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Human growth hormone-loaded nanogels composed of cinnamoyl alginate, cinnamoyl Pluronic F127, and cinnamoyl poly(ethylene glycol)

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ABSTRACT: Cinnamoyl alginate (CinAlg), cinnamoyl Pluronic F127 (CinPlu), and cinnamoyl poly(ethylene glycol) (CinPEG) could be self-assembled into nanogels, possibly due to hydrophobic interaction among cinnamic acid (CA) residues. The nanogels were nearly spherical particles on TEM photographs. The photodimerization of CA residue had little effect on the integrity and the size of nanogel. The stability of the nanogels in blood plasma for 48 h at 37°C did not depend on UV irradiation, however they were stable in deoxycholate solution (1 and 10 m*M*) only when they were UV-treated. Human growth hormone (hGH) could readily be loaded in the nanogel under an acidic condition possibly due to the electrostatic interaction between hGH and CinAlg. hGH hardly released from the nanogel at body temperature (37°C) when the release medium was at acidic condition (e.g., pH 3.0, pH 5.0; <10% in 170 h). However, it readily released at the same temperature when the medium was at physiological pH (e.g., pH 7.4; around 45% in 170 h) and at an alkaline condition (e.g., pH 9.0; >50% in 170 h). However, slow release of hGH was observed for 100 h possibly due to the layer of thermally condensed Pluronic F127 chains. In addition, initial burst release was suppressed as the CinPlu content increased. For example, the release degree at pH 9 in 60 min decreased from 15.3 to 5.9% as the CinPlu content increased from 20 to 60%. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42446.

KEYWORDS: drug delivery systems; photopolymerization; proteins; self-assembly; thermal properties

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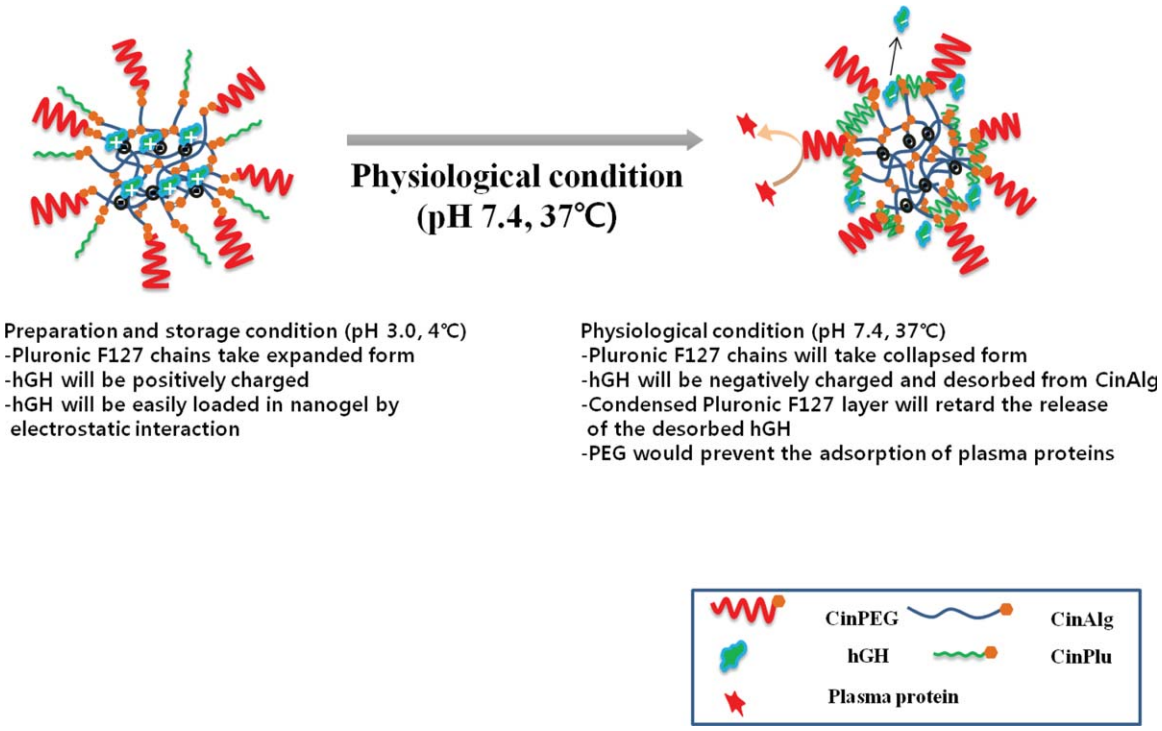
INTRODUCTION

Human growth hormone (hGH) is a therapeutic agent for the hGH deficiency-caused growth retardation, Turner syndrome, the abnormality of sex chromosome, and chronic renal failure.[1] hGH is daily injected subcutaneously because it is cleared from plasma very fast. To avoid the daily injection, which leads to poor patient compliance, the formulations which allow hGH to release in a sustained manner and maintain its efficacy for a long time have been developed. PEGylated hGH,[2,3] microsphere,[4] microparticle,[5] and hydrogel[6] are the examples of the sustained-release formulation. hGH loaded in poly(lactic acid-co-glycolic acid) (PLGA) microsphere, whose trade name is Nutropin depot®, was the first commercially available sustained preparation.[7] The microsphere was reported to release hGH in a sustained manner for 1 month. However, high initial burst release and protein denaturation are known to be the short comings of the PLGA microsphere,[8] and it is not on the market any more due to its high cost. hGH loaded hyaluronate microparticle was developed as a weekly injection formulation for the sustained-release and for now it is the only sustained-release formulation on the market.[9] It was reported that the hyaluronate microparticle exhibits more drug loading and higher bioavailability than PLGA microparticles, however it maintains hGH level in blood only for 30 h in cynomolgus monkeys.[10]

In this study, microgels composed of alginate, Pluronic F127, and polyethylene glycol were developed for the sustained release of hGH. Cinnamic acid (CA), a hydrophobic photodimerizable compound, was covalently attached to each polymer to obtain cinnamoyl alginate (CinAlg), cinnamoyl Pluronic F127 (CinPlu) and cinnamoyl polyethylene glycol (CinPEG) through the reaction between the carboxylic group of CA and the hydroxyl groups of each polymer. Due to the phenyl group, the conjugation of CA to the water-soluble polymers will produce amphiphilic polymers. When CinAlg, CinPEG, and CinPlu are dispersed together in an aqueous phase, they will be assembled into particles-like polymeric mixed micelles (nanogel) owing to the hydrophobic interaction of CA residues. Upon UV irradiation, the CA residue will be dimerized, and the polymer chains constituting the

Additional Supporting Information may be found in the online version of this article.



