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Preparation and Biodegradation of Thermosensitive Chitosan Hydrogel as a Function of pH and Temperature

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Abstract: We have developed an injectable thermosensitive hydrogel for local drug delivery to treat cancers clinically. We selected chitosan as a polymer matrix because of its biocompatibility and biodegradability. Glycerol 2-phosphate disodium salt hydrate (β-GP) was used to neutralize the chitosan solution to physiological pH. The chitosan solution displayed a sol-gel phase transition in a pH- and temperature-dependent manner and formed an endothermic hydrogel after subcutaneous injection into mouse in the presence of β-GP. Additionally, we evaluated the biodegradation of chitosan hydrogel in mice by measuring the volume of injected chitosan hydrogel after subcutaneous injection. The injected chitosan hydrogel in mice was sected and stained with hematoxylin-eosin reagent for histological observation to confirm biodegradation of the hydrogel by the infiltrated cells. Chitosan hydrogel systems that possess biocompatibility and biodegradability could be promising thermosensitive injectable materials useful as depot systems for local anti-cancer drug delivery.

Keywords: sol-gel transition, chitosan hydrogel, biodegradation.

Introduction

Injectable gel-forming biopolymer has been developed increasingly for the therapeutic depots and vehicles.[1-4] These biocopolymers in aqueous solution are well-known thermoset gel-forming materials but lack of physiological degradability, which is a hurdle for clinical applications. Recently, one of the approaching in the field of gel-forming materials, copolymers such as poly(ethylene oxide), poly(lactic acid) and poly(*N*-isopropylacrylamide) were proposed as materials to provide injectable drug delivery system because of their biodegradability or thermosensitivity.[5-8] However, copolymers still have problems that the need of high polymer concentration and high injection temperature may limit the benefits of such systems. Additionally, their biodegradability and biocompatibility have been concerned with clinical applications.

On the other hand, natural biopolymer such as chitosan is currently receiving a great deal of attention for medical and pharmaceutical applications.[9,10] The main reasons for using chitosan are undoubtedly due to its appealing intrinsic properties in medical applications such as topical ocular application, implantation or injection.[11,12] Chitosan is metabolized by certain human enzymes such as lysozyme and should be considered as biodegradable and biocompatible.[13,14] In addition, chitosan biodegradability has been reported that chitosan acts as a penetration enhancer by opening epithelial tight-junctions. Due to its positive charges at physiological pH, chitosan is also bioadhesive, which increases retention at the site of application.[15,16] Importantly, chitosan is abundant in nature, and its production is of low cost and is ecologically interesting. For these reason, chitosan has been used as pharmaceutical and medical materials including hydrogel system.[9,17]

In this study, a novel thermosensitive chitosan hydrogel was prepared in order to use as an injectable depot system. We found that the condition for hydrogelation of chitosan where chitosan shows the gradual phase transition from solution to hydrogel at body temperature (37 °C) and physiological pH in the presence of *β*-GP. Additionally, the biodegradation of chitosan hydrogel was tested by measuring of chitosan volume and H&E staining hydrogel section after subcutaneous injection into mice. In this result, the chitosan hydrogel showed good biocompatibility and biodegradability *in vivo* mouse system, indicating it as a promising clinical device for local drug delivery.

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Experimental

Materials. Chitosan (medium molecular weight of 161 kDa, viscosity of 200,000 cps and a degree of deacetylation of 80%) and glycerol 2-phosphate disodium salt hydrate (*β*-GP) were purchased from Sigma-Aldrich Co. (Louis, MO, USA). All other materials were of analytical grade and were used without further purification.

Preparation of Chitosan Solution. Clear solutions of chitosan were obtained by dissolving chitosan of 200 and 400 mg in 18 mL of 0.1 M HCl solution. A series of *β*-GP solutions were prepared by dissolving from 0 to 3 g of *β*-GP in 1 mL distilled water. The chitosan solutions were cooled down to 4 °C and continuously stirred while adding *β*-GP solution of 1 mL and the final volume fill up to 20 mL with distilled water. Thus, the final 20 mL solutions also contained chitosan of 1 or 2% w/v.

