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Research paper

Enhancement of sustained and controlled protein release using polyelectrolyte complex-loaded injectable and thermosensitive hydrogel

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ABSTRACT

In this study, we aimed at developing controlled and sustained protein release formulations using a combination system of polyelectrolyte complexes (PECs) and thermosensitive poly(organophosphazene) hydrogels as an injectable gel-depot system. In the protein-loaded hydrogel system, the loaded proteins were released rapidly through diffusion regardless of viscosities and mass loss of the gels because of the small hydrodynamic size of the proteins. To suppress protein diffusion and increase protein size, we induced PECs between negatively charged proteins (BSA, gelatin-type B 75 bloom, α-amylase, and hGH) and polycations (protamine, polyethylenimine, poly-L-lysine, and poly-L-arginine (PLA)) via an electrostatic interaction and loaded the PECs into the hydrogels. The formations of PECs were affected by molecular weight, p*I* (or pK_a), and types of amine group of the used polycations. Unlike other polycations, PLA formed a large uniform complex with BSA, and the PLA/protein complex-loaded hydrogel showed the slowest protein release behavior. In the PEC-loaded hydrogel system, the protein release could also be controlled by viscosities of the gel and weight ratios of polycations and proteins, although the activities of the proteins were decreased in proportion to the PLA amounts. These results suggest that the PEC-loaded injectable and thermosensitive poly(organophosphazene) hydrogel has considerable potential for creating a sustained protein delivery system by using the PEC via electrostatic interaction.

1. Introduction

Protein therapeutics is a rapidly developing area within the biopharmaceutical industry [1]. The use of protein drugs has increased more quickly than that of small-molecule drugs because of their high specificities and fewer adverse side effects [1,2]. However, most protein drugs are frequently administered by the subcutaneous injection due to the inherent instability and extremely short half-life *in vivo* [3–5]. This leads to poor patient convenience, high dose, non-specific toxicity, and increased cost [4]. Therefore, the optimization of pharmacokinetic properties of the proteins is a main issue in this area, and many approaches such as mutagenesis, chemical modification, and sustained delivery system to overcome the obstacles have been suggested [1].

Among these approaches, a sustained protein delivery system can improve the pharmacokinetic properties of the proteins by maximizing the efficacy–dose relationship and decreasing adverse side effects without changes in their own structures of the proteins. The protein drugs are formulated mainly by encapsulation or entrapment in the polymeric delivery system. The most investigated system is poly(lactic-*co*-glycolic acid) (PLGA) microsphere, and it has advantages such as extended release period and biodegradability [6,7]. However, the development of sustained protein delivery system using the PLGA microsphere has been hampered due to the initial burst release of proteins, use of organic solvents, hydrophobic environment, and acidic degradation products [5,6,8–10].

As an alternative delivery system, injectable and thermosensitive hydrogels have been receiving extensive attention because of their temperature-dependent gelation properties without any additional external factors [11]. The advantages of this system are easy administration [12] and simple drug loading without organic solvents as well as their hydrophilicity and compatibility with proteins and living tissues [13,14]. However, the protein release from the hydrogels is generally diffusion controlled, and a long-term sustained release is not expected due to the small hydrodynamic size of the proteins [15].

The combination of more than two different delivery systems such as microsphere and hydrogel can be one of the formulation methods for controlled drug release [16]. The diffusion rate of the drug is controlled by two different release steps: release from

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the microsphere and release from the hydrogel. With this composite system, several groups combined PLGA microsphere and hydrogel together and obtained controlled and sustained protein release profiles, although the microsphere preparation often has low protein loading efficiency and shows protein instability from the use of organic solvents [17–19].

The use of water soluble, biodegradable, and polymeric polyelectrolyte complexes (PECs) instead of the PLGA microsphere can circumvent these limitations. PECs are formed in solution by strong electrostatic interactions between two oppositely charged polyelectrolytes. The preparation method of PEC is less complicated, and the use of water as a solvent is a major advantage as a drug delivery system in humans [20]. This system has many potential applications including gene delivery and surface modification [20,21]. Like genes, some protein drugs with isoelectric points (p*I*) below 7.4 exhibit negative charges at the physiological condition (pH 7.4). Mao et al. successfully induced PECs between chitosan derivatives and insulin [22]. The formations of PECs were influenced by a variety of factors such as the system pH, polymer/protein ratio, and polymer molecular weight. The PEC could protect insulin from degradation and be lyophilized successfully without influencing the complex property. Huang et al. induced PECs using dextran sulfate and various polycations for controlled release of vascular endothelial growth factor (VEGF) [23]. The resulting PECs showed high VEGF entrapment efficiency and controlled release of bioactive VEGF.

Recently, we have examined injectable and thermosensitive poly(organophosphazene) hydrogels as a protein delivery system [24,25]. The poly(organophosphazene) hydrogel showed a temperature-dependent sol–gel transition behavior, and it is known that the degradation products are non-toxic [26–28]. However, some of the entrapped proteins were released rapidly from hydrogel by diffusion. To suppress this initial burst release, we tried to make a complex of protein and chitosan to increase protein size and loaded them into the hydrogel [25]. The electrostatic interactions between negatively charged proteins (pI < 6) and positively charged chitosan decreased the initial release rate, but the initial burst release behaviors of gelatin and BSA were still observed. For this reason, we assumed that because the pK_a value of chitosan is 6.5, the ionic interaction between chitosan and protein was weakened by deionization of the amine group at pH 7.4, producing protein dissociation and diffusion.

Here, we first examined the effects of protein molecular weight, gel viscosities, and gel mass loss on the protein release behaviors from the hydrogels. Second, to inhibit the initial diffusion of proteins, we induced PECs of the negatively charged proteins by mixing with several polycations (protamine, polyethylenimine (PEI), poly-L-lysine (PLL), and poly-L-arginine (PLA)), which have pI (or pK_a) values above 7.4, and then characterized. Third, we loaded the PECs into the hydrogel and evaluated the effects of the types of polycation, gel viscosities, and weight ratios of polycation and protein on the protein release behaviors. Finally, we chose α-amylase and human growth hormone (hGH) as therapeutic proteins and examined the effect of PECs on release kinetics and the activities of released proteins.

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). Poly(dichlorophosphazene) was prepared as described previously [29]. α-Amino-ω-methoxy-PEG (AMPEG) was prepared by a published method [30]. The L-isoleucine ethyl ester hydrochloride (IleOEt·HCl) was prepared according to the literature [31]. Ethyl-2-(*O*-glycyl) lactate ammonium oxalate (GlyLacOEt·AO) was prepared as described by Crommen et al. [32]. Tetrahydrofuran (THF) was dried by reflux over sodium metal and distilled, and triethylamine (TEA) was distilled over barium oxide under dry nitrogen. Bovine serum albumin (BSA, Wako, purity: 95%, Mw 66 kDa), gelatin-type B 75 bloom (GB 75, Mw 20–25 kDa, Sigma), lysozyme grade I (Mw 14 kDa, Sigma), α-amylase from *Bacillus subtilis* (Mw 68 kDa, Sigma), immunoglobulin G (IgG, Mw 150 kDa, Sigma), water-soluble chitosan (Mw 120 kDa, Ja Kwang, Korea), protamine (Mw 7 kDa, Sigma), polyethylenimine (PEI, Mw 25 kDa, Aldrich), poly-L-lysine hydrobromide (PLL, Mw > 70 kDa, Sigma), and poly-L-arginine hydrochloride (PLA, Mw > 70 kDa, Sigma) were used without further purification. Recombinant human growth hormone (hGH) was kindly provided by Chong Ken Dang Pharmaceutical Corp. (Seoul, Korea). In case of hGH, six molar ratio of zinc acetate was added to 10 mg/ml of hGH solution to stabilize hGH. After precipitation, the suspension was lyophilized. All other reagents were purchased from commercial suppliers and used as received.

