Development of Cell-Implantable Scaffolds Using Polyion Complex Gel of Chitosan and Succinylated Poly(Pro-Hyp-Gly)

(コハク酸化 poly(Pro-Hyp-Gly)とキトサンからなる
ポリイオンコンプレックスゲルを用いる細胞封入型足場材料)

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CONTENTS

GENERAL INTRODUCTION

1. Hydrogel as Three-Dimensional Scaffold in Tissue Engineering

Tissue engineering is "an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain or improve tissue function" [1]. This technology is expected to resolve issues surrounding organ transplantation such as donor shortage, immune rejection from the host, and limited ability to recover functions of defected tissues or organs [2,3]. The concept of tissue engineering is illustrated in Fig. 1 [4].

Beside cells and growth factors, scaffold fabrication is also a deciding factor for success in tissue engineering. The scaffold is expected to have similar functions with natural extracellular matrices (ECMs) that support cell survival, contribute in mechanical properties, provide biological or physical cues to facilitate cell activities, and support the formation of new blood vessels. Therefore, the scaffold should be biocompatible and biodegradable, have a porous structure and adequate mechanical properties, and promote cell proliferation and differentiation [3].

Many studies have been done using two-dimensional (2D) cell culture systems. In

2D culture, cells are placed on the flat surfaces such as Petri dishes, micro-well plates, and tissue culture flasks [5]. Although the 2D culture notably improved the understanding of basic cell biology, it cannot provide the native environment. The limitation of 2D culture is clearly observed in the cell differentiation of the epithelial cells. Bissel et al. showed that human breast epithelial cells grow like tumor cells when they were cultured on 2D scaffold, while they become normal cells when cultured in three-dimensional (3D) matrix [6,7]. Therefore 3D matrices mimicking native environment are more preferable.

There are several approaches to develop prefabricated 3D scaffolds that apply in tissue engineering, including electrospinning, freeze-drying, gas foaming, and porogen process [5]. Although these processes produce porous scaffolds, they are unable to produce homogeneous cell distributions in scaffold. Recently, there has been much attention in cell-implantable and injectable hydrogels as 3D scaffolds that can be used in cell-printing systems and resulted an uniform cell seeding [8,9]. Therefore, the hydrogel that can undergo gelation during cell incorporation seems preferable.

Hydrogel is a crosslinked hydrophilic polymer and it can absorb a large amount of water up to thousand times of its dry weight [10,11]. Material of hydrogel can be classified into two groups, natural and synthetic hydrophilic polymers. Natural polymers

include collagen, chitosan, hyaluronate (HA), alginate, pectin, carrageenan, chondroitin sulfate, dextran sulfate, gelatin, fibrin, dextran, and agarose. Poly(hydroxyethyl methacrylate) (polyHEMA), poly(ethylene oxide) (PEO), poly(*N*-vinyl-2-pyrrolidone) (PVP), poly(vinyl alcohol) (PVA), poly(acrylic acid) (PAA), polyimide (PI), and polyurethane (PU) are classified as synthetic polymers [10,12].

Collagen is a representative fibrous protein of mammalian ECM. It comprises of three alpha chains. Each of polypeptide alpha chains consists of amino acid sequences, where the glycine (Gly) is located at every third residue [13]. Due to its biocompatibility, biodegradability, and low antigenicity, collagen is widely applied for bone and cartilage regeneration, cardiac and vascular reconstruction, burn dressings, peripheral nerve regeneration, barrier membrane of incorporated drug, and drug reservoir [13-16].

HA is a non-sulfated glycosaminoglycan and consists of altering disaccharide, β-1,4-*D*-glucuronic acid and β-1,3-*N*-acetyl-*D*-glucosamine. HA is a highly hydrated polyanionic molecule, ranging from 100 kDa to 8000 kDa of molecular weight [17].

Alginate consists of *L*-guluronate (G) and *D*-mannuronate (M), and it contains consecutive –G–G–G–G–, –M–M–M–M– or –G–M–G–M– sequences. This linear polysaccharide is obtained from brown algae. By interacting G block with divalent cation such as Ca^{2+}, alginate hydrogels are formed. Alginate is possible to be applied to

wound dressings, drug delivery systems, and tissue engineering [18].

Chitosan is a partially de-acetylated derivative of chitin and is a linear polysaccharide comprised of *D*-glucosamine residues with a various number of randomly located *N*-acetyl-*D*-glucosamine [19-21]. The advantages of chitosan are its biological properties such as biocompatibility, biodegradability, non-toxicity, ability to form hydrogel, good mucoadhesive properties, haemostatic properties, and antibacterial properties [22-25].

As synthetic materials for hydrogel, polyHEMA, PEO and PVA, either alone or combined, have been widely used. PolyHEMA was prepared by free-radical copolymerization of HEMA as a monomer and a crosslinking reagent in the presence of water or hydro-organic media (mixtures of water and organic solvent) [26]. The polyHEMA either alone or combined has been proposed as promising biomaterial in many biomedical applications ranging from cartilage regeneration [27], skin and wound healings [28] and contact lens [29,30]. PEO consists of repeating unit of ethylene and oxygen and it contains two terminal groups, H and OH. The hydrophobic ethylene units and the hydrophilic oxygen, are identified as amphiphilic character [31]. Another type of synthetic material for hydrogel is PVA. PVA has pendant hydroxyl group and is produced by polymerization of vinyl acetate and then continued by hydrolysis of

polyvinyl acetate [32]. PVA hydrogel has several advantages include non-toxic, bioadhesive, high swelling degree, therefore this hydrogel has potential as biomaterial.

2. Collagen-Like Polypeptide, Poly(Pro-Hyp-Gly)

Collagen, a fibrous structural protein consisting of a triple-helical structure, is a main component in ECM. The existence of glycine (Gly) at every third residue in amino acid sequences (Xaa−Yaa−Gly) contributes for formation of the unique collagen triple-helical structure. The primary force to stabilize the triple-helical structure of collagen is interstrand hydrogen bonding [$N–H_{(Gly)}\cdots\cdots O=C_{(Pro)}$]. The most common amino acid sequence in collagen triple-helical region is proline-hydroxyproline-glycine (Pro-Hyp-Gly) [33]. Although animal-derived collagen is obtained abundantly, it has a possibility to transfer pathogenic substances [34]. Therefore, the synthetic materials showing collagen characteristic has been desired. Kishimoto et al. chemically synthesized polypeptides containing Pro-Hyp-Gly sequences, poly(Pro-Hyp-Gly), which forms triple-helical structure and shows high thermal stability up to 80 °C [35]. Poly(Pro-Hyp-Gly) shows good biodegradability, does not cause inflammatory reaction when embedded subcutaneously into a rat dorsal area, and accelerates the

epithelialization of full-thickness wound created on rabbit ears [36]. Moreover, a copolymer of peptides having Pro-Hyp-Gly sequence and an elastin derived Val-Pro-Gly-Val-Gly sequence can change from transparent liquid to turbid gel upon raising temperature [37]. Furthermore, poly(Pro-Hyp-Gly) film that simultaneously conjugated with Gly-Arg-Gly-Asp-Ser and Pro-His-Ser-Arg-Asn, which are the cell-adhesion motif from fibronectin, enhanced a NIH3T3 cell adhesion and migration [38]. Because of the above advantages, poly(Pro-Hyp-Gly) is a promising material to be a main component of synthetic 3D scaffolds for tissue regeneration. Although the poly(Pro-Hyp-Gly) hydrogel can be obtained at over 1 w/v% concentration, such preformed hydrogel cannot encapsulate cells homogeneously.

3. Hydrogel Formation

There are two general approaches to describe gelation phenomena, Flory-Stockmayer model and percolation model [39]. Based on Flory-Stockmayer model, gelation occurs when the polymerization reaction reaches a critical value. This critical stage that defines as gel point is a state when the largest molecular clusters tend to diverge. Practically, at this gel point, the product of polymerization transforms

suddenly from a viscous solution to gel. In Florry-Stockmayer model, the large network polymers are built by successive addition of branches at polymer backbone (Fig. 2a).