Formation of Chitosan Hydrogel. Both pH and temperature dependence in chitosan hydrogel formation were determined by measuring the turbidity change and gelling time at various pH and temperatures. In addition, the formation of chitosan hydrogel was confirmed *in vitro* and *in vivo* tests. In case of the *in vitro* test, the formed of the 2% w/v chitosan solution containing 10% w/v *β*-GP was observed at various temperatures from 25 to 37 °C. In case of the *in vivo* test, the form of the chitosan hydrogel was observed after subcutaneous injection in a mouse flank.

Measurement *in vivo* Biodegradation of Chitosan Hydrogel. *In vivo* biodegradation of chitosan hyrogel was examined by measuring the volume of chitosan hydrogel in mice (female, 6 week, KRIBB, Korea). The chitosan solution was injected into both the right and left sides of the back subcuits of mice. The volume of chitosan hydrogel in mice was measured using vernier calipers (Mitutoyo, Japan) and was calculated by following eq. (1)[18]:

$$\text{Volume (mm}^3\text{)} = \text{length} \times \text{width}^2 \times 1/2 \quad (1)$$

Histological Examination. Histological examination was performed to observe the cell growth within chitosan hydrogel and to predict the biodegradation procedure of chitosan hydrogel. The mice were sacrificed after 10, 20 and 30 days. Chitosan hydrogel was retrieved whole, along with adjacent hydrogel and fixed in 10% v/v neutral buffered formalin solution (Sigma Aldric Co.). Each chitosan hydrogel was then bisected manually with a blade macroscopic localization of the implant site. This portion of the chitosan hydrogel was embedded in paraffin, and serially sectioned by 5-6 μm thickness. These samples were stained with H&E reagent.[19] In each samples, a randomized area (microscopic fields, × 40 or 100) showing the largest capillary density, was photographed. Histological analysis of stained chitosan hydrogel sections was examined using light microscopy with an Olympus CX40 (Japan), and images were captured using a Samsung color digital camera (Korea).

Results and Discussion

Characteristics of Chitosan Hydrogel. The chitosan was added in 0.1 M HCl solution to dissolve it by protonation of its amine groups in acidic environments. In order to neutralize the physiological pH, *β*-GP solutions having various concentrations were added into the chitosan solution. The change of pH in chitosan solutions (1 or 2% w/v) as function of *β*-GP concentration at 20 °C was shown in Figure 1. The pH of these chitosan solutions approached the physiological region when the molar concentration of *β*-GP exceeded the molar concentration of the amine group of chitosan. The 1% w/v chitosan solution was neutralized at pH 7.3 by addition of 15% w/v *β*-GP. In the case of 2% w/v chitosan solution, addition of 10% w/v of *β*-GP neutralized chitosan solution to pH 7.2. Therefore we used these formulations for optimization of hydrogel formation. Notably, in the range of pH 6.8-7.2, there was not any sign of precipitation or gelation at 20 °C, indicating that the presence of *β*-GP at physiological pH and 20 °C was not only to prevent chain aggregation and formation of hydrogel but also to increase the pH of chitosan solution to physiological condition.

The 2% w/v chitosan solutions were found to be thermosensitivity and induced gelation upon adding *β*-GP at 37 °C. Hydrogel formation was monitored by measuring turbidity in chitosan solutions at 400 nm, which shown in Figure 2. In the case of pH 4.5 (pure chitosan solution) at 37 °C, turbidity of chitosan solution was not changed. This implies that the chitosan solution could not form hydrogel at low pH. However, pH 7.2 (neutralized by adding 10% w/v *β*-GP) at 37 °C, the turbidity change was rapidly increased within 5 min, indicating that chitosan solution forms hydrogel. Thus, we observed that the chitosan solution forms a hydrogel having thermosensitivity in the presence of *β*-GP at physio-

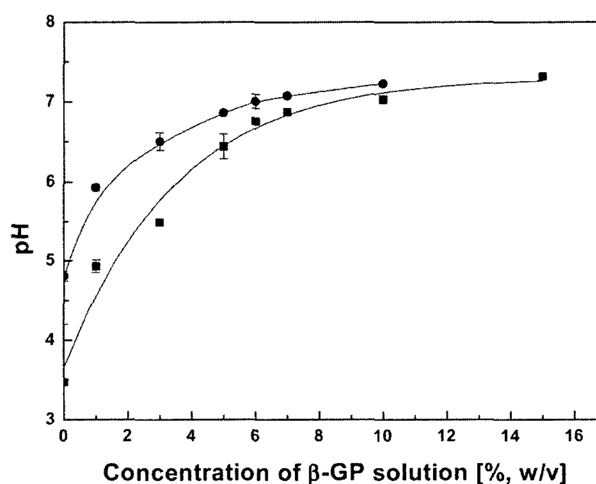

Figure 1. The pH change of chitosan solution as a function of *β*-GP concentration at 20 °C: (■) 1 and (●) 2% w/v chitosan solution. The data is shown as mean ± S.D. (n=3).

logical pH.