2.2. Synthesis of poly(organophosphazenes)

All reactions were carried over an atmosphere of dry nitrogen by using standard Schlenk-line techniques.

2.2.1. $[NP(IleOEt)_{1.23}(AMPEG550)_{0.77}]_n$ (**1**)

Polymer **1** was synthesized according to the procedure of the previous report [33]. In detail, IleOEt·HCl (16.21 g, 82.84 mmol) suspended in anhydrous THF (200 ml) containing TEA (46.18 ml, 331.35 mmol) was added slowly to poly(dichlorophosphazene) (8.00 g, 69.03 mmol) dissolved in dry THF (200 ml). The reaction mixture was stirred placed in a dry ice bath for 12 h and then at room temperature for 36 h. To this mixture, AMPEG 550 (60.75 g, 110.45 mmol) dissolved in dry THF (200 ml) containing TEA (61.58 ml, 441.80 mmol) was added to the polymer solution, and the reaction mixture was stirred at room temperature for 12 h and then at 40–50 °C for 36 h. 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 4 days and then in distilled water at 4 °C for 4 days. The dialyzed solution was freeze-dried to obtain polymer **1**. Yield: 69%. ^{31}P NMR ($CDCl_3$), δ (ppm): 18.4. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.0 (s, 6H), 1.1–1.3 (br, 3H), 1.3–1.6 (br, 2H), 1.6–1.9 (br, 1H), 2.8–3.1 (br, 2H), 3.4 (s, 3H), 3.5–3.9 (br, 42H), 3.9–4.1 (br, 2H), 4.1–4.3 (br, 3H).

2.2.2. $[NP(IleOEt)_{1.30}(AMPEG550)_{0.70}]_n$ (**2**)

Polymer **2** was synthesized by the procedure of polymer **1** with poly(dichlorophosphazene) (8.00 g, 69.03 mmol), IleOEt·HCl (16.21 g, 82.84 mmol), and AMPEG 550 (60.75 g, 110.45 mmol) were used. Yield: 62%. ^{31}P NMR ($CDCl_3$), δ (ppm): 19.0. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.0 (s, 6H), 1.1–1.3 (br, 3H), 1.3–1.6 (br, 2H), 1.6–1.9 (br, 1H), 2.8–3.1 (br, 2H), 3.4 (s, 3H), 3.5–3.9 (br, 42H), 3.9–4.1 (br, 2H), 4.1–4.3 (br, 3H).

2.2.3. $[NP(IleOEt)_{1.34}(AMPEG750)_{0.66}]_n$ (**3**)

Polymer **3** was synthesized by the procedure of polymer **1** with poly(dichlorophosphazene) (8.00 g, 69.03 mmol), IleOEt·HCl (18.24 g, 93.19 mmol), and AMPEG 750 (67.30 g, 89.74 mmol). Yield: 70%. ^{31}P NMR ($CDCl_3$), δ (ppm): 18.9. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.0 (s, 6H), 1.1–1.3 (br, 3H), 1.3–1.6 (br, 2H), 1.6–1.9 (br, 1H), 2.8–3.1 (br, 2H), 3.4 (s, 3H), 3.5–3.9 (br, 62H), 3.9–4.1 (br, 2H), 4.1–4.3 (br, 3H).

2.2.4. $[NP(IleOEt)_{1.21}(GlyGlyOEt)_{0.01}(AMPEG550)_{0.78}]_n$ (**4**)

Polymer **4** was synthesized according to the procedure of the previous report [24]. In detail, IleOEt·HCl (4.02 g, 20.54 mmol) suspended in anhydrous THF (50 ml) containing TEA (11.42 ml, 82.15 mmol) was added slowly to poly(dichlorophosphazene) (2.0 g, 17.26 mmol) dissolved in dry THF (50 ml). The reaction mixture was stirred placed in a dry ice bath for 12 h and then at room temperature for 36 h. To this mixture, TEA (0.29 ml, 2.07 mmol) and GlyLacOEt·AO (0.11 g, 0.52 mmol) dissolved in dry acetonitrile (30 ml) were added, and the reaction mixture was stirred at room temperature for 8 h. AMPEG 550 (14.81 g, 26.92 mmol) dissolved in dry THF (50 ml) containing TEA (14.97 ml, 107.69 mmol) was added to the polymer solution, and the reaction mixture was stirred at room temperature for 12 h and then at 40–50 °C for 36 h. 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 4 days and then in distilled water at 4 °C for 4 days. The dialyzed solution was freeze-dried to obtain polymer **4**. Yield: 61%. ^{31}P NMR ($CDCl_3$), δ (ppm): 18.0. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.0 (s, 6H), 1.1–1.3 (br, 6H), 1.3–1.6 (br, 5H), 1.6–1.9 (br, 1H), 2.8–3.1 (br, 2H), 3.4 (s, 3H), 3.5–3.9 (br, 42H), 3.9–4.1 (br, 4H), 4.1–4.3 (br, 5H), 5.0–5.1 (br, 1H).

2.2.5. $[NP(IleOEt)_{1.11}(GlyGlyOEt)_{0.06}(AMPEG550)_{0.83}]_n$ (**5**)

Polymer **5** was synthesized by the procedure of polymer **4** with poly(dichlorophosphazene) (8.00 g, 69.03 mmol), IleOEt·HCl (15.53 g, 79.39 mmol), and GlyLacOEt·AO (0.92 g, 4.16 mmol), and AMPEG 550 (60.75 g, 110.45 mmol). Yield: 66%. ^{31}P NMR ($CDCl_3$), δ (ppm): 18.5. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.0 (s, 6H), 1.1–1.3 (br, 6H), 1.3–1.6 (br, 5H), 1.6–1.9 (br, 1H), 2.8–3.1 (br, 2H), 3.4 (s, 3H), 3.5–3.9 (br, 42H), 3.9–4.1 (br, 4H), 4.1–4.3 (br, 5H), 5.0–5.1 (br, 1H).

2.2.6. $[NP(IleOEt)_{1.17}(GlyGlyOEt)_{0.04}(AMPEG550)_{0.79}]_n$ (**6**)

Polymer **6** was synthesized by the procedure of polymer **4** with poly(dichlorophosphazene) (4.00 g, 34.52 mmol), IleOEt·HCl (7.70 g, 39.35 mmol) and GlyLacOEt·AO (0.46 g, 2.07 mmol), and AMPEG 550 (30.37 g, 55.23 mmol). Yield: 66%. ^{31}P NMR ($CDCl_3$), δ (ppm): 19.2. ^{1}H NMR ($CDCl_3$), δ (ppm): 0.8–1.0 (s, 6H), 1.1–1.3 (br, 6H), 1.3–1.6 (br, 5H), 1.6–1.9 (br, 1H), 2.8–3.1 (br, 2H), 3.4 (s, 3H), 3.5–3.9 (br, 42H), 3.9–4.1 (br, 4H), 4.1–4.3 (br, 5H), 5.0–5.1 (br, 1H).

2.3. Characterization of poly(organophosphazenes)

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 spectral measurements were taken with the same spectrometer operating at 300 MHz in the Fourier transform mode. The viscosity measurements on the aqueous polymer solutions were taken on a Brookfield RVDV-III + viscometer between 5 and 60 °C under 0.1^{-1} shear rate. The measurements were taken with a set spindle speed of 0.2 rpm and with a heating rate of 0.33 °C/min. Molecular weights (Mw) of polymers were measured by gel permeation chromatography (GPC) system (Waters 1515) with a refractive index detector (Waters 2410) and two stryagel columns (Waters styragel HR 4E and HR 5E) connected in line at a flow rate of 1 ml/min at 35 °C. THF containing 0.1 wt.% of tetrabutylammonium bromide was used as an eluent. Polystyrenes (Mw: 1270; 3760; 12,900; 28,400; 64,200; 183,000; 658,000; 1,050,000; 2,510,000; 3,790,000) were used as standards to calibrate the column.