From the point of view of percolation theory, the gelation point is identified as the critical point of percolation. Percolation is related with the probability to occupancy cites. There are two types of percolation models, bond percolation and site percolation. The gel phase occurs in the system when the number of bond higher than the critical value and lower than its critical value, the system is in the sol phase (Fig. 2b).

Hydrogel can be prepared either by chemical crosslinking or physical crosslinking [40]. Chemically crosslinked hydrogel has covalent bonds that result in irreversible networks. Xanthan-PVA hydrogel in the present of epichorohydrin [41], chitosan-PVA hydrogel crosslinked with glutaraldehyde [42] and guar gum-polyacrylamide hydrogel crosslinked with glutaraldehyde [43] are types of chemically crosslinked hydrogels. Although chemically crosslinking can improve the mechanical strength, some of chemical crosslinking requires cytotoxicity chemicals such as dialdehydes and diisocianates. These can cause considerable damage to the encapsulated cells and surrounding tissues.

Another method to produce chemically crosslinked hydrogel is radiation crosslinking. This method is widely used to create chemically crosslinked hydrogel

because it is free from chemical additives. The free radicals are generated directly or indirectly in the polymer after irradiation. The irradiation can be done using gamma ray, X-ray or electron beam. In dilute solution, the radiation is absorbed by water and then creates reactive species including hydroxy radicals, hydrogen atoms, and hydrated electrons. Those reactive species can react with the polymer chains. In the case of high concentration of polymers, the radiation produces radicals directly on the polymer chain. Crosslinked polymer can be formed by radical reactions between polymers chains [40]. For example, carboxymethyl cellulose and poly(acrylic acid) hydrogel that is formed by electron beam irradiation [44].

The physically crosslinked hydrogel forms reversible hydrogel by physical crosslinking with heating or cooling the polymer solution. There are two types of critical temperature in thermosensitive hydrogel, upper critical solution temperature (UCST) and lower critical solution temperature (LCST) [45]. A homogeneous polymer solution is clear below LCST and turns to be cloudy when the temperature is raised to over LCST. Poly(N-isopropylacrylamide) (PNIPAAm), one of thermosensitive polymer, is commonly used in biomedical application because its LCST is relatively close with body temperature.

Freeze-thawing is a physical gelation method that involves freezing and thawing

for several times. In this cycle, the stable hydrogel is created. The PVA hydrogel is one of the examples of freeze-thawed gel [46].

Another physically crosslinked hydrogel is formed by existing of ionic bonds, hydrogen bonds, and hydrophobic bonds. The physically crosslinked hydrogel has the advantages of using no toxic agent. Hydrogel using low molecular weight ion crosslinking, such as Ca-crosslinked alginate hydrogels, have almost no cytotoxicity, but resulting hydrogel with low stability. On the other hand, polyion complexes can show remarkable stability in physiological conditions because of multiple bonds between two high molecular weight polyion precursors.

4. Polyion Complex

PIC can be formed by mixing oppositely-charged polymers, a cationic polymer such as chitosan with an anionic polymer such as alginate [47-51], xanthan gum [52-54], γ-poly(glutamic acid) [55,56] and hyaluronate [57,58]. This interaction is driven by electrostatic forces that are influenced by ionic strength and pH of solution, ratio of precursors, and counterions. The complex formation results in precipitate, film, microparticle, or hydrogel. Most of the complexation of chitosan-alginate is

additionally crosslinked with $CaCl_2$ solution and resulted in porous complex by freeze drying [48-51]. The existing of PIC hydrogel promises much higher stability than a low-molecular weight ion-crosslinked hydrogel such as Ca^{2+}alginate. The complexation of chitosan-xanthan gum forms hydrogel capsules with average diameter size of 3 mm [54]. Previous reports utilized the chitosan-based polyion complex to seed the cells into porous sponges [49, 55] and hydrogel [56]. All of these PIC hydrogel studies report the incorporation of the cells after scaffold fabrication. Besides the preparation is time consuming, the incorporation of the cells after scaffold fabrication might have limited cell penetration and it causes an inhomogeneous cell distribution. In addition, there has been no report on pre-incorporation of the cells with simple preparation into precursor solutions toward hydrogel through polyion complex formation.

To improve the cell entrapment in scaffold, it is preferable that the cells are incorporated within two high molecular weight polyion in a liquid form and then undergo the gelation by ionic interaction. Using this type of scaffold formation, cells can be entrapped into the scaffold with a simple step enabling simultaneous cell encapsulation and the homogeneous cell distribution might be achieved. In addition, it also provide remarkable stability in physical condition due to existing of multiple bonds

between polyion precursors. This preparation can be used also as an injectable scaffold that provides minimally invasive treatments and has the ability to repair irregular shaped defects in tissue engineering.

5. Purpose of This Work

As described previously, the synthetic polymer having collagen characteristics, poly(Pro-Hyp-Gly), is a promising material for developing new 3D scaffold. The substitution of hydroxy group in Hyp residue by esterification with succinic anhydride resulted in succinylated poly(Pro-Hyp-Gly) [suc-poly(Pro-Hyp-Gly)]. In this study, first, I fabricated the hydrogel through PIC gel formation between cationic polymer chitosan and anionic polymer Suc-poly(Pro-Hyp-Gly) in a simple procedure of mixing. Second, the obtained PIC hydrogels were characterized by gelation and swelling ratios. Third, the surface morphology of the lyophilized hydrogel was observed using a scanning electron microscope (SEM). Fourth, the effects of counterions such as sodium, ammonium, acetate and chloride on the hydrogel formation were discussed. Finally, the viability of encapsulated rat bone marrow stromal cells (rBMSCs) into PIC hydrogel was assessed.

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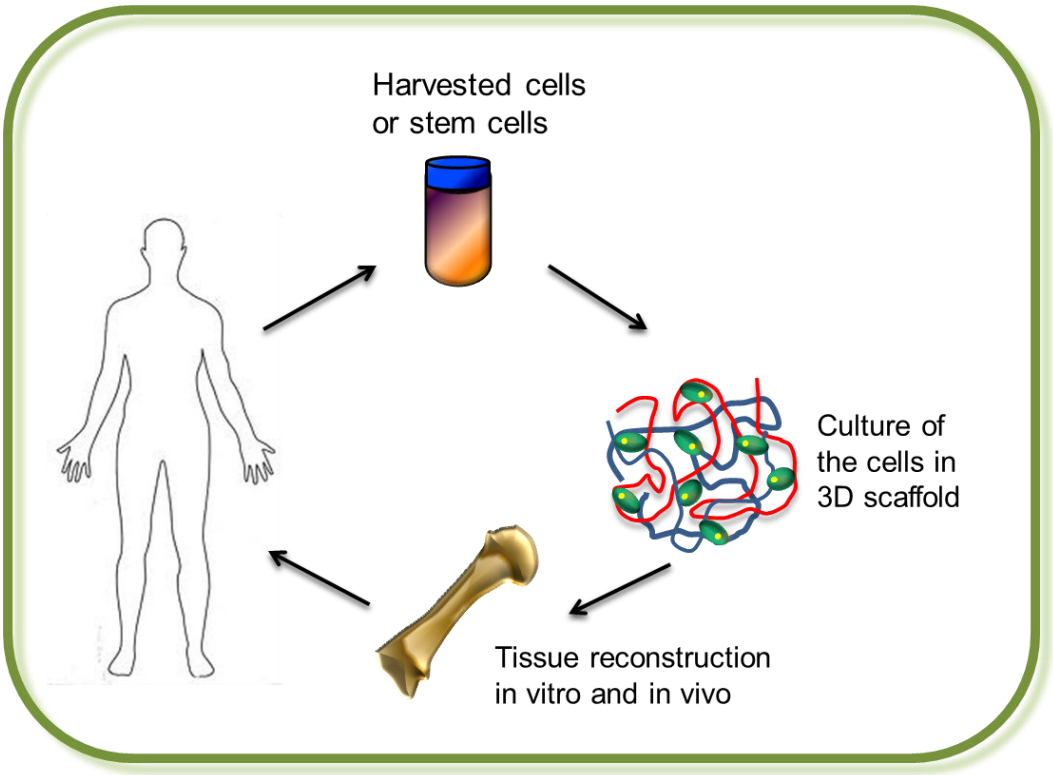