Scheme 1. A schematic diagram illustrating the release of the nanogel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

self-assembly will be chemically linked. When hGH was loaded in the nanogel, the pH value of the aqueous medium was adjusted to less than the pI value of the protein drug to take advantage of electrostatic interaction between the protein and CinAlg and maximize the loading amount. In addition, the temperature of the medium was kept below the gelation temperature of Pluronic F127 so that the thermo-sensitive polymer chains take an expanded form and the protein drug can easily access to CinAlg. However, when the nanogel is exposed to physiological condition (e.g., pH 7.4 and 37°C, this is the case when the nanogel is injected to human body), hGH will be negatively charged and it will be desorbed from CinAlg chain. At the same time, Pluronic F127 chains will thermally collapse to form condensed layer and they will retard the release of the desorbed hGH. As a result, the condensed layer will allow hGH to release in a sustained manner. Meanwhile, PEG is reported to prevent the adsorption of plasma proteins on drug delivery carriers and help them to avoid reticuloendothelial system and circulate systemically for a long time.[11,12] A schematic diagram illustrating the release of the nanogel is shown in Scheme 1. This is the reason why PEG was included in the self-assembly. The nanogel was characterized in terms of photodimerization, electron microscopy, surface potential, stability in blood plasma, and bile salt solution, and hGH-releasing property. The *in vitro* release profiles of hGH were examined at body temperature (37°C) at different pH values of release medium.

EXPERIMENTAL

Materials

Poly(ethylene glycol) (PEG, MW 8,000), triethylamine and cinnamoyl chloride (CA) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Alginate-cinnamic acid conjugate (CinAlg) was obtained from a previous work and the molar ratio of pyranose to CA was 1 : 0.09.[13] Pluronic F127-cinamic acid conjugate (CinPlu) was obtained from a previous work and the molar ratio of Pluronic F127 chain to CA was 1 : 1.4. hGH was purchased from Pfizer (New York).

Preparation of PEG-CA Conjugate

PEG (5g. 0.6 mmol) and triethylamine (0.14 g, 1.0 mmol) were dissolved in 100 mL dichloromethane contained in a 250 mL 1-neck round bottom flask, then cinnamoyl chloride (0.3g, 1.8 mmol) was put in the PEG solution.[14,15] For the conjugation, the reaction mixture was stirred for 12 h in an ice bath then stirred for 24 h at room temperature. For the precipitation of PEG-CA conjugate (CinPEG), the reaction mixture was poured into dimethyl ether (700 mL) contained in a 1000 mL beaker. The precipitate was filtered through a filter paper (Whatman, No. 2) and it was dried in an oven thermostated at 55°C.

^{1}H NMR Spectroscopy of PEG-CA Conjugate

CinPEG was dried with P_2O_5 in a vacuum oven thermostated at 40°C. Dry CinPEG was dissolved in $CDCl_3$ and the ^{1}H NMR spectrum was taken on a Bruker Avance 400 spectrometer (Karlsruhe, Germany, installed in the Central Laboratory of Kangwon National University).

Colorimetric Study of Conjugates

Alg, CinAlg, Plu, CinPlu, PEG, and CinPEG were extensively dried in a vacuum oven thermostated at 40°C. Each of dry samples were contained in an aluminum pan and it was pressed using a Tzero press (Tzero, TA Instruments, New Castle, DE). The DSC curves were obtained by thermally scanning them on a differential scanning calorimeter (DSC Q2000, TA

Instruments, New Castle, DE) in the range of 20–80°C at the heating rate of 2°C/min.

Preparation of CinAlg/CinPlu/CinPEG Nanogels

For the preparation of nanogels, 50 mg of CinAlg, variable amount of CinPlu, and 50 mg of CinPEG were codissolved in 4 mL of glycine buffer solution (30 m*M*, pH 3.0) contained in a 10 mL vial so that the mass ratio of CinAlg/CinPlu/CinPEG was 1/0.5/1, 1/1/1, 1/2/1, and 1/3/1. Nanogel of which CinAlg/CinPlu/CinPEG mass ratio was 1/0.5/1, 1/1/1, 1/2/1, and 1/3/1 will be termed as CinAlPlPe NG(1/0.5/1), CinAlPlPe NG(1/1/1), CinAlPlPe NG(1/2/1), and CinAlPlPe NG(1/3/1), respectively. For the photodimerization of CA residues contained in CinAlPlPe NGs, the suspensions of the nanogels were subjected to UV irradiation (365 nm, 400 W) for 1 h.

Photodimerizaion of CA Residue of Nanogel

The suspension of CinAlPlPe NG(1/0.5/1), CinAlPlPe NG(1/1/1), CinAlPlPe NG(1/2/1), and CinAlPlPe NG(1/3/1) was diluted with distilled water so that the concentration of CA residue was 0.05 % (w/v). UV (365 nm, 400 W) was irradiated for 60 min to 5 mL of nanogel suspension contained in a 10 mL vial. The dimerization degree was determined using the following equation.[16]

$$\text{Dimerization (\%)} = (1 - A_t/A_0) \times 100\%$$

where, A_0 is the absorbance at 275 nm before the nanogel suspension was subjected to UV irradiation, and A_t is the absorbance at the same wavelength after the nanogel suspension was subjected to UV irradiation during a given period. The nanogels treated with 60 min-UV irradiation were used in the subsequent sections if UV treatment was not mentioned.

TEM of Nanogels

The TEM photos of nanogels were obtained using a native staining technique.[17,18] Nanogel suspension was mixed with phosphotungstic acid solution [2% (w/v)] in equi-volumetric ratio and the mixture was allowed to stand at room temperature for 2 h. Then, a drop of stained nanogel suspension was transferred onto a formvar/copper-coated grid (200 mesh) and it was air-dried for 48 h. The TEM photographs of stained nanogels were taken on a transmission electron microscope (LEO 912AB OMEGA, Germany, installed in Korea Basic Science Institute, Chuncheon, Republic of Korea).

