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공학박사학위논문

# **Injectable Hydrogel Systems Based on Thermo-Sensitive Polymer for Delivering Bioactive Agents**

생체 활성 물질 전달을 위한 온도 민감성 고분자  
기반의 주사 가능한 수화젤 시스템

2016 년 8 월

서울대학교 대학원

재료공학부

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# **Abstract**

## **Injectable Hydrogel Systems Based on Thermo-Sensitive Polymer for Delivering Bioactive Agents**

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Pluronic F127, poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer, is well known material for its thermally induced reversible sol-gel transition behavior above critical gelation concentration owing to micelle packing mechanism. Because of different low critical solution temperature of each block, the micelle structure is obtained above around 15 °C and more micelles are formed as

temperature increases. This temperature sensitive property have attracted a lot of interests in biomedical field especially for therapeutic agent delivery carriers due to high water contents and soft texture similar with extracellular matrix, but its low mechanical strength limits clinical applications.

The micelle packing behaviors in Pluronic F127 hydrogel can be controlled by simple end group modification, resulting to drastic changes in gelation behavior. In chapter 2, the micelle packing abilities were compared by observing gelation behaviors with different interactions. After the adamantane groups were conjugated to the terminal end of Pluronic F127, the critical gelation concentration was increased, suggesting hindered micelle packing. The introduction of  $\beta$ -cyclodextrin polymer, however, significantly facilitated hydrogel formation owing to host-guest interactions between  $\beta$ -cyclodextrin and adamantane groups. The interactions between micelles are considered as a key factor to determine overall gelation behavior of Pluronic F127 hydrogel.

From the understandings of Pluronic F127 hydrogel, the possibility of delivery carriers was evaluated. In chapter 3, Pluronic F127 multiblock copolymers were conjugated to several functional proteins for delivering bioactive agents including proteins and cells. Gelatin was utilized for cell delivery carrier due to its abundant Arg-Gly-Asp sequences which can

provide cell adhesion site and albumin and lysozyme were introduced into the hydrogel matrix for charge interaction mediated sustained protein release behavior. To overcome the viscosity problem caused by high molecular weight of Pluronic F127 multiblock copolymer, differently modified Pluronic F127 blend system was designed in chapter 4. The resultant hydrogel showed highly increased stability in physiological environment and used for islet cell delivery system. From *in vivo* experiments, the high glucose level of mice with diabetes was controlled around 200 mg/dl for 2 weeks. Overall, the hydrogels are thought to be a promising delivery carrier for proteins and cells.

**Keywords :** Pluronic F127, hydrogel, injectable system, micelle packing mechanism, protein delivery system, cell delivery system

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# **Chapter 1.**

## **Introduction**

## **1.1. Hydrogels for Biomedical Application**

Hydrogels have been of great interest in biomedical field for many years since Wichterle developed crosslinked poly(hydroxyethyl methacrylate) (HEMA) hydrogels for soft contact lens <sup>[1]</sup>. Hydrogels are three-dimensional hydrophilic polymeric networks which absorb up to thousands of times water in their contents. Their high water contents, soft nature and porosity enable them to be similar with natural living tissues, so they have been extensively applied for pharmaceutical purposes such as tissue engineering <sup>[2], [3], [4]</sup>, biosensors <sup>[5]</sup>, wound dressing <sup>[6]</sup> and various delivery systems <sup>[7], [8], [9]</sup>.

Hydrogels can be prepared in two different ways, physically and chemically crosslinking methods (Table 1-1). The physically crosslinked hydrogels utilize molecular entanglements or secondary interactions including ionic, hydrogen bonding or hydrophobic interaction as a driving force for obtaining network structures. Physically crosslinked hydrogels usually display reversible sol-gel transition behavior, so they can be dissolved by changing external environments such as pH, ionic strength and temperature. For example, alginate gel beads are commonly formed by adding sodium or potassium alginate solution into an aqueous solution of

**Table 1-1.** Comparison of physically and chemically crosslinked hydrogels

Physical Crosslinking	Chemical Crosslinking
Ionic interactions (alginate etc.)	Polymerization (acryloyl group etc.)
Hydrophobic interactions (PEO-PPO-PEO etc.)	Radiation ( $\gamma$ -ray etc.)
Hydrogen bonding (PAAc etc.)	Small-molecule crosslinking (glutaraldehyde etc.)
Stereocomplexation (enantiomeric lactic acid etc.)	Polymer-polymer crosslinking (condensation etc.)
Supramolecular chemistry (inclusion complex etc.)	
Biocompatible, Low cytotoxicity	Toxic due to additional crosslinkers or residual monomers (additional purification is required.)
Reversible sol-gel transition	Irreversible
Low physical property	High mechanical strength

calcium ions like calcium chloride ( $\text{CaCl}_2$ ). By controlling gelation rate with calcium ion solution, the crosslinking density and polymer concentration within the gel bead can be varied <sup>[10]</sup>. Song et al. also fabricated hydrogels using aqueous acrylamide (AAm) solution in the presence of poly(*N*-vinylpyrrolidone) (PVP) without any chemical initiators or covalent crosslinking agents. The gel formation was achieved by strong cooperative hydrogen bonding between the PVP and PAAm chains <sup>[11]</sup>. Physically crosslinked hydrogel, however, displays relatively low gel modulus, so its application in biomedical field is limited. Chemically crosslinked hydrogels can be obtained by using polyfunctional crosslinking agents in polymerization process or direct chemical crosslinking of water soluble polymers. The polymerization using polyfunctional crosslinker is usually achieved by free radical generating initiators such as 2,2-azobisisobutyronitrile (AIBN) and benzoyl peroxide or by UV irradiation. Wang et al. reported about hydrogel with PHEMA and gelatin by synthesizing methacrylic anhydride modified gelatin and irradiating UV light to facilitate cell behavior on the hydrogels <sup>[12]</sup>. The chemically crosslinked hydrogel exhibits high mechanical strength enough to be used for pharmaceutical purposes, but the hydrogel with chemical conjugation often reveals problems due to unreacted residual monomers and additional crosslinkers



which can cause severe toxicity and therefore additional purification procedure is necessary.

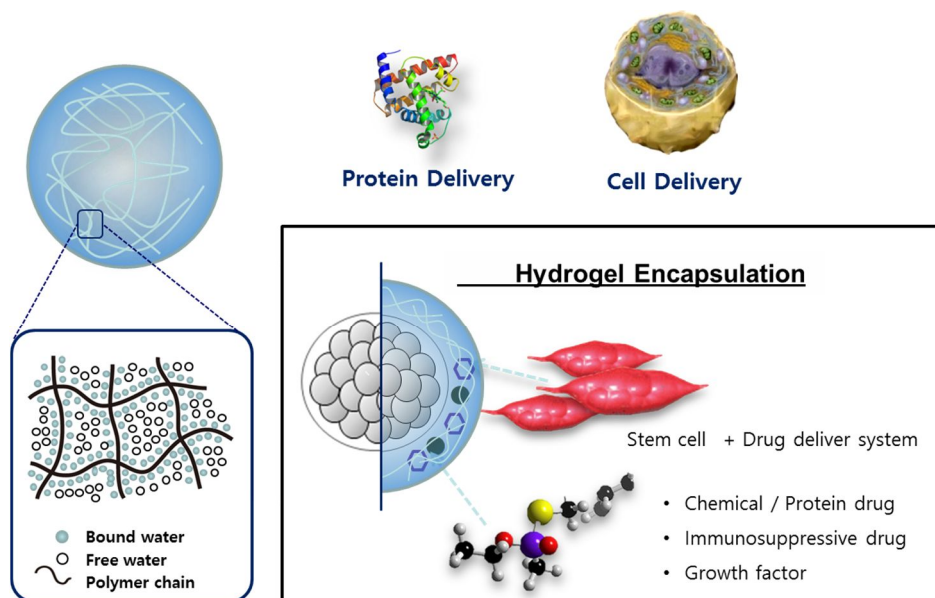
## **1.2. Hydrogels for Protein and Cell Delivery**

The character of the water in hydrogel matrix can determine the overall permeation of nutrients and oxygen. Generally, when a dry hydrogel starts to absorb water, the first water molecule enters to the matrix and hydrates the most polar and hydrophilic groups in polymer chain, often called '*primary bound water*'. As the polymer chain is further hydrated, the network structure swells and additional water molecule interacts with exposed hydrophobic groups, leading to hydrophobically bound water or '*secondary bound water*'. After the hydrophilic polar groups and hydrophobic sites fully interact with water molecule, additional water can be imbibed to the network structure by osmotic forces, and the hydrogel will meet an equilibrium swelling state. This additional swelling water molecule is typically referred as '*free water*' or '*bulk water*' which fills the space between the polymer network chains. The amount of bound water and free water will determine the average pore size and distribution in hydrogel matrix <sup>[13]</sup>. These unique properties of hydrogels have raised enormous

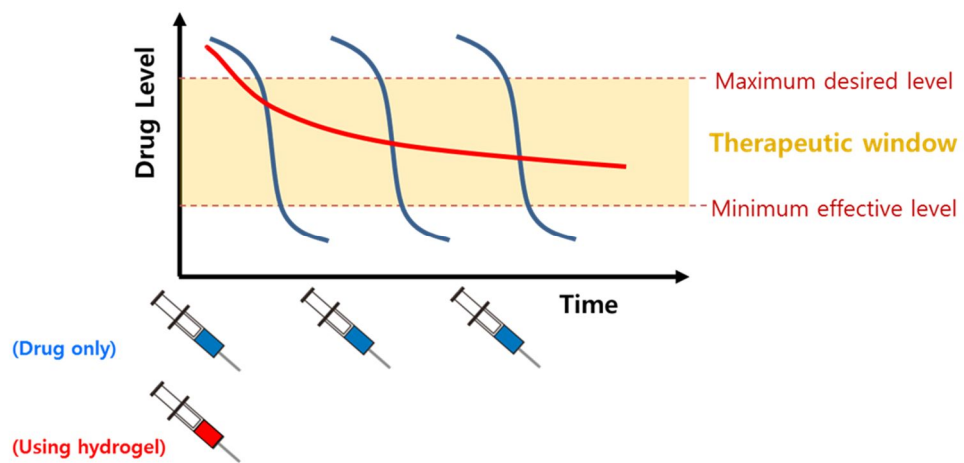
attentions in their use as reservoir systems especially for proteins and cells since the free water in hydrogel matrix can prevent them from denaturation (Figure 1-1).

Ideal delivery system should be able to provide controlled release behavior to maintain therapeutic levels for an extended period of time and ensure the stability of encapsulated proteins. In order to achieve the desired therapeutic effect, an appropriate amount of bioactive agents should be delivered to the target site in the body. However, due to their poor solubility, fast proteolysis and short half-lives, the effective delivery is limited <sup>[14]</sup>. The protein drugs undergo enzymatic degradation process readily in the blood stream and degraded drugs are cleared by the kidney. Accordingly, frequent injections of high doses of bioactive therapeutic agents are required to maintain the therapeutic effect but another significant problems such as toxic side effects and immune response can be caused. The frequent multiple administrations can also be burdensome to patient with discomfort and expensive treatment (Figure 1-2).

To overcome this limitation, it is necessary to develop delivery systems which can release the loaded bioactive agents with controlled manner over a prolonged period of time <sup>[15]</sup>. In this point of view, hydrogels have gained considerable interests as an ideal delivery vehicle for proteins and cells



**Figure 1-1.** Hydrogels for delivery carrier



**Figure 1-2.** The use of hydrogel for prolonged therapeutic window

because of their unique properties. The high water contents and highly porous structure of hydrogel enable them to carry bioactive agents with preserving the native structures and bioactivities <sup>[16]</sup>. The component of delivery carrier also must be non-immunogenic, non-toxic, biodegradable and biocompatible for clinical uses. Protein release behavior from hydrogel structure is generally governed largely by diffusion and surface erosion mechanism and a lot of the gel matrices exhibit the diffusion controlled release profile with proportional to square root of time, the Higuchi's kinetics <sup>[17]</sup>. In the case of cell delivery system, on the other hand, cell viability as well as protein stability should be considered. The highly hydrated hydrogel nature can mimic the environment of extracellular matrix (ECM) and therefore provide ideal cellular microenvironment for cell proliferation and differentiation. A lot of scientists applied hydrogels for the purpose of tissue engineering, the *ex vivo* cultivation of cells on polymeric hydrogel scaffolds in order to generate tissues for transplantation <sup>[18]</sup>. Recently, to maximize the therapeutic efficacy, several studies about increasing cell viability have been reported using peptide with Arg-Gly-Asp (RGD) sequences. The RGD amino acid sequence is known to be able to function as cell attachment sites recognized by many integrins in cells, facilitating cell adhesion and proliferation and enabling protein secretion for

a long period of time <sup>[19]</sup>.

### **1.3. Injectable Hydrogel Systems**

For the ease of hydrogel administration, injectable hydrogels have been emerged as promising biomaterials. These injectable systems can suggest an effective and convenient way of administration of various bioactive agents such as proteins, genes and living cells to minimize surgical process and prevent the discomfort of patient. To make injectable systems, various methods using external stimuli such as temperature and pH have been investigated.

Triblock copolymer of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO), also called Poloxamer or Pluronic, is the most widely used material for injectable hydrogel systems because it shows thermally induced reversible gelation behavior in an aqueous environment above critical gelation concentration. Each block is readily soluble in water at low temperature, but the solubility gradually decreases as temperature increases, similar with the polymers with low critical solution temperature (LCST). Although the LCST of PEO and PPO are reported above 100 °C and around 10 °C, respectively, so it can form micellar

structure above the LCST of PPO middle block. As temperature goes higher, the more micelles are formed and eventually the physically crosslinked network structure, the gel state with no fluidity, is obtained. This physically crosslinked structure can be disrupted by decreasing temperature, suggesting the reversible gelation behavior, and this fancy property has attracted a lot of attentions in biomedical field. However, the Pluronic hydrogel reveals fatal disadvantages to be used in clinical applications, limitation of deep site injection and its low mechanical strength. Temperature-sensitive hydrogels have severe problem in the case of deep site injection due to the rapid temperature changes during injection, so the blockage of syringe needle hinders successful administration. Even if the Pluronic hydrogel is successfully transplanted into the body, the gel formation disappears within one day, so the function as a delivery carrier is hard to be expected.

To increase the hydrogel stability, multiblock copolymerization method was reported. Cohn et al. synthesized Pluronic multiblock copolymers using hexamethylene diisocyanate as a linker and the Pluronic multiblock copolymer hydrogel showed highly increased stability and the hydrogel formation was maintained for about 40 days <sup>[20]</sup>. To make stable hydrogel in physiological condition, stereocomplex method was also investigated <sup>[21]</sup>. Park et al. fabricated a series of multiblock Pluronic

copolymers linked by D-lactide and L-lactide oligomers with various degree of polymerization and the stereocomplexed hydrogels displayed far increased mechanical strength compared to multiblock copolymer hydrogels due to the additional crystalline domains.

