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Effect of chitosan on the release of protein from thermosensitive poly(organophosphazene) hydrogels

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Abstract

Poly(organophosphazenes) have been suggested as a potential thermosensitive hydrogel for use in the development of an injectable gel-depot system. Under biological conditions, hydrophilic model protein drugs, including bovine serum albumin (BSA), gelatin type B (MW 20,000) (GB20), and fluorescein isothiocyanate albumin (FITC-albumin) loaded in the hydrogels were released for 1–2 weeks, showing an initial burst release. However, this initial burst release could be suppressed when the proteins were couched in a complex with chitosan, and under these conditions evidenced a prolonged release period. BSA, GB20, and FITC-albumin, all of which are negatively charged at a pH of 7.4, interacted with chitosan harboring positive amine groups. The formation of these protein/chitosan complexes were confirmed via measurements of changes in zeta-potential and high-performance liquid chromatography. We determined the appropriate ratio of proteins to chitosan for suppression of the initial burst to be 1:5 to 1:10. From these findings, we were able to conclude that both the release rate and release period could be controlled via the formation of protein/chitosan complex.
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Keywords: Poly(organophosphazenes); Thermosensitive hydrogel; Drug delivery system; Protein/chitosan complex; Ionic interaction

1. Introduction

Recently, remarkable developments in biotechnology have made mass production of bioactive proteins (Persson et al., 2005). In general, protein drugs exhibit an extremely short plasma half-life, due to their clearance via glomerular filtration (Sytkowski et al., 1998), and tend to be unstable *in vivo* due to their denaturation or degradation occurring in the gastrointestinal tract. For this reason, drug delivery systems to protect proteins against external stimuli and maintain the pharmaceutical effects of proteins for extended periods via sustained release mechanism, have been tremendously improved concomitantly to address these issues. Among these drug delivery systems, *in situ* gel-forming systems are a promising choice for the delivery of proteins. Photo-reactive hydrogel, which involves light irradiation (Sawhney et al., 1994; Hill-West et al., 1994) is known to be a very effective system for protein sequestration. However, the *in vivo* irradiation necessary for the process requires a relatively complicated set of procedures. Chemical crosslinking hydrogel, which involve a chemical reaction between two reactive precursors, results in fast transition, but also have been associated with unexpected side reactions and limited selectivity, and also require the immediate injection of the reactive liquid (Elbert et al., 2001). The precipitation method is also fairly favorable in terms of an *in situ* drug depot forming system (Dunn et al., 1994). Highly concentrated PLGA solutions containing protein synthesized from organic solvents may be precipitated by exposure to water, however, disadvantages are associated with this process including the fact that it requires a high concentration of organic solvents and accompanied denaturation of proteins by the organic solvent. Finally, thermosensitive polymer hydrogels are formed from aqueous polymer solutions by changes in temperature, originating primarily from the packing of polymeric micelles or from physical associations between polymer segments in aqueous solution (Jeong et al., 2002). Therefore, thermosensitive polymer hydrogels do not require the use of toxic organic crosslinkers which have in the past been employed in hydrogel formation. In this system, a variety of drugs can be incorporated via simple mixing and the drug-containing solution is injected locally into specific body

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sites. The resulting solution is converted instantly to a hydrogel at the injected site, and the drugs are slowly released via a three-dimensional hydrogel network persisting for extended period. There are several thermosensitive polymers that are sufficient for this system, including the following: copolymers of *N*-isopropylacrylamide (P*Ni*PAAM) (Durand and Hourdet, 1999; Lin and Cheng, 2001), PEO–PPO–PEO triblock copolymer (Poloxamer) (Rassing and Attwood, 1983; Glatter et al., 1994), and PLGA–PEG–PLGA triblock copolymer (Zentner et al., 2001; Chen et al., 2005). Ruel-Gariepy et al. (2000, 2004) have constructed a thermosensitive hydrogel for the local delivery of antineoplastic agents. This hydrogel consists of a chitosan/glycerophosphate complex. PEG-grafted chitosan has also been advanced as a possible injectable thermosensitive hydrogel for use in a sustained protein release system (Bhattarai et al., 2005). In a recent study, we described a set of thermosensitive and biodegradable poly(organophosphazene) hydrogels (Lee et al., 2002; Lee and Song, 2004). These injectable hydrogels can be safely applied as a delivery matrix with a bioactive protein, due to their inherent biocompatibility and inertness with regard to heating, sonication, and organic solvents.

Despite the many advantages of thermosensitive hydrogels, the problem of initial burst release and short-term release period of hydrophilic protein make the development of a thermosensitive hydrogel-based protein delivery system difficult. In most drug delivery systems, the pattern of initial release depends on the rate at which a given drug diffuses through the inner gel pathway. We assumed that the narrowing of the inner gel pathway or the enlarging of the drug itself would both circumvent the issue of initial burst release, as diffusion rates can be disturbed by steric hindrance (Tae et al., 2005; Desai and Blanchard, 1998). Some protein drugs with low isoelectric points ($pI < 6$) exihibit negative electrical charge at the biological condition (pH 7.4). Negatively charged proteins electrostatically interact fairly with positively charged molecules, such as chitosan, that harbor abundant cationic charge in the primary amine groups.