Stability of Nanogel in Blood Plasma and Bile Salt Solution

A volume of 0.1 mL of each of UV-untreated CinAlPlPe NG(1/1/1) suspension (1%) and UV-treated one (1%) was added to 2.9 mL of blood plasma contained in a 10 mL vial. In parallel, 0.1 mL of each of UV-untreated CinAlPlPe NG(1/1/1) suspension (1%) and UV-treated one (1%) was added to 2.9 mL of deoxycholate solution (in distilled water, 1 and 10 m*M*) contained in a 10 mL vial. After the vials were rolled on a roller mixer (205RM, Hwashin Technology, Seoul, Korea) for 48 h at 37°C, the integrity of CinAlPlPe NG(1/1/1) was examined on TEM using a negative staining technique described previously.

Determination of Size and Zeta Potential of Nanogels

The zeta potential of nanogels was determined on a dynamic light scattering equipment (ZetaPlus 90, Brookhaven Instrument, NY) at room temperature in the range of pH 3.0–9.0, where glycine buffer solution was used for pH 3.0, pH 4.0, and pH 9.0, MES buffer solution was used for pH 5.0 and pH 6.0, and PBS buffer solution was used for pH 7.0 and pH 8.0.

Loading of hGH in Nanogels

hGH (5 mg) was dissolved in 1 mL of glycine buffer solution (30 m*M*, pH 3.0) and the hGH solution (1 mL) was put in 4 mL of nanogel suspensions (3%, w/v). For the interaction of hGH and the nanogels, the mixtures of hGH and the nanogels were stirred for 24 h at 4°C. To remove unloaded hGH, 5 mL of the nanogel suspension was put in a dialysis bag (MWCO 100,000) and it was dialyzed against 500 mL of glycine buffer solution (30 m*M*, pH 3.0) until no hGH was released out of the dialysis bag. The dialysis medium was exchanged two times with fresh buffer solution during the dialysis period.

Release of hGH from Nanogels

hGH (1 mL)-loaded nanogel suspension (3%, w/v) in glycine buffer (pH 3.0) was put in a dialysis bag (MWCO 100,000) and it was immersed in 40 mL buffer solution (pH 3.0, pH 5.0, pH 7.4, and pH 9.0) contained in a 50 mL conical tube. Glycine buffer solution was used for pH 3.0 and pH 9.0, MES buffer solution was used for pH 5.0, and PBS buffer solution was used for the pH 7.4. The dialysis medium was gently stirred at 37°C for 7 days and an aliquot (50 mL) of the dialysis medium was taken at a given time for the assay of hGH. The amount of hGH was determined using a kit for hGH assay (R&D System Elisa).

RESULTS AND DISCUSSION

^{1}H NMR Spectroscopy of CinPEG

Figure 1 showed the ^{1}H NMR spectrum of CinPEG. Cinnamoyl proton signals were found in 6.4 to 7.9 ppm, and the ethylene proton signals of PEG were found at 3.7 ppm. Using the signal areas, the molar ratio of PEG chain to CA residue was calculated to be 1 : 0.68.

Calorimetric Study of Conjugates

Figure 2 shows the thermograms of Plu, CinPlu, PEG, CinPEG, Alg, and CinAlg. Plu showed an endothermic peak around 54°C, which is believed to be due to the melting because the melting point of Plu was reported to be 56°C.[19,20] Plu-CA showed an endothermic peak around 43°C, about 10°C lower than the peak position of Plu. CA residues would hydrophobically attract one another to form microdomains, which, in turn, might interfere with the crystalline structure of the PPO hydrophobic blocks, leading to a lower melting point. PEG showed an endothermic peak around 61.5°C. The peak position was in a good agreement of the melting point of PEG (MW. 8,000; 61°C).[21] CinPEG showed an endothermic peak around 61°C, almost the same as the peak position of PEG. CA residues would form microdomains due to their hydrophobic interaction, but the microdomains might hardly interact with PEG chains, which are hydrophilic. This may account for why CA residue had little effect on the melting point of PEG. However, neither Alg nor CinAlg showed calorimetric peak in the scan range (20–80°C), because the melting point of Alg is known to be >300°C.[22]

Photodimerizaion of CA Residue of Nanogel

Figure 3 shows the dimerization degree of CA residue contained in CinAlPlPe NG(1/0.5/1), CinAlPlPe NG(1/1/1), CinAlPlPe

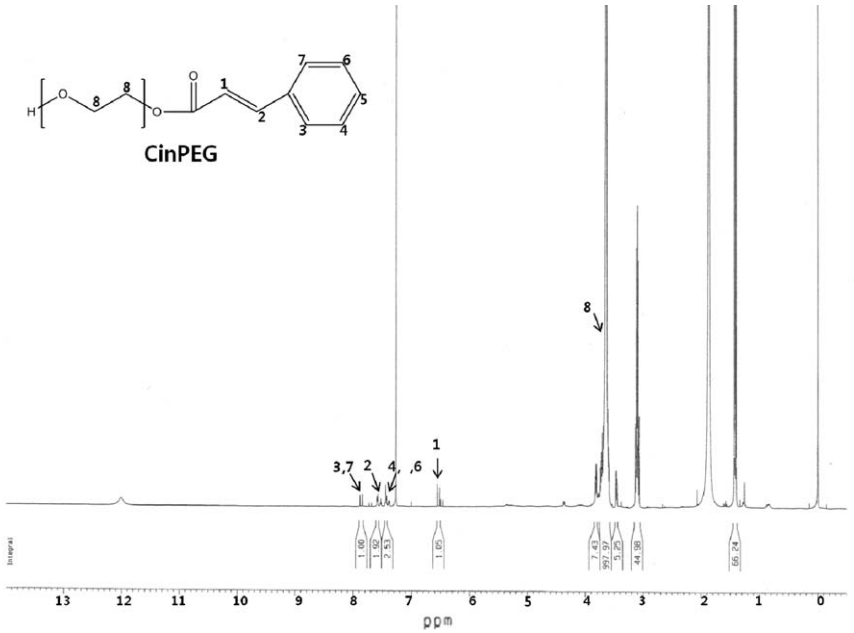


Figure 1. ^{1}H NMR spectrum of CinPEG.