2.4. Preparation of polycation/protein complexes

BSA and GB 75 were used as negatively charged protein drugs for making complexation with polycations. All polycation/protein complexes were prepared in phosphate-buffered saline (PBS) at pH 7.4 and freshly prepared before use. The complexes were prepared at various polycation/protein weight ratios by adding polycation solutions to protein solution with gentle vortexing and incubated at room temperature for 20 min.

2.5. Characterization of polycation/protein complexes

To confirm the complex formation of different polycations and BSA, we performed NATIVE-PAGE using a Bio-Rad Mini-Protean 3 cell electrophoresis system. Formation of complexes was induced at various weight ratios from 0.1 to 50, and the final volume with the 5× sample buffer was 25 μl. Gels were prepared discontinuously with stacking and running gel of 5% and 10% polyacrylamide, respectively. After loading of the complexes onto the polyarylamide gel, electrophoresis was carried out in a constant voltage mode at 80 V using a Bio-Rad power supply in a Tris/glycine buffer at pH 8.3. The gels were stained with Coomassie brilliant blue R-250 staining solution (Bio-Rad, USA) and destained with an aqueous solution of 10% methanol and 10% acetic acid.

The particle sizes of the polycation/BSA complexes were measured using a zetasizer nano particle analyzer (Zetasizer Nano ZS, Malvern, UK). The measurements were taken in triplicate.

2.6. In vitro protein release

Poly(organophosphazenes) and different polycations were dissolved in PBS with stirring at 4 °C. After dissolving, protein solution was added to the polymer solutions with stirring at 4 °C (final concentration of protein: 0.1% (w/v)). In order to transform the solutions into hydrogels, 0.5 ml of the final solution was transferred to a millicell (diameter: 12 mm, Millipore), and the millicells containing the solution were incubated for 10 min at 37 °C. The millicells containing polycation/protein complex-loaded hydrogels were soaked in 10 ml of PBS and incubated in a water bath (KMC-1205SW1, Vision, Korea) at 37 °C, with a mild shaking motion (50 rpm). The PBS was renewed periodically with a fresh buffer. The released protein was quantified via micro BCA (bicinchoninic acid) assays (Pierce, USA), and the total amount of released proteins was calculated from each of the established standard curves. The bioactivity of released α-amylase was measured by the procedure of Sigma (Enzymatic assay of α-amylase, EC 2.1.1.1). The biological binding activity of released hGH was analyzed by a commercial enzyme-linked immunosorbent assay (ELISA) kit (BioCheck, Inc.). All release experiments were conducted in triplicate.

3. Results and discussion

3.1. Characterization of poly(organophosphazenes)

We obtained injectable and thermosensitive poly(organophosphazenes) by a substitution reaction of poly(dichlorophosphazene) with L-isoleucine ethyl ester (IleOEt), α-amino-ω-methoxy-PEG (AMPEG), and ethyl-2-(*O*-glycyl)lactate (GlyLacOEt) (Fig. 1). IleOEt contributes to the hydrophobic interactions occurring between hydrophobic groups of neighboring polymer molecules. AMPEG is a hydrophilic part of the polymer, which forms hydrogen bonds with water molecules. GlyLacOEt is a hydrolysis-sensitive moiety that functions in the acid-catalyzed degradation of the polymer

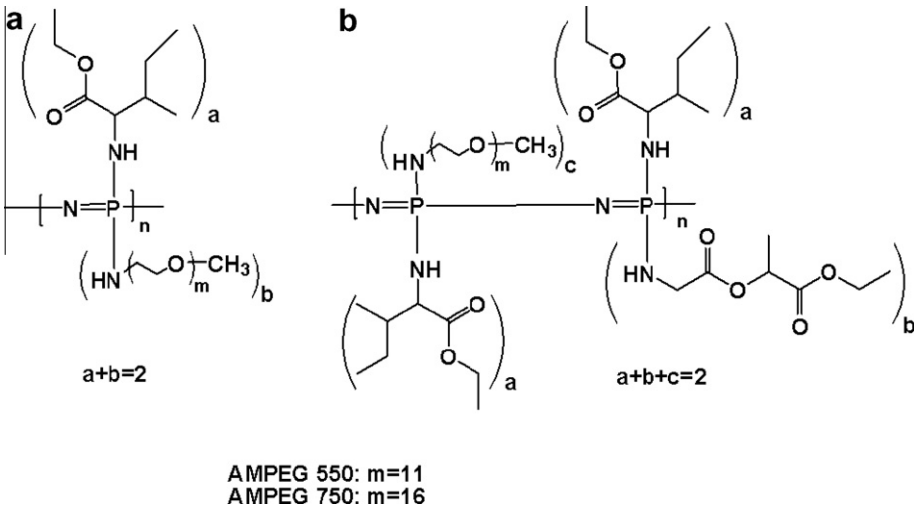

Fig. 1. Structures of synthesized poly(organophosphazenes). (a) Polymers **1**–**3**; (b) polymers **4**–**6**.

[25]. By adjusting the compositions of the substituents, the gelation temperature and degradation rate can be controlled as well.

The synthesized polymers with different substituents and molar ratios are listed in Table 1. The polymers were characterized using NMR spectroscopy, GPC, and viscometer. The molar ratio of the substituents of the polymers was calculated from the integration ratios of ^{1}H NMR spectra between methyl protons (6 H) of the IleOEt, CH (1 H) of GlyLacOEt, and ethylene protons (42 H or 62 H) of AMPEG 550 or AMPEG 750 appearing at 0.8–1.0, 5.0–5.1, and 3.5–3.9 ppm, respectively, according to our previous works [23,24,32]. The polymers exhibited average molecular weights in the range of 1.9–4.3 $\times$ 10^4 g/mol. The polymers were soluble in cold water, and the aqueous solutions of the polymers exhibited sol–gel phase transition properties according to the temperature changes. The gelation properties were controlled by hydrophilic/hydrophobic balances of the polymer composition. Increasing the molar ratio of hydrophobic IleOEt in the polymer increased the maximum viscosity (V_{max}) and decreased the maximum temperature (T_{max}), except for polymer **2**. The longer chain length of AMPEG (AMPEG 750) of the polymer (polymer **3**) required more IleOEt contents to compensate increased hydrophilicity, and this led to high V_{max} and T_{max} of hydrogel.

The hydrophobicity of a loaded drug can change the gelation property as it changes the hydrophobic/hydrophilic ratio. We previously mixed doxorubicin or paclitaxel with the polymer solution and measured gelation behaviors [34]. Doxorubicin increased T_{max} and decreased V_{max} because of the hydrophilic glucosamine, whereas paclitaxel, a hydrophobic drug, increased V_{max}. When we mixed the proteins to the polymer solution, however, the gelation behaviors were very similar with the polymer solution alone. In case of BSA, the gelation behaviors of polymer **5** solutions with 0%, 0.1%, and 0.2% BSA, the same concentrations as doxorubicin did not vary dramatically (Fig. 2), although the V_{max} was decreased by 20 Pa s in both BSA-loaded hydrogels, and T_{max} was increased by 2 °C in the 0.2% BSA-loaded hydrogel. The polymer **5** solutions loaded with lysozyme and IgG also showed the same V_{max} and T_{max}, and almost similar gelation behaviors with the polymer solution alone (data not shown). These results indicate that the prepared polymer hydrogels can be used as an injectable protein delivery system.