In this study, in order to prepare large molecules containing bioactive proteins, to retard the initial burst, and to prolong the release period, we designed a protein/chitosan complex characterized by ionic interactions between the chitosan and protein. This designed protein/chitosan complex was verified via zeta-potential analysis, HPLC, and light scattering analysis. Thermosensitive gels bearing hydrophobic L-isoleucine ethyl ester (IleOEt), hydrophilic α-amino-ω-methoxy-PEG with a molecular weight of 550 Da (AMPEG550), and ethyl-2-(*O*-glycyl)lactate (GlyLacOEt) as a hydrolysis-sensitive depsipeptide was synthesized, the gelation properties and release behaviors of the polymeric protein/chitosan complexes were constructed by the polymer hydrogels.

2. Materials and methods

2.1. Materials

Hexachlorocyclotriphosphazene was acquired from Aldrich and purified by sublimation at 55 °C under vaccum (about 0.1 mmHg). α-Amino-ω-methoxy-PEG (AMPEG) with molecular weights of 550 Da were prepared by a published method (Bromberg and Temchenko, 1999). The isoleucine ethyl ester was prepared according to the literature (Greenstein and Winitz, 1961). Ethyl-2-(*O*-glycyl)lactate was prepared as described by Crommen et al. (1993). Tetrahydrofuran (THF) was dried by reflux over sodium metal and distilled under a nitrogen atmosphere. Bovine serum albumin (BSA, MW 66,000), gelatin type B (MW 20,000) (GB20), and fluorescein isothiocyanate albumin (FITC-albumin, MW 66,000) were purchased from Sigma and water-soluble chitosan (MW 120,000) was purchased from Ja Kwang (Korea).

2.2. Polymerization

2.2.1. $[NP(IleOEt)_{1.10}(AMPEG550)_{0.88}(GlyLacOEt)_{0.02}]_n$ (**1**)

Poly(dichlorophosphazene) was prepared as described previously (Sohn et al., 1995). Polymer **1** was then synthesized according to the elucidated procedure (Lee and Song, 2004). In details, L-isoleucine ethyl ester hydrochloride (7.43 g, 37.97 mmol) suspended in anhydrous THF (100 mL) containing triethylamine (15.37 g, 151.88 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 (0.28 g, 2.76 mmol) and ethyl-2-(*O*-glycyl)lactate (0.15 g, 0.69 mmol) dissolved in acetonitrile (50 mL) were added, and the reaction mixture was stirred for 19 h in an ice-water bath. AMPEG550 (33.42 g, 60.76 mmol) dissolved in THF (100 mL) containing triethylamine (24.59 g, 243.04 mmol) was added to the polymer solution, 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 polymer **1**. 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, 6H), 1.3–1.6 (b, 5H), 1.6–1.9 (b, 1H), 2.8–3.1 (b, 2H), 3.4 (s, 3H), 3.5–3.9 (b, 42H), 3.9–4.1 (b, 4H), 4.1–4.3 (b, 4H), 5.0–5.1 (b, 1H).

2.2.2. $[NP(IleOEt)_{1.14}(AMPEG550)_{0.84}(GlyLacOEt)_{0.02}]_n$ (**2**)

IleOEt (39.35 mmol), AMPEG 550 (57.99 mmol), and GlyLacOEt (0.69 mmol) were used. Yield: 86%. ^{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, 6H), 1.3–1.6 (b, 5H), 1.6–1.9 (b, 1H), 2.8–3.1 (b, 2H), 3.4 (s, 3H), 3.5–3.9 (b, 42H), 3.9–4.1 (b, 4H), 4.1–4.3 (b, 4H), 5.0–5.1 (b, 1H).

2.2.3. $[NP(IleOEt)_{1.12}(AMPEG550)_{0.86}(GlyLacOEt)_{0.02}]_n$ (**3**)

IleOEt (38.66 mmol), AMPEG 550 (59.37 mmol), and GlyLacOEt (0.69 mmol) were used. Yield: 82%. ^{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, 6H), 1.3–1.6 (b, 5H), 1.6–1.9 (b, 1H), 2.8–3.1 (b,

2H), 3.4 (s, 3H), 3.5–3.9 (b, 42H), 3.9–4.1 (b, 4H), 4.1–4.3 (b, 4H), 5.0–5.1 (b, 1H).