NG(1/2/1), and CinAlPlPe NG(1/3/1). The dimerization degree increased fast for the first 5 min-irradiation then it increased slowly for the rest period of irradiation. CA is photodimerized and the dimer is also photocleaved under the irradiation of UV light.[23] Since monomeric CA residue is abundant in the early stage of UV irradiation, the dimerization will be dominant and the dimerization degree will increase fast in the early stage. As the irradiation goes on, the dimer will be built up, the dedimerization rate will become comparable to the dimerization rate, and an equilibrium between the dimerization and the dedimerization will be reached. This can account for why the dimerization degree increased in a saturation manner with UV irradiation time. The dimerization degree in 60 min of CA residue contained in CinAlPlPe NG(1/0.5/1), CinAlPlPe NG(1/1/1), CinAlPlPe NG(1/2/1), and CinAlPlPe NG(1/3/1) was about 49%, 53.5%, 57%, and 53%, respectively.

TEM of Nanogels

Figure 4 shows the TEM photographs of CinAlPlPe NG(1/0.5/1), CinAlPlPe NG(1/1/1), CinAlPlPe NG(1/2/1), and CinAlPlPe

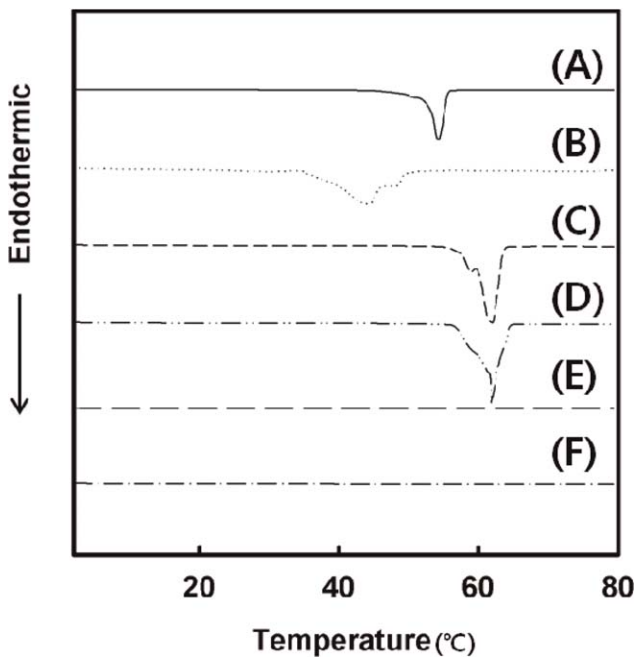


Figure 2. Thermograms of Plu (A), CinPlu (B), PEG (C), CinPEG (D), Alg (E), and CinAlg (F).

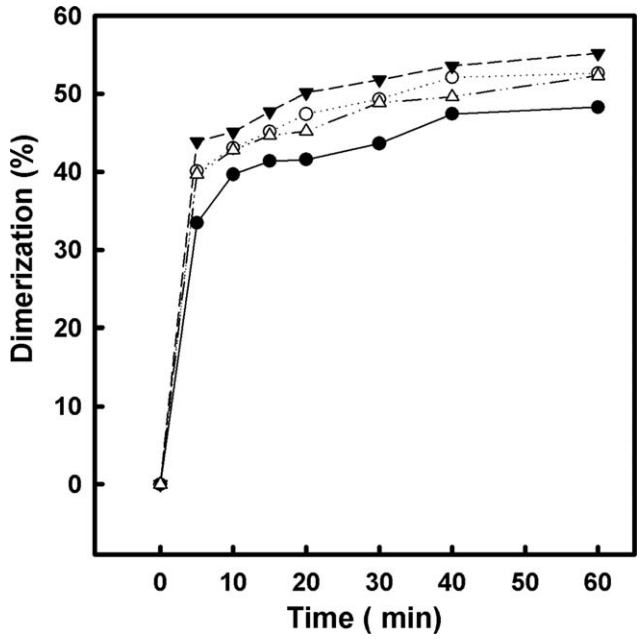


Figure 3. Dimerization degree of CA residue contained in CinAlPlPe NG(1/0.5/1) (•), CinAlPlPe NG(1/1/1) (○), CinAlPlPe NG(1/2/1) (▼), and CinAlPlPe NG(1/3/1) (△).

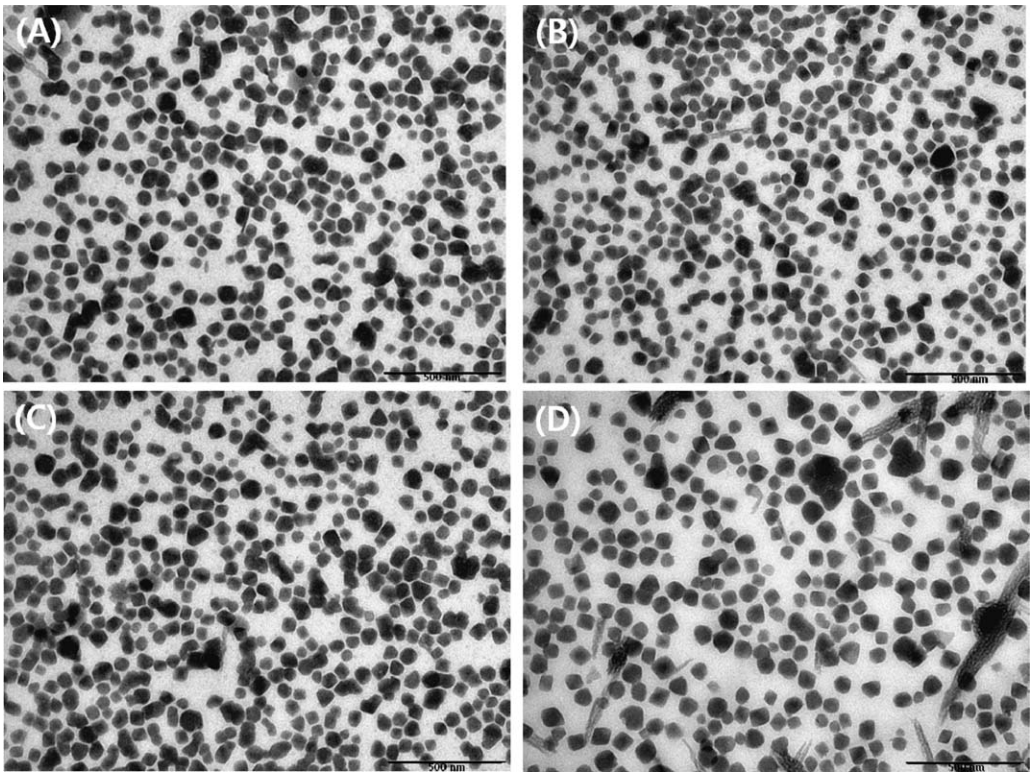


Figure 4. TEM photographs of CinAlPlPe NG(1/0.5/1) (A), CinAlPlPe NG(1/1/1) (B), CinAlPlPe NG(1/2/1) (C), and CinAlPlPe NG(1/3/1) (D), which were subjected to no UV irradiation.