Another type of temperature-sensitive hydrogel is poly(lactic acid-*co*-glycolic acid)-poly(ethylene oxide)-poly(lactic acid-*co*-glycolic acid) (PLGA-PEO-PLGA) triblock copolymer. This hydrogel displays better biodegradability, higher gelation temperatures and longer periods of sustained loaded therapeutics release compared to Pluronic hydrogel system [22]. For other synthetic thermally induced gelling polymers, poly(*N*-isopropylacrylamide) (PNIPAAm) is one of the most widely investigated polymers for further prolonged drug release with low concentration and it has been reported that PNIPAAm-poly(phosphorylcholine)-PNIPAAm triblock copolymers form gel structure at around 7 wt% in aqueous solution [23]. Poly(caprolactone) (PCL) has also been utilized for copolymerization with PEO in diblock and triblock formations to facilitate the prolonged release behavior. The PEO-PCL diblock copolymers exhibited enhanced sustained release behavior over a period of 30 days owing to the stability of network structures compared to Pluronic hydrogels [24]. It is also reported about poly(organophosphazenes) which can produce mechanically strong



hydrogels at concentration below 15 wt%. Various hydrophobic, hydrophilic and other functional polymers were introduced to the poly(organophosphazenes) polymer backbone to control the gelation behavior and degradation rate <sup>[25]</sup>.

Injectable pH- and temperature-sensitive hydrogel systems were developed to prevent the gelation in syringe needle during injection. Lee et al. fabricated pentablock copolymers by conjugating the sulfamethazine oligomers (OSMs) to the thermo-sensitive poly(caprolactone-*co*-lactic acid)-PEO-poly(caprolactone-*co*-lactic acid) (PCLA-PEO-PCLA) triblock copolymers <sup>[26]</sup>. The resultant polymer hydrogel showed reversible sol-gel transition behavior by changing pH in the range of 7.4 ~ 8.0 in aqueous solution. In pH 8.0, the hydrogel formation was not obtained in the range of 4 ~ 60 °C because of the ionized sulfonamide oligomer groups. In the condition of pH 7.4, however, the thermo-sensitive gelation behavior was recovered because the sulfonamide groups become neutral, resulting to facilitate hydrophobic interactions between PCLA and OSM blocks. This hydrogel can be readily injected without premature gelation in the syringe needle, and rapidly forms strong gel after injection. Paclitaxel (PTX) was utilized for evaluating this pH and temperature sensitive hydrogel by *in vivo* experiment with tumor bearing mice and showed antitumor efficacy for up

to 2 weeks with one injection.

There also have been studies about injectable hydrogels with shear-thinning properties. Leeuwenburgh et al. designed the system with mixing oppositely charged gelatin nanospheres <sup>[27]</sup>. They fabricated cationic and anionic gelatin nanospheres using glutaraldehyde as a crosslinker with around 200 nm diameter. Above 15 w/v% condition, the mixed suspension formed gel state owing to electrostatic interactions and the gel displayed shear-thinning property, suggesting to be applicable to injectable systems. Also, Burdick et al. developed shear-thinning hydrogels with host-guest interactions using  $\beta$ -cyclodextrin and adamantane complex <sup>[28]</sup>. They conjugated  $\beta$ -cyclodextrin and adamantane to the hyaluronic acid backbone and developed self-assembling hyaluronic acid hydrogels based on host-guest interactions. The resultant hydrogel permitted shear-thinning behavior for the ease of injection and the gel displayed sustained release behavior for over 60 days.

#### **1.4. Effect of End Group Modification**

Simple modification at the end groups of stimuli-sensitive triblock copolymers has been reported to make dramatic changes in the macroscopic

physical gelation behavior. The PLGA-PEO-PLGA triblock copolymer displays thermally induced gelation behavior and the critical gelation concentration and temperature are strongly dependent to the composition and the length of each block. Ding and coworkers conjugated various hydrophobic molecules with different length at the terminal end of PLGA-PEO-PLGA triblock copolymers <sup>[29]</sup>. The PLGA-PEO-PLGA derivatives showed quite different gelation behavior because the subtle hydrophobicity changes caused surprisingly different macroscopic behaviors. Pluronic F127 also exhibited the drastic different gelation behaviors by end group modification. Park et al. modified Pluronic F127 using succinic anhydride to make carboxylic acid groups at the end of Pluronic F127 <sup>[30]</sup>. After modification, the resultant hydrogel showed pH-sensitivity owing to the electrostatic interactions and PEO dehydration, controlling macroscopic micelle packing abilities. In our previous studies, we also confirmed the effect of end group modification of Pluronic F127 <sup>[31]</sup>. Various diamines were conjugated to the end group of Pluronic F127 and the changes on gelation behavior were monitored. When diethylenetriamine (DETA) and triethylenetetramine (TETA) were conjugated to Pluronic F127, closed-loop phase transition behavior was observed as pH increases. The protonated amine groups cause repulsion between micelles at low pH, hindering

hydrogel formation with tightly packed micelle structure. As pH increases, the positive charges in amine groups become neutral and the repulsion forces disappear, recovering tightly packed structure. At high pH condition, the sol state was observed due to the dehydration of PEO chain. For the purpose of increase hydrogel modulus, oppositely charged Pluronic F127 was blended and the gelation behavior was analyzed <sup>[32]</sup>. Hydrogels with negatively charged Pluronic F127 or positively charged one showed lower storage modulus values because of repulsion interactions between micelles. The oppositely charged Pluronic F127 blend system with optimized composition ratio, however, exhibited much higher mechanical strength compared to Pluronic F127 hydrogel since the attraction forces between micelles act dominantly in the blend system.

## **1.5. Research Objectives**

During the last decades, injectable hydrogels have attracted lots of attentions in biomedical field due to their high water contents because they are considered as a potential candidate for delivery carriers to minimize immune response and maintain therapeutic window for an extended period of time. Pluronic F127 exhibits thermally induced reversible sol-gel

transition behavior above critical gelation concentration caused by micelle packing mechanism. For clinical applications, the low mechanical strength of Pluronic F127 hydrogel should be increased, so a lot of methods were investigated including multiblock copolymerization<sup>[20]</sup> and stereocomplexation<sup>[21]</sup>. Also, it has been already reported that the effect of end group modification cause dramatic changes in gelation behavior. In chapter 2, the micelle packing abilities were compared by observing gelation behaviors with different interactions to understand gelation mechanism of Pluronic F127, and the relationship between macroscopic micelle behaviors and hydrogel stability was investigated.

In chapter 3, the function of hydrogel as a delivery carrier was evaluated. For cell delivery, Pluronic F127 multiblock copolymer was conjugated to gelatin with a lot of Arg-Gly-Asp sequences which can facilitate cell adhesion and proliferation. Albumin and lysozyme were also utilized for protein delivery carrier because they display negative and positive charges, respectively. When the proteins are incorporated into hydrogel matrix, the highly sustained release behavior is expected due to electrostatic attraction forces with loaded proteins. By *in vitro* experiments, the clinical application possibility of Pluronic F127 multiblock copolymer-protein conjugate hydrogel was evaluated.

In chapter 4, the blend system with differently modified Pluronic F127 was designed for low viscosity and high mechanical strength. The gelation behavior was confirmed by observing phase diagram transition curves and monitoring the hydrogel dissolution rate. The resultant hydrogel was applied to islet cell delivery carrier for treating diabetes and the possibility for cell delivery carriers was estimated.

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## **Chapter 2.**

### **Host-Guest Interaction Mediated Pluronic F127**

### **Hydrogel Based on Enhanced Micelle Packing**

### **Mechanism**

## 2.1. Introduction

Temperature sensitive polymers displaying sol-gel transition behavior have attracted a lot of interests in biomedical field due to their possibility for injectable reservoir for protein delivery and cell therapy <sup>[1], [2]</sup>. Various block copolymers including poly(ethylene oxide) (PEO), poly(propylene oxide) (PPO) <sup>[3], [4]</sup>, poly(lactic acid), poly(glycolic acid) <sup>[5], [6]</sup>, poly( $\epsilon$ -caprolactone) <sup>[7], [8]</sup> and poly(*N*-isopropylacrylamide) <sup>[9], [10]</sup> have been widely investigated to induce gelation behavior using temperature as a stimulus and the temperature and concentration for the onset of gelation can be changed by controlling length and composition of each block <sup>[11]</sup>. Pluronic F127, PEO-PPO-PEO triblock copolymer, is one of the materials showing thermo-gelling property in aqueous solution above 16 wt% polymer concentration and the gelation is reported to be based on physical micelle packing mechanism associated by hydrophobic interactions <sup>[12], [13]</sup>. However, Pluronic hydrogel with self-assembled structure exhibits low mechanical property and poor stability *in vivo*, which limit the application as a delivery carrier <sup>[14], [15]</sup>.

$\beta$ -cyclodextrin (CD) is a cyclic oligosaccharide consisting of 7 dextrose units connected *via*  $\alpha$ -1,4-glucosidic linkages and employed in

various chemical applications using amphiphilic characteristics of the molecule <sup>[16]</sup>. 21 hydroxyl groups in  $\beta$ -CD are located at the outer shell of the molecule and interior cavity displays hydrophobic property which offers binding sites to a hydrophobic molecules such as adamantane (Ad) or cholesterol *via* hydrophobic as well as van der Waals interactions <sup>[17]</sup>. Ritter et al. polymerized  $\beta$ -CD (CDP) in a linear or a globular structure and proposed physically crosslinked hydrogels based on host guest interactions between  $\beta$ -CD cavity and Ad groups <sup>[18]</sup>. Burdick et al. also developed a hydrogel system by introducing  $\beta$ -CD and Ad into hyaluronic acid backbone and applied the hydrogel as a protein delivery carrier <sup>[19]</sup>. Interestingly, previously reported hydrogels using host guest interactions as a driving force for three-dimensional network formation exhibited shear-thinning thixotropic properties, which endowed them with easy administration into a body with simple injection.

Here, we applied the host guest interactions to conventional Pluronic F127 hydrogel to increase gel stability. The Ad molecule was introduced into two terminal hydroxyl groups of Pluronic F127 to provide binding ability using host guest interactions with CDP. The gelation behavior was observed by confirming phase diagram and the gelation mechanism was elucidated by monitoring dynamic light scattering (DLS)

and rheometrics mechanical spectrometry (RMS) analysis. The stability of F127-Ad and CDP blend hydrogel in physiological environment was finally evaluated by *in vitro* and *in vivo* experiments.

## 2.2. Experimental

**Materials.** Pluronic F127 [(PEO)<sub>99</sub>-(PPO)<sub>69</sub>-(PEO)<sub>99</sub>], *p*-nitrophenyl chloroformate (*p*-NPC) (96.0 %), 1-adamantane (Ad) methylamine (98.0 %),  $\beta$ -cyclodextrin ( $\beta$ -CD) ( $\geq$  97.0 %), triethylamine (TEA) ( $\geq$  99.0 %), epichlorohydrin ( $\geq$  99.5 %) and toluene (99.8 %) were purchased from Sigma Aldrich (St. Louis, MO) and methylene chloride (99.5 %), isopropanol (99.0 %), diethyl ether (99.0 %), hydrochloric acid (HCl) (35.0 %), sodium hydroxide (NaOH) (99.0 %), sodium chloride (NaCl) (99.0 %) and magnesium sulfate (99.0 %) were obtained from Daejung (Korea). Methylene chloride and TEA were dried over calcium hydride before use and all other chemicals were used as received.

**Activation of Pluronic F127 with *p*-NPC (F127-NPC).** 10.00 g (0.79 mmol) of Pluronic F127 was placed into a 250 mL two-neck round bottom flask and dried under vacuum at 120 °C for 3 h before reaction. After the polymer was clearly dissolved in 100 mL anhydrous methylene chloride, *p*-NPC (0.48 g, 2.38 mmol) and TEA (0.24 g, 2.38 mmol) were introduced and the reaction continued for 24 h at room temperature. The

mixture was washed with 150 mL brine solution and the organic layer was dried over magnesium sulfate. After concentration, the product was isolated by precipitating into 10-fold excess volume of cold diethyl ether and dried under vacuum for 3 days (yield : 89 %).  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ ,  $\delta$ , ppm) = 4.3 (t, 4 H,  $-\text{CH}_2\text{CH}_2\text{OC}(=\text{O})-$ ), 7.5-8.5 (d, 8 H, Ar of *p*-NPC), 3.2-3.8 (br, 2 H of PEO and 1 H and 2 H of PPO), 1.0-1.3 (br, 3 H of PPO).

**Conjugation of 1-adamantane (Ad) methylamine with F127-NPC (F127-Ad).** F127-NPC (5.07 g, 0.39 mmol) was dissolved in 50 mL anhydrous methylene chloride in a 100 mL two-neck round bottom flask equipped with a magnetic stirrer under nitrogen atmosphere. After addition of 1-adamantane (Ad) methylamine (0.19 g, 1.18 mmol) to the solution, the reaction was allowed to proceed for 12 h at room temperature. To the reaction mixture was poured 100 mL brine solution and stirred for additional 1 h. The organic layer was separated and dried over magnesium sulfate. After concentration, the solution was precipitated into 10-fold excess volume of cold diethyl ether and the white power product was collected and dried under vacuum for 3 days (yield : 76 %).  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ ,  $\delta$ , ppm) = 4.3 (t, 4 H,  $-\text{CH}_2\text{CH}_2\text{OC}(=\text{O})-$ ), 1.4-2.0 (br, 15 H of Ad), 3.2-3.8 (br, 2 H of PEO and 1 H and 2 H of PPO), 1.0-1.3 (br, 3 H of PPO).



**CD polymerization.** 10.00 g (8.81 mmol) of  $\beta$ -CD was dissolved in 15 mL NaOH aqueous solution (15 wt%) in a 100 mL two-neck round bottom flask at 35 °C for 2 h. After adding 2 mL toluene, 3.86 g (41.70 mmol) of epichlorohydrin was introduced to the mixture. The polymerization reaction proceeded for 3 h and the solution was poured into 200 mL isopropanol. The precipitate was dissolved again in water and neutralized using 1 M HCl solution. The product was isolated by dialysis (MWCO : 5,000) for 5 days and lyophilization (yield : 75 %).  $^1\text{H-NMR}$  ( $\text{D}_2\text{O}$ ,  $\delta$ , ppm) = 5.2 (d, 7 H of  $\beta$ -CD), 3.5-4.0 (br, 1H and 2H of epichlorohydrin and  $\beta$ -CD).

**Phase Diagram of Sol-Gel Transition.** Pluronic F127 and F127-Ad hydrogels were prepared by dissolving in pH 7.4 phosphate buffered saline (PBS, 10 mM, 0.138 M NaCl) solution with various concentrations at 4 °C. To make homogeneous F127-Ad and CDP blend solution (F127-Ad/CDP), CDP with desired amount was introduced to the solution of F127-Ad and immersed in a water bath for additional 6 h at 4 °C. The sol-gel transition was monitored using a test tube tilting method by gradual temperature increase, and the gel state was determined when no fluidity is

observed for 1 min <sup>[20]</sup>.

***In Vitro* Gel Dissolution Rate.** Pluronic F127 and F127-Ad/CDP blend solution were prepared by dissolving each polymers in PBS solution and 1 mL of each solution was placed in 4 mL vials. After all samples were incubated in a water bath with 37 °C for 3 h, a 3 mL PBS solution was added to the vial with preformed hydrogel and the height of the gel was measured with changing PBS solution at determined periods. The remaining gel volume was calculated by comparing with the initial one.