Fig. 1. Concept of tissue engineering

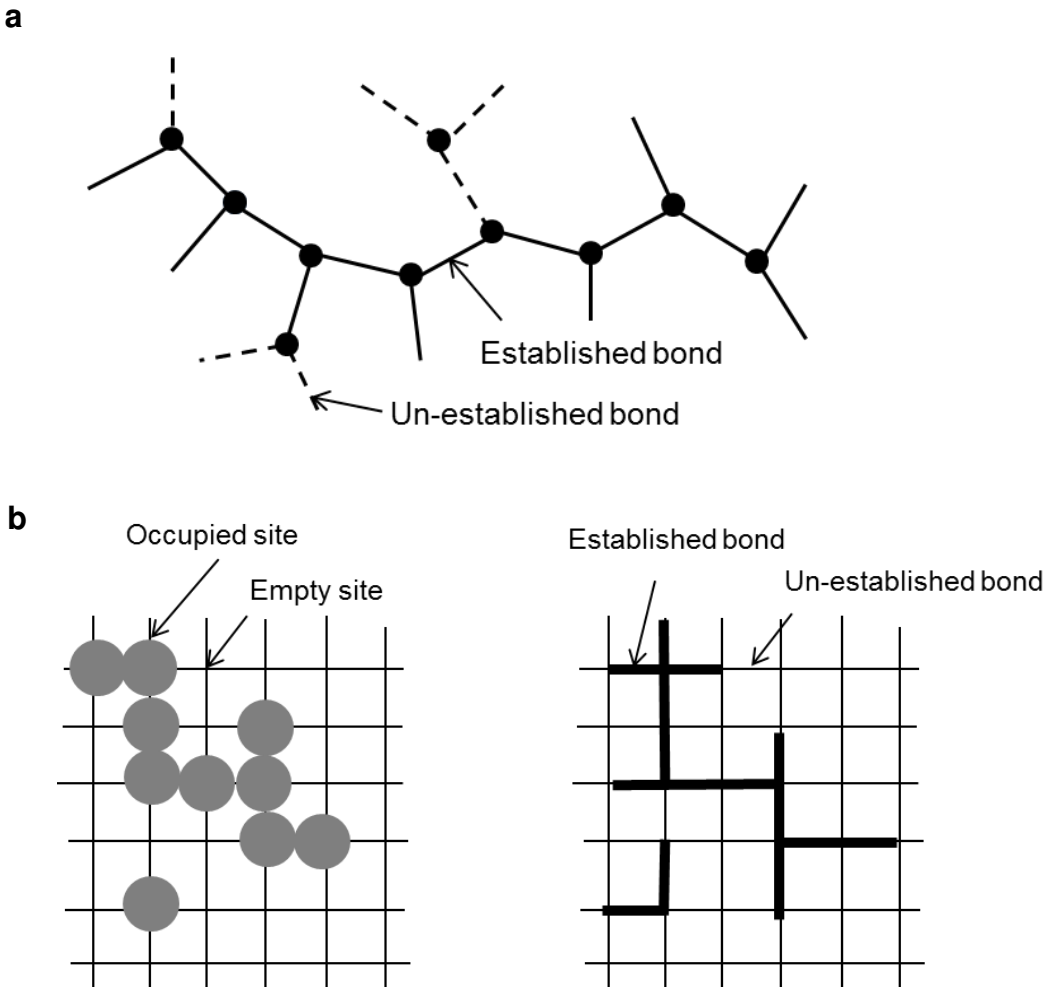

Fig.2. Flory-Stockmayer model (a) and percolation model (b).
(Pierre A.C., 1998)

CHAPTER 1

Synthesis and Characterization of Succinylated Poly(Pro-Hyp-Gly)

1-1. Introduction

Modification of functional group is necessary to improve the physical properties of the polymer. Generally, there are two approaches for introduction of carboxy group in polymer backbone, carboxymethylation and succinylation. Many researchers have been utilized carboxymethylation to modify the hydroxy group of polymer chain into carboxylate group, such as in cellulose, hemi-cellulose, and starch; and results in such carboxymethyl cellulose [1,2], carboxymethyl starch [3], carboxymethyl xylan [4], and carboxymethyl *kappa*-carrageenan [5]. Commonly, the introduction of carboxymethyl group utilizes monochloroacetate or its sodium salt in the presence of alkaline solution. Although the reaction occurred in the presence of strong base that increased the nucleophilicity of the hydroxy group, the carboxymethylation resulted in sodium glycolate as undesired by-products [1,3]. It was reported that the by-product was obtained from reaction between sodium hydroxide and sodium monochloroacetate.

Various organic solvents including isopropyl alcohol [4,6,7], dimethyl sulfoxide (DMSO) [8], ethanol [9], and acetone [9] have been used for carboxymethylation. Utilization of organic solvents and existing of undesired side reaction are notified as drawbacks of carboxymethylation.

Another method to introduce the carboxy group is succinylation with anhydride as a reagent. Anhydride reagents are highly reactive electrophiles for attachment of various functional groups in macromolecules [10,11]. Succinic anhydride, one of anhydride reagents, has a five-atom cyclic that is highly reactive with nucleophiles. When nucleophile attacks to one of carbony group, the cyclic ring opens and results in ester bond and free carboxylic acid [11]. Comparing with carboxymethylation, succinylation seems preferable by considering side reactions.

As mentioned in General Introduction, the collagen-like polypeptide, poly(Pro-Hyp-Gly), is a promising material for developing new 3D scaffold. Because poly(Pro-Hyp-Gly) does not have any ionic groups except for both terminus, ionic interaction cannot be occurred by itself. Therefore, modification of poly(Pro-Hyp-Gly) is necessary to create anionic species. In this study, the succinylation of poly(Pro-Hyp-Gly) was conducted using succinic anhydride for introducing a carboxy group into the Hyp residue, and then resulted in succinylated poly(Pro-Hyp-Gly)

[Suc-poly(Pro-Hyp-Gly)] as anionic species.

1-2. Materials and Methods

1-2-1. Materials

Pro-Hyp-Gly, 1-hydroxybenzotriazole (HOBt), and 1-ethyl-3-(3-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC·HCl) were purchased from the Peptide Institute (Osaka, Japan). *N,N*-Diisopropylethylamine (DIPEA) was purchased from Applied Biosystems (Carlsbad, CA, USA). Chitosan (1000 sugar units; 80% deacetylation ratio) and other reagents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Succinic anhydride was recrystallized from 2-propanol before use.

1-2-2. Synthesis of Succinylated Poly(Pro-Hyp-Gly)

The synthesis of poly(Pro-Hyp-Gly) was conducted as reported previously with slight modifications [12,13]. Briefly, Pro-Hyp-Gly (0.70 mmol) and HOBt (0.14 mmol) were dissolved in 4 mL of 10 mM phosphate buffer (PB, pH 7.4). EDC·HCl (3.50 mmol) was added and stirred at 400 rpm for 2 h at 4° C and then stirred for 46 h

at 20° C. The reaction was terminated by adding an aliquot of Dulbecco's phosphate buffered saline (PBS, pH 7.4). The reaction mixture was homogenized using a Waring blender (Waring Products Division, New Hartford, CT, USA) five times for 1 min at the maximum speed. The mixture was stirred for 1 day at room temperature and dialyzed against Milli-Q (Merck-Millipore, Billerica, MA, USA) water for 7 days at 4° C using a dialysis membrane (UC20-32-100, Sanko Junyaku Co., Ltd., Tokyo, Japan) to remove any residual reagents.