3.2. In vitro release of proteins

We examined protein release behaviors from poly(organophosphzene) hydrogels based on the molecular weights of the proteins used and viscosities and mass loss behaviors of the hydrogels. Fig. 3 shows *in vitro* release behaviors of lysozyme, BSA, and IgG, which have different molecular weights (14 kDa, 66 kDa, and 150 kDa, respectively) from hydrogels. As expected, the protein release rate was decreased, and the prolonged release period was observed with increasing molecular weight of the protein. The cumulative released amounts from polymer **5** hydrogel for lysozyme, BSA, and IgG after 2 days were 87%, 61%, and 45%, respectively. However, all proteins showed a rapid initial release behavior, indicating that a precise controlled release is still required.

The influence of gel viscosity on protein release was also investigated using three polymers with viscosities of 1025, 582, and

Table 1
Characteristics of poly(organophosphazenes).

Polymer	Structure[a]	T_{ass} (°C)[b]	T_{max} (°C)[c]	$V_{37°C}$ (Pa s)[d]	V_{max} (Pa s)[e]	Mw ($\times 10^4$)[f]	PDI
1	$[NP(IleOEt)_{1.23}(AMPEG550)_{0.77}]_n$	5.0	37.7	1025	1085	2.6	2.2
2	$[NP(IleOEt)_{1.30}(AMPEG550)_{0.70}]_n$	19.8	34.7	400	405	1.9	1.8
3	$[NP(IleOEt)_{1.34}(AMPEG750)_{0.66}]_n$	7.8	45.9	557.5	652.5	2.2	1.7
4	$[NP(IleOEt)_{1.21}(GlyLacOEt)_{0.01}(AMPEG550)_{0.78}]_n$	10.8	39.8	582	617.5	4.3	2.9
5	$[NP(IleOEt)_{1.11}(GlyLacOEt)_{0.06}(AMPEG550)_{0.83}]_n$	15.8	39.7	177.5	190.0	3.7	2.7
6	$[NP(IleOEt)_{1.17}(GlyLacOEt)_{0.04}(AMPEG550)_{0.79}]_n$	19.6	38.9	187.5	205	2.0	2.0

[a] The substituted ratios were determined by ^{1}H NMR.
[b] The association temperature at which the viscosity starts to increase. Viscosity was measured at 10 wt.% of polymer concentration in PBS (pH 7.4).
[c] The temperature at which viscosity reaches the maximum value.
[d] Viscosities at 37 °C.
[e] Viscosities at T_{max}.
[f] Mw was measured by GPC.

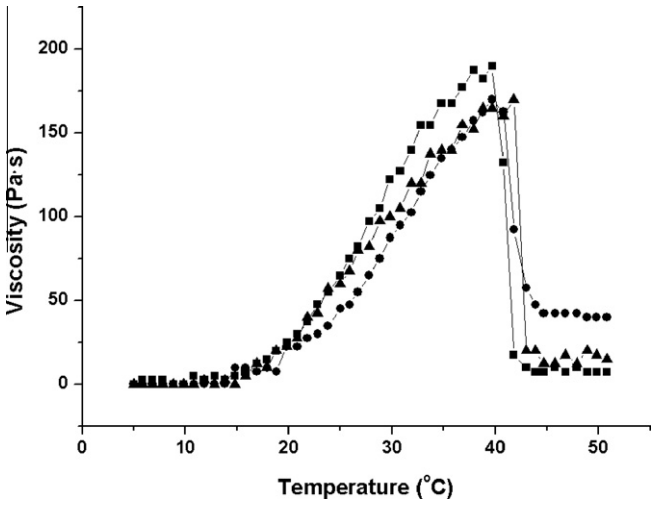

Fig. 2. Viscosity changes of polymer **5** hydrogel with BSA (■: polymer **5** only, ●: polymer **5** with 0.1% (w/v) BSA, ▲: polymer **5** with 0.2% (w/v) BSA).

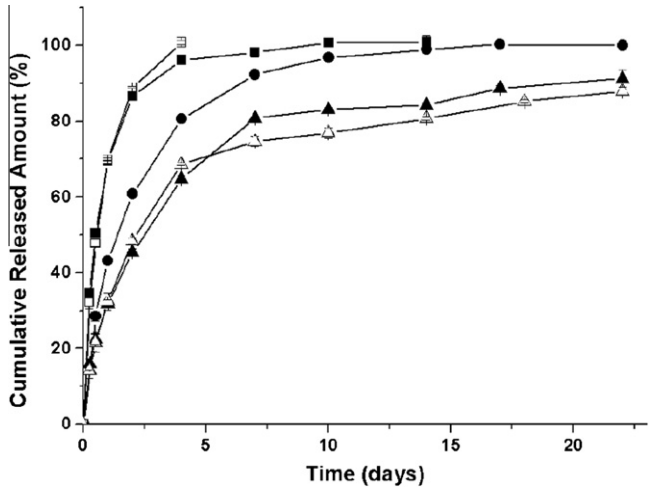

Fig. 3. *In vitro* release behaviors of various proteins from two hydrogels which have different molecular weight of AMPEG. *In vitro* cumulative release behaviors of proteins by time increasing (■: lysozyme from polymer **5** hydrogel, ●: BSA from polymer **5** hydrogel, ▲: IgG from polymer **5** hydrogel, □: lysozyme from polymer **3** hydrogel, △: IgG from polymer **3** hydrogel). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

177.5 Pa s at 37 °C (Fig. 4). We expected to see more sustained release behaviors at the higher viscosities, but the release rates of the three polymer systems were similar, as shown in Fig. 4. Theoretically, no solute diffusion is possible within the hydrogel matrix when the mesh size approaches the size of the solute [35]. This result indicates that the mesh sizes of the hydrogel networks were larger than the hydrodynamic size of BSA (~7.2 nm) [36]. Therefore, BSA was released by diffusion, and the release rate did not decrease, even in the gel with the highest viscosity.

Fig. 3 also indicates that the proteins were released from hydrogels by diffusion. The swelling and mass loss behaviors of the hydrogels were affected by the length of AMPEG. In the polymer **5** hydrogel, which has AMPEG 550, the swelling was observed for 22 days, and then the mass of the polymer was decreased gradually. On the other hand, the polymer **3** hydrogel, which has AMPEG 750, showed faster swelling and mass loss than polymer **5** hydrogel. To examine a relationship between swelling and mass loss properties of hydrogels and protein release, *in vitro* release exper-

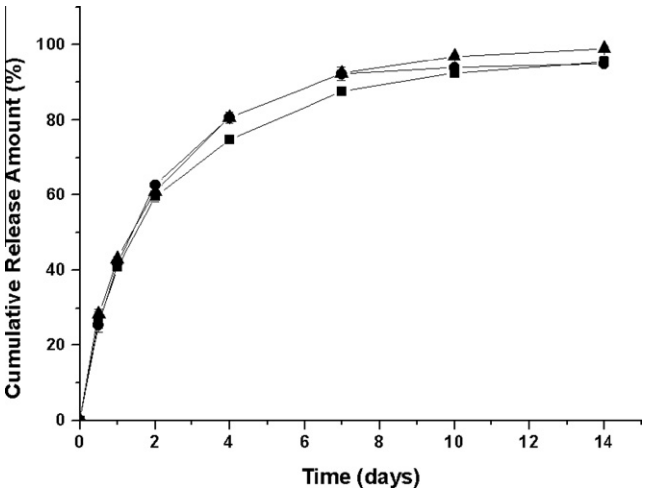

Fig. 4. *In vitro* release of BSA from various hydrogels with different gel viscosities (■: polymer **1** hydrogel, ●: polymer **4** hydrogel, ▲: polymer **5** hydrogel).

iments of lysozyme and IgG were performed using the hydrogels of polymers **3** and **5**. Both hydrogels showed similar release behaviors for both proteins despite different swelling rates and mass loss behaviors (Fig. 3). This result indicates that the mesh sizes of hydrogels are larger than the hydrodynamic sizes of the proteins (lysozyme: 4.1 nm, IgG: 10.7 nm) [36] and the proteins are released by diffusion, regardless of swelling and mass loss of hydrogel. To suppress the initial burst release and obtain sustained release, we prepared polyelectrolyte complexes (PECs) by mixing negatively charged proteins and polycations to enlarge the protein sizes.