2.3. Instruments and measurements

All reactions were carried over an atmosphere of dry nitrogen by using standard Schlenk-line techniques. 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. A higher resolution NMR spectrometer (Varian UI-500) was used for ^{1}H NMR studies on the phase transition behaviors in the range 5–60 °C. The viscosity measurements on the aqueous polymer solutions were performed on a Brookfield RVDV-III+ viscometer between 5 and 60 °C. Gel permeation chromatography was carried out using a GPC system (Waters 1515) with a refractive index detector (Waters 2410). Chromatographic condition includes The connection of two stryagel columns (Waters styragel HR 5E) connected in line at a flow rate of 0.8 mL/min at 35 °C. THF containing 0.1 wt% of tetrabutylammonium bromide was used as an eluent. Polystyrenes (MW: 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. Preparation and characterization of protein/chitosan complex

BSA, FITC-albumin, and GB20 were selected as hydrophilic and negatively charged model protein drugs. After the dissolution of the proteins and chitosan in distilled water individually, the aqueous protein solution was mixed with chitosan solution at various ratios and stirred for 24 h in a cooling chamber to obtain a homogeneous polymer complex. Zeta-potential analysis (Malvern Zetasizer, Malvern Ins.) and HPLC (Waters HPLC systems) analysis were conducted for physicochemical characterization of the complexes, and to determine its relevant characteristics.

2.5. In vitro release of model protein

Initially the protein/chitosan complex was made in distilled water at mass ratios of 1:0, 1:1, 1:5, and 1:10, and then diluted with phosphate buffer (pH 7.4). Because the direct dissolution of protein and chitosan in phosphate buffer tends to result in the precipitation of chitosan by phosphate anions, thereby resulting in formation of an incomplete or heterogeneous complex. Poly(organophosphazenes) were dissolved in the phosphate buffer solution prepared via the above process in a cooling chamber, the temperature of which had been adjusted to 4 °C. 0.5 mL of the final solution was transferred to a millicell (Ø12 mm, Millipore) and millicells containing the solution were incubated for 30 min at 37 °C, in order to transform the solutions into hydrogels. The millicells containing hydrogels were soaked in 10 mL of PBS buffer and incubated in a water bath (KMC-1205SW1, Vision, Korea) at 37 °C, with a mild shaking motion (50 rpm). The PBS buffer was renewed periodically with fresh buffer. FITC-albumin was analyzed via UV–vis spectroscopy (Optizen 2120UV, Mecasys, Korea) at 495 nm, and the BSA and GB20 were quantified via BCA assays, and the total amount of released proteins was calculated from each of the established standard curves. All release experiments were conducted in triplicate.

2.6. In vitro hydrolytic degradation

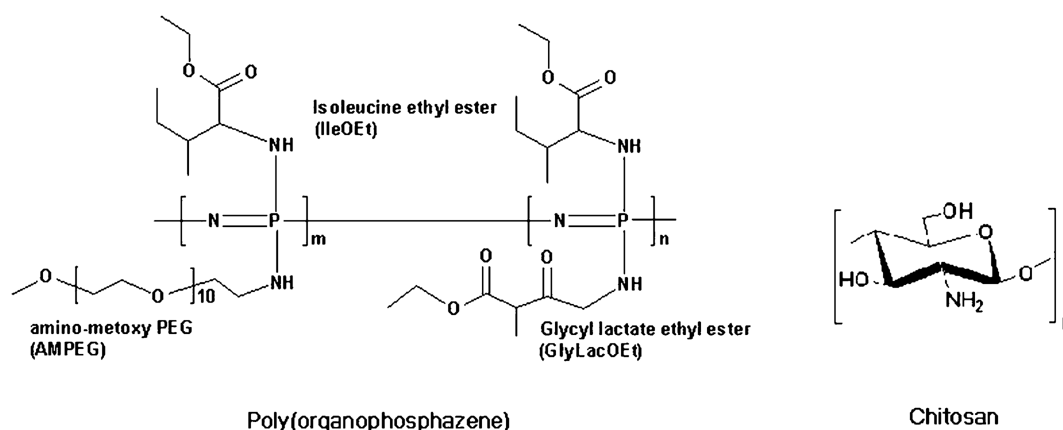
The time-dependent degradation behavior of the polymer **1** was measured in terms of decrease in hydrogel mass and molecular weight. 0.2 g of hydrogel of polymer **1** was soaked in 10 mL of PBS buffer and then incubated at 37 °C. After 1, 3, 7, 14, 30, 45, and 60 days, the hydrogel was lyophilized and weighed. In order to measure the changes in molecular weight, 12 mL of polymer **1** solution (2 wt%) was incubated at 37 °C. One milliliter of the solution was taken after 1, 3, 5, 7, 10, 14, 21, 30, 45, and 60 days and molecular weights of each solutions were measured by GPC.