The temperature-dependence of gelling time for chitosan solution was shown in Figure 3. In the case of 1% w/v chitosan solution neutralized with 15% w/v β-GP at pH 7.3, gelling time appeared to display an exponential decrease from 2,280 sec at 35 °C to 70 sec at 45 °C. The gelling time of 2% w/v chitosan solution neutralized with 10% w/v β-GP at pH 7.2 appeared to display an exponential decrease with temperature varying from 530 sec at 35 °C to 50 sec at 45 °C. For the neutralized 1% or 2% w/v chitosan solution, the hydrogels were formed, respectively, in 900 and 300 sec at 37 °C. The temperature is, therefore, a factor that modulates the gelling process probably by formation of junction zones of the polymers in a temperature dependent manner. Thus, taken together these observations, the gelation process of chitosan solutions appears to be controlled by a coupling among pH, temperature and neutralization degree of the chitosan chain in the presence of β-GP. For the neutralized 1% w/v chitosan solution, however, the time to make the hydrogel was too long because chitosan solution should be quickly made a hydrogel form in body. The gelling time was an important parameter for hydrogel formation in body because the solution was washed out from injection site unless quickly making hydrogel. Thus, we used 2% w/v chitosan solution neutralized with 10% w/v β-GP for further optimization procedures instead of 1% w/v chitosan solution.

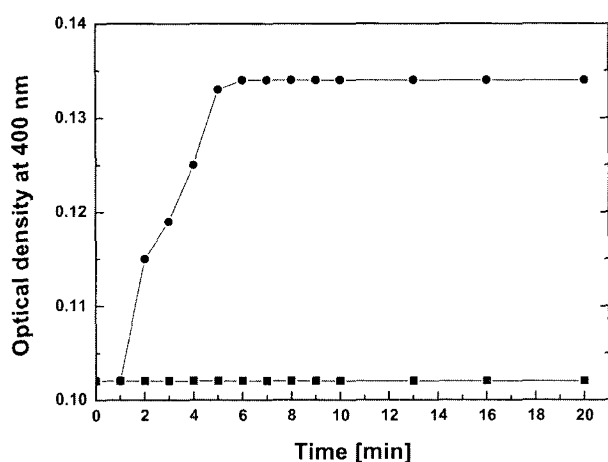

Figure 2. Turbidity change with time of chitosan solution (2% w/v) incubated at 37 °C: (●) in the presence of β-GP at pH 7.2; (■) in the absence of β-GP at pH 4.5.

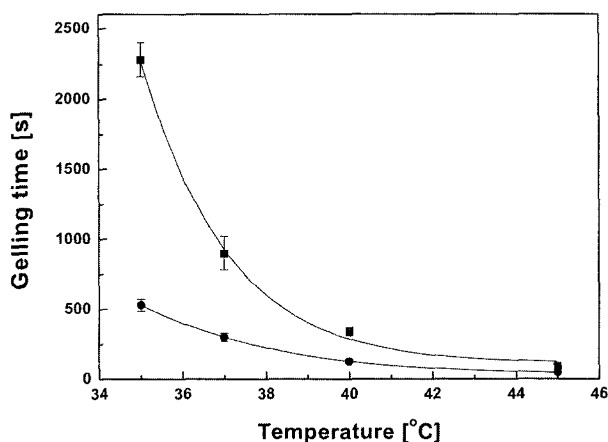

Figure 3. Temperature-dependence of gelling time of chitosan solution. The chitosan solutions: (■); 1% w/v neutralized with 15% w/v β-GP at pH 7.3, (●); 2% w/v neutralized with 10% w/v β-GP at pH 7.2. The data is shown as mean ± S.D. (n=3).