3.3. Preparation and characterization of protein-loaded PECs

We chose BSA as a model protein because it is a negatively charged protein under physiological conditions with a p*I* of 5.6. To control the release behavior from hydrogel, we induced polyelectrolyte complexes (PECs) by mixing BSA in PBS at pH 7.4 with polycations such as protamine, polyethylenimine (PEI), poly-L-lysine (PLL), and poly-L-arginine (PLA) and then examined the complex formation, particle size, and any effects on the gelation behaviors. These polycations have different molecular weights and charge densities and have been used in the gene delivery system mainly for gene condensation and nanoparticle formation [37–40].

The complex formation between BSA and polycations was confirmed by a gel retardation assay (Fig. 5). In the previous study, we prepared similar complexes between cationic chitosan and several negatively charged proteins and performed *in vitro* studies of protein [25]. The chitosan/protein complex reduced the initial release rate compared with free protein, but gelatin and BSA still showed an initial burst release. It might be due to the weak ionic interaction between chitosan and the proteins. Because the pK_a of chitosan is 6.5, the ionic interaction between chitosan and the proteins can be weakened at pH 7.4 by the deionization of the amine groups, leading to protein diffusion. To confirm the complex formation at pH 7.4, we induced chitosan/protein complex in PBS at pH 7.4 and performed an electrophoresis assay at various weight ratios of chitosan and BSA. As expected, chitosan and BSA did not form a complex, even at a weight ratio of 50 (Fig. 5a).

In contrast to chitosan, other polycations, which have higher pK_a than 7.4, formed complexes with BSA, but all of the PECs were required considerable positive charges for complex formation (Table 2), and the weight ratio for the complex formation was

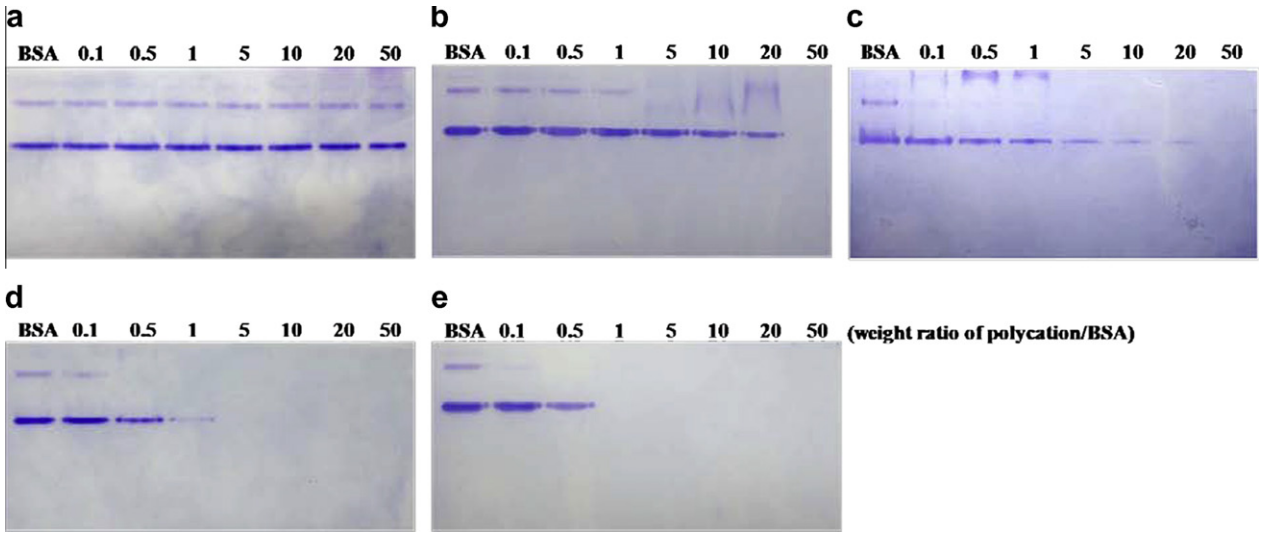

Fig. 5. NATIVE-PAGE for the confirmation of various polycation/BSA complexes. (a) Chitosan; (b) protamine; (c) PEI; (d) PLL; (e) PLA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

varied by polycations used. Protamine and PEI required a polycation/BSA weight ratio of 50 for the complete complex formation, whereas PLL and PLA required a lower weight ratio. Although the weight ratio for the complex formation was the same as in protamine and PEI, the band intensities of BSA were varied at the weight ratios of less than 50. Protamine started to retard BSA migration at a weight ratio of 5, whereas PEI only required a weight ratio of 0.1. This is maybe due to the high positive charges of PEI with high molecular weight (Table 2). In the cases of PLL and PLA, they more strongly blocked BSA migrations than PEI, even though they have a lower amount of positive charge than PEI. This result indicates that the molecular weight of polycation is important for the complex formation with the number of positive charge. In the PECs of PLL and PLA, the complex formation of PLA was a little stronger than that of PLL. This difference might be explained by p*I*. Because arginine has a higher p*I* than lysine, arginine can be ionized more easily than lysine to create stronger ionic interactions with BSA (p*I*: 5.6). The p*I*s of arginine and lysine are 10.76 and 9.74, respectively.

The polycation/BSA complexes were also characterized by measuring their size and distribution. Fig. 6 shows the particle size distribution of polycation/BSA complexes at a weight ratio of 1. The peak around 8 nm corresponds to free BSA. In the protamine, PEI, and PLL groups, multimodal peaks were observed in a broad range, indicating that varied sizes of complexes were formed. With NATIVE-PAGE result, the intensity of free BSA peak decreased with the order of protamine > PEI > PLL. We also measured the particle size of all kinds of PECs at different weight ratios (0.1 and 10), but multimodal size distributions were still observed except protamine/BSA at the ratio of 10, which produced a uniform complex with a hydrodynamic diameter of 1721 nm (data not shown). In

Table 2
Conversion of weight ratio to mole ratio and number of charges.

	BSA[a]	Protamine[b]	PEP[c]	PLL[d]	PLA[d]
Weight ratio	1	1	1	1	1
Mole ratio	1	10.8	2.6	0.9	0.9
Charges at weight ratio of 1 (ca.)	−17	248	512	492	404

[a] The charges were calculated by total number of positively charged residues (Arg + Lys) minus total number of negatively charged residues (Asp + Glu).
[b] The number of charged amino acids was calculated by the result of amino acid analysis.
[c] The charges correspond to the number of primary amines.
[d] The molecular weights were regarded as 70 kDa. The charges were corresponded to all ε-amino or guanidine groups of PLL or PLA.