Suc-poly(Pro-Hyp-Gly) was obtained by reacting poly(Pro-Hyp-Gly) with a 50 times the molar excess of both succinic anhydride and DIPEA with the hydroxy group of poly(Pro-Hyp-Gly). The reaction was conducted on ice for 2 h with stirring, and the product was then stirred at room temperature for 24 h. The obtained Suc-poly(Pro-Hyp-Gly) was dialyzed first with 1 M NaCl solution for 1 day and then with Milli-Q water for 3 days to obtain sodium Suc-poly(Pro-Hyp-Gly) [NaSuc-poly(Pro-Hyp-Gly)]. To obtain the ammonium Suc-poly(Pro-Hyp-Gly) [NH_4 Suc-poly(Pro-Hyp-Gly)], a 1 M NH_4Cl solution was used instead of the 1 M NaCl solution. The concentration was determined by freeze drying method. A summary of the synthetic procedure of Suc-poly(Pro-Hyp-Gly) is illustrated in Scheme 1-1.

1-2-3. Characterization of Succinylated Poly(Pro-Hyp-Gly)

The obtained both poly(Pro-Hyp-Gly) and Suc-poly(Pro-Hyp-Gly) were analyzed by gel permeation chromatography (GPC) and circular dichroism (CD) spectroscopy. The GPC analysis was conducted using an AKTA purifier system on a Superdex 200 HR 10/300 GL column (GE Healthcare Biosciences, Piscataway, NJ, USA) at a concentration of 0.5 mg/mL in Milli-Q water. The flow rate was 0.5 mL/min at room temperature, with the detection wavelength of 215 nm with PBS as the elution buffer. The molecular weight of the obtained polypeptides was calculated based on poly(ethylene glycol) standards (Waters, Milford, MA, USA). The CD spectra were recorded on a Jasco model J-820 spectropolarimeter (Jasco, Tokyo, Japan) using a quartz cell with an optical path length of 0.1 cm. The concentration of each sample was 0.25 mg/mL in Milli-Q water. The molecular weight containing counterions (sodium or ammonium) was used to calculate the mean residual molar concentration in CD measurement of Suc-poly(Pro-Hyp-Gly). In the measurement of temperature dependence, the temperature of the cell was controlled by a Peltier thermoelectric temperature controller (PTC-423L, Jasco). The 50 mM glycine-HCl buffer (pH 3.0), 10 mM phosphate buffer (pH 7.4), and 0.1M NaOH solution (pH 12.1) were used as

solvent for measuring pH dependence of the obtained Suc-poly(Pro-Hyp-Gly).

Fourier transform infrared (FTIR) spectra of both poly(Pro-Hyp-Gly) and Suc-poly(Pro-Hyp-Gly) were measured using a Spectrum One FTIR spectrometer (PerkinElmer, Wellesley, MA, USA) based on the KBr method with 16 scans and resolution of 2 cm^{-1}.

Potentiometric titration was conducted using a DL 58 Titrator (Mettler-Toledo, Schwerzenbach, Switzerland) to determine the degree of succinylation of Suc-poly(Pro-Hyp-Gly). An aliquot of Suc-poly(Pro-Hyp-Gly) containing 5 mL of 0.02 M HCl solution was titrated with 0.02 M NaOH. Blank titration result without Suc-poly(Pro-Hyp-Gly) was subtracted from this to determine the amount and pKa of the carboxy group obtained.

1-3. Results and Discussions

1-3-1. Characterization of Succinylated Poly(Pro-Hyp-Gly)

The GPC profiles shows that the molecular weights of dialyzed poly(Pro-Hyp-Gly) (Fig.1-1) and Suc-poly(Pro-Hyp-Gly) (Fig.1-2) peaked at over 120 kDa, similar to that of poly(Pro-Hyp-Gly) synthesized by Kishimoto et al [12]. The appearance of the dialyzed polypeptides was transparent solution, where poly(Pro-Hyp-Gly) showed higher viscosity than Suc-poly(Pro-Hyp-Gly).

The CD spectrum of the poly(Pro-Hyp-Gly) (Fig. 1-3) showed a positive Cotton effect at 225 nm and negative Cotton effect at near 197 nm, showing the formation of a triple-helical structure. Both NaSuc-poly(Pro-Hyp-Gly) and NH_4Suc-poly(Pro-Hyp-Gly) showed similar CD spectra (Fig. 1-4). These results suggest that succinylation and counterions had almost no effect on the molecular weight or triple-helical structure of the obtained polypeptides.

The FTIR spectra of freeze-dried poly(Pro-Hyp-Gly), NaSuc-poly(Pro-Hyp-Gly), and NH_4Suc-poly(Pro-Hyp-Gly) are shown in Fig. 1-5**.** The ester peak (C=O) was observed at 1735 cm^{-1}. The existence of the ester peak in the spectrum of

Suc-poly(Pro-Hyp-Gly) revealed that the hydroxy group of the Hyp residue in poly(Pro-Hyp-Gly) might be replaced successfully by a succinyl group through ester formation.

From the potentiometric titration, the obtained pKa of both Na Suc-poly(Pro-Hyp-Gly) and NH_4Suc-poly(Pro-Hyp-Gly) was 4.8 – 4.9 with 49.4 ± 1.7% (mean ± standard deviation) of the hydroxy groups at the Hyp residue in poly(Pro-Hyp-Gly) (Fig. 1-6). The pKa values of acetic acid and propanoic are 4.76 and 4.82, respectively [14]. Therefore, no difference in pKa between low molecular weight carboxylic acid and Suc-poly(Pro-Hyp-Gly) was observed.

1-3-2. Temperature Dependence of CD Spectra of Succinylated Poly(Pro-Hyp-Gly)

The CD spectra of NaSuc-poly(Pro-Hyp-Gly) and NH_4 Suc-poly(Pro-Hyp-Gly) at various temperatures are shown in Fig. 1-7 and the temperature dependence of the mean residual molar ellipticity at 225 nm is shown in Fig. 1-8. The measurement of thermal stability of CD spectra of Suc-poly(Pro-Hyp-Gly) was limited up to 80 °C because water was used as solvent. The mean residual molar ellipticity at 225 nm of Suc-poly(Pro-Hyp-Gly) showed constant below 40 °C, however it slightly decreased

when increased temperature over 40 °C to 60 °C. The thermal stability of triple-helical structure of collagen is enhanced by the hydroxylation of Pro residues in the Yaa position with configuration in 4R (2S,4R)-4-hydroxyproline [15]. As described by Suzuki et al., the 4R configuration of Hyp residue has possibility to form the water-mediated hydrogen bonds, thus forming an interchain link that stitch together with the folded triple helix [15,16]. This Hyp residue also stabilizes the triple-helical structure of collagen trough a stereoelectronic effect. Therefore, it suggests that the slight instability of Suc-poly(Pro-Hyp-Gly) is caused by the 49.4 ± 1.7% substitution of Hyp residue in poly(Pro-Hyp-Gly) through esterification.

The spectrum at 197 nm is more intense at low temperature than at high temperature. It suggests that the increasing of the intensity arises from greater chain rigidity, resulted in more intense of spectra at lower temperature [17]. The heating tends to make a weaker interaction between polypeptides chains, contributes to enhance polypeptide chain motion, and tends to make polypeptide chain more flexible. Therefore, it resulted in weaker intensity in CD signal when raising the temperature.