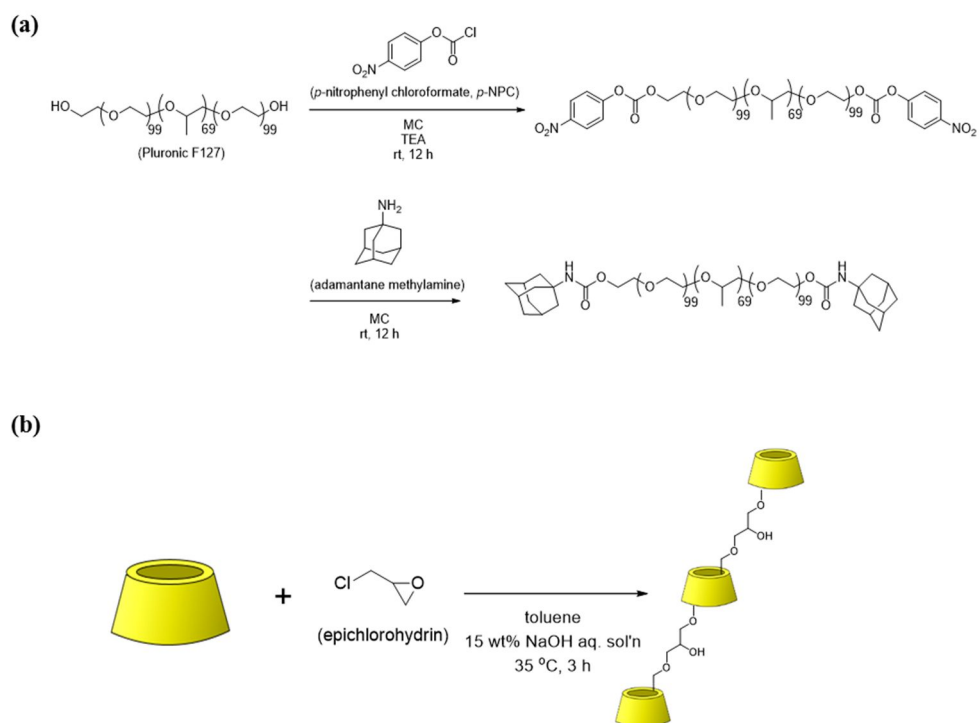
***In Vivo* Gel Dissolution Rate.** The samples with desired concentration of F127-Ad and CDP were prepared and injected subcutaneously to C57BL/6 mice to confirm the stability of each hydrogel again using syringe with 31 gauge needle. The transplanted gel formation was monitored every day.

**Instruments.** <sup>1</sup>H-NMR analysis was performed using Bruker Advance 300 MHz spectrometer in CDCl<sub>3</sub> and D<sub>2</sub>O. Measurement of

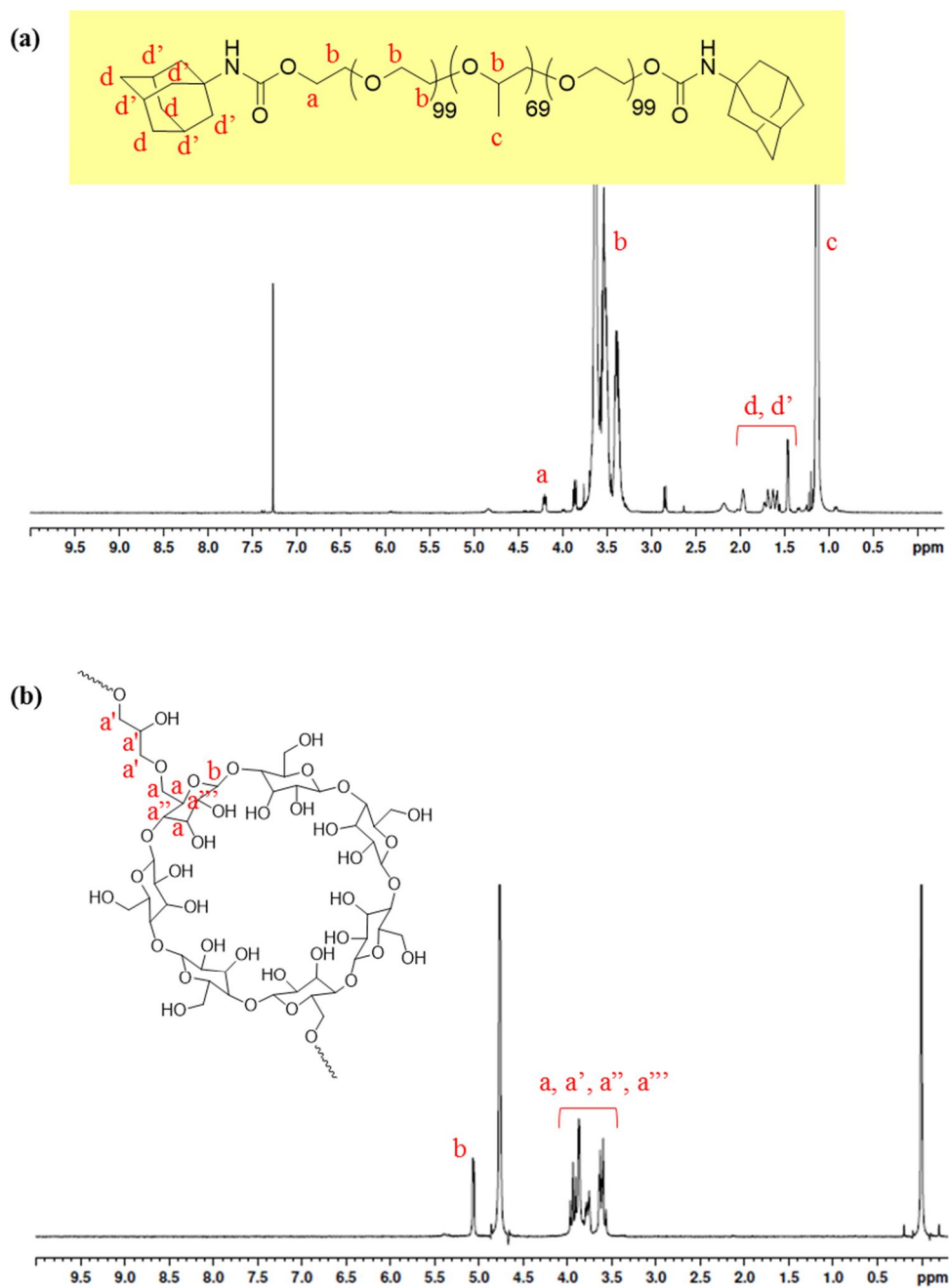
micelle size was carried out using Otsuka ELS-Z instrument and the viscosity was analyzed by advanced rheometric extended systems (ARES, The Rheometric Science Inc., NI)

## 2.3. Results and Discussion

To compare gelation behaviors from different micelle packing abilities, Ad group was introduced into two distal chain ends of Pluronic F127 and  $\beta$ -CD was polymerized using epichlorohydrin as shown in Scheme 2-1. Two hydroxyl groups of Pluronic F127 were firstly activated by *p*-NPC and followed by reacting with 1-adamantane (Ad) methylamine. The resultant product was successfully modified with Ad groups *via* carbamate linkage and the degree of functionalization was around 100 % when confirmed by  $^1\text{H}$  NMR (Figure 2-1(a)). The peak at 4.2 ppm and peaks from 1.5 to 2.0 ppm indicate that the Ad groups were successfully conjugated at the end of Pluronic F127 and undesired nitrophenyl chloroformate and nitrophenol peaks were completely disappeared. From the Figure 2-1(b), CDP contains protons relevant to both  $\beta$ -CD and epichlorohydrin and the amount of  $\beta$ -CD and epichlorohydrin were calculated almost the same, as expected. It is already reported that the linear structured CDP is obtained instead of globular structure by adding toluene during polymerization process because of the steric hindrance of CD-toluene complex <sup>[18]</sup>. The solubility of CDP in water was also highly increased due to the lack of crystalline domains compared with  $\beta$ -CD, so it



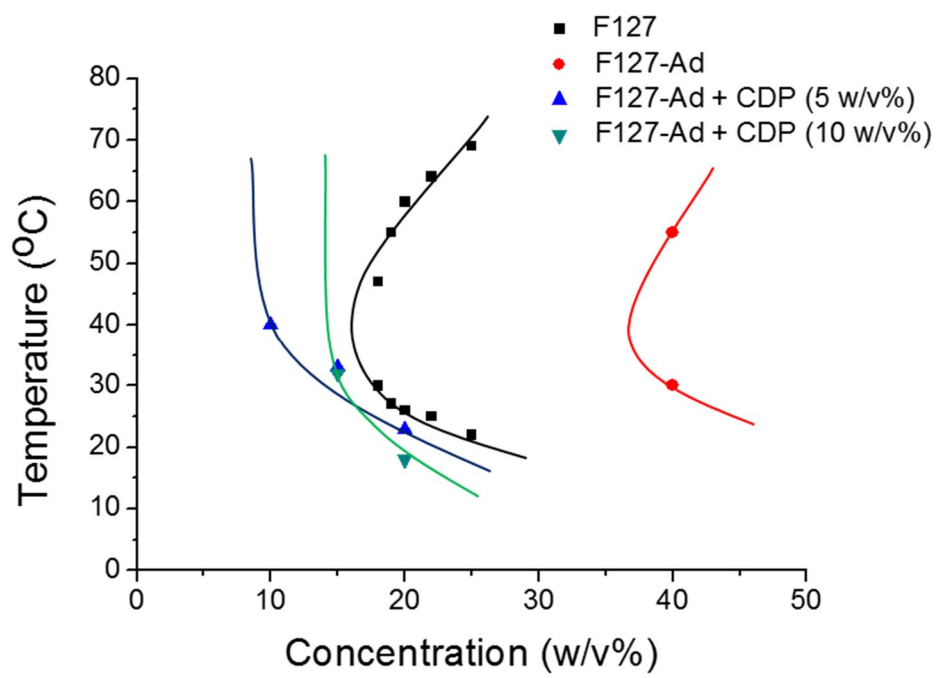
**Scheme 2-1.** Synthetic scheme of (a) F127-Ad and (b) CDP



**Figure 2-1.**  $^1\text{H}$ -NMR spectrum of synthesized (a) F127-Ad and (b) CDP

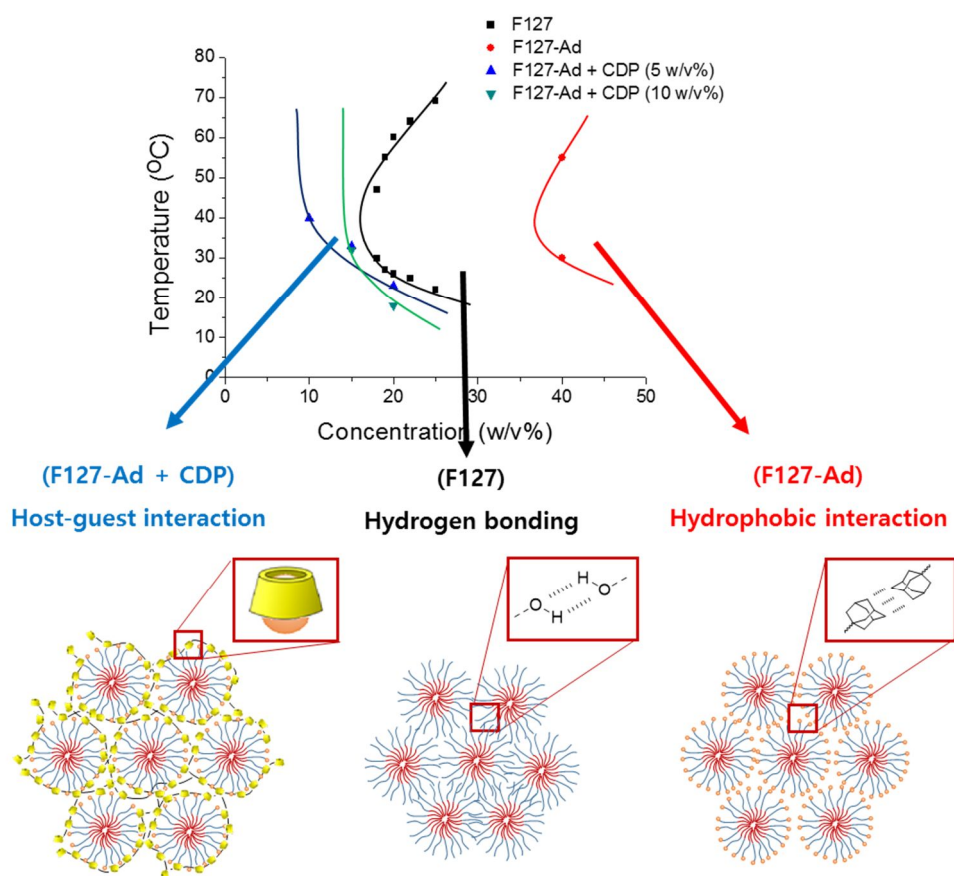
is appeared that the  $\beta$ -CD was successfully polymerized in a linear structure with around 100 kDa molecular weight.

Hydrogels were prepared by dissolving each polymers in pH 7.4 PBS solution with various concentrations at 4 °C water bath for 1 day and sol-gel phase transition behaviors were observed using vial converting method (Figure 2-2). All the samples exhibited reversible sol-gel transition behavior as temperature increases above critical gelation concentration. When the hydroxyl groups were modified with Ad groups, the critical gelation concentration was significantly increased with narrow gel windows compared with unmodified Pluronic F127 hydrogel, requiring higher polymer concentration for gelation. In the presence of CDP, however, the sol-gel transition curve of F127-Ad was substantially changed with much less critical gelation concentration. This phenomenon is of great interest in understanding gelation behavior of Pluronic F127 caused by micelle packing mechanism. As shown in Figure 2-3, Pluronic F127 is composed of PEO-PPO-PEO triblock copolymer and the property of hydrophilic PEO shell is a key factor in forming micelle packing structure for gelation. It is well known that the Pluronic F127 copolymer assembles in the form of spherical micelles by accelerated hydrophobic interactions between PPO