NG(1/3/1), which were subjected to no UV irradiation. The nearly spherical particles were observed and the diameter was 50–150 nm. CA is a kind of hydrophobic compound and the conjugation of CA with a water-soluble polymer can lead to the formation of an amphiphile. In fact, CinAlg and CinPlu were reported to be amphiphilic and surface-active.[13,24] According to the result of air/water interfacial tension measurement (Supporting Information), CinPEG was also found to be surface-active. When they were dispersed in an aqueous phase, they could be self-assembled into particles (nanogels), possibly due to the hydrophobic interaction of CA residues. CinAlg could be the backbone of the nanogel because alginate used in this study is a high molecular weight polymer (MW 10,000–600,000) and one molecule was calculated to have 153 CA residues when the average molecular weight was assumed to be 300,000. In this circumstance, CinPEG and CinPlu could be associated to CinAlg along its backbone owing to the hydrophobic interaction among CA residues. It is our speculation that CinAlg having CinPEG and CinPlu along its chain could be one of the major building blocks for the formation of CinAlPlPe NG. The shape and the size of UV-treated nanogels were not markedly different from those of nanogel (Supporting Information), which were not treated with UV irradiation. CA residues in nanogels were readily dimerized under the irradiation of UV light (Figure 3). As the result of photodimerization, cyclobutane bridge is formed between two CA residues.[25] The bridge formation will take place between two CA residues which may have already been in close contact with each other, it would hardly have an effect on the size and the shape of the nanogels. The molar ratio of alginate to CA of CinAlg was 1 : 153, that of Pluronic F127 to CA of CinPlu was 1 : 1.4, and that of PEG to CA of CinPEG was 1 : 0.68 Thus, the CA residue molar ratio of CinAlg/CinPlu/CinPEG of CinAlPlPe NG(1/0.5/1), CinAlPlPe NG(1/1/1), CinAlPlPe NG(1/2/1), and CinAPP MI(1/3/1) was calculated to be 4.7 : 0.55 : 1.23, 4.7 : 1.10 : 1.23, 4.7 : 2.20 : 1.23, and 4.7 : 3.30 : 1.23, respectively. Whichever CinAlg/CinPlu/CinPEG mass ratio was in the nanogels, the number of CA residue in CinAlg was greater than the number of CA residue in CinPlu plus the number of CA residue in CinPEG. Thus, if the CA residue in CinAlg interacts, in 1 : 1 molar ratio, with the CA residue in CinPlu and the CA residue in CinPEG, CinAlg molecules could accommodate all the molecules of CinPlu and CinPEG, and all the CA residues associating alginate with Pluronic F127 and PEG could participate in the photodimerization. However, intermolecular dimerization between two molecules of the same kind, intermolecular dimerization between CinPlu and CinPEG, and intramolecular dimerization within one molecule of each conjugate would also take place. In summary, whether CA residues were photodimerized or not and whether the photodimerization was intramolecular or intermolecular, CinAlg/CinPlu/CinPEG could be associated one another through the hydrophobic interaction among CA residues to form nanogel.

Stability of Nanogel in Blood Plasma and Bile Salt Solution

Figure 5 shows the TEM photo of UV-untreated CinAlPlPe NG(1/1/1) and UV-treated CinAlPlPe NG(1/1/1) after the nanogels were exposed to blood plasma for 48 h at 37°C. Nanogels remained intact whether they were subjected to UV irradiation.

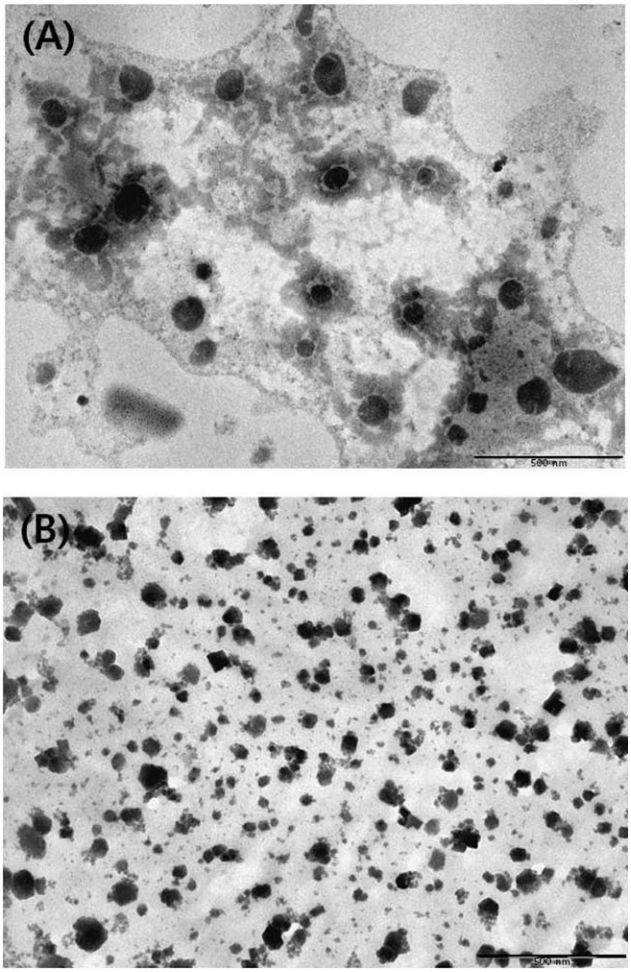


Figure 5. TEM photograph of UV-untreated CinAlPlPe NG(1/1/1) (A) and UV-treated CinAlPlPe NG(1/1/1) (B) after the nanogels were exposed to blood plasma for 48 h at 37°C.