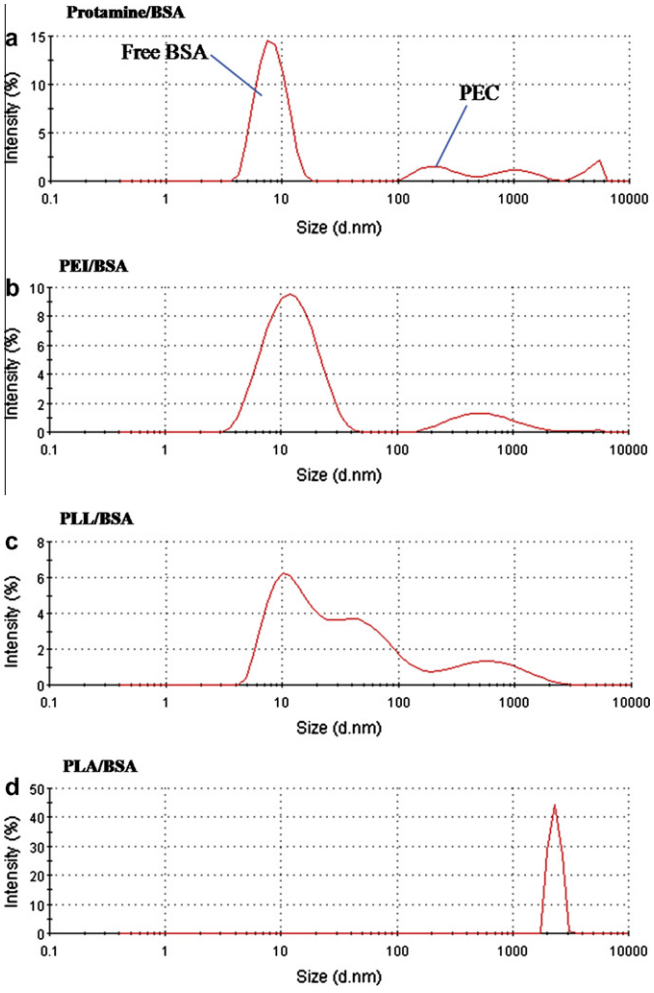

Fig. 6. Particle size distributions of various polycation/BSA complexes at weight ratio of 1. (a) Protamine/BSA; (b) PEI/BSA; (c) PLL/BSA; (d) PLA/BSA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

contrast, the size distribution of PLA/BSA complexes was monomodal at all weight ratios, and the size of the complexes was larger than those of other polycation/BSA complexes. At a weight ratio of 1, the hydrodynamic diameter of the PLA/BSA complex was 6840 nm, the largest particle size.

This result may be due to the high molecular weight and high charge density of PLA. The molecular weight of PLA is 10-times higher than that of protamine, and it has the highest p*I*. The pK_a values of PEI are nine for the primary amine, eight for the secondary amine, and 6–7 for the tertiary amine [41]. Another possible reason may be that higher propensity of arginine form larger clusters than any other amino acids containing aliphatic chain or many methylene groups. Arginines form supramolecular clusters due to their multiple polar interactions and planar structures of the guanidine group, and these clusters help in stacking and having strong interactions with neighboring molecules in the aqueous medium. These clusters also present a hydrophobic surface by the alignment of their three methylene groups. This hydrophobic surface provided by the clusters might also contribute to the formation of large complexes with BSA as well as multiple ionic interactions [42].

As mentioned in Section 3.1, the gelation behavior of a polymer solution can be affected by the hydrophobicity of the loaded drug, and the polymer solution with drugs must become a gel for the sustained protein release at the body temperature. We, therefore, tested the gelation behaviors of the various PECs-loaded polymer **5** solutions (Fig. 7). PEI/BSA complexes showed the same T_{max} but higher V_{max} than hydrogel alone, whereas PLL/BSA and PLA/BSA complexes showed increased T_{max} but similar V_{max}. PEI is a viscous liquid and its structure is highly branched, whereas PLL and PLA are homopolymers of lysine or arginine, the most hydrophilic amino acids, which might influence the gelation behavior. Although arginine exists as hydrophobic clusters in solution, the hydrophobic surface of clusters might be masked by the complexation with BSA.

Protamine/BSA complexes showed higher T_{max} and V_{max} than hydrogel only and the same T_{max} and higher V_{max} than PLL and PLA complexes. The hydrophobic amino acids of protamine such as valine seem to interact with the hydrophobic regions of the polymer, and the hydrophilic amino acids such as arginine allocate hydrophilicity separately, altering the gelation behavior. All groups showed a sol status at low temperature and a gel status at 37 °C, indicating that the PECs-loaded polymers can still be used as an injectable depot system.

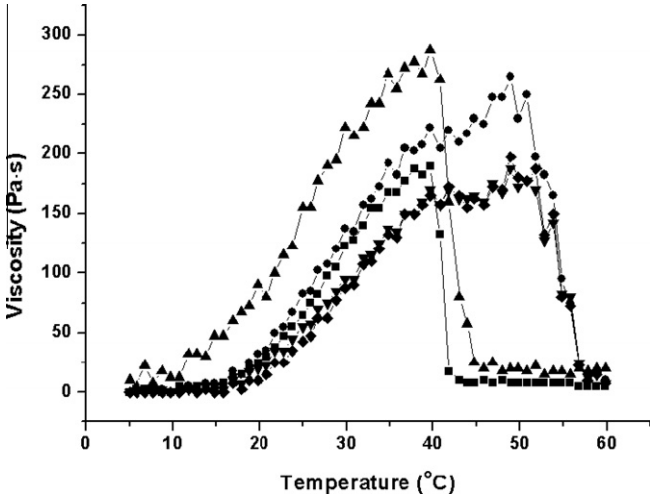

Fig. 7. Viscosity changes in polymer **5** hydrogel according to the loading of the various polycation/BSA complexes (■: polymer **5** only, ●: protamine, ▲: PEI, ▼: PLL, ♦: PLA).

3.4. In vitro release of protein from PEC-loaded hydrogel

The *in vitro* protein release studies were performed to examine the effect of the PECs on the protein release from hydrogel. Fig. 8a shows *in vitro* BSA release behaviors from polymer **5** hydrogel loaded with various kinds of PECs at a weight ratio of 1. Because the largest particle size was observed at a weight ratio of 1 in PLA/BSA complexes, we used this ratio in all kinds of PECs and compared the release rate. All PEC systems showed slower release than BSA alone, and the release rate of BSA was decreased in the order of BSA alone > PEI > PLL > protamine > PLA. The cumulative released amounts of BSA from PEC-loaded polymer **5** hydrogel for BSA alone, PEI, PLL, protamine, and PLA after 2 days were 61%, 38%, 37%, 28%, and 23%, respectively. This result indicates that the release rate is affected by the size and distribution of complexes. The PLA/BSA complexes, which showed a narrow distribution of large particles, showed the slowest release. Otherwise, the release rate was increased with the number of smaller particles, except for protamine. Although protamine/BSA complexes showed the highest intensity of small particles, they also showed the largest particles, and this might decrease the initial burst release of BSA (Fig. 6).