**Figure 2-2.** Sol-gel phase diagram of F127, F127-Ad and F127-Ad/CDP blend hydrogel

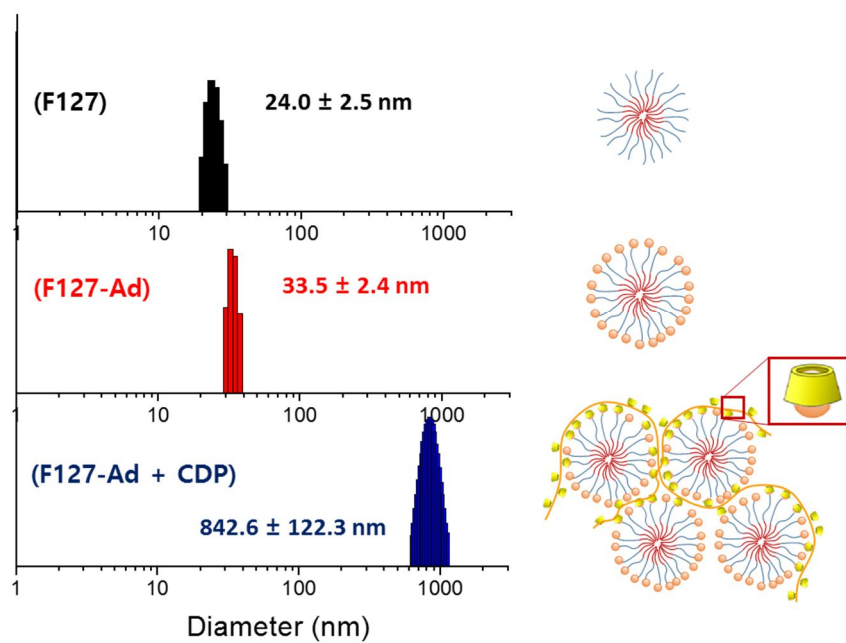




**Figure 2-3.** Schematic illustration of gelation mechanism of F127, F127-Ad and F127-Ad/CDP blend hydrogel

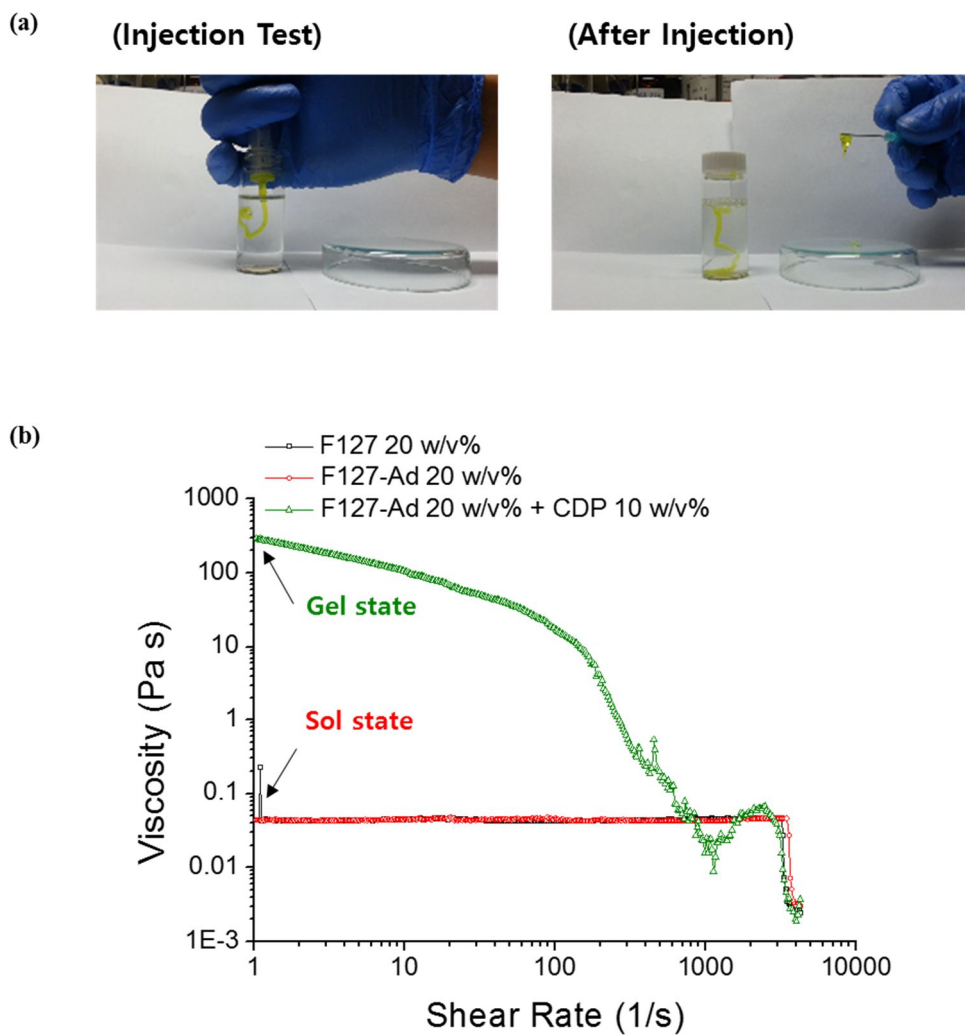
blocks in copolymer and generates physically crosslinked hydrogel structure, but the stability of micelles and interactions between them are also important for determining critical gelation concentration. The hydroxyl groups in Pluronic F127 will be located at the periphery in micelle structure, so the hydrogen bonding between micelles can facilitate tightly packed structure. After modification with Ad groups, the hydrogen bonding no longer exists due to the absence of hydroxyl groups, and only hydrophobic interaction functions as a driving force in three dimensional micelle packed network structure, so higher polymer concentration is required for gelation. In the case of CDP and F127-Ad blend system, the interactions between F127-Ad micelles are significantly increased due to host-guest interactions between  $\beta$ -CD and Ad molecules, resulting in much lowered critical gelation concentration. Interestingly, the solution with 10 w/v% of F127-Ad and 5 w/v% CDP exhibited thermally induced gelation behavior although 10 w/v% of F127-Ad solution with 10 w/v% CDP did not. It appears that too much CDP concentration hinders host-guest interaction mediated tightly packed structure because the number of F127-Ad micelles interacting with one CDP chain decreases, thereby showing higher critical gelation concentration.

The interactions between F127-Ad and CDP were confirmed by



**Figure 2-4.** DLS analysis of F127, F127-Ad and F127-Ad/CDP blend solution (0.1 w/v%)

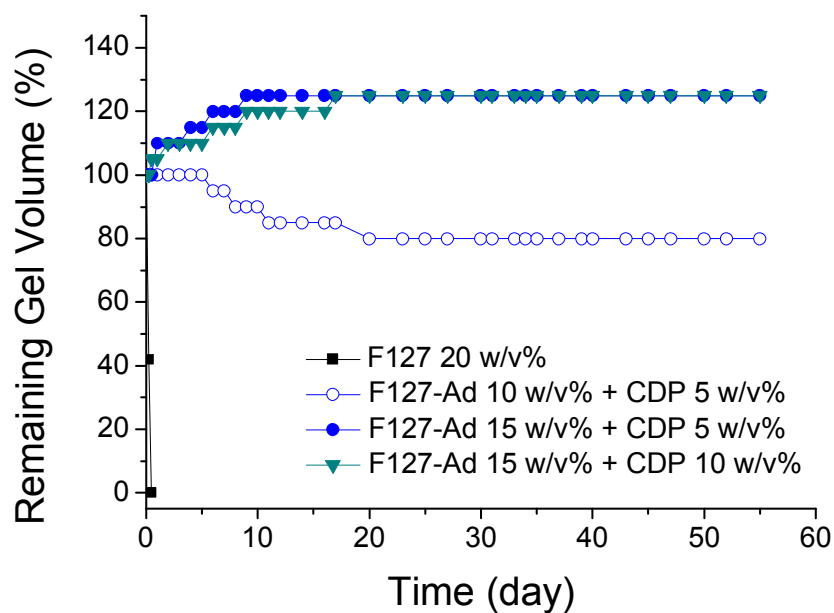
measuring micelle size and monitoring rheological behavior to support the proposed gelation mechanism. As shown in Figure 2-4, the DLS results revealed that the micelle size of unmodified Pluronic F127 was measured to 24 nm and the slight increase up to 33 nm was observed after end group modification with Ad. After introduction of CDP in the solution of F127-Ad, the peak with about 1  $\mu\text{m}$  size was detected, and it is appeared that the host-guest interactions between F127-Ad and CDP caused large aggregates. The injectability was firstly confirmed by simple test using F127-Ad 20 w/v% hydrogel with CDP 10 w/v% because host-guest assembly is known as reversible dynamic interactions (Figure 2-5). The gel was able to be easily injected to the PBS solution at room temperature although it exhibited gel state before injection and the injected solution recovered its original gel property after injection which is the same with typical behavior of thixotropic materials. The hydrogels were further subjected to continuous flow experiments and the viscosity change was monitored. The viscosity was drastically decreased when the shear force was applied, and the value was almost similar with F127 20 w/v% and F127-Ad 20 w/v% solutions with sol state. This result suggests that the host-guest interaction mediated hydrogels maintained their thixotropic properties, facilitating easy administration even in high concentrations although the gelation is already



**Figure 2-5.** (a) Photographs of injection test of F127-Ad 20 w/v% and CDP 10 w/v% and (b) viscosity measurement of F127-Ad/CDP blend hydrogel

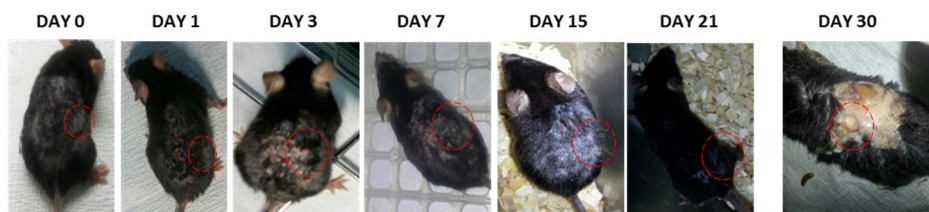
occurred.

To evaluate the gel stability, swelling and erosion behaviors of F127-Ad/CDP blend hydrogel were observed with different F127-Ad and CDP concentrations at 37 °C in pH 7.4 PBS solution (Figure 2-6). The complete erosion of prepared blend samples was not observed over 50 days while Pluronic F127 hydrogel, as expected, was disappeared within 1 day. During around 20 days, the blend hydrogel with 15 w/v% F127-Ad showed slight swelling and that with 10 w/v% F127-Ad exhibited a little volume erosion, but the overall gel structure was maintained without any additional volume changes. For *in vivo* gel dissolution rate experiment, F127-Ad/CDP blend hydrogels were injected subcutaneously into C57BL/6 mice. As shown in Figure 2-7, the blend hydrogels maintained the gel structure for more than 3 weeks, and this reveals that the host-guest interaction mediated micelle packing structure is stable in physiological conditions and can be applied to carriers for delivering bioactive agents.

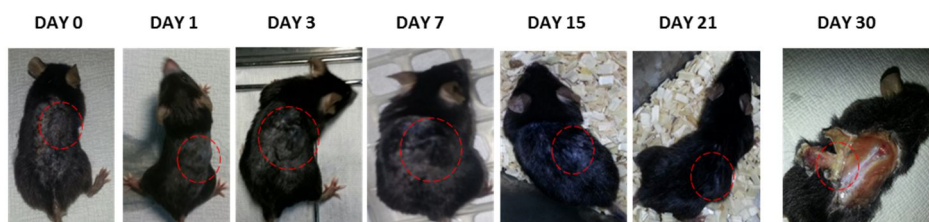


**Figure 2-6.** *In vitro* gel dissolution rate of F127, F127-Ad and F127-Ad/CDP blend hydrogel

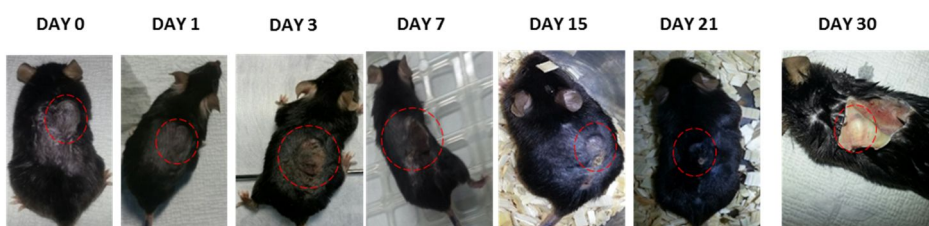
(a) F127-Ad 10 w/v% + CDP 5 w/v%



(b) F127-Ad 15 w/v% + CDP 5 w/v%



(c) F127-Ad 15 w/v% + CDP 10 w/v%



**Figure 2-7.** *In vivo* gel dissolution experiment of F127-Ad/CDP blend hydrogel with various concentrations



## 2.4. Conclusion

In conclusion, the gelation behaviors with different micelle packing mechanisms were observed by modifying the hydroxyl groups of Pluronic F127 into Ad groups and introducing CDP for blend system. The F127-Ad/CDP hydrogel exhibited significantly increased mechanical property by host-guest interactions between  $\beta$ -CD and Ad although F127-Ad hydrogel itself showed lower stability than Pluronic F127 hydrogel due to the loss of hydroxyl groups. It is expected that the F127-Ad/CDP hydrogel can be applied to various delivery systems considering the *in vitro* and *in vivo* gel stability experiment results. Also, functional polymers and proteins bearing  $\beta$ -CD moieties will be able to replace the CDP to maximizing therapeutic effect for the future study.

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## **Chapter 3.**

# **Functional Hydrogels Based on Pluronic F127 Multiblock Copolymer-Protein Conjugates for Delivering Bioactive Agents**

### 3.1. Introduction

Hydrogels are three dimensional crosslinked networks of hydrophilic polymers capable of retaining large amount of water. Since Wichterle et al. discovered and applied them to biomedical area in 1950s <sup>[1]</sup>, a number of hydrogels have been extensively investigated for pharmaceutical applications including contact lenses <sup>[2]</sup>, tissue engineering <sup>[3]</sup>, <sup>[4]</sup>, drug delivery <sup>[5]</sup>, <sup>[6]</sup>. Hydrogels are considered as biocompatible materials because their high water contents and soft environment make them similar to natural extracellular matrices (ECM). Also, their porous structure allows them to accommodate high load of water soluble bioactive agents such as therapeutic proteins and cells without denaturation.

Proteins can be physically incorporated in hydrogel matrix and the release behavior is determined by several mechanisms including swelling, diffusion and degradation, so it can be controlled by tailoring crosslink density *via* changes in polymer structure, concentration or molecular weight. The majority of the hydrogel, however, showed diffusion controlled release behavior according to the Higuchi's kinetics implying that the release profile is proportional to the square root of time <sup>[7]</sup>. In cell delivery system, on the other hand, more complicated substantial challenges are still

remaining like cell migration, proliferation, differentiation and interactions between cell and hydrogel matrix which is hardly controllable. A large number of trials have been reported about hydrogel mediated cell delivery for the regeneration of cartilage<sup>[8]</sup>, cornea<sup>[9]</sup>, liver<sup>[10]</sup>, islet<sup>[11]</sup> and nerve<sup>[12]</sup>. The most representative examples are their use for the cartilage regeneration.

To increase the efficiency of hydrogel, we utilized several functional proteins for enhancing cell viability and increasing sustainability of the bioactive molecules entrapped in the hydrogel matrices. Gelatin is a mixture of proteins obtained by partial acid or alkaline hydrolysis of collagen extracted from the skin, bone and connective tissues of animals. It contains abundant Arg-Gly-Asp (RGD) sequences which can serve as cell attachment sites recognized by a lot of integrins, so the cell adhesion and spreading is expected to be facilitated in the presence of RGD sequences<sup>[13]</sup>. Alexandra Grigore et al. already reported that the MG-63 osteosarcoma cells encapsulated gelatin modified alginate hydrogel showed highly proliferated and migrated structures making it a promising material for cell encapsulation<sup>[14]</sup>. Serum albumin is the most abundant protein in blood stream whose main function is to regulate the colloidal osmotic pressure of blood and Lysozyme (Lys), also known as muramidase or *N*-acetylmuramide glycanhydrolase, acts by attacking peptidoglycans found in

the cell walls of bacteria. They are water soluble and exhibit negative and positive charges, respectively, in physiological condition, so there are several reports about their binding affinity to various drugs <sup>[15]</sup>, proteins <sup>[16]</sup> and other bioactive compounds <sup>[17], [18]</sup>. To incorporate these proteins in hydrogel constituents, they are conjugated with Pluronic F127 multiblock copolymer (F127 MBC) by Michael reaction between thiol groups in cysteine residue and double bond in acrylated F127 MBC although the lysine groups in gelatin, in the case of cell delivery carrier, was reacted with F127 MBC. These F127 MBC-protein conjugates were successfully synthesized and the possibility of carriers for delivering bioactive agents was evaluated by confirming *in vitro* stability test and monitoring release profiles of loaded proteins.