Plasma proteins might be adsorbed onto the nanogel and they could disrupt the integrity of the nanogel. PEG chains are believed to prevent the adsorption of plasma protein and stabilize the nanogel. PEG is known to be flexible, act as a spring, and prevent the adsorption of plasma protein by entropy-driven process.[26] Figure 6 shows the TEM photo of UV-untreated CinAlPlPe NG(1/1/1) and UV-treated CinAlPlPe NG(1/1/1) after the nanogels were exposed to DOC solution (1 m*M*) for 48 h at 37°C. Smaller and disintegrated particles were observed when the nanoparticle was not treated with UV irradiation. The nanogels are believed to be formed due to the hydrophobic interaction among CA residues of polymer conjugates (CinAlg, CinPlu, CinPEG). DOC is a kind of surfactant and it would interfere with the hydrophobic interaction among CA residues. As a result, the nanogel could be disintegrated into mixed micelles composed of polymer conjugates and DOC molecules. However, the nanogels remained intact when they were treated with UV irradiation. As shown in Figure 3, AC residues of nanogel were readily photodimerized under the irradiation of UV light. Thus, it is believed that the polymer conjugates (CinAlg, CinPlu, and CinPEG) of nanogel were chemically linked one another. In this circumstance, the UV-treated nanogel is hardly likely to be disintegrated by the surfactant (DOC). Even when the nanogels were exposed to DOC solution of higher concentration (10 m*M*) (Supporting Information), the UV-treated microgels remained intact while the UV-untreated microgels were disintegrated.

Determination of Zeta Potential of Nanogels

Figure 7(A) shows the zeta potential of UV-treated nanogels [CinAlPlPe NG(1/0.5/1), CinAlPlPe NG(1/1/1), CinAlPlPe NG(1/2/1), CinAlPlPe NG(1/3/1)] in the pH value range of 3–9. The zeta potential was negative in the full range of pH value tested because the carboxylate of alginate is permanent negative charge. The absolute value of zeta potential increased with increasing pH value because the carboxylic acid of alginate is deprotonated as the pH value increased. In the full range of pH value tested, the absolute value of zeta potential was less in the order of CinAlPlPe NG(1/3/1) > CinAlPlPe NG(1/2/1) > CinAlPlPe NG(1/1/1) > CinAlPlPe NG(1/0.5/1). Pluronic F127 chains and PEG chains, which are nonionic polymers, could shield the negative charge of alginate chain, and the shielding effect will be greater when the content of the nonionic polymer chains is higher. Figure 7(B) shows the zeta potential

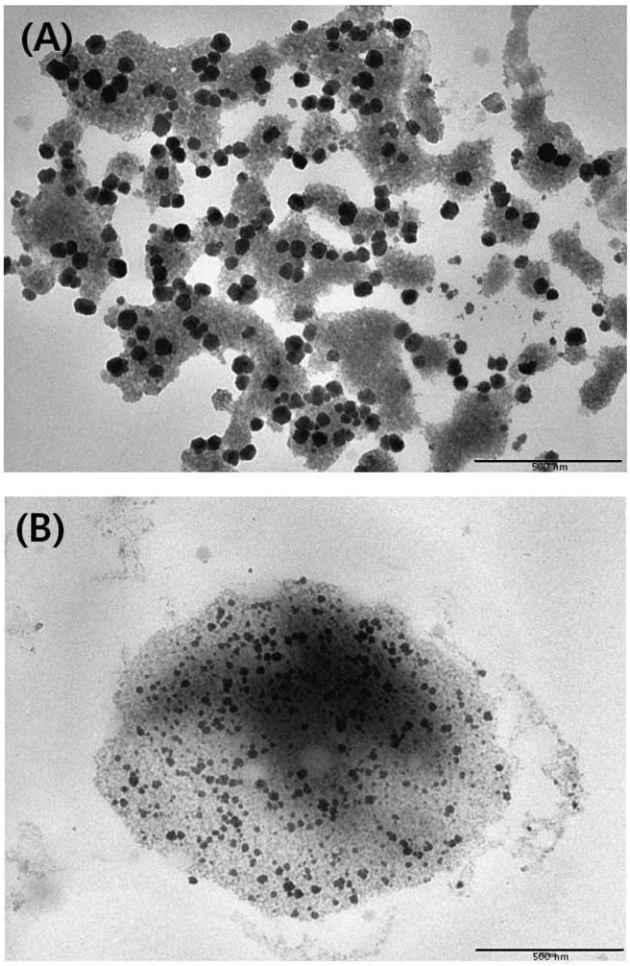


Figure 6. TEM photograph of UV-untreated CinAlPlPe NG(1/1/1) (A) and UV-treated CinAlPlPe NG(1/1/1) (B) after the nanogels were exposed to DOC solution (1 m*M*) for 48 h at 37°C.