1-3-3. pH Dependence of CD Spectra of Succinylated Poly(Pro-Hyp-Gly)

The pH dependence of the mean residual molar ellipticity at 225 nm of both Na Suc-poly(Pro-Hyp-Gly) and NH_4Suc-poly(Pro-Hyp-Gly) are shown in Fig. 1-9. The content of triple-helical structure that implicated by the peak height of positive Cotton effect did not significantly change at basic condition. However, in acidic condition it resulted in many insoluble precipitate because of the protonation of carboxy group of Suc-poly(Pro-Hyp-Gly). Microscopic observation revealed that small particles formed for Suc-poly(Pro-Hyp-Gly) solution at acidic condition. No particles were found at neutral pH and basic condition.

1-3-4. Stability of Succinylated Poly(Pro-Hyp-Gly)

Electrostatic interaction from the substitution of carboxy group in hydroxy group of Hyp residue of poly(Pro-Hyp-Gly) did not highly destabilize triple-helical structure of Suc-poly(Pro-Hyp-Gly). The estimated crystal structures of Suc-poly(Pro-Hyp-Gly) with two repeating units of Pro-Hyp-Gly are shown in Fig. 1-10. In addition, only a half of hydroxy group of Hyp is changed to carboxylic acid. There are many

possibilities of the substitution locations in Suc-poly(Pro-Hyp-Gly). To calculate the average repulsion strength, two possibilities of substitution location of carboxy group are shown in Fig. 1-10B and Fig. 1-10C. The strength of hydrogen bond in water is 1 kcal/mol [18]. There are four hydrogen bonds exist in the triple-helical structure of two repeating units of Pro-Hyp-Gly. Therefore, the total strength of hydrogen bond is 4 kcal/mol. With Coulomb law, the repulsion energy between carboxy group is proportional to Coulomb constant ($k = 9.0 \times 10^9$ Nm^2/C^2), each electrical charge (1.602×10^{-19} C), and invers of the distance between two charges. Using the dielectric constant in water (80) and Avogadro number (6.0×10^{23} mol^{-1}), the repulsion energies of both possibilities of Hyp substitution are shown in Table 1. The total average of repulsion energy of both possibilities is 0.68 kcal/mol. Comparing both energy bonding, the strength of hydrogen bond are six times larger than the repulsion energy, therefore the repulsion between carboxy group cannot highly influence the stability of Suc-poly(Pro-Hyp-Gly).

1-4. Conclusion

Poly(Pro-Hyp-Gly) was synthesized by polycondensation of Pro-Hyp-Gly. The succinylation of the obtained poly(Pro-Hyp-Gly) resulted in Suc-poly(Pro-Hyp-Gly) with sodium or ammonium as counterion. From the FTIR spectra, it clearly shows the introduction of succinyl group through esterification. From the CD spectra of Suc-poly(Pro-Hyp-Gly), it was shown that the mean residual molar ellipticity at 225 nm of Suc-poly(Pro-Hyp-Gly) was constant below 40°C, however it was slightly decreased when increased temperature over 40°C to 80°C. Therefore, Suc-poly(Pro-Hyp-Gly) is slightly unstable than poly(Pro-Hyp-Gly) due to substitution of Hyp residue. Furthermore, the mean residual molar ellipticity at 225 nm of Suc-poly(Pro-Hyp-Gly) showed no significant change even at pH 12. However, in acidic condition, it resulted in many insoluble precipitated because of the protonation of carboxy group of the Suc-poly(Pro-Hyp-Gly).

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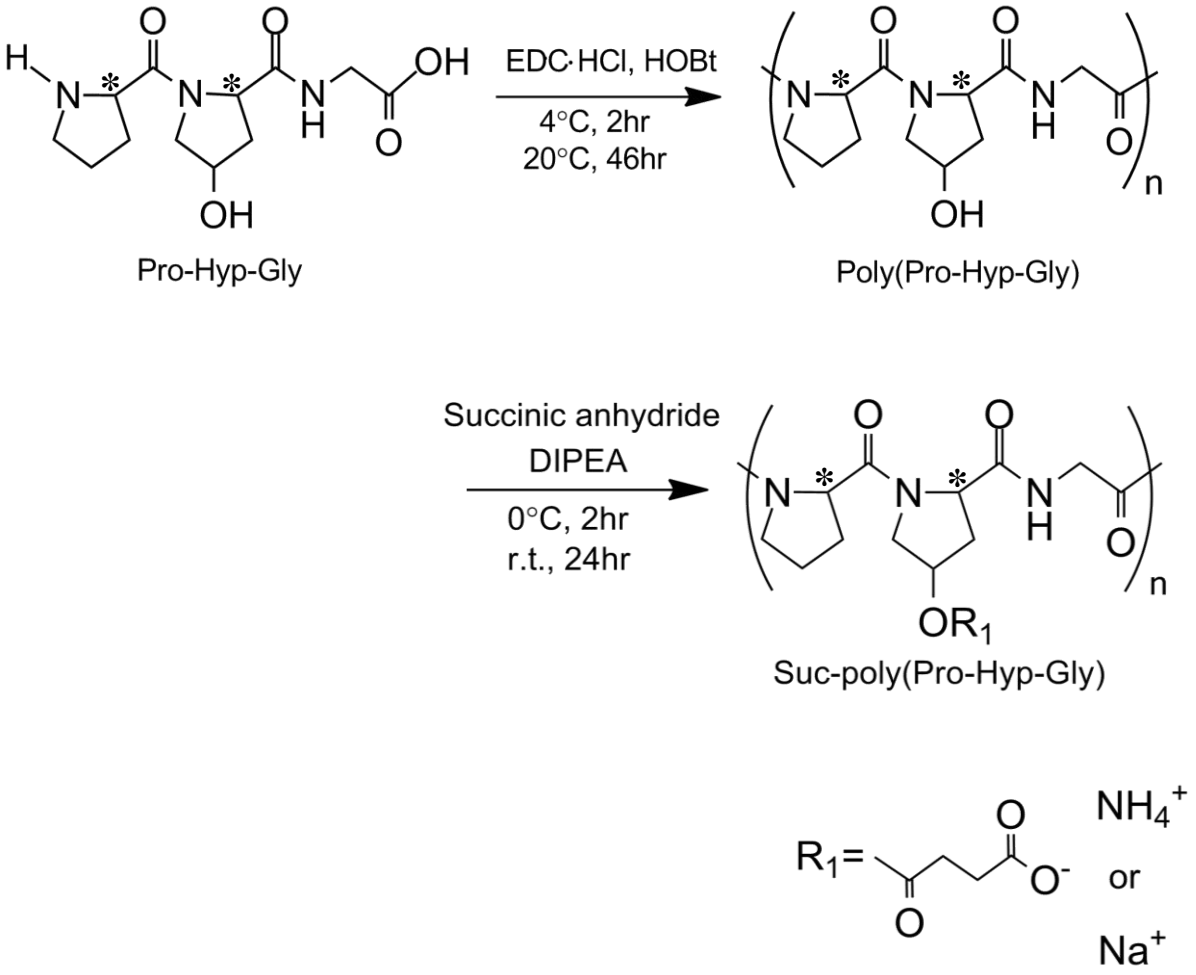

Scheme 1-1. Synthesis of Suc-poly(Pro-Hyp-Gly). "*" indicates asymmetric carbon.