### 3.2. Experimental

**Materials.** Pluronic F127 [(PEO)<sub>99</sub>-(PPO)<sub>69</sub>-(PEO)<sub>99</sub>], *p*-nitrophenyl chloroformate (*p*-NPC) (96.0 %), diethylenetriamine (DETA) (≥ 99.0 %), triethylamine (TEA) (≥ 99.0 %), tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) (≥ 98.0 %), dimethyl sulfoxide (DMSO) (anhydrous, ≥ 99.9 %), gelatin from porcine skin (gel strength 300, Type A), bovine serum albumin (BSA) (lyophilized powder, ≥ 96.0 %), lysozyme (Lys) from chicken egg white (lyophilized powder, ≥ 90.0 %), bicinchoninic acid solution (BCA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (≥ 98.0 %) and urea (≥ 98.0 %) were commercially available from Sigma Aldrich (St. Louis, MO) and acryloyl chloride (≥ 98.0 %) and picrylsulfonic acid hydrate (≥ 98.0 %) was obtained from Tokyo Chemical Industry (TCI, Japan). Methylene chloride (99.5 %), diethyl ether (99.0 %), sodium chloride (99.0 %) and magnesium sulfate (99.0 %) were purchased from Daejung (Korea). Methylene chloride, acryloyl chloride and TEA were refluxed with calcium hydride to remove undesired water and all other chemicals were used without purification.

**Activation of Pluronic F127 with *p*-NPC (F127-NPC).** 10.00 g (0.79 mmol) of Pluronic F127 was dried under vacuum at 120 °C for 3 h in a 250 mL two-neck round bottom flask before reaction, and 100 mL anhydrous methylene chloride was introduced to dissolve the polymer. The reaction was allowed to proceed for 24 h at room temperature after adding *p*-NPC (0.48 g, 2.38 mmol) and TEA (0.24 g, 2.38 mmol). The solution was extracted from 150 mL brine solution, and the organic layer was dried over magnesium sulfate. The product was isolated by precipitating into 10-fold excess volume of cold diethyl ether before drying in vacuum for 3 days (yield : 89 %). <sup>1</sup>H-NMR (CDCl<sub>3</sub>, δ, ppm) = 4.3 (t, 4 H, -CH<sub>2</sub>CH<sub>2</sub>OC(=O)-), 7.5-8.5 (d, 8 H, Ar of *p*-NPC), 3.2-3.8 (br, 2 H of PEO and 1 H and 2 H of PPO), 1.0-1.3 (br, 3 H of PPO).

**General Synthesis Procedure of F127 multiblock copolymer with DETA (F127 MBC, F127 MBC-NH<sub>2</sub>).** F127-NPC (5.07 g, 0.39 mmol) was dissolved into 50 mL anhydrous methylene chloride in a 100 mL two-neck round bottom flask and DETA was added to the solution under nitrogen condition for 5 days (F127 MBC). In the case of conjugation with cysteine groups in protein, the same amount of DETA was further added and the reaction proceeded additional 1 day to terminate multiblock chain end

with amine groups (F127 MBC-NH<sub>2</sub>). The reaction solution was then washed with 100 mL brine solution, dried over magnesium sulfate and concentrated before precipitating in 10 fold excess volume of cold diethyl ether. The product was isolated by filtration and dried under vacuum for 3 days. (yield : 72 % (F127 MBC) and 68 % (F127 MBC-NH<sub>2</sub>))

**Synthesis of F127 MBC-Gelatin conjugates.** Gelatin from porcine skin (0.50 g, 0.006 mmol) was placed into 500 mL 2-neck round bottom flask and dissolved in 100 mL anhydrous DMSO at 50 °C for 2 h, followed by adding 100 mL DMSO solution of F127 MBC (6.61 g, 0.18 mmol). The reaction continued at 50 °C for 24 h and the reaction mixture was dialyzed against distilled water for 5 days (MWCO : 50,000) and freeze dried.

**Conjugation F127 MBC-NH<sub>2</sub> with BSA and Lys (F127 MBC-BSA and F127 MBC-Lys).** F127 MBC-NH<sub>2</sub> was firstly reacted with acryloyl chloride before conjugation with BSA and Lys. 4.27 g of F127 MBC-NH<sub>2</sub> was dissolved in 50 mL anhydrous methylene chloride in 100 mL 2-neck round bottom flask with magnetic stirrer and acryloyl chloride (0.08 g, 0.83 mmol) and TEA (0.08 g, 0.83 mmol) was added. After 12 h,

the reaction mixture was precipitated in cold diethyl ether and dried under vacuum for 3 days for further reaction. (yield : 92 %)  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ ,  $\delta$ , ppm) = 4.3 (t,  $-\text{CH}_2\text{CH}_2\text{OC}(=\text{O})-$ ), 3.2-3.8 (br,  $-\text{OC}(=\text{O})\text{NHCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NHC}(=\text{O})\text{O}-$ , 2 H of PEO and 1 H and 2 H of PPO), 1.0-1.3 (br, 3 H of PPO), 2.6 (t,  $-\text{CH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2-$ ), 5.5-6.5 ( $\text{CH}_2=\text{CHC}(=\text{O})-$ ). The conjugation reaction with proteins, BSA and Lys, was achieved according to the following procedure. The TCEP-HCl was dissolved in the pH 7.4 phosphate buffered saline (PBS, 10 mM, 0.138 M NaCl) aqueous solution of protein with 8 M urea (molar ratio 2:1 TCEP-HCl to cysteine residue in protein) and the solution of acrylated F127 MBC with various amount was added. The reaction continued for 24 h at room temperature and the mixture was poured in acetone. After dissolved again in PBS solution with 8 M urea, the conjugates were dialyzed against PBS solution (MWCO : 12,000) and lyophilized.

**Phase Diagram of Sol-Gel Transition.** Each F127 MBC-protein conjugate was prepared by dissolving in pH 7.4 phosphate buffered saline (PBS, 10 mM, 0.138 M NaCl) solution with various concentrations at  $4\text{ }^\circ\text{C}$  for 2 days. The solutions were immersed into water bath for 6 h at  $4\text{ }^\circ\text{C}$  and

the temperature was gradually increased. The sol-gel phase transition behavior was observed using a test tube inverting method.

***In Vitro* Gel Dissolution Rate.** The F127 MBC-protein conjugate hydrogels were prepared by dissolving each polymers in PBS solution and 1 mL of each solution was placed in 4 mL vials. All samples were incubated in a water bath with 37 °C for 3 h before a 3 mL PBS solution was added to the vial with preformed hydrogel. The remaining gel volume was estimated by measuring the height of the gel at determined periods with changing PBS solution. The remaining hydrogel volume was calculated by comparing with initial gel height.

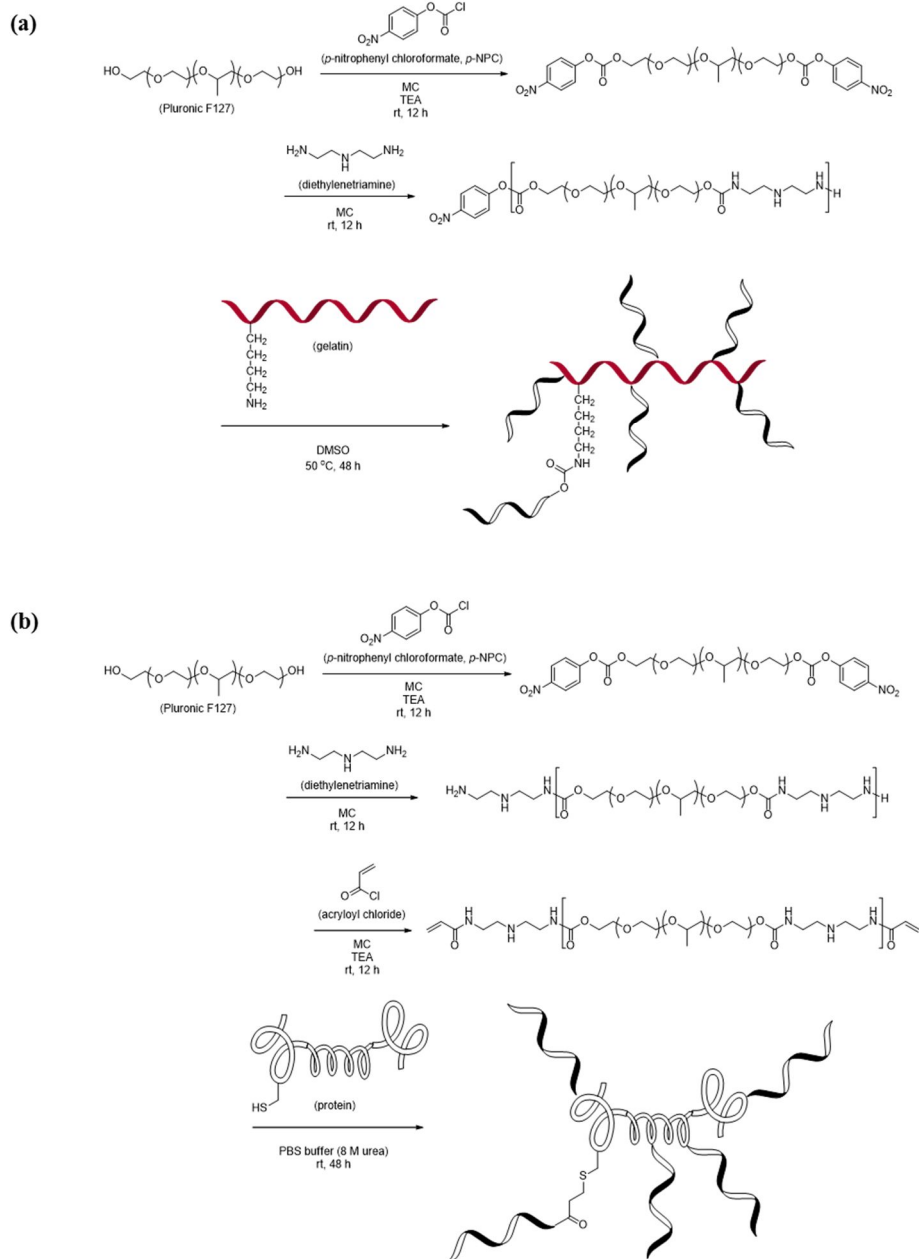
***In Vitro* Protein Release Profile.** The samples were prepared by dissolving F127 MBC-protein with 20 w/v% concentration in 1 mL PBS solutions at 4 °C and the model proteins were mixed at a final concentration of 10 mg/mL. After incubating in water bath with 37 °C for 6 h, 3 mL PBS solution was added on to the hydrogel contained vial. At determined time period, the release medium was replaced with fresh PBS

solution and the release profile was measured by BCA method <sup>[19]</sup>. The hydrogels with only F127 MBC-protein were utilized as background and the amount of released proteins was calculated by subtracting from those of protein loaded hydrogels.

**Instruments.** <sup>1</sup>H-NMR analysis was performed using Bruker Advance 300 MHz spectrometer in CDCl<sub>3</sub>. Gel permeation chromatography (GPC) was used to determine molecular weights and molecular weight distribution of F127 MBC using poly(ethylene glycol) standards (Polymer Laboratories a part of VARIAN). The system configuration was consisted of refractive index detector (Shimadzu RID-10A refract meter) and Styragel HR3, HR4 and HR 4E column in series, and the dimethylformamide (DMF) was used as an eluent with 1 mL/min flow rate at 40 °C. To calculate the degree of conjugation, UV-visible spectra were obtained by Shimadzu UV-1650 PC at the wavelength of 345 nm and 450 nm.

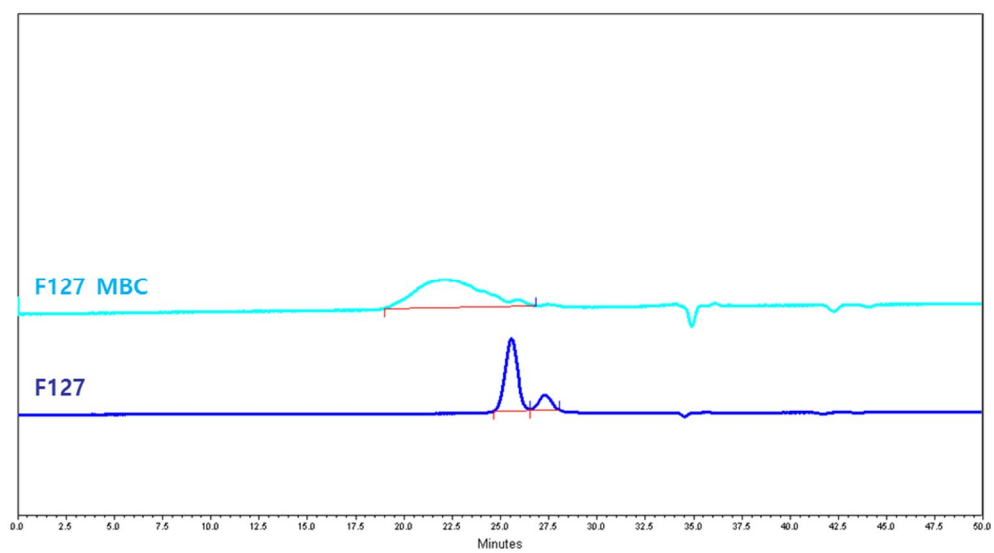
### 3.3. Results and Discussion

To make functional hydrogels with high stability in physiological conditions, Pluronic F127 multiblock copolymer was conjugated with several proteins as shown in Scheme 3-1. Pluronic F127 was firstly activated using *p*-NPC and multiblock formation was achieved using DETA as a linker. It is expected that the stability of final hydrogel after conjugated with functional peptides will be maximized as the degree of polymerization increases, so the reaction for multiblock formation proceeded for 5 days. The degree of polymerization was concluded around 3.3 from GPC analysis (Figure 3-1). In this study, three proteins, gelatin, BSA and Lys were utilized for preparing functional hydrogels. The lysine contents in gelatin and the cysteine residues in BSA and Lys were used for conjugation with F127 MBC, so further acrylation step was performed for F127 MBC-BSA and F127 MBC-Lys conjugates although F127 MBC-gelatin was synthesized without further functionalization after multiblock copolymerization. Gelatin contains 36 lysine residues and there are 35 and 8 cysteine groups in BSA and Lys in one molecule, respectively, and the reaction condition and molecular weight analysis results were summarized in Table 3-1. The degree of conjugation in each F127 MBC-protein conjugate was measured using



