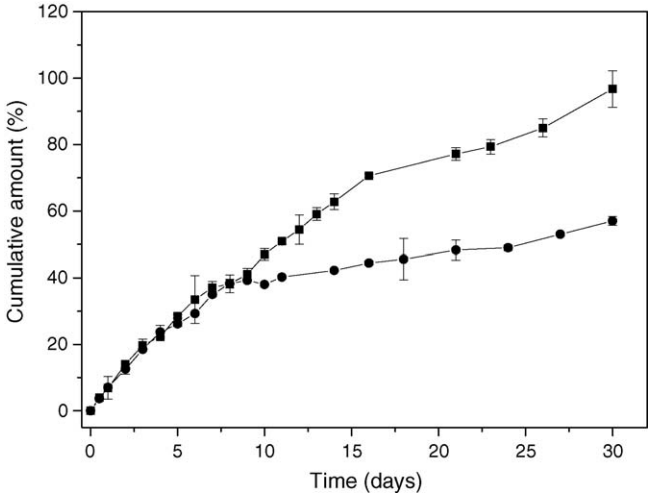


Fig. 5. Cumulative release profile of doxorubicin from the hydrogel of polymer **1** at 37 °C with various drug concentrations (0.2%, ● and 1%, ■). The concentrations of synthesized poly(organophosphazene) was 10 wt.%.

which were quite low compared with 430 Pa s of polymer **1**. In the present hydrogel system, gel strength is closely related to the inner network structure of hydrogel and loaded drugs in the polymer hydrogels were released by diffusion through hydrogels. The more tightly packed polymer hydrogels provides more sustained release of drugs. With respect to the gel strength, similar tendency was observed in the results of Fig. 4. Fig. 4 shows the effect of polymer concentration on the release of doxorubicin from the hydrogels of polymer **1**. The more sustained release of doxorubicin was observed at a higher concentration of polymer **1**. The effect of polymer concentration on the release of doxorubicin was as follows. In the case of 7 wt.% of the polymer concentration, 175 Pa s of $V_{37\,^\circ C}$ was investigated (Fig. 1A), initial burst release was observed and half of the amount of loaded drug was released after 3 days. However, in case of 10 wt.% hydrogel system a viscosity of 430 Pa s at 37 °C was observed, release period was prolonged without initial burst release and a half amount of drug was released after 11 days. When the polymer concentration was 15 wt.%, the $V_{37\,^\circ C}$ was 1600 Pa s and the release behavior was more sustained than any other groups. Moreover, it has taken 14 days for release of half amount of loaded doxorubicin.

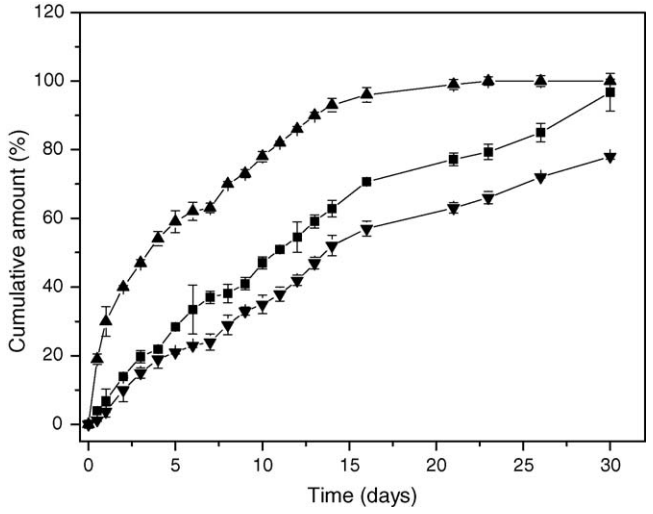


Fig. 4. Cumulative release of doxorubicin from the hydrogel of polymer **1** with various polymer concentrations (7 wt.%: ▲; 10 wt.%:■; 15 wt.%: ▼) at 37 °C. Each hydrogel contained 1% (w/v) of doxorubicin.

Drug concentration was also affected the release behavior as shown in Fig. 5. When 0.2 wt.% of doxorubicin was loaded in the hydrogel, a fast release rate was observed for first 7 days due to the diffusion of drugs on the hydrogel surface or the drugs existed outside of hydrophobic chambers in hydrogel. After 7 days, the release rate was significantly reduced over 30 days. On the other hand, the hydrogel loaded with 1 wt.% of doxorubicin was observed to provide a constant release rate for almost 30 days and the initial release rate for 7 days was similar in the case of 0.2 wt.% of doxorubicin. This result seems to be due to the two possible reasons; one is the change of hydrogel strength with different drug concentration and another is hydrophobic interactions between doxorubicin and polymers. As explained in Fig. 2, doxorubicin plays an important role in the hydrogel strength of the polymer resulted in lowered viscosity at higher concentration of doxorubicin. Therefore, doxorubicin loaded in the hydrogels in the higher drug concentration can be released in a faster rate compared to the lower drug concentration. Additionally, since doxorubicin can hydrophobically interact with the polymer, some amount of drugs may be strongly captured in the hydrogel will be difficult to be released.