3. Results and discussion

3.1. Characterization of poly(organophosphazenes)

Poly(organophosphazenes) were synthesized via the substitution reaction of three different groups; hydrophobic isoleucine ethyl esters (IleOEt), which contribute to the hydrophobic interactions occurring between hydrophobic groups of neighboring polymer molecules, hydrophilic α-amino-ω-methoxy-PEG (AMPEG), which attract water molecules, and hydrolysis-sensitive ethyl-2-(*O*-glycyl)lactate (GlyLacOEt), which perform important functions for acid-catalyzed degradation of the polymer (Lee et al., 1999). The chemical structure of the poly(organophosphazene) prepared is shown in Scheme 1. Three different poly(organophosphazenes) were prepared with different the mole ratios of the three substituents and they are listed in Table 1. The obtained polymers were then characterized via ^{1}H and ^{31}P NMR spectroscopies, GPC, and viscometry. All the polymer show temperature-dependent sol–gel transitions and their gelation characteristics are shown in Table 1. The polymer solutions were maintained in an aqueous state up to room temperature, and transformed into the hydrogel up on increasing the temperature above room temperature. The 10 wt% solution of polymer **1** began to show a viscosity increase from 23 °C, and the maximum viscosity (115 Pa s) was observed at 38 °C (Table 1). Polymer **2** followed a behavioral trend similar to that of polymer **1**. The physical property of polymer **3** was stronger than those of the other polymers as evidenced by 425 Pa s of gel strength at 37 °C. Gel strength is intimately dependent on the structure of the inner network. Therefore, the strength of the gel properties increases directly with the density of the inner structure, whereas the pathway size varies inversely with inner structure density.

Fig. 1 shows ^{1}H NMR spectra of polymer **3** at 47 °C in D_2O and room temperature in $CDCl_3$. The gelation property of polymer **3** solution (4 wt%) in distilled water were investigated. T_{ass} and T_{max} for polymer **3** in PBS (10 wt%) were changed from 14 and 37 °C to 37 and 47 °C in distilled water, respectively.



Scheme 1. Chemical structures of poly(organophosphazene) and chitosan.

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

Polymer	Structure	MW (×10^4)	T_{ass} (°C)[b]	T_{max} (°C)[c]	$V_{37°C}$ (Pa s)[d]	V_{max} (Pa s)[d]
1	$[NP(IleOEt)_{1.10}(GlyLacOEt)_{0.02}(AMPEG550)_{0.66}]_n$	4.5	23	38	110	115
2	$[NP(IleOEt)_{1.14}(GlyLacOEt)_{0.02}(AMPEG550)_{0.64}]_n$	2.8	20	38	215	245
3	$[NP(IleOEt)_{1.12}(GlyLacOEt)_{0.02}(AMPEG550)_{0.65}]_n$	1.3	14	37	425	425

[a] Viscosity was measured at 10 wt% of polymer concentration.
[b] The association temperature at which viscosity begins to increase sharply.
[c] The temperature at which viscosity reaches the maximum value.
[d] Viscosities at 37 °C and T_{max}.

This change of gelation property was due to desalting effect and low polymer concentration. Therefore, ^{1}H NMR spectrum of polymer **3** in D_2O was measured at 47 °C. ^{1}H NMR spectra of polymer **3** solutions were dependent on solvents. The ^{1}H NMR spectrum of polymer **3** in $CDCl_3$ showed sharp peaks of isoleucine ethyl ester and depsipeptide ethyl ester at 0.8–1.3 ppm and AMPEG at 3.3–3.7 ppm, respectively. The mole ratios of those substituents were calculated from the integration ratios of the peaks. The ^{1}H NMR spectrum of the polymer in D_2O was quite different from that in $CDCl_3$. The resonance peaks at 0.8–1.3 ppm assigned to methyl protons of isoleucine ethyl ester and depsipeptide ethyl ester were remarkably reduced and broadened, suggesting the hydrophobic association of isoleucine ethyl esters. Amphiphilic block copolymers having phospholipid polymer sequences (Yusa et al., 2005) showed a similar tendency. The resonance peaks at 0.8–2.0 ppm assigned to hydrophobic poly(*n*-butyl methacrylate) block were sharply appeared in methanol-d_4, while they were remarkably reduced in D_2O. These results indicate that the gelation of the present polymer solution is attributed to the hydrophobic interaction between the side chain fragments (–$CH(CH_3)CH_2CH_3$) of IleOEt which act as the physical junction in the polymer aqueous solution.