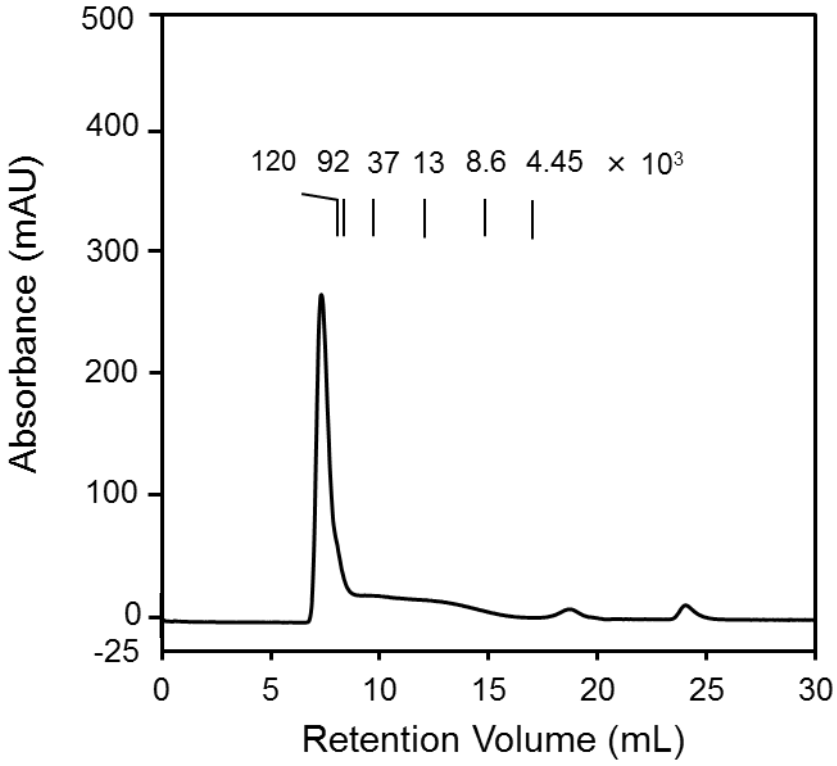

Fig. 1-1 GPC profile of poly(Pro-Hyp-Gly). GPC was carried out using an AKTA purifier system [column = Superdex 200 HR 10/300 GL, elution buffer = PBS (pH = 7.4), flow rate = 0.5 mL/min, detection wavelength = 215 nm].

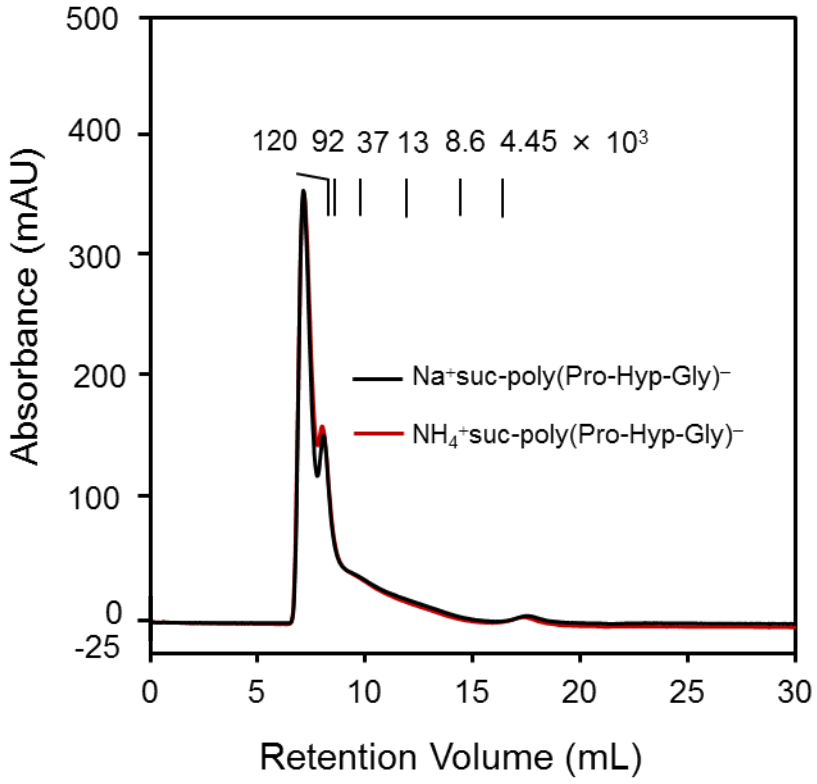

Fig. 1-2 GPC profile of NH_4Suc-poly(Pro-Hyp-Gly) and NaSuc-poly(Pro-Hyp-Gly) GPC was carried out using an AKTA purifier system [column = Superdex 200 HR 10/300 GL, elution buffer = PBS (pH = 7.4), flow rate = 0.5 mL/min, detection wavelength = 215 nm].

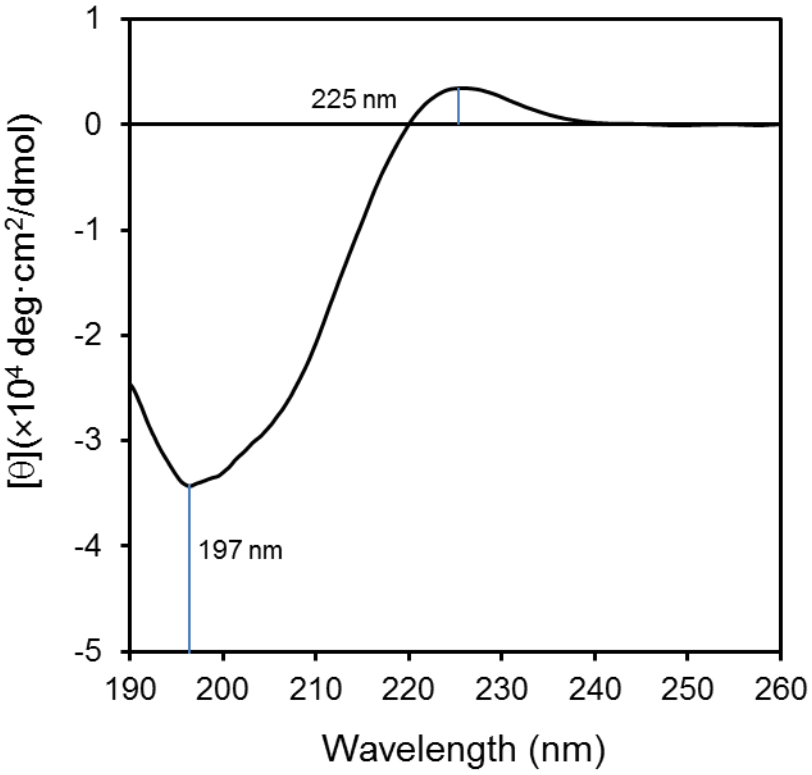

Fig. 1-3 CD spectra of poly(Pro-Hyp-Gly) were recorded using a Jasco model J-820 spectropolarimeter [optical path length = 0.1 cm, number of scans = 5, resolution = 0.2 nm, scan speed = 50 nm/min].

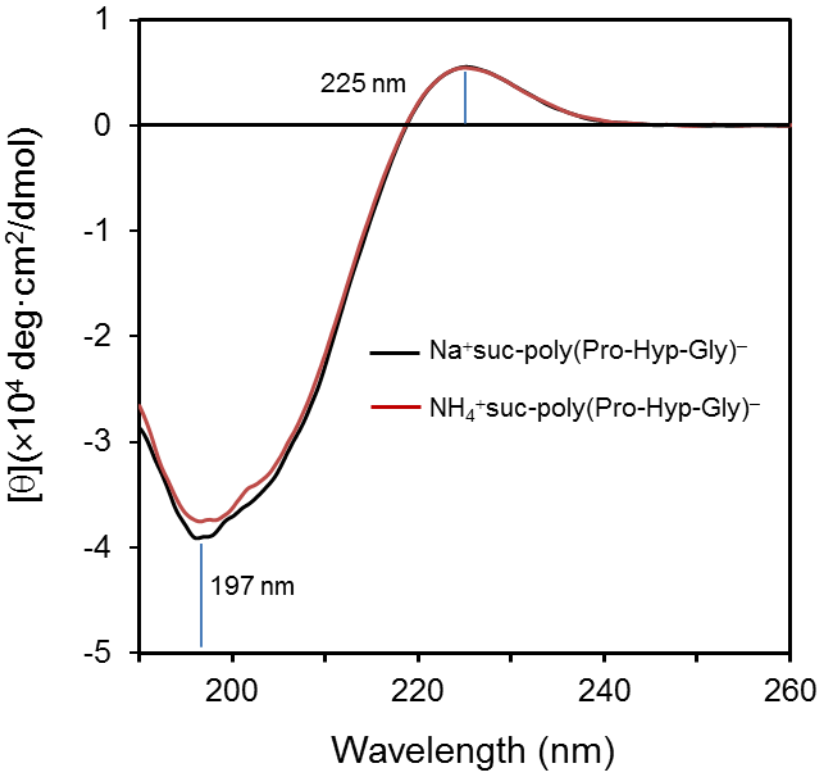

Fig.1-4 CD spectra of NH_4Suc-poly(Pro-Hyp-Gly) and NaSuc-poly(Pro-Hyp-Gly) were recorded using a Jasco model J-820 spectropolarimeter [optical path length = 0.1 cm, number of scans = 5, resolution = 0.2 nm, scan speed = 50 nm/min].