3.3. In vitro anticancer efficacy of doxorubicin hydrogel

The anticancer efficacy of doxorubicin hydrogel made of polymer **1** was investigated using P388D1, mouse lymphoblast cell line. In control group (without doxorubicin treatment), the

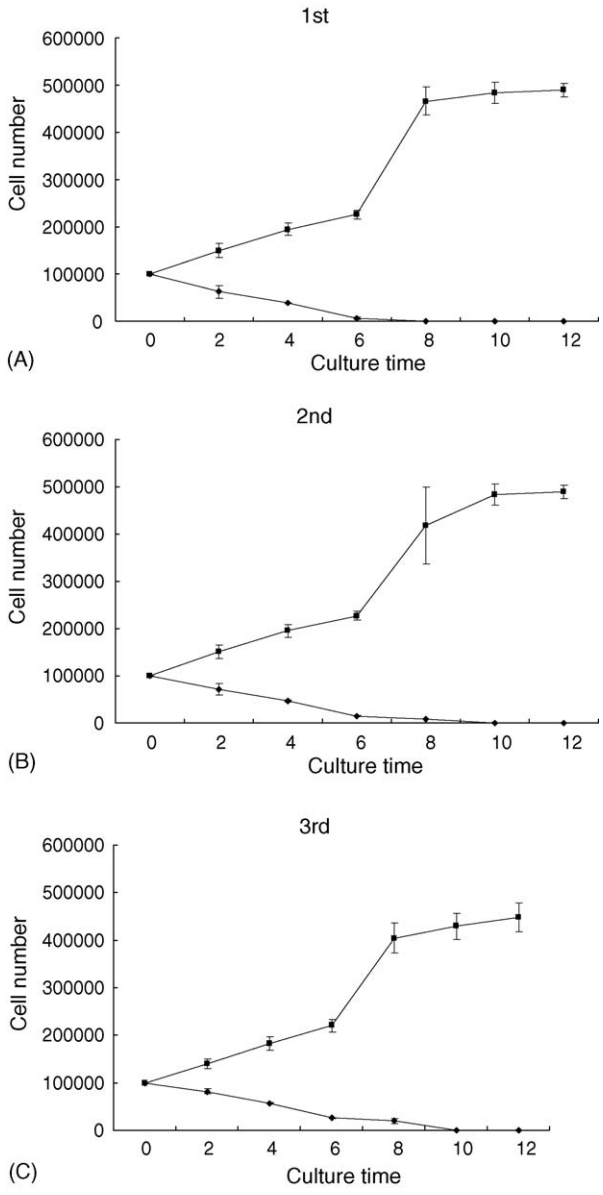


Fig. 6. Anticancer activity of DOX loaded in polymer **1** hydrogel (treated, ● and control, ■) for first 12 days (A), for second 12 days from 13 to 24 days (B), and for third 12 days from 25 to 36 days (C). Cell line was changed by new one, but release system was continued from 1 to 36 days.

cells were found to proliferate continuously without any cell death as shown in Fig. 6. On the other hand, the gradient death of cancer cells were observed in the group treated with hydrogel loaded with doxorubicin. In order to check the release period and its efficiency, cell lines were was replaced by new ones after 12 and 24 days under same doxorubicin loaded hydrogel. As shown in Fig. 6, the death of the cancer cell lines were continued even after 12 days (second group, Fig. 6B) and 24 days (third group, Fig. 6C) of cell culture and death pattern in the later groups were observed similar to that of starting group (first group, Fig. 6A). This result indicated that doxorubicin was released from the polymeric hydrogel in a controlled rate over 24 days without any loss of its antitumor activity.

4. Conclusions

In this work, several different poly(organophosphazenes) were synthesized and they were observed to exhibit thermosensitive reversible gelation property. The poly(organophoaphazene) hydrogel provided the excellent increase of solubility of hydrophobic anticancer drug, doxorubicin, in PBS solution by 40-fold. From the doxorubicin/poly(organophosphazene) hydrogel system, doxorubicin was released in a controlled rate over 20 days. The release of doxorubicin from the polymer hydrogel was being influenced by their gel strength. The rigid hydrogels provided the slower release rate. Antitumor activity of doxorubicin released from the polymer hydrogels was continued for 30 days, evaluated by monitoring the cancer cell line death. From the results, it can be concluded that the thermosensitive poly(organophosphazene) gels can be a candidate for locally injectable drug delivery systems.

Acknowledgements

This research was supported by the Ministry of Science and Technology in Korea (M104140300001-05N1403-00110).

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