3.2. In vitro release of model proteins

The release behaviors of hydrophilic proteins, including GB20, BSA, and FITC-albumin from the polymer **1** hydrogel are shown in Fig. 2. After 1 day, the amount of GB20 released was 32%, and 64% of the GB was cumulatively released after 2 days. In the case of BSA, after 1 and 2 days, the cumulative amounts of released protein were 44 and 64%, respectively. The release behavior of the FITC-albumin was observed to be similar to that of BSA after 1 day. However, after 1 day, the release rate of the FITC-albumin was less than that evidenced by the GB20 and BSA. This may be attributable to the differences in the molecular weights and hydrophobicities of the proteins. The molecular weight of the gelatin used in this study was approximately 20 kDa, which is much less than that of BSA or FITC-albumin. Additionally, the hydrophobicity of the FITC-albumin was expected to be much stronger than that of BSA or GB20, as the result of the fluorescein isothiocyanate groups conjugated to the albumin. Accordingly, GB20, with the smallest molecular weight and increased hydrophilicity was released at a rate greater than those of other proteins. BSA, which has the same molecular weight but is more hydrophilic than FITC-albumin, was released at the second-highest rate, and FITC-albumin was released at the least rapid rate. By way of contrast, protein concentrations did not appear to exert significant effects on the release properties of the hydrophilic proteins (data not shown). These results indicated that the hydrophilic proteins which dissolved completely in water, are released freely via diffusion with water from the hydrogel, as the motions of water molecules are not restricted between the inside of the hydrogel and the release medium. Unlike the release behaviors observed at protein conditions and concentrations, hydrogel strength was found to play an important role in protein release profiles (Fig. 3).

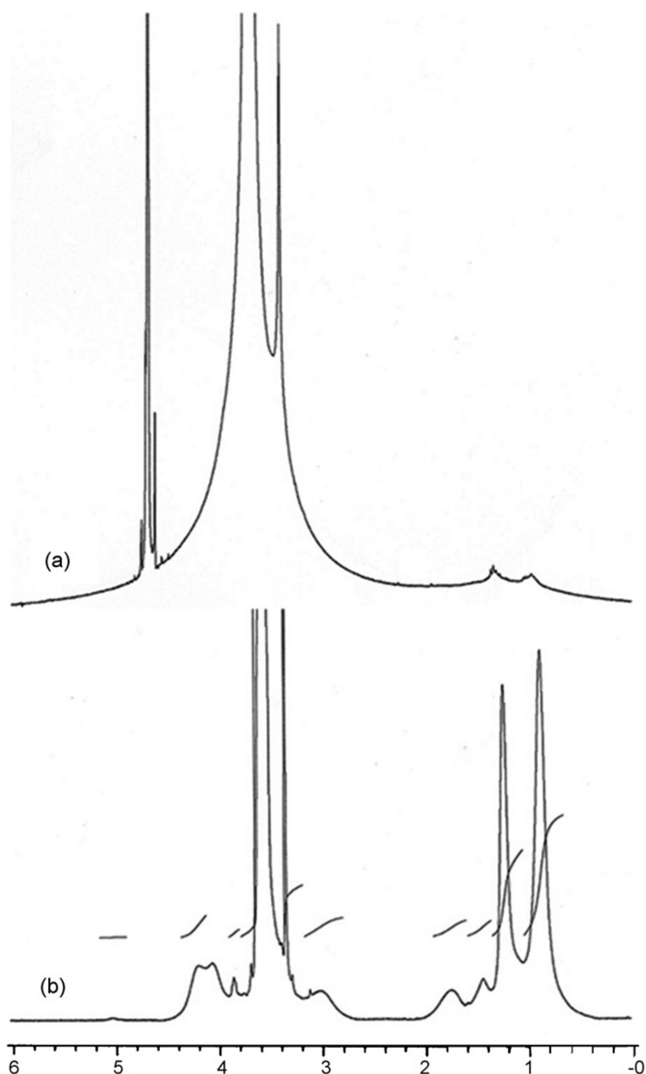


Fig. 1. ^{1}H NMR spectra for polymer **3** solution (3 wt%) in D_2O at 47 °C (a) and in $CDCl_3$ at 20 °C (b).

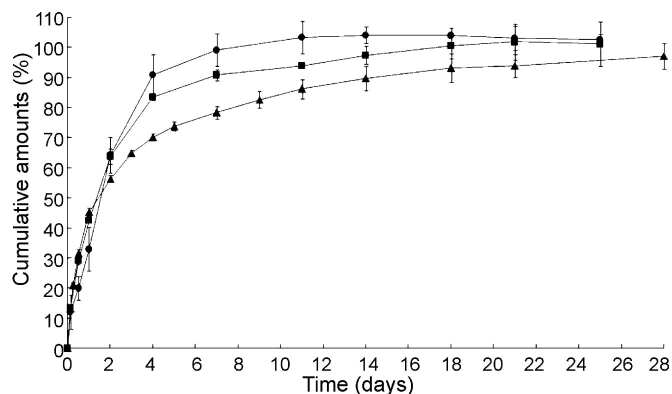


Fig. 2. Release profiles of various proteins, including GB20 (●), BSA (■), and FITC-albumin (▲) from 10 wt% polymer **2** hydrogel at 37 °C. The concentration of proteins was 0.1% (w/v).