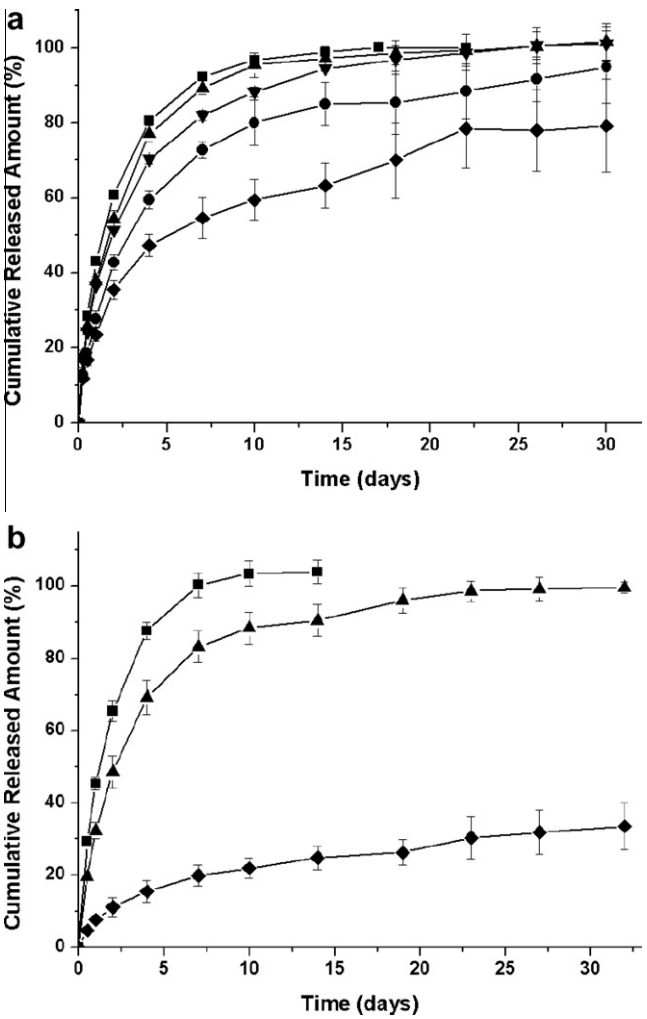

Fig. 8. *In vitro* BSA release behaviors from various polycation/BSA complexes-loaded polymer hydrogels at a weight ratio of 1. (a) Polymer **5** hydrogel (■: BSA only, ●: protamine, ▲: PEI, ▼: PLL, ♦: PLA). (b) Polymer **2** hydrogel (■: BSA only, ▲: PEI, ♦: PLA).

To examine the viscosity effect of hydrogel on the protein release from the PEC-loaded hydrogel system, we also used polymer **2** hydrogel, which has a higher viscosity than polymer **5** hydrogel, with the same complex systems except of protamine and PLL (Fig. 8b). When compared with the result of Fig. 8a, the release rate of BSA alone was slightly increased, while that of PEI or PLA complex systems was suppressed. The cumulative released amounts of BSA from PEC-loaded polymer **2** hydrogel for BSA alone, PEI, and PLA after 2 days were 66%, 48%, and 11%, respectively. Especially, PLA/BSA complexes in polymer **2** hydrogel showed more suppressed release rate than those in polymer **5** hydrogel, perhaps because of different mesh sizes. When the mesh size approaches the size of the solute, no solute diffusion is possible within the hydrogel matrix [36]. Because the viscosity of polymer **2** is higher than that of polymer **5**, the three-dimensional network of the polymer **2** may be tighter than that of the polymer **5** and affect BSA release. The initial release rate of BSA was decreased in the order of particle sizes (BSA alone > PEI/BSA complex > PLA/BSA complex) like the polymer **5** hydrogel.

As shown in Fig. 3, the initial release rate was increased with decreasing protein molecular weight. To control the release rate of low molecular weight of protein, we also performed *in vitro* GB 75 release using PLA and polymer **2** hydrogel, which showed the lowest initial BSA release, at different weight ratios (Fig. 9). GB 75 has a similar p*I* (4.5–5.2) but a threefold smaller molecular weight than BSA. The PLA/GB 75 complexes showed slower release than GB 75 alone, like BSA, but all groups of GB 75 showed faster release rate than those of BSA. BSA alone and GB 75 alone were completely released after 10 days and 4 days, respectively. With PLA, the cumulative release amounts of BSA and GB 75 from the complexes at a weight ratio of 1 after 36 days were 35.3% and 97.1%, respectively. Increasing the weight ratio of PLA also decreased the initial release rate of the protein. When we increased the weight ratio to 10, the GB 75 was released steadily without initial burst release until 36 days. The cumulative released amounts of GB 75 from PEC-loaded polymer **2** hydrogel for GB 75 alone, PLA/GB 75 complex at a weight ratio of 1, and 10 after 2 days were 54%, 42%, and 16%, respectively.

Finally, we chose α-amylase (p*I*: 5.59, Mw: 68 kDa) and human growth hormone (hGH) (p*I*: 5.27, Mw: 22 kDa) as negatively charged therapeutic proteins and performed *in vitro* release studies to examine the effect of PECs on the release kinetics and the activities of released proteins. The PLA was used to form a complex with proteins because of the reason mentioned above. Fig. 10a showed cumulative release behavior of α-amylase. The p*I* and Mw of α-amylase were similar with BSA, but the release behavior was quite different. The PLA decreased the release rate of α-amylase in proportion to the PLA amounts, as the result of BSA, but the difference in the release rate was negligible at the beginning, although that was expanded as time goes on. The cumulative released amounts

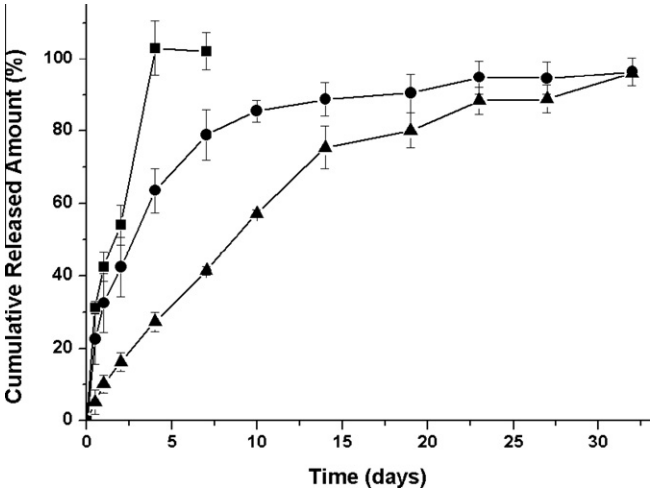

Fig. 9. *In vitro* release behaviors of GB 75 from the hydrogel of polymer **2** at various PLA/GB 75 weight ratios (■: gelatin only, ●: PLA/GB 75 weight ratio of 1, ▲: PLA/GB 75 weight ratio of 10).