**Scheme 3-1.** Synthetic scheme of (a) F127 MBC-gelatin and (b) F127 MBC-BSA and F127 MBC-Lys





**Figure 3-1.** GPC traces of F127 and F127 MBC

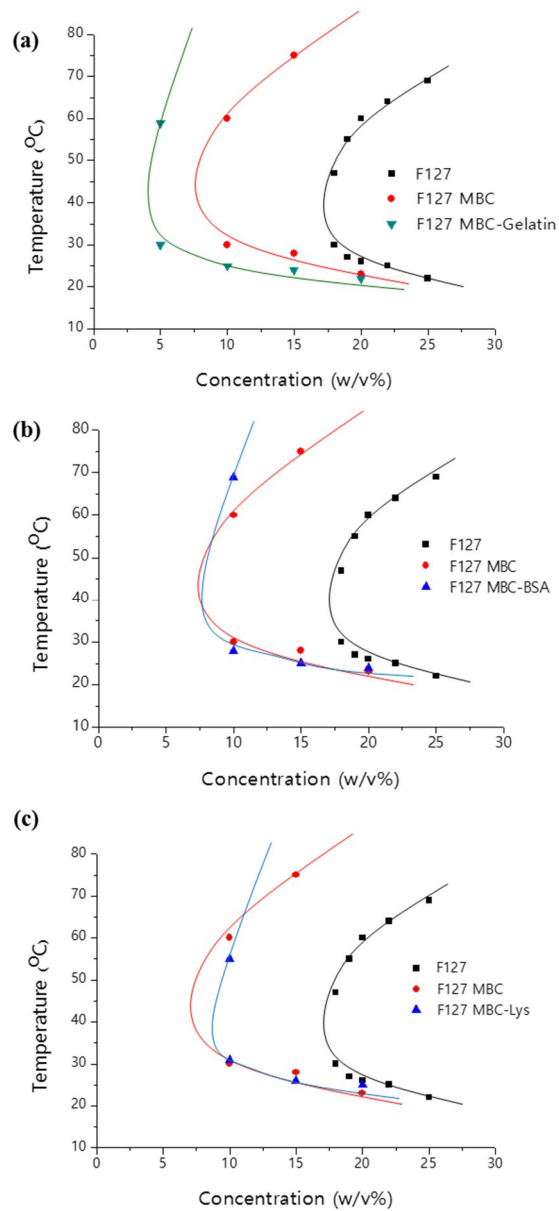
**Table 3-1.** Characterization of F127 MBC-protein conjugates

	Feed Ratio [mol]			# of conjugation in one protein
	F127 MBC	lysine	cysteine	
F127 MBC-Gelatin	1	1		13
F127 MBC-BSA	1.5		1	21
F127 MBC-Lys	1.5		1	2

2,4,6-trinitrobenzene sulfonic acid (TNBS) assay and DTNB (Ellman's Reagent) assay, respectively. The TNBS component reacts readily with primary amino groups of amino acids making the appearance of absorption peak at 345 nm and the DTNB has an ability to analyzing free thiol groups by monitoring absorbance at 420 nm.

The gelation behavior of each synthesized product was confirmed by sol-gel phase transition diagrams, and all samples showed thermally induced reversible gelation behaviors as shown in Figure 3-2. The synthesized F127 MBC, as expected, exhibited much lowered critical gelation concentration than Pluronic F127. After conjugation with functional proteins, the phase diagram curves showed similar sol-gel transition behaviors with that of F127 MBC, so the transition behavior of multiblock copolymer determines the overall thermal phase transition property of F127 MBC-protein conjugates although the amounts of F127 MBC in F127 MBC-protein conjugates are different from each other.

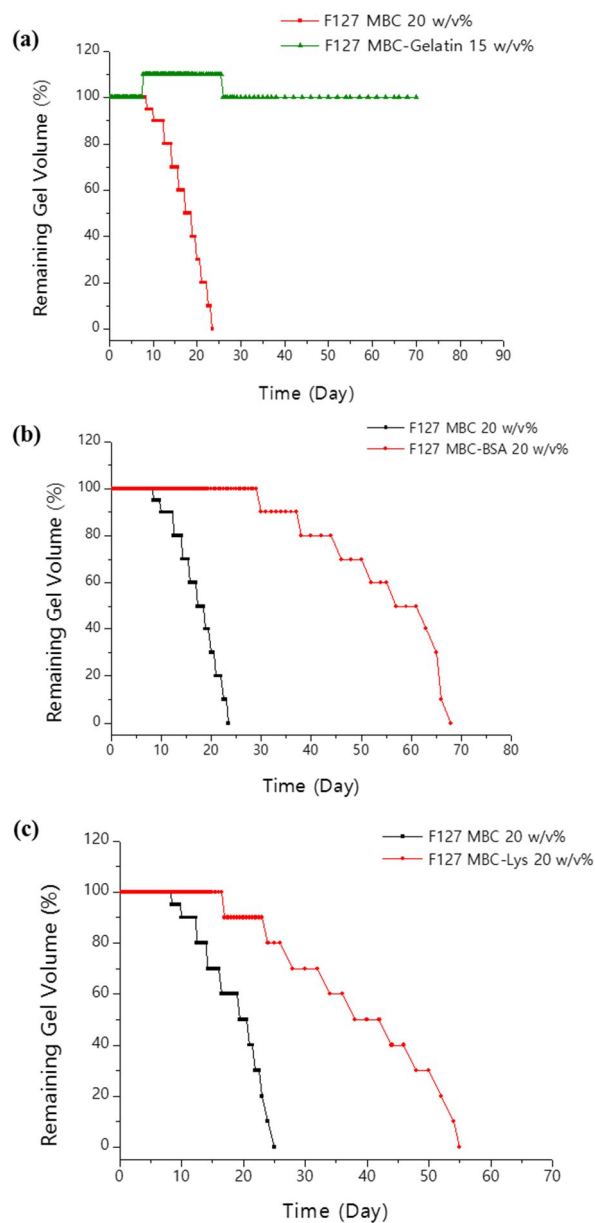
To evaluate the stability in physiological environment of the resultant hydrogels, the dissolution rate was monitored at 37 °C in PBS solutions. The formation of F127 MBC hydrogel was maintained for about 2 weeks although Pluronic F127 hydrogel was completely disappeared within



**Figure 3-2.** Phase diagrams of (a) F127 MBC-gelatin, (b) F127 MBC-BSA and (c) F127 MBC-Lys hydrogels

1 day. In general, Pluronic F127 hydrogel has been found to have poor mechanical integrity which makes it poor strength, high permeability and limited stability with quick dissolution, so its biomedical application as a delivery reservoir is limited. After multiblock copolymerization, however, the effect of forming bridges between adjacent micelles increase the modulus of multiblock copolymer hydrogels which can be explained by Percolation theory <sup>[20]</sup>. In the case of F127 MBC-protein conjugates, the hydrogel did not eroded until around 2 month and it is appeared that this further increased stability comes from increased molecular weight and hydrophobicity. Gelatin has relatively high molecular weight and hydrophobicity than BSA and Lys, so this can attribute to more robust hydrogel formation in physiological conditions compared with F127 MBC-BSA and F127 MBC-Lys hydrogels. These hydrogels with highly increased mechanical strength is expected to be applied to delivery systems with ability of charge interactions in F127 MBC-BSA and F127 MBC-Lys hydrogels or RGD sequences for cell adhesion in F127 MBC-gelatin hydrogel. Especially, the possibility of protein delivery carrier was estimated using F127 MBC-BSA and F127 MBC-Lys hydrogels.

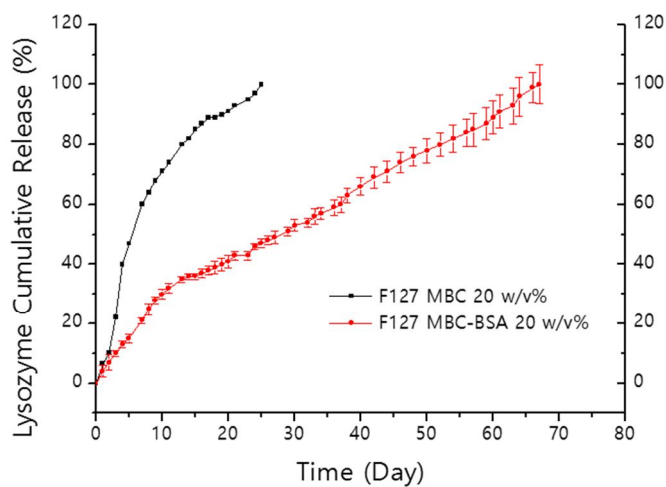
The isoelectric points (PI) of BSA and Lys are known as 4.9 and 9.3,



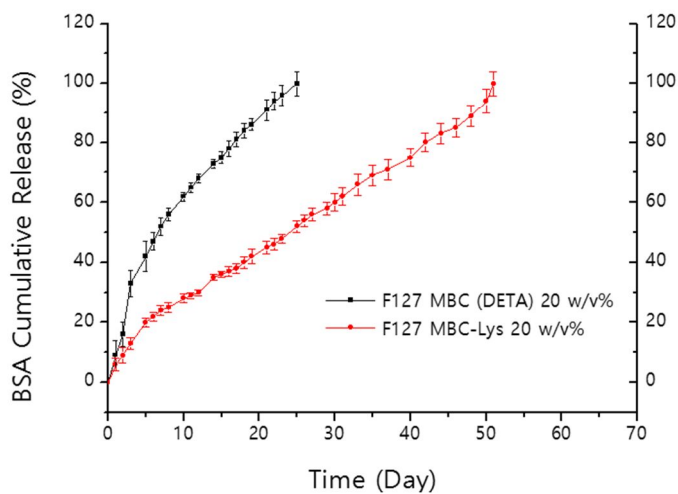
**Figure 3-3.** *In vitro* dissolution rate of (a) F127 MBC-gelatin, (b) F127 MBC-BSA and (c) F127 MBC-Lys

respectively, so they exhibit negative and positive charges in blood stream, and the charge of resultant F127 MBC-protein conjugates will not be changed because F127 MBC was conjugated to the thiol groups of cysteine residues in each protein instead of lysine or aspartic acid moieties. This charge is expected to be able to function for the enhanced sustained protein release when oppositely charged protein is physically loaded into the F127 MBC-protein hydrogel matrix. The BSA, representative anionic protein, release profile was observed using F127 MBC-Lys hydrogel and the release behavior of cationic Lys protein in F127 MBC-BSA hydrogel was also monitored. The release behaviors of loaded protein in F127 MBC-protein conjugates were sustained for about 2 month without any noticeable burst release although that of F127 MBC hydrogel was controlled for only 2 weeks due to the relatively weak stability.

(a)



(b)



**Figure 3-4.** *In vitro* oppositely charged protein release profile of (a) F127 MBC-BSA and (b) F127 MBC-Lys



### 3.4. Conclusion

In sum, the hydrogels with functional proteins were synthesized and the possibilities for delivering bioactive agents were evaluated. Pluronic F127 was copolymerized up to 3.3 degree of polymerization and the F127 MBC was conjugated with BSA, Lys and gelatin. The F127 MBC-protein hydrogels exhibited highly increased mechanical properties from *in vitro* gel dissolution rate measurements. The F127 MBC-gelatin hydrogel is expected to enhance the viability of incorporated cells when applied to cell delivery carriers due to the RGD sequences in gelatin. The BSA and Lys show negative and positive charges in physiological condition, respectively, so the F127 MBC-BSA and F127 MBC-Lys hydrogels can be applied to oppositely charged protein delivery carriers for more sustained release behaviors.

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## **Chapter 4.**

# **Blend System Hydrogel with Enhanced Mechanical Properties for Islet Cell Carrier**

## 4.1. Introduction

The main characteristic of type 1 diabetes is the autoimmune destruction of the  $\beta$ -cells that produce insulin in the pancreas. Due to the lack of endogenous insulin, the glucose builds up in the bloodstream instead of moving into the cells to be consumed as an energy source<sup>[1], [2]</sup>. The accumulated glucose in the blood stream leads to a high blood viscosity and this can cause many severe complications such as retinopathy and nephropathy<sup>[3]</sup>. The optimal treatment of type 1 diabetes mellitus is to acquire normoglycemia at all times. In this respect, several studies report possible candidates for treating type 1 diabetes, including protein delivery systems<sup>[4], [5], [6]</sup> and cell delivery systems<sup>[7], [8]</sup>. In particular, cell delivery systems are considered as a permanent treatment method compared to protein delivery systems.

In cell delivery systems, it is essential to avoid immune reaction because introduced cells are considered as foreign materials; the cell surface modification method using biocompatible polymers such as poly(ethylene glycol) (PEG) has therefore been proposed. Although cell viability can significantly increase after coating of the cell surfaces, PEG itself can cause cell death by penetrating the cell surface. To overcome this problem,

dendrimer structured polymers were evaluated to maximize the cell surface coverage ability <sup>[9]</sup>. In addition, hydrogel was considered as the most appropriate cell carrier due to its high water content <sup>[10], [11]</sup>. When cells and proteins are incorporated into hydrogels, cell death and protein denaturation can be prevented because hydrogel functions as a water reservoir. Another advantage of hydrogel is that various drugs and stem cells can also be loaded to maximize the cell viability.

Several important conditions need to be met before hydrogels can be used as cell carriers. Hydrogels should have minimal cell toxicity and should maintain hydrogel formation for a long period in a physiological environment. Hydrogels usually contain crosslinkers to form network structures, and the crosslinking mechanisms are generally divided into two categories: chemical crosslinking and physical crosslinking <sup>[12], [13], [14], [15]</sup>. In the case of physical crosslinking, cell toxicity is relatively low, but the strength of the hydrogel is also very low. In contrast, in a physiological condition, the chemically crosslinked hydrogel is stable due to its high modulus; however, most of the chemical crosslinkers induce high toxicity in the cells, resulting in cell death. While the ideal cell carriers should simultaneously satisfy these two conditions, most of the conventional

hydrogels can meet only one condition.

Pluronic F127, poly(ethylene oxide) (PEO) – poly(propylene oxide) (PPO) – PEO triblock copolymer, is a well-known copolymer because it is a Food and Drug Administration (FDA) approved material and it exhibits thermally induced gelation behavior in an aqueous condition <sup>[16]</sup>. While its thermal reversible gelation behavior can be applied to the injectable system with minimal surgical process, its weak gel stability hinders its use for applications of cell delivery. Several reports on increasing the gel stability like multiblock copolymerization have been presented because this can raise gel stability without using additional crosslinkers. The major disadvantage of the multiblock copolymerization method, however, is that high concentration solution cannot be injected because of the high viscosity, limiting its application <sup>[17], [18]</sup>.