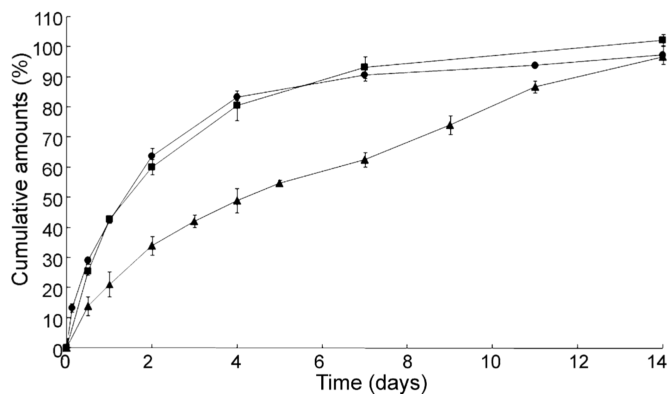


Fig. 3. Release of BSA from the 10 wt% hydrogels of poly(organophosphazenes) 1 (●), 2 (■), and 3 (▲). The concentration of proteins was 0.1% (w/v).

The release behavior of proteins from the polymer **3** delivery system was well controlled and did not exhibit initial burst behavior. The inside of the polymer **3** hydrogel was considered to be fairly tightly packed, relative to the strongest gel property (425 Pa s) among the synthesized polymers. Correspondingly, we expected to see more sustained release behavior with the polymer **2** system than with the polymer **1** system, as the gel strength of polymer **2** (215 Pa s) was higher than the gel strength of polymer **1** (110 Pa s) at 37 °C. However, there was no significant difference between these two polymer systems. This result indicates that the three-dimensional inner network structure in the hydrogel was not sufficiently tightly packed to prevent protein diffusion, although the gel strength was at 215 Pa s. These results showed that if the polymer network structure was highly packed, release properties could be controlled without modulating any other factors. In order to prevent initial burst release and to prolong release period from the hydrogels that were not having enough rigid structure such as the polymer **1** and **2** systems, should also employ other processes, such as the enlargement of the hydrodynamic radii of drugs or the reinforcement of gel strength. In this study, we focused on enlargement of the hydrodynamic radii of proteins via the formation of an ionic complex.

3.3. Preparation and characterization of protein/chitosan complex

The isoelectric point (p*I*) of BSA is 5.6, according to the Swiss-Prot service, and the p*I* of the GB20 used in this study was 4.7–5.2, according to the product description provided by Sigma. This indicates that the electrical charges of these compounds under biologically neutral pH conditions (7.4) are negative. The zeta-potential of FITC-albumin (−26.1) was much lower than that of BSA (−11.7) or GB20 (−13.3), as the result of its fluorescein isothiocyanate conjugated groups that also harbor carboxyl groups. FITC-dextran was also charged negatively, for the same reason, and its zeta potential in PBS was −19.3. The chitosan, with its abundant amino groups as shown in Scheme 1, exhibited a high degree of positive electrical charge in pure water with a high value (81.8). Accordingly, the negative charge of chitosan in the PBS buffer is attributable to the presence of

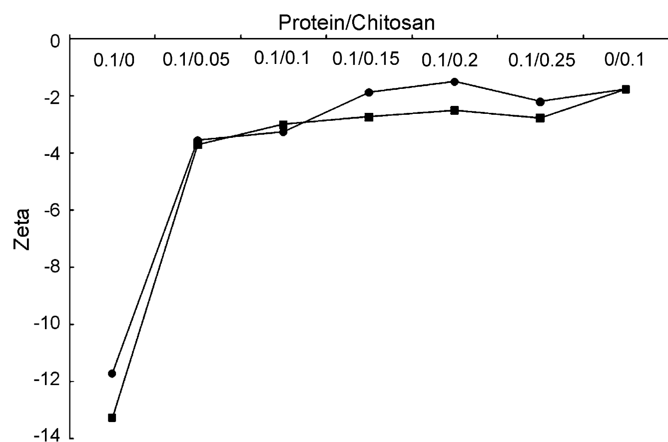


Fig. 4. Changes in the zeta-potentials of BSA (●) and GB20 (■) with increasing amount of added chitosan in PBS (pH 7.4).

phosphate anions. According to these results, negatively charged proteins appear to interact with the positively charged, amino group-rich chitosan in water. If the protein/chitosan complex is prepared via ionic interaction, the complex functions as a different, novel macromolecule, as the polymer complex is not readily dissociated, as is an electrolyte. Whereas free protein molecules can be released at a relatively rapid rate, the release of chitosan-entrapped proteins may be more sustained, as a result of the widening of the protein's hydrodynamic radius of drug. Fig. 4 shows the changes in the zeta-potential of the protein solution, in accordance with the content of added chitosan. As the amount of chitosan added to the solution increased, the zeta-potential values of both the BSA solution and gelatin solution also increased, indicating the formation of a complex between the negative proteins and the positive chitosan. HPLC measurements provided direct evidence against the formation of the BSA/chitosan complex via ionic interaction. Fig. 5 shows changes in the intensity and shifts of peaks in accordance with increases in the amount of BSA. When the BSA and chitosan were mixed in water, the peak corresponding to free BSA was detected at a shifted position (0.7 min), while in pure solution it was detected at 1.5 min. Additionally, the BSA/chitosan complex could be apparently observed at 1.0 min. The intensities of the free BSA and the BSA/chitosan complex increased with increasing amount of BSA. By way of contrast, when differing quantities of chitosan were added to BSA solution, similar results were obtained (data not shown). As the amount of added chitosan increased, intensity of free BSA decreased, indicating that the formation of BSA/chitosan complex increased with increasing the amount of added chitosan. Also, new complicated peaks were seen in conjunction with increases in the quantity of added chitosan. According to theses results, it could be concluded that some portion of negatively charged proteins in water had interacted with the positively charged chitosan to form a complex, and that this complex was stable, not easily dissociated in water. Also, in the delivery system, the diffusion rate of protein was expected to be effectively sustained and the initial burst release problem was expected to be obviated.