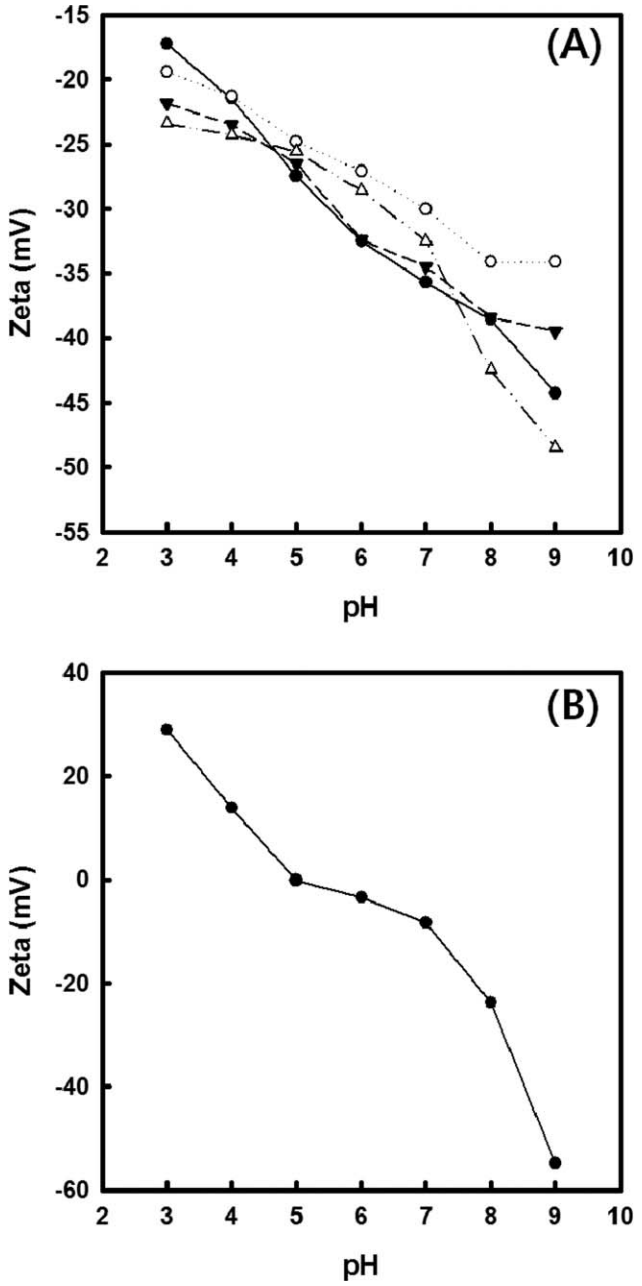


Figure 7. Zeta potential of UV-treated nanogels (CinAlPlPe NG(1/0.5/1) (△), CinAlPlPe NG(1/1/1) (▼), CinAlPlPe NG(1/2/1) (○), CinAlPlPe NG(1/3/1) (•) (A), and hGH (B) in the pH value range of 3–9.

of hGH in the pH value range of 3–9. The zeta potential decreased from + 30 mV to −55 mV when the pH value increased from pH 3 to pH 9. The isoelectric point was found around pH 5 and the result was in a good agreement with the value reported in a literature.[27,28]

Loading of hGH in Nanogels

The specific loading of hGH in CinAlPlPe NG(1/0.5/1), CinAlPlPe NG(1/1/1), CinAlPlPe NG(1/2/1), and CinAlPlPe NG(1/3/1) was 3.83%, 3.10%, 2.4%, and 1.8% (hGH/nanogel, w/w), respectively. As the content of CinPlu increased, the specific loading decreased. At pH 3.0 where hGH was loaded in the nanogels, the zeta potential of the nanogels was −17 to −24 mV, depending on their composition [Figure 7(A)], and the zeta potential of hGH was + 30 mV [Figure 7(B)]. Accordingly, hGH will be loaded in the nanogels by electrostatic interaction. The absolute value of the negative zeta potential of the nanogels was lower when the content of CinPlu was higher [Figure 7(A)]. For example, the zeta potential value at pH 3.0 of CinAlPlPe NG(1/0.5/1), CinAlPlPe NG(1/1/1), CinAlPlPe NG(1/2/1), and CinAlPlPe NG(1/3/1) was −24, −22.5, −20, and −17 mV, respectively. Therefore, the electrostatic interaction between hGH and the nanogel would be more unfavorable when the content of CinPlu is higher. This could account for the reason why the specific loading was higher in the order of CinAlPlPe NG(1/0.5/1) > CinAlPlPe NG(1/1/1) > CinAlPlPe NG(1/2/1) > CinAlPlPe NG(1/3/1).

Release of hGH from CinAlg/CinPlu/CinPEG Nanogels

Figure 8(A) shows the release profile of hGH from CinAlPlPe NG(1/0.5/1) for 170 h at pH 3.0, pH 5.0, pH 7.4, and pH 9.0 at 37°C. When the pH value of release medium was 3.0 and 5.0, the release rate was so low that the release degree was <10% in 170 h. When the pH value of release medium was 7.0, a fast release was observed for the first 24 h, followed by a slow release for the rest of the release period. And the release degree in 170 h was around 45%, much higher than that observed under acidic condition (e.g., pH 3.0 and pH 5.0). A similar release profile was observed at pH 9.0, and the release degree was further promoted. Under an acidic condition (e.g., pH 3.0 and pH 5.0), hGH will be positively charged (the isoelectric point is 5.0), so it will electrostatically interact with negatively charged alginate. However, when the pH value of medium was 7.4 and 9, hGH will be negatively charged, so it will hardly electrostatically interact with alginate. This may explain why the release degree at pH 7.4 and pH 9 was much higher than the release degree at the acidic conditions. The pattern of pH-dependent hGH release profile of CinAlPlPe NG(1/1/1), CinAlPlPe NG(1/2/1), and CinAlPlPe NG(1/3/1) was similar to the pattern obtained with CinAlPlPe NG(1/0.5/1) [Figure 8(B–D)]. However, the initial burst release was more suppressed as the CinPlu content increased. At pH 9, for example, the release degree in 60 min of hGH from CinAlPlPe NG(1/0.5/1), CinAlPlPe NG(1/1/1), CinAlPlPe NG(1/2/1), and CinAlPlPe NG(1/3/1) was 15.3%, 10.6%, 8.2%, and 5.9% respectively. That is, the release was close to be first order when the CinPlu content of nanogel was low (e.g. CinAlPlPe NG(1/0.5/1)) and it approached zero order when the CinPlu content of nanogel increased. In fact, the initial slope of release curve of CinAlPlPe NG(1/3/1) at pH 7.4 and pH 9 was much less than that of CinAlPlPe NG(1/0.5/1) at the same pH values. Since the release profiles were obtained at 37°C, Pluornic F127 chains would form a condensed layer due to their thermal dehydration and collapse. In this circumstance, hGH could be trapped by the thermo-responsive polymer layer. As the CinPlu content is higher, the more condensed layer would be formed thus the initial release could be suppressed more. In addition, the hydrophobic domain of hGH could interact with the thermally hydrophobicized Plutonic F127 chains, and the hydrophobic interaction would be stronger as the content of