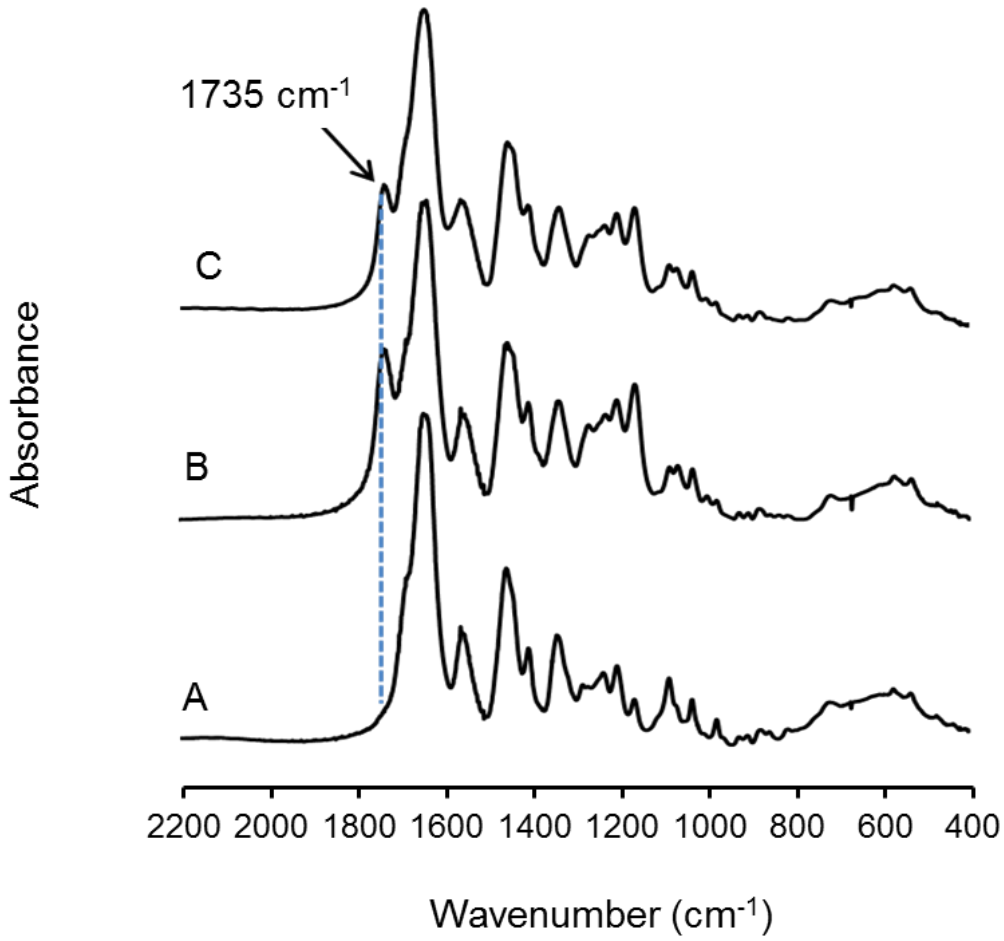

Fig. 1-5 FTIR spectra of freeze-dried poly(Pro-Hyp-Gly) (A), NH_4Suc-poly(Pro-Hyp-Gly) (B), and NaSuc-poly(Pro-Hyp-Gly) (C). KBr method with resolution 2 cm^{-1} and 16 times of scans at room temperature.

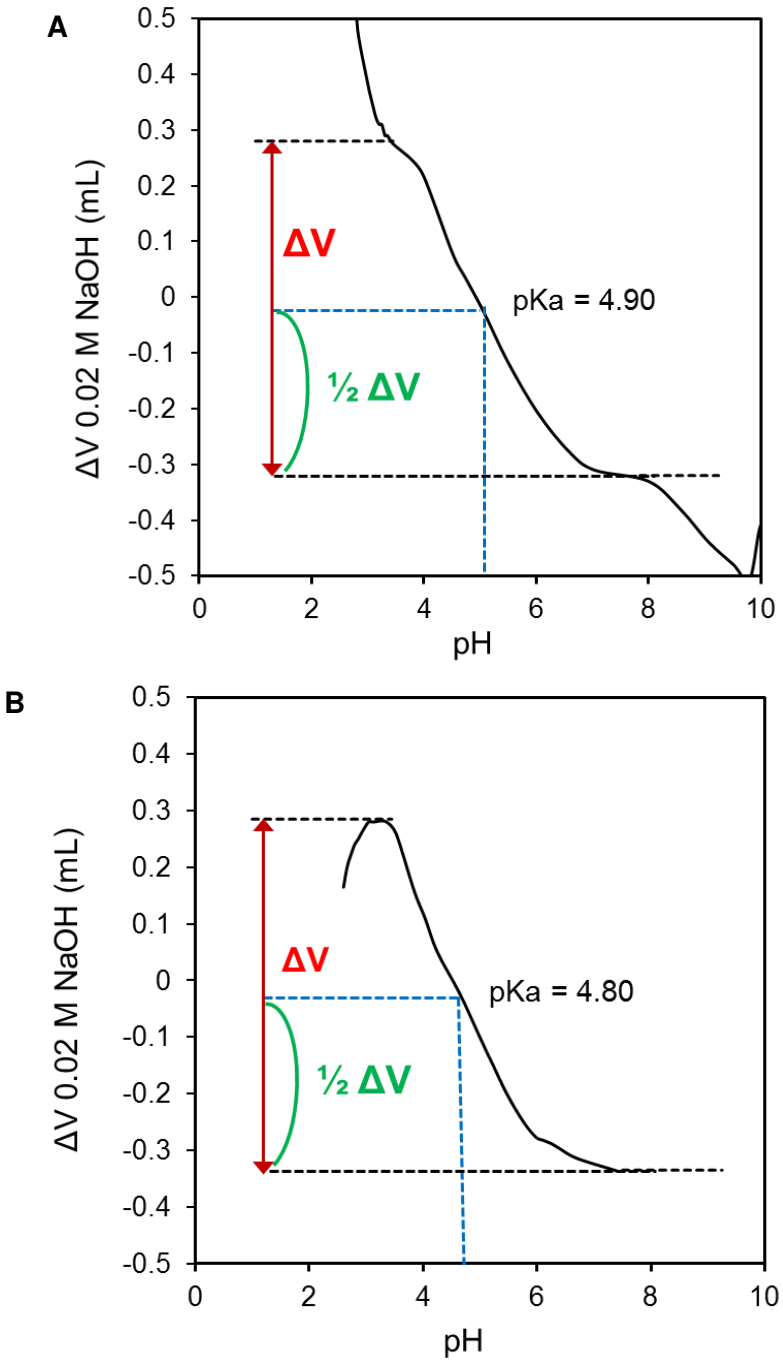

Fig. 1-6 Potentiometric titration of NH_4Suc-poly(Pro-Hyp-Gly) (A) and NaSuc-poly(Pro-Hyp-Gly) (B) with 0.02 M HCl as blank.