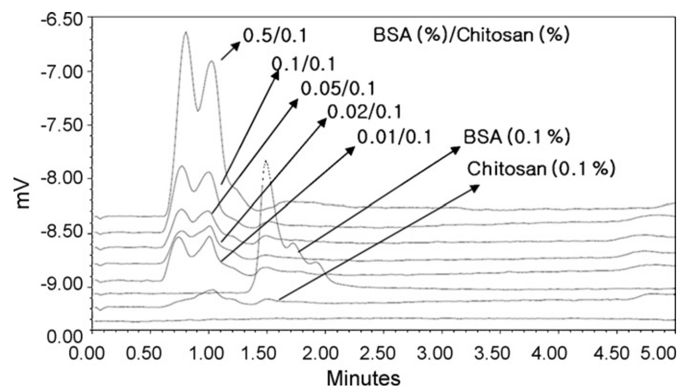


Fig. 5. Formation of the protein/chitosan complex with variable concentrations of BSA and chitosan, via the HPLC measurement.

3.4. Sustained release of protein via formation of complex

As per our expectations, the design of the protein/chitosan complex was partially successful in sustaining the release rate and preventing the problem of initial burst release. When the GB20/chitosan complex was prepared, no significant effects on release behavior were observed in conjunction with the formation of the chitosan complex during the initial release period (Fig. 6). After 1 day, however, the cumulative amounts of released GB20 from the free protein system and the chitosan/GB20 complex system were 33 and 29%, respectively. As time progressed, the release rate of the complex system was observed to decline substantially. After 4 days, the cumulative release amounts of the complex system was 68% (Chitosan:GB20 = 5:1) and 73% (1:1), but the cumulative release amount of free GB20 system was 91%. In the case of the BSA/chitosan complex delivery system, the effect of chitosan on the release rate of BSA release rate was similar to that of GB20 with regard to initial fast release rate, later slow release rate, and periodical cumulative release amount, as is

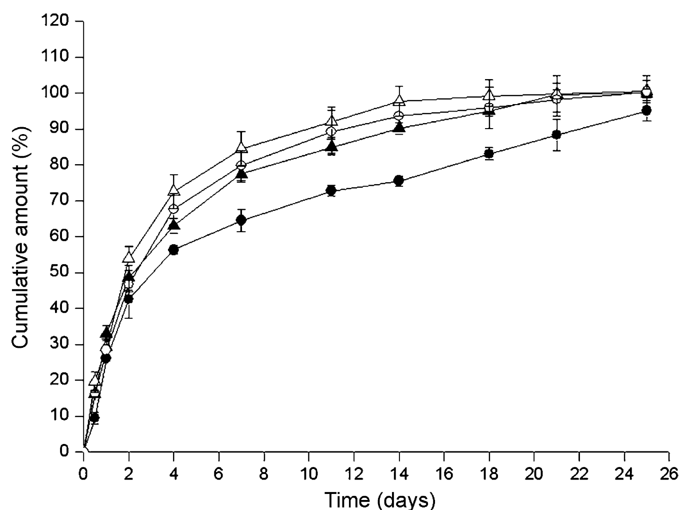


Fig. 6. Effects of chitosan on the release of GB20 and BSA from polymer **1** hydrogel. The weight ratios of GB20 and chitosan were 0.1/0.1 (△), and 0.1/0.5 (◯). The BSA and chitosan weight ratios were 0.02/0.1 (▲), and 0.02/0.2 (●).

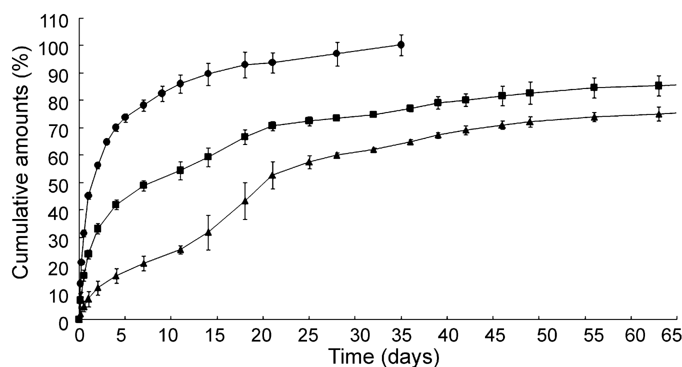


Fig. 7. Effect of chitosan on the release of FITC-albumin from polymer **1** hydrogel. The protein and chitosan weight ratios were 0.1/0 (●), 0.1/0.1 (■), and 0.1/0.5 (▲).