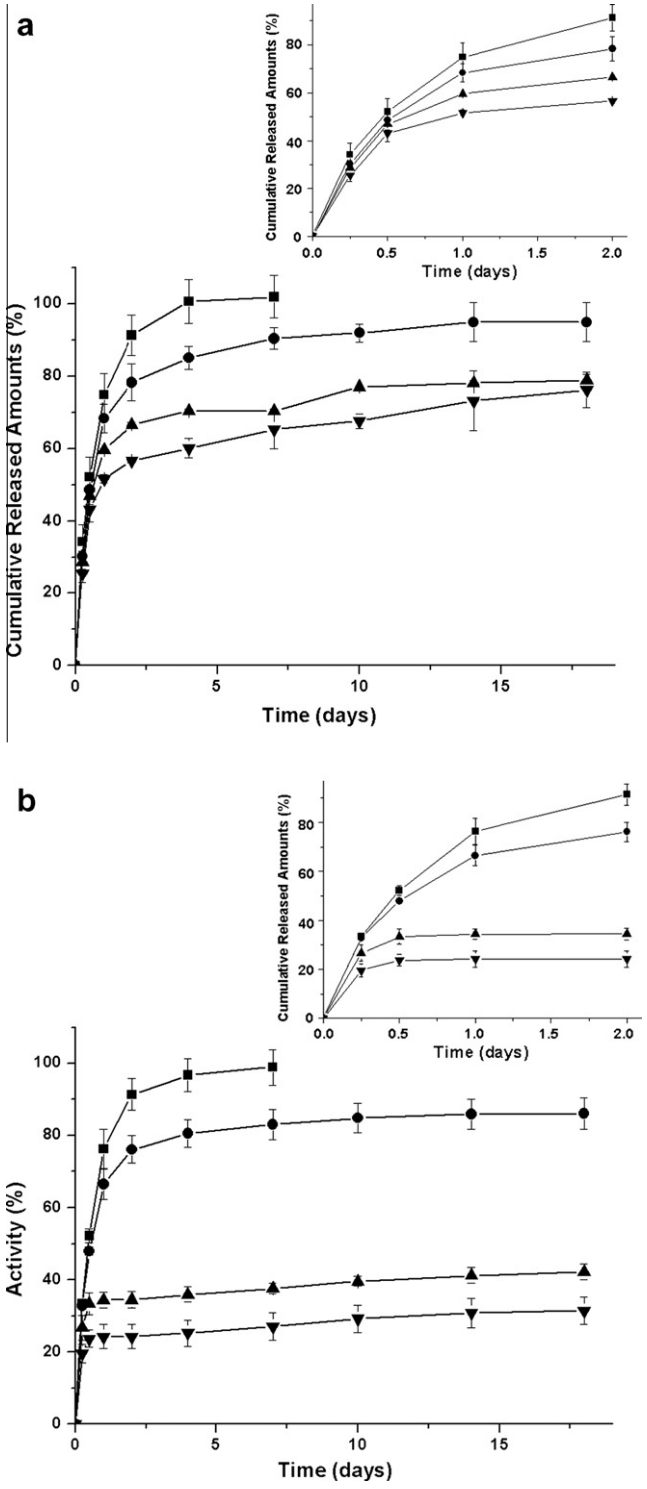

Fig. 10. *In vitro* release behaviors of α-amylase (a) and bioactivities of released α-amylase (b) from the hydrogel of polymer **5** at various PLA/α-amylase weight ratios (■: α-amylase only, ●: PLA/α-amylase weight ratio of 0.1, ▲: PLA/α-amylase weight ratio of 0.5, ▼: PLA/α-amylase weight ratio of 0.75).

of α-amylase from PEC-loaded polymer **5** hydrogel for α-amylase alone, PLA/α-amylase complex at a weight ratio of 0.1, 0.5, and 0.75 after 2 days were 91%, 78%, 67%, and 57%, respectively, which were higher than those of BSA. This difference may be come from their own characteristics of proteins, indicating that the formulation should be oriented to the each protein drug.

The bioactivity of released α-amylase is shown in Fig. 10b. The release behavior of bioactive α-amylase was similar to total α-amylase released. The bioactivities of α-amylase alone and PLA/α-amylase complex at a weight ratio of 0.1 were maintained, relatively. However, from the weight ratio of 0.5, the difference was increased between the amount of released α-amylase and the activities of that. The activities of α-amylase from PEC-loaded polymer **5** hydrogel for α-amylase alone and PLA/α-amylase complexes at weight ratios of 0.1, 0.5, and 0.75 after 2 days were 91%, 76%, 34%, and 24%, respectively.

In case of hGH, PLA suppressed initial release rate and showed controlled release behaviors compared to α-amylase (Fig. 11a). In contrast to the hGH alone, which showed complete release of hGH after 2 days, the PLA/hGH complex at weight ratio of 1 released 31% of loaded hGH. The release period of hGH from PEC was also extended more than hGH alone, and 80% of hGH was released after 14 days. With quantification of totally released amount of hGH, the biological binding activity of released hGH was also quantified by ELISA (Fig. 11b). The release behavior of quantitatively active hGH as a biological functionality was similar with the result of Fig. 11a, but the amount of that was decreased compared to the total released amount of hGH. Among the released hGH, 61% of hGH showed biological binding activity quantitatively after 14 days. It may be due to the associated PLA with the hGH because the hGH alone showed 100% of activities at the same condition. Although the biological binding activity of released hGH from PEC was decreased more than hGH alone, the release period was extended with controlled release rate. These results show that the sustained delivery of charged proteins may be possible by using PEC-loaded hydrogel systems. We are underway for applying this system to therapeutic proteins and evaluating PEC formations, protein release behaviors, and their bioactivities from the released proteins. Furthermore, because the polycations are known to be cytotoxic generally, the biocompatibility test will be performed together for the development of safe and effect delivery system.

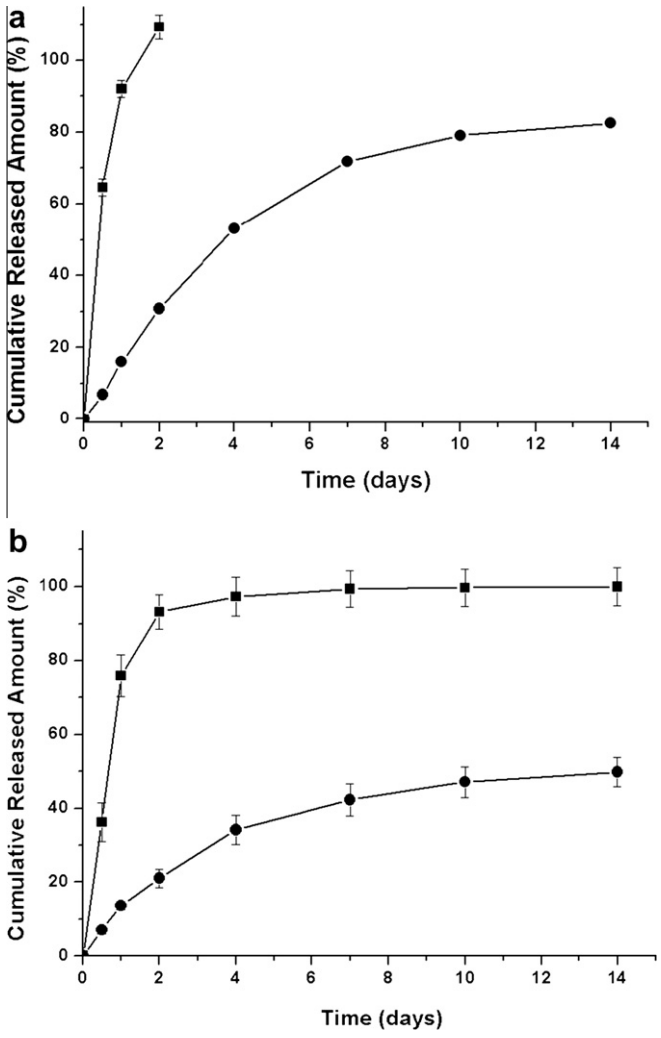

Fig. 11. *In vitro* release behaviors of hGH by BCA assay (a) and quantitatively binding activities of released hGH by ELISA (b) from the hydrogel of polymer **6** (■: hGH only, ●: PLA/hGH weight ratio of 1).

4. Conclusions

We have developed a combined system of polyelectrolyte complex (PEC) and injectable and thermosensitive poly(organophosphazene) hydrogel to suppress initial burst release and improve sustained and controlled protein release. The hydrophilic proteins were readily released by diffusion at the initial time period, and the release rate was increased with small proteins, but the release rate was not affected by viscosities or mass loss of the polymer. To circumvent this problem, we induced PECs between anionic proteins and various polycations. The formations of PECs were affected by the p*I*, molecular weight, and types of amine group of the used polycations. Among polycations, PLA formed the largest complex uniformly with BSA and showed the most suppression of the initial release. In the PEC-loaded hydrogel system, protein release could also be controlled by the viscosities of the gel and the weight ratios of polycations and proteins, although the activities of the proteins were decreased in proportion to the PLA amounts. These results suggest that PEC-loaded, injectable, and thermosensitive poly(organophosphazene) hydrogels have considerable potential as a sustained and controlled protein delivery system. We are underway for applying this system to therapeutic proteins and evaluating PEC formations, protein release behaviors, biocompatibility, and their bioactivities from the released proteins.

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