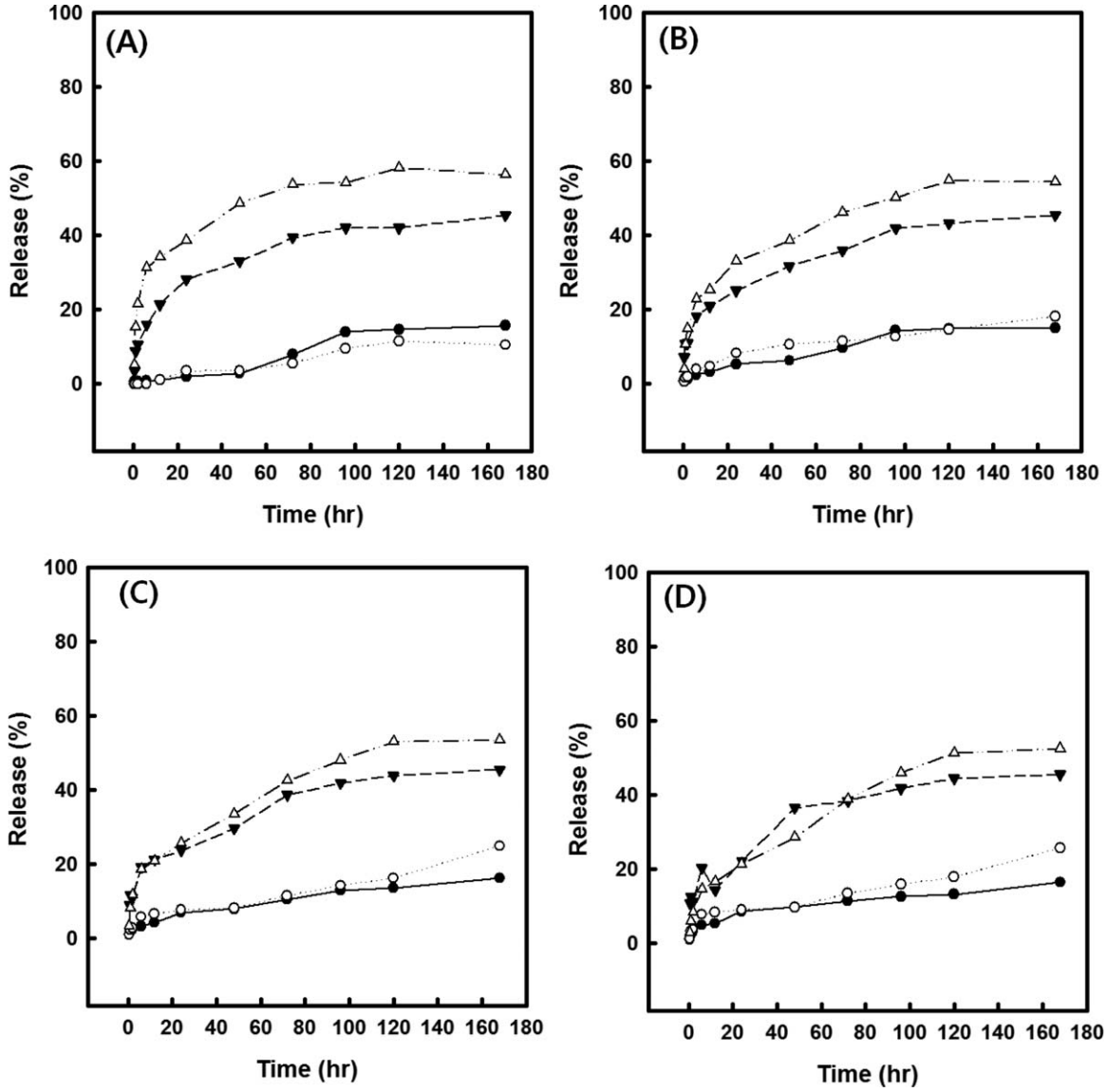


Figure 8. Release profile of hGH from CinAlPlPe NG(1/0.5/1) (A), CinAlPlPe NG(1/1/1) (B), CinAlPlPe NG(1/2/1) (C), and CinAlPlPe NG(1/3/1) (D) for 170 h at pH 3.0 (•), pH 5.0 (○), pH 7.4 (▼), and pH 9.0 (△) at 37°C.

thermoresponsive polymer is higher. This may be another reason for the initial release suppression.

Many parameters and different materials were considered in the present study to obtain the best value for each of them. It is known that the optimization of two or more parameters is easier, more statistically correct, and more functional if performed with a multivariate study.[29,30] A multivariate will be done in our future works to take into account all the relevant variables and their interactions, and to find the best experimental conditions for the proper functioning of materials used.

CONCLUSIONS

CinPEG was prepared by the condensation reaction between the acid chloride of cinnamoyl chloride and the hydroxyl group of PEG. On the thermograms, the melting point of CinPlu was about 10°C lower than that of Plu. CA residues are believed to form hydrophobic microdomains and they disrupt the hydrophobic crystalline structure of PPO segment of Plu. However, the melting point of CinPEG was almost the same as that of PEG, possibly because hydrophobic CA residues could hardly interact with hydrophilic PEG chains. CinAlg, CinPlu, and CinPEG could be building blocks for the formation of self-assembled nanogels in aqueous phase. The nanogels (CinAlPlPe NG(1/0.5/1), CinAlPlPe NG(1/1/1), CinAlPlPe NG(1/2/1), CinAlPlPe NG(1/3/1) were <100 nm on TEM photographs. The photodimerization degree of CA residue of nanogels increased in a saturation manner with UV irradiation time and the dimerization degree in 60 min was 48–55%, depending on the composition of nanogels. The integrity and the size of nanogels were not appreciably changed after the photodimerization. The integrity of nanogels [CinAlPlPe NG(1/1/1)] was maintained in blood plasma for 48 h at 37°C regardless of whether or not they were UV-treated; however, the integrity was maintained in

deoxycholate solution (1 m*M* and 10 m*M*) only when they were UV-treated. By taking advantage of the electrostatic interaction between hGH and CinAlg, hGH could readily be loaded in the nanogel under an acidic condition (e.g., pH 3.0). When the release medium was at acidic condition (e.g., pH 3.0, pH 5.0), no significant release of hGH from nanogels was observed at the body temperature (37°C). When the medium was at physiological pH (e.g., pH 7.4) and at an alkaline condition (e.g., pH 9.0), marked release was observed at the same temperature. However, hGH was released slowly from microgels for 100 h possibly because Pluronic F127 chain of CinPlu may form thermally condensed layers at the body temperature. And, initial burst release was suppressed as the CinPlu content increased. That is, the release was close to be first order when the CinPlu content of nanogel was low and it approached zero order when the CinPlu content of nanogel increased. The nanogel developed in this study could be used not only for injection but also for oral administration. When orally administered, hGH will hardly be detached from CinAlg chain in acidic gastric juice and it will be protected from the juice by condensed layer of Pluronic chains. In small intestine (pH 7.0), hGH will be discharged from CinAlg chain and would be absorbed into the endothelium.

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