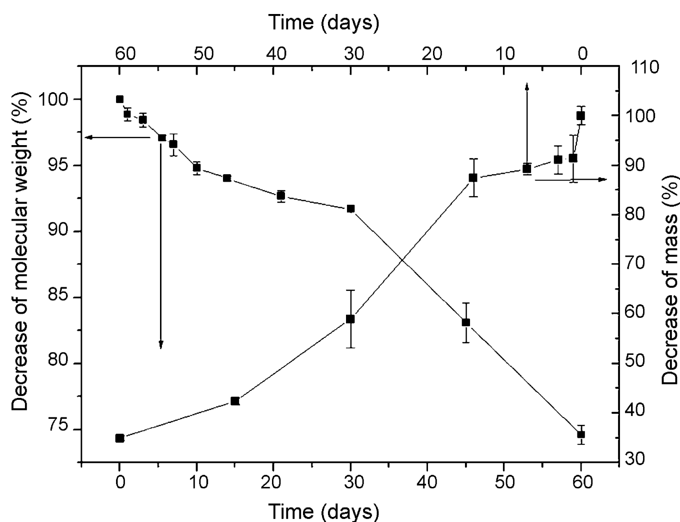


Fig. 8. Hydrolytic degradation properties of polymer **1** in PBS (pH 7.4, 37 °C).

shown in Fig. 6. When the ratio of chitosan:BSA was 10:1, half of the BSA was released in approximately 3 days, and the BSA was slowly released another 4 weeks. We previously discussed the fact that the molecular weight of BSA (66 kDa) was higher than that of gelatin (20 kDa), the release behavior of BSA was sustained to a greater degree than the release behavior of GB20 in the free protein delivery system. However, it was observed no significant difference in release behavior between the BSA/chitosan and GB20/chitosan systems. From the reports of dynamic light scattering study, the hydrodynamic volume of chitosan was 1000–1500 nm (Kang et al., 2004), while that of BSA was known as 3.6 nm (Tae et al., 2005), therefore the hydrodynamic volume of the complex was expected to be affected by chitosan to a greater degree than by proteins. As different from Fig. 6 results, significant effect of chitosan on sustaining release rate of FITC-albumin was investigated. Fig. 7 shows that 7 days were required for a half-release at an FITC-albumin:chitosan ratio of 1:1, whereas a half-release of free protein was achieved in only 2 days. As well, when the weight ratio of protein and chitosan was 1:5, 20 days was required for a half release. From the aforementioned zeta-potential values of proteins in PBS, FITC-albumin is more negatively charged in water than other proteins, and therefore, it could interact strongly with chitosan via ionic charges, thereby forming the most stable complex among the protein/chitosan complex systems. In the FITC-albumin/chitosan complex system, the release mechanism was characterized in terms of the initial diffusion and secondary degradation of the hydrogel. The referenced initial diffusion was attributable to the free protein that did not participate in complex formation, and the second step resulted from the hydrolytic properties of the poly(organophosphazenes). Fig. 8 illustrates the time-dependent decreases of molecular weight and gel mass for polymer **2** in the PBS buffer solution (10%) of pH 7.4 at 37 °C. Significant decreases of molecular weight and gel mass were observed along with time and decrease of gel mass was faster than that of molecular weight. About 50% mass decrease was attained after 30 days incubation, while about 10% molecular weight decrease was observed under same condition. This result seems to be due to the hydrolysis of pendant depsipeptide ethyl ester (ethyl-2-(*O*-glycyl)lactate) of polymer **2** which caused to gel dissolution. It has been reported that depsipeptide ethyl ester is more hydrolysis labile than amino acid esters. The carboxylic acid group generated by the hydrolysis of depsipeptide ester group resulted in Tmax increase and backbone cleavage. The basic charged protein/chitosan complex may not increase the degradation of polymer gel.

4. Conclusion

In a thermosensitive hydrogel system, hydrophilic protein were readily released exhibiting an initial burst behavior as the result of the weak physical properties of this system compared to those of other delivery systems. In order to circumvent this problem, we prepared a set of protein complexes formed via ionic interaction, using negatively charged proteins and positively charged chitosan. This complex existed as a stable macromolecule in water, and the release rates of the complex-entrapped proteins were found to be more precisely controlled than in the free protein delivery system. As the charge interval between proteins and chitosan increased, the complex became more tightly packed, and the release behavior was sustained more effectively. According to these results, the protein–polymer complex system can be an effective tool for sustained protein delivery.

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