The gelling temperature as function of the pH of the 2% w/v chitosan solution by adding β-GP in 5 min was shown in Figure 4. Gelling temperature was greatly affected by the change of pH of a chitosan solution since the neutralization behavior of chitosan solutions was obviously a main characteristic which determines their solubility and phase transition phenomena. The decrease of gelling temperature at high pH suggests that the number of charged ammonium groups on the chitosan chain is an important parameter controlling gelation in this system. A reduction in charge density on the chitosan chain seems to associate with reduction of interchain electrostatic repulsion and permit to initiate gelation. In considering molecular mechanisms of gelation for thermally responsive polymer systems, it is important a broad range of physicochemical interactions between the cationic polyelectrolyte chitosan and the divalent anionic base glycerol phosphate includes: (1) electrostatic repulsion between positive charged chitosan chains; (2) electrostatic attraction between oppositely charged chitosan and the phosphate moiety of β-GP; (3) attractive hydrophobic and hydrogen bonding between chitosan chains; and (4) the hydrophobic or water-structuring character of the glycerol moiety of β-GP.[20]

We tried to visualize the formation of chitosan hydrogel at

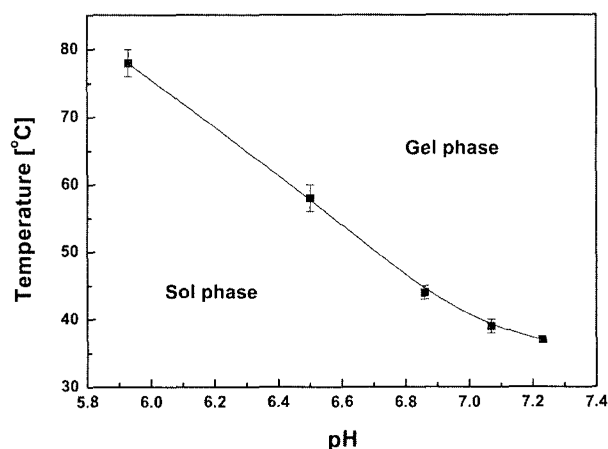

Figure 4. Gelation temperature as a function of the pH of 2% w/v chitosan solution by adding β-GP. The data is shown as mean ± S.D. (n=3).

optimized pH and temperature. The formation of solution-hydrogel phase transition was shown in Figure 5. In this experiment, 2% w/v chitosan solution neutralized with 10% w/v β-GP at pH 7.2, (A) formed a solution phase at room temperature and (B) a hydrogel phase at body temperature (37 °C). This result clearly showed that chitosan solution was changed from solution to hydrogel as a function of temperature.

In addition, the formation of chitosan solution *in vivo* mouse system was shown in Figure 6. (A) shows the formation of chitosan solution after subcutaneous injection into a mouse and (B) shows the separated chitosan hydrogel from mouse at body temperature after 10 h. This result represents in situ hydrogel formation after subcutaneous injection of 0.2 mL of chitosan solution when concentration of chitosan solution is 2% w/v neutralized with 10% w/v β-GP at pH 7.2.

Measurement *in vivo* Biodegradation of Chitosan Hydrogel. The biodegradation of biopolymers is important in clinical application as a drug delivery carrier in body. Therefore, the biodegradation of a chitosan hydrogel was estimated by measuring a decrease of hydrogel volume in mice. The change of volume of chitosan hydrogel to evaluate the biodegradation rate after subcutaneous injection in mice was shown in Figure 7. The decreased volume of chitosan hydrogel *in vivo* should be associated with the biodegradation of the hydrogel. The volume of chitosan hydrogel was decreased from 800 to 200 mm^3 after subcutaneous injection during 50 days. In 12 days, the volume of chitosan hydrogel was rapidly decreased, whereas the volume of chitosan hydrogel was slowly decreased from 12 to 57 days. Thus, this result showed that the injected chitosan hydrogel was partially biodegraded after subcutaneous injection into mice.

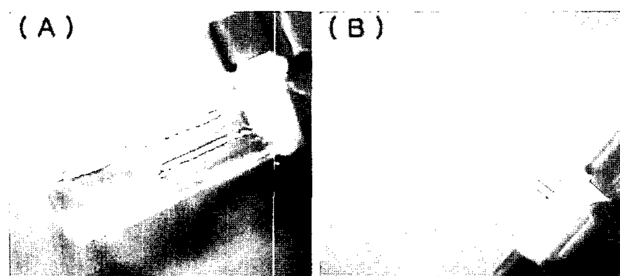

Figure 5. Formation of chitosan hydrogel at different temperatures *in vitro*. (A) a room temperature (25 °C) and (B) a body temperature (37 °C).