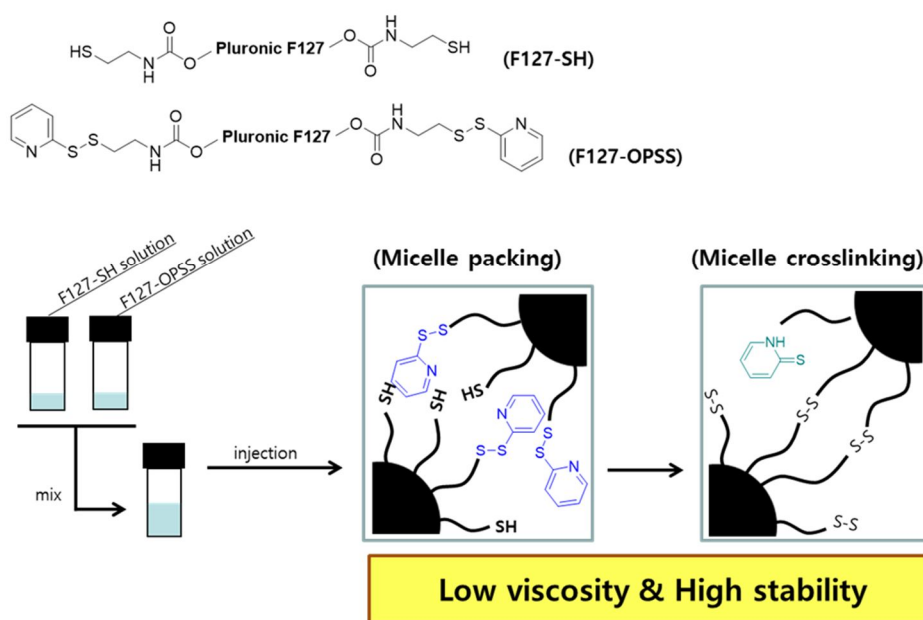
In this study, we developed a novel blend system hydrogel based on Pluronic F127. Two hydroxyl end groups of Pluronic F127 were simply modified into thiol groups (SH) and *ortho*-pyridinyl disulfide groups (OPSS), respectively. When these two modified Pluronic F127 are mixed together in an aqueous solution, they can form a new disulfide linkage. This linkage can work as a crosslinker between micelles, resulting in high gel



stability in the physiological condition (Figure 4-1). The reaction between SH and OPSS is known as the thiol-disulfide exchange reaction. This reaction occurs in an aqueous solution without any further catalyst, and the byproduct has little cell cytotoxicity. Another advantage of this reaction is that we can monitor the degree of exchange by measuring the ultra-violet (UV) absorbance. After the reaction, the OPSS group changes into pyridine-2-thione as a byproduct, and the UV absorbance peak changes from 290 nm to 343 nm. By measuring the absorbance at 343 nm, we can calculate the degree of exchange; it has been reported that most of the exchange reaction occurs within 100 min <sup>[19]</sup>. Thus, when the mixed solution is injected into the body, hydrogel is first formed by the physical micelle packing mechanism, which is similar to the Pluronic F127 gelation mechanism. After forming the hydrogel, the crosslinking between micelles occurs due to the thiol-disulfide exchange reaction; it is therefore expected that the resultant hydrogel can maintain its gel formation for a long period in the body condition. This property was confirmed by phase diagram, *in vitro* and *in vivo* experiments. Further, we transplanted normal islets into diabetes and monitored the glucose level to evaluate this blend system hydrogel as a cell carrier.

✓ **Pluronic F127 End Group Modification**

- ✓ SH : thiol
- ✓ OPSS : *ortho*-pyridinyl disulfide



**Figure 4-1.** Schematic illustration of gelation mechanism of F127-SH and F127-OPSS blend system hydrogel

## 4.2. Experimental

**Materials.** Pluronic F127 [(PEO)<sub>99</sub>-(PPO)<sub>69</sub>-(PEO)<sub>99</sub>], *p*-nitrophenyl chloroformate (*p*-NPC) (96.0 %), cysteamine hydrochloride (98.0+ %), anhydrous methanol (99.8 %), triethylamine (TEA), and aldrithiol-2 (98.0%) were purchased from Sigma-Aldrich. Methylene chloride (99.5 %), diethyl ether (99.0 %) and sodium chloride (NaCl) (99.0 %) were obtained from Daejung. Methylene chloride and TEA were dried over calcium hydride and all other chemicals were used as received.

### **Synthesis of 2-(pyridin-2-yl)disulfanylethanaminium chloride.**

Aldrithiol-2 (17.46 g, 79.23 mmol) was placed into a 500 mL 2-neck round-bottom flask and dissolved in 150 mL anhydrous methanol. Cysteamine hydrochloride (3.00 g, 26.41 mmol) was added and the reaction proceeded at room temperature under a nitrogen atmosphere. After 12 h, the reaction mixture was concentrated under reduced pressure and precipitated in 300 mL of cold diethyl ether. The precipitates were collected by filtering and dried in a vacuum for 3 days. The yield was 83.5 %. <sup>1</sup>H NMR (DMSO-

$d_6$ ,  $\delta$ , ppm) = 3.1 (t, 4 H,  $-\text{SCH}_2\text{CH}_2\text{NH}_2$ ), 7.3-8.5 (m, 4 H, Ar of aldrithiol).

**Synthesis of F127-SH.** Pluronic F127 (30.00 g, 2.38 mmol) was placed in a 500 mL 2-neck round-bottom flask and dried at 80 °C under vacuum for 5 h before reaction. After drying, the flask was cooled to room temperature and purged with nitrogen. To dissolve Pluronic F127, 250 mL of anhydrous methylene chloride was introduced to the flask and *p*-NPC (2.88 g, 14.29 mmol) and TEA (1.45 g, 14.29 mmol) were added. The reaction mixture was stirred for 12 h at room temperature. To isolate the product, the mixture was extracted with 300 mL of brine solution and the organic layer was separated, dried over magnesium sulfate, and filtered. Finally, the methylene chloride was removed using a rotary evaporator and dried in a vacuum oven for 3 d. The yield of this reaction was 81.3 %. Activated Pluronic F127 (10.00 g, 0.77 mmol) was first dissolved in 100 mL of anhydrous methylene chloride under a nitrogen atmosphere. Cysteamine hydrochloride (0.88 g, 7.73 mmol) was then added to the reactor, and the TEA (0.78 g, 7.73 mmol) was finally introduced. The reaction proceeded overnight at room temperature. After the reaction, the reaction mixture was extracted using 150 mL brine solution. The organic layer was isolated, dried

over magnesium sulfate and filtered. Each final product was obtained by removing the solvent, and was finally dried in a vacuum oven at room temperature for 3 days. The average isolation yield was 83.7 %.  $^1\text{H}$  NMR (DMSO-*d*6):  $\delta$  (ppm) = 4.0 (t, 4 H,  $-\text{CH}_2\text{CH}_2\text{OC}(=\text{O})-$ ), 3.0 (t,  $-\text{OC}(=\text{O})\text{NHCH}_2\text{CH}_2\text{SS}-$ ), 3.2-3.8 (br, 2 H of PEO and 1 H and 2 H of PPO), 0.7-1.3 (br, 3 H of PPO).

**Synthesis of F127-OPSS.** The activation process using p-NPC and the following procedure were similar to the case of the F127-SH synthesis, with the only exception that 2-(pyridin-2-yl)disulfanyl)ethanaminium chloride (1.45 g, 7.73 mmol) was used instead of cysteamine hydrochloride. The yield of this reaction was 76.4 %.  $^1\text{H}$  NMR (DMSO-*d*6,  $\delta$ , ppm) = 4.0 (t, 4 H,  $-\text{CH}_2\text{CH}_2\text{OC}(=\text{O})-$ ), 2.7-3.1 (t, 4 H,  $-\text{OC}(=\text{O})\text{NHCH}_2\text{CH}_2\text{SH}-$ ), 7.2-8.5 (m, 8 H, pyridine), 3.2-3.8 (br, 2 H of PEO and PPO), 0.7-1.3 (br, 3 H of PPO).

**Sol gel phase transition diagram.** Each modified Pluronic F127 polymer and unmodified Pluronic F127 were dissolved in 1 mL pH 7.4

phosphate buffered saline (PBS, 10 mM, 0.138 M NaCl) with various concentrations. Before analyzing, all samples were immersed in a water bath for 6 h at 4 °C and the temperature was gradually increased. The sol gel phase transition was performed using a test tube inverting method, and the gel state was determined at no fluidity for 1 min. The accuracy of this method is known to have a precision of  $\pm 1$  °C.

***In vitro* gel dissolution rate.** Modified Pluronic F127 polymers and unmodified Pluronic F127 were dissolved separately at certain concentrations in a PBS solution. 1 mL of resultant solutions were placed in 4 mL vials at various ratios. All samples were then incubated in a 37 °C water bath to form a gel and the height of the gel was measured. A 3 mL PBS solution was added to the vial, and the solution was changed at determined periods, and the remaining gel height was then measured. The remaining gel volume was calculated by dividing the remaining gel height with the initial gel height <sup>[20]</sup>.

***In vivo* experiment.** Blend system hydrogel stability was also

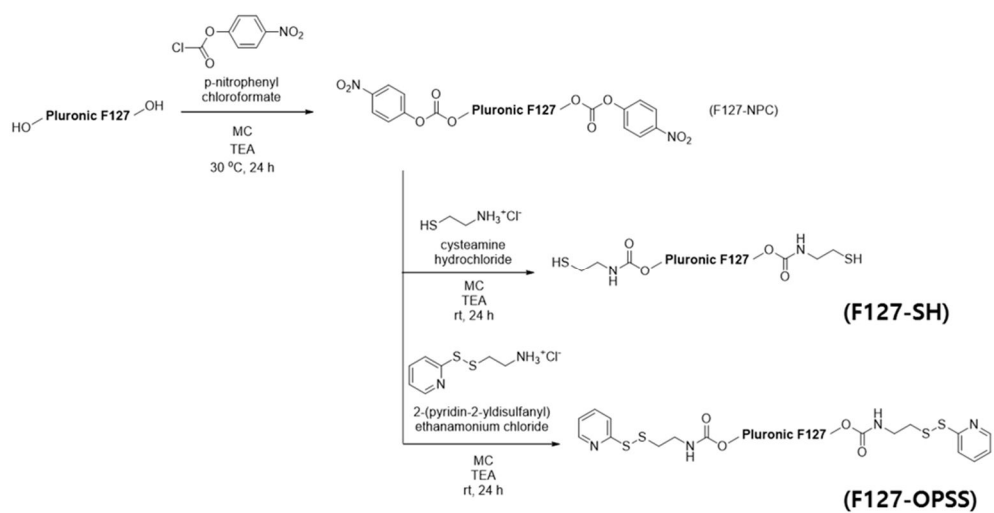
confirmed using C57BL/6 mice. The Pluronic F127 solution of 1 mL with 16 wt% and the blend system of 1 mL with 10 wt% were injected to the mice subcutaneously using 31 gauge needle syringe, respectively, and the gel formation was confirmed every day. After 3 days, the mouse with Pluronic F127 hydrogel was incised to check the remaining hydrogel, and the blend system hydrogel in another mouse was extracted after 24 days. To monitor the effect of controlling blood glucose level, the islets were isolated from Spraque-Dawley (SD) rat as described in the previous method [21]. After isolation, 300  $\mu$ L of 10 wt% blend system hydrogel solution with 2000 islet equivalents (IEQ) was subcutaneously injected into three nude mice with diabetes chemically induced by streptozotocin (STZ), and non-fasting blood glucose levels (NBG) were measured using portable glucometer (Super glucocard II, Arkray, Kyoto, Japan) from the tail vein of recipient mice every day.

### 4.3. Results and Discussion

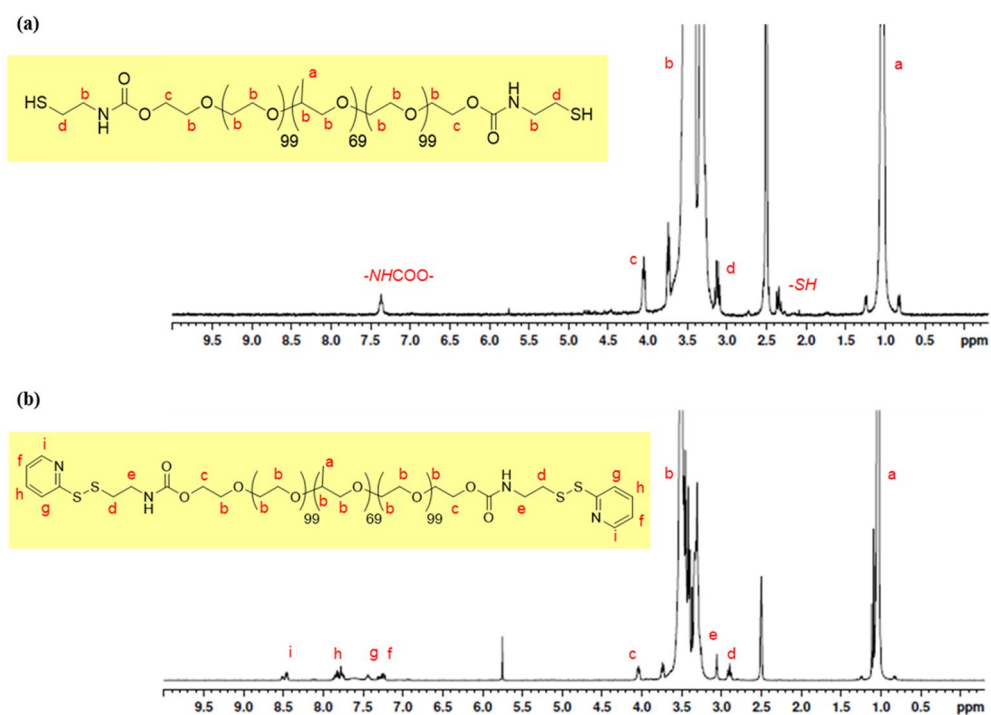
SH and OPSS modified Pluronic F127 were prepared by the end group modification of Pluronic F127 as shown in Figure 4-2. Two hydroxyl groups of Pluronic F127 were first activated using *p*-NPC to conjugate with the amine groups of cysteamine hydrochloride or 2-(pyridine-2-yl)disulfany)ethanaminium chloride. After the activation process, conjugation reaction proceeded in the presence of TEA, and the reaction mixture exhibited a yellow color, verifying the appearance of the byproduct, *p*-nitrophenol. The degree of substitution of each final product was calculated by  $^1\text{H-NMR}$  and it was concluded that all the hydroxyl groups of Pluronic F127 were replaced with by SH or OPSS without any noticeable impurities.