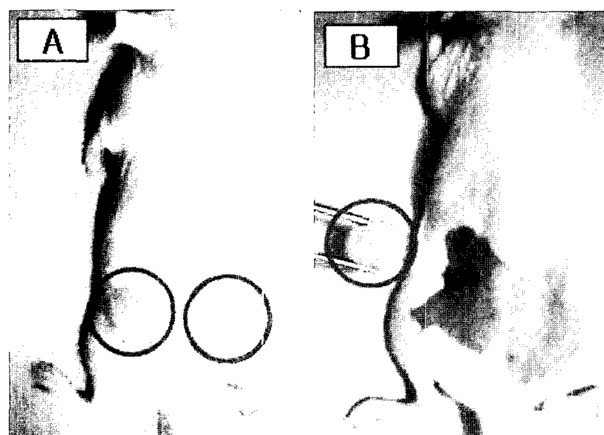

Figure 6. Formation of chitosan hydrogel in a mouse. (A) a formation after subcutaneous injection and (B) a formation of chitosan hydrogel.

Histological Examination of Chitosan Hydrogel. To confirm the degradation of the chitosan hydrogel in mice, histological observation of hydrogel have been carried out after H&E staining and represented in Figure 8. The 0 day chitosan hydrogel have not contained any infiltrated cells. However, various type of cells infiltrated into the hydrogel at 10, 20 and 30 days after injection. Hydrogels were encapsulated by fibrous tissue and covered with regenerated thick pleura-like cell membrane. After 30 days, the chitosan hydrogel was almost disappeared. It has been reported that the growth of infiltrated cells in hydrogel is highly related with biodegradation of chitosan hydrogel.[21,22] On the basis of these observations, biodegradation of the chitosan hydrogel may be caused by infiltrated cells. During this experimental procedure, the mice did not have side effect such as pus and inflammation, and they were in a good health condition. Thus, this result clearly showed that the chitosan hydrogel had good biocompatibility and biodegradability *in vivo* system.

Conclusions

The formation of thermosensitive chitosan hydrogel,

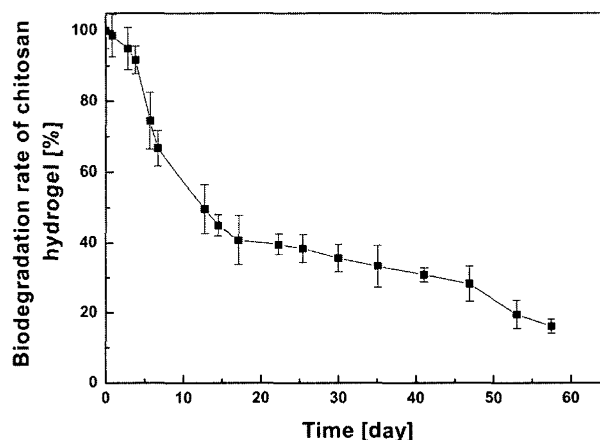

Figure 7. Estimation of biodegraded chitosan hydrogel in mice. The concentration of chitosan solution is 2% w/v neutralized with 10% w/v β-GP at pH 7.2. The data is shown as mean ± S.D. (n=3).

Figure 8. The histological observation of the chitosan hydrogel after subcutaneous injection in mice (original magnification; a, b and d (× 40), c (× 100)). (a) 0 day, (b) 10 days, (c) 20 days, and (d) 30 days.

which does not involve organic solvents or detergents, was induced at optimized pH and temperature. In this report, we showed that chitosan solution can be neutralized up to physiological pH by using β-GP. The sol-gel transition of thermosensitive chitosan hydrogel appears to be controlled by interaction between pH and temperature. As a consequence, chitosan hydrogel was successfully formed at body temperature and physiological pH. In addition, this chitosan hydrogel showed an *in vivo* degradation after subcutaneous injection into mouse, which may be caused by infiltrated cells. Importantly, the mice did not have severe side effect such as pus and inflammation and maintained a healthy appearance after implantation of the hydrogel. Thus, the chitosan hydrogel having good biocompatibility and biodegradability *in vivo* system had the great potential as a thermosensitive injectable depot system for local drug delivery especially to treat cancer.

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