The sol-gel phase transition diagrams of Pluronic F127, each modified Pluronic F127, and the blend systems were confirmed using a simple test tube inverting method. As shown in Figure 4-3, the critical gelation concentration of the blend system had shifted from 15 wt% to 5 wt%. This means that the blend systems can form a hydrogel at a much lower concentration than Pluronic F127, and it is expected that the stability of the blend system will be much higher than Pluronic F127 at the same

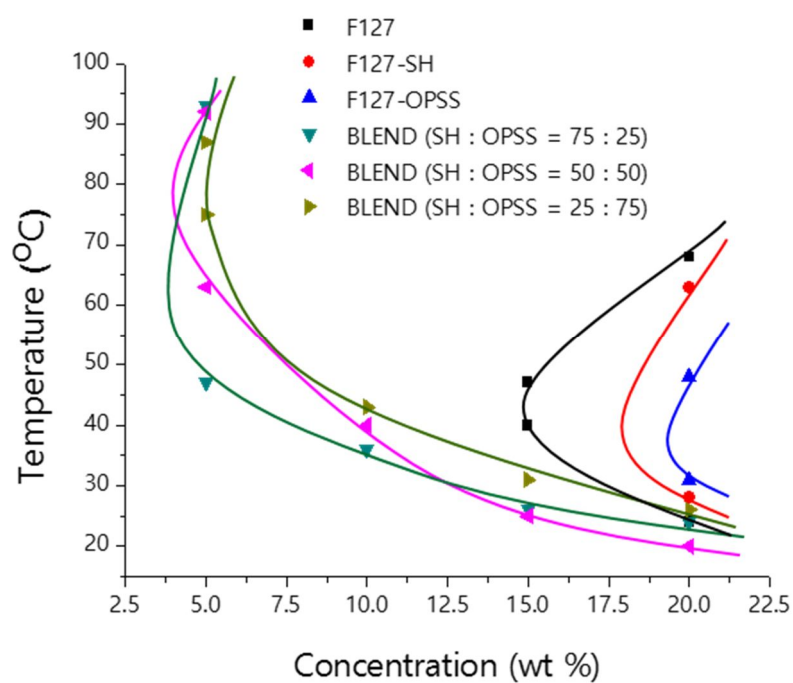




**Scheme 4-1.** Synthetic scheme of F127-SH and F127-OPSS



**Figure 4-2.**  $^1\text{H}$ -NMR spectrum of synthesized (a) F127-SH and (b) F127-OPSS



**Figure 4-3.** Sol-gel phase diagram of F127, F127-SH, F127-OPSS and blend system hydrogel

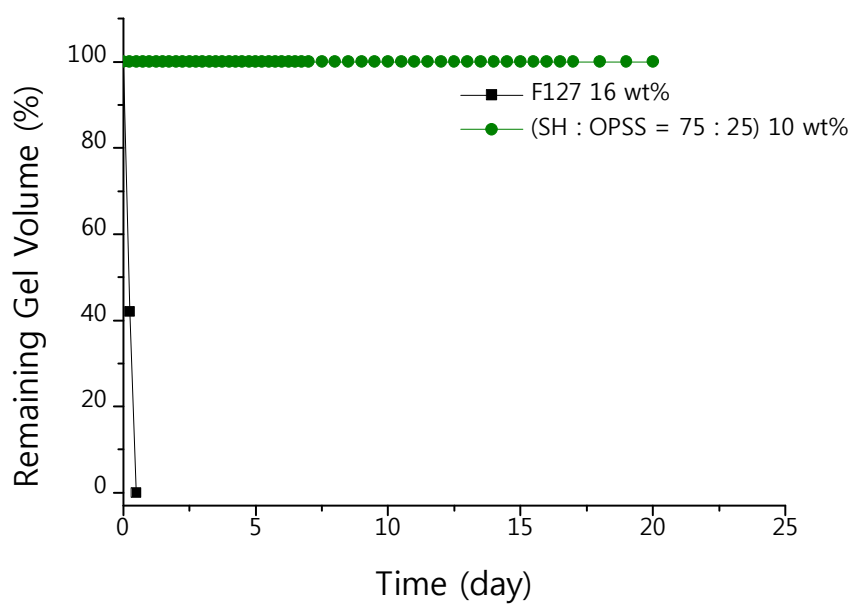
concentration. This lowered critical gelation concentration is thought to be caused by the chemically linked micelles that can maintain the crosslinked structure, even in diluted concentrations. The newly introduced potential functional groups, SH and OPSS groups, in the blend system can react with each other, so the covalently crosslinked micelle formation can function similarly to chemically crosslinked hydrogels. The ratio of SH to OPSS with a 75 to 25 blend system showed the least critical gelation concentration, which can be expected to have the highest gel strength. Theoretically, the blend system hydrogel with the same amount of each modified polymer should exhibit the lowest critical gelation concentration. While this result remains controversial, it is thought that an optimum ratio exists between SH and OPSS after micelle formation. However, in the case of each modified Pluronic F127, the phase diagram was shifted with a higher critical gelation concentration, implying that more polymers are required for gelation behavior. This result was due to the hindered physical crosslinking formation resulting from the end group modification. After modification, the SH group and OPSS group will be located at the surface of the micelles; however, these groups function as obstacles in the micelle packing for hydrogel formation. Thus, the resultant phase diagram was shifted and the critical gelation concentration was around 18 wt% and 20 wt%, which is

higher than Pluronic F127 <sup>[22]</sup>. Interestingly, blend system hydrogels maintain their gel structures even at high temperatures, although Pluronic F127 hydrogel shows a sol state. It is known that, at high temperatures, cracks will appear micelle structures that are too highly packed and the gel state thus cannot be maintained. However, hydrogels with chemically crosslinked micelles can hold the network structure because they are linked together by a covalent bond, and this results in a stable hydrogel formation, even at high temperatures.

The tendency of the blend system hydrogel seems to be very similar to that of the conventional Pluronic F127 multiblock, although the major advantage of the blend system hydrogel is its lower viscosity than that of the Pluronic F127 multiblock copolymers. The Pluronic F127 multiblock copolymers also show thermally induced sol-gel transition behavior, although they are difficult to but it is hard to inject using a syringe due to their high viscosity. The molecular weight of Pluronic F127 multiblock copolymers with the degree of polymerization of 3 is around 40,000 kDa; this high molecular weight causes high viscosity. Thus, Pluronic F127 multiblock with a concentration of over 15 wt% concentration is impossible to transplant into the body using a syringe, although it is in a sol state. The

blend system hydrogel, however, can be injected using a syringe, even at a 20 wt% concentration because the molecular weight of the polymer is not excessive. The reaction between SH and OPSS occurs after blending each solution, so the initial molecular weight of the polymer is similar to unmodified Pluronic F127. In this state, it can be injected into the body even in high concentrations, and this was also confirmed using rheological analysis (data not shown).

To confirm the stability of the blend system hydrogel in a physiological condition, the *in vitro* gel dissolution rate of each hydrogel was measured at the critical gelation concentrations at 37 °C. As shown in Figure 4-4, the blend system hydrogels showed highly increased stability, although the concentration was much lower than that of Pluronic F127. The Pluronic F127 hydrogel with 16 wt% was completely eroded within a day, implying that the tightly packed micelles had dissipated in the presence of a large lot of water content. However, in the case of blend system hydrogels with 10 wt% concentrations, the formation of hydrogel was maintained for more than 10 days. This indicates that it is difficult to separate the covalently crosslinked micelle structure in diluted conditions because the experiments were performed at critical gelation concentrations. The stability

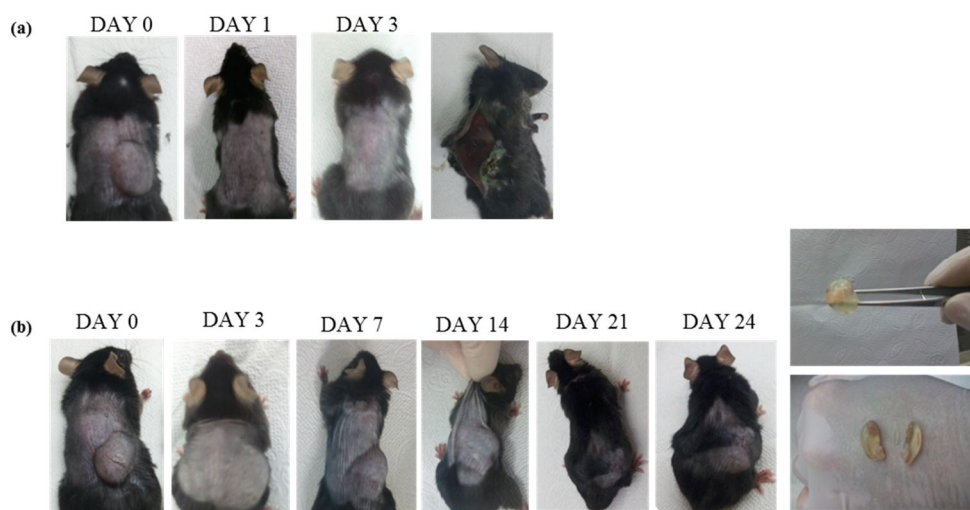


**Figure 4-4.** *In vitro* gel dissolution rate of F127 and blend system hydrogel

of the blend system hydrogel was also checked by *in vivo* experiment (Figure 4-5). Pluronic F127 hydrogel with 16 wt% and blend system hydrogel (75/25) with 10 wt% were injected subcutaneously. The formation of the Pluronic F127 hydrogel disappeared within 1 day and the mouse died after 4 days. This toxicity is caused by the dissipated Pluronic F127 polymer because the hydrogel contains a high polymer content, even though Pluronic F127 is an FDA approved material. In contrast, the blend system hydrogel (75/25) maintained its formation during the 24 days, and the mouse survived. After sacrifice, we confirmed the remaining hydrogel by extracting from the mouse and concluded that the majority of the polymers remained in the hydrogel. Because the polymers were not dissolved, but were located inside the gel, toxicity was not induced, even though the concentration of the blend system hydrogel (75/25) is also too high, and any significant inflammation phenomenon was not observed.

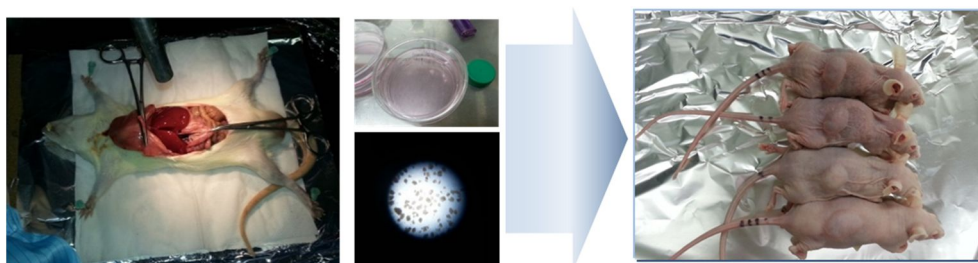
To assess the potentiality of this blend system hydrogel (75/25) as a cell carrier, *in vivo* blood glucose levels were monitored for a certain time period from diabetic nude mice recipients transplanted with islets containing hydrogel (Figure 4-6). Pancreatic islets were isolated from the pancreases of outbred male SD rats. The isolated cells suspended



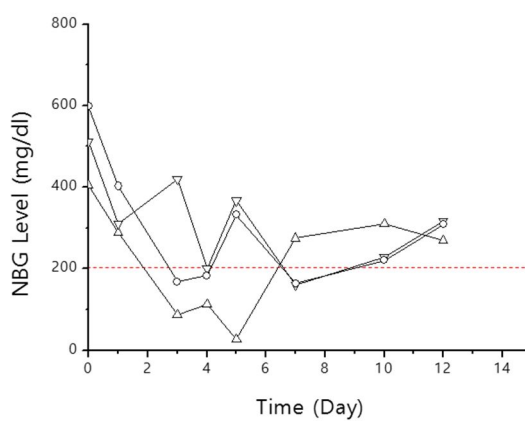


**Figure 4-5.** *In vivo* gel dissolution experiment of (a) F127 16 wt% hydrogel and (b) blend system 10 wt% hydrogel

in PBS were then mixed with prepared blend system hydrogel (75/25) solution. The concentration of the final solution was 10 wt% and the solution was freely flowing at room temperature. We injected the blend system hydrogel (75/25) solution containing isolated islet cells subcutaneously and monitored the glucose levels every day in the blood of the transplanted mice. 2 days after transplantation, high glucose level seemed to be lowered, but it still remained in hyperglycemia state. After 2 more days, however, the glucose level was successfully controlled within the range between 200 and 300 mg/dl which is correspondent with normoglycemia and this normal glucose level was maintained for around 10 days. It is thought that the transplanted islet cells in hydrogel secret insulin to lower blood glucose level down, which is the indicator of well functioned islet cells. Moreover, the hydrogel barrier protects islet cells from immune responses like macrophage invasion into the transplanted site.



✓ **Glucose Level Control**



**Figure 4-6.** *In vivo* glucose control experiment after 10 wt% blend system hydrogel subcutaneous injection with islet cells

#### **4.4. Conclusion**

In summary, we investigated the potential of hydrogels as cell carriers. The carriers need to be non-toxic and stable in physiological conditions; we therefore designed a modified Pluronic F127 blend system hydrogel. While this blend system does not affect the viscosity, it significantly increases gel strength highly. In addition, when this blend system hydrogel with islet cells was transplanted subcutaneously, the hyperglycemia levels of diabetes mellitus were controlled in the range of the normoglycemia level for more than 10 days. Overall, the results show that hydrogel with high strength and non-toxicity is a promising cell carriers.

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## 국문 요약

폴리 에틸렌 옥사이드 - 폴리 프로필렌 옥사이드 - 폴리 에틸렌 옥사이드 공중합체로 이루어진 플루로닉 고분자는 마이셀 패킹 메커니즘에 의해 일정 농도 이상의 수용액 상에서 온도 변화에 따라 가역적으로 졸 젤 거동을 변화하는 물질로 알려져 있다. 각 블록의 다른 저 임계 용액 온도로 인해 약 15 도 이상의 온도에서 마이셀 구조를 형성하게 되고, 온도가 증가함에 따라 마이셀이 점점 더 많이 생성되어 물리적으로 가교가 되는 수화젤을 형성하는 특징을 갖는 물질이다 이러한 온도 민감성 수화젤 특징 이외에 세포 외기질과 같은 많은 수분 함량 및 부드러운 재질로 인해 치료제 전달을 위한 전달체로서 생체 의료용 분야에 많은 관심을 끌어왔지만, 낮은 물성으로 인해 임상으로의 응용에 제약이 있다.

플루로닉 고분자의 마이셀 패킹 거동은 간단한 말단기 개질을 통해 조절될 수 있으며, 이는 젤 거동에 큰 영향을 미치게 된다. 두 번째 단원에서는 서로 다른 상호 작용을 갖는 젤 거동의



관찰을 통해 마이셀 패킹 능력을 비교하였다. 아다만탄 기를 플루로닉 고분자 말단에 결합시킨 후에는 마이셀 패킹이 방해되어 임계 젤 형성 농도의 증가를 관찰할 수 있었다. 하지만 싸이클로덱스트린 고분자의 도입으로 호스트 게스트 상호작용을 유도하여 젤 형성이 촉진되는 것을 확인하였고, 이는 마이셀 간의 상호작용이 전체적인 플루로닉 젤 거동을 결정짓는 요소로 간주되는 것으로 판단된다.

이러한 플루로닉 수화젤의 이해를 통해 전달체로서의 응용 가능성을 평가하였다. 3 단원에서는 플루로닉 고분자의 멀티블록 공중합체를 단백질과 세포와 같은 생체 활성 물질을 전달하기 위하여 기능성 단백질에 결합시켰다. 젤라틴 단백질은 세포의 접합부로 작용할 수 있는 RGD 단백질 서열이 풍부하여 세포 전달체로 응용하였고, 알부민과 리소자임 단백질은 정전기적 상호작용을 이용한 서방성 단백질 방출 거동을 위하여 수화젤 내부에 도입되었다. 분자량 증가로 야기되는 점도 문제를 극복하기 위하여 다른 물질로 각기 개질된 플루로닉 고분자의 혼합 기법에 대하여 4 단원에서 언급하였다. 제조된 수화젤은

생체 조건에서 크게 향상된 안정성을 나타내었으며, 이를 체도 세포 전달체로 적용하였다. 동물 실험을 통하여 당뇨병이 유도된 쥐들의 혈당 수치가 약 2 주 동안 200 mg/dl 수준으로 조절되는 것을 알 수 있었다. 전체적인 결과를 통하여 수화젤이 단백질과 세포의 전달을 위한 유망한 전달체로 판단된다.

**주요어** : 플루로닉, 수화젤, 주사가능한 시스템, 마이셀 패키징 메커니즘, 단백질 전달 시스템, 세포 전달 시스템

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