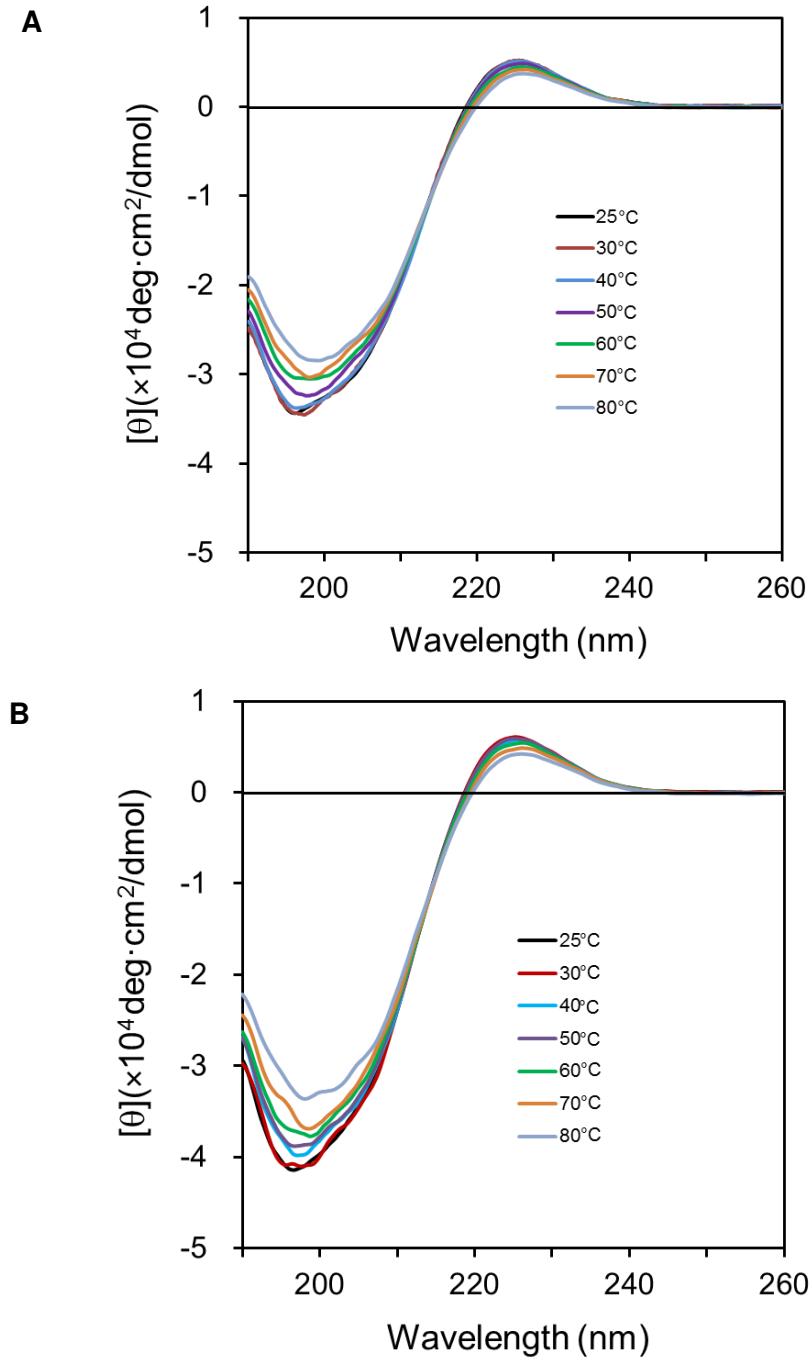

Fig. 1-7 CD spectra of NH_4Suc-poly(Pro-Hyp-Gly) (A) and Na Suc-poly(Pro-Hyp-Gly) (B) at pH 7.4 with various temperature were recorded using a Jasco model J-820 spectropolarimeter [optical path length = 0.1 cm, number of scans = 5, resolution = 0.2 nm, scan speed = 50 nm/min].

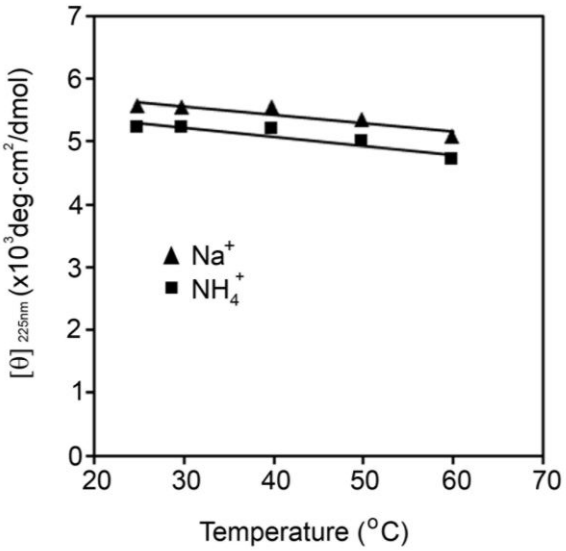

Fig.1-8 Temperature dependence of the mean residual molar ellipticity at 225 nm of NaSuc-poly(pro-Hyp-Gly) and NH_4Sucpoly(Pro-Hyp-Gly) were recorded using a Jasco model J-820 spectropolarimeter equipped with a Peltier thermoelectric temperature controller [optical path length = 0.1 cm, number of scans = 5, resolution = 0.2 nm, scan speed = 50 nm/min].

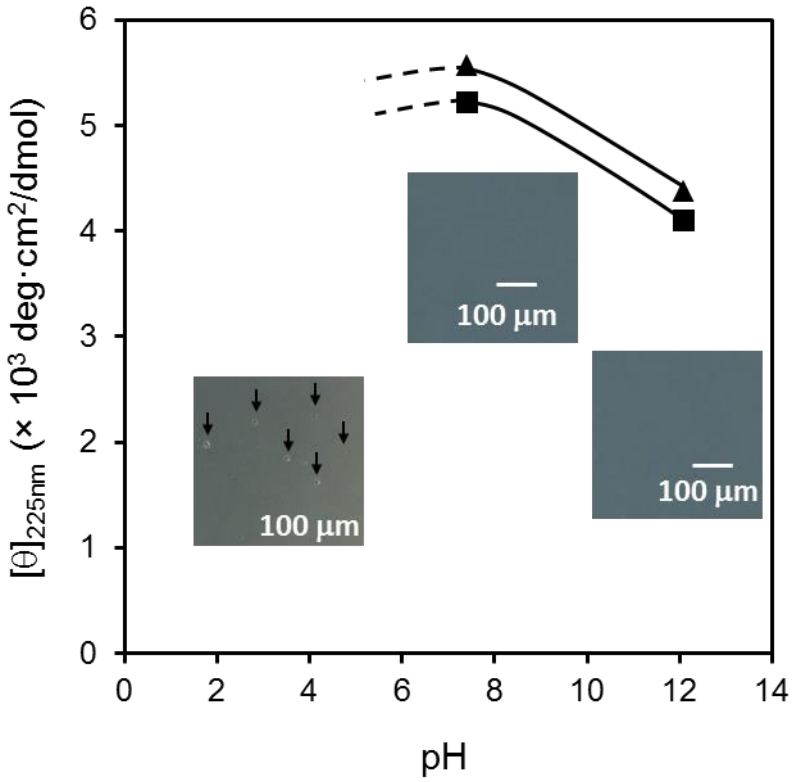

Fig. 1-9 pH dependence of the mean residual molar ellipticity at 225 nm were recorded using a Jasco model J-820 spectropolarimeter [optical path length = 0.1 cm, number of scans = 5, resolution = 0.2 nm, scan speed = 50 nm/min].

(■) NH_4 Suc-poly(Pro-Hyp-Gly); (▲) NaSuc-poly(Pro-Hyp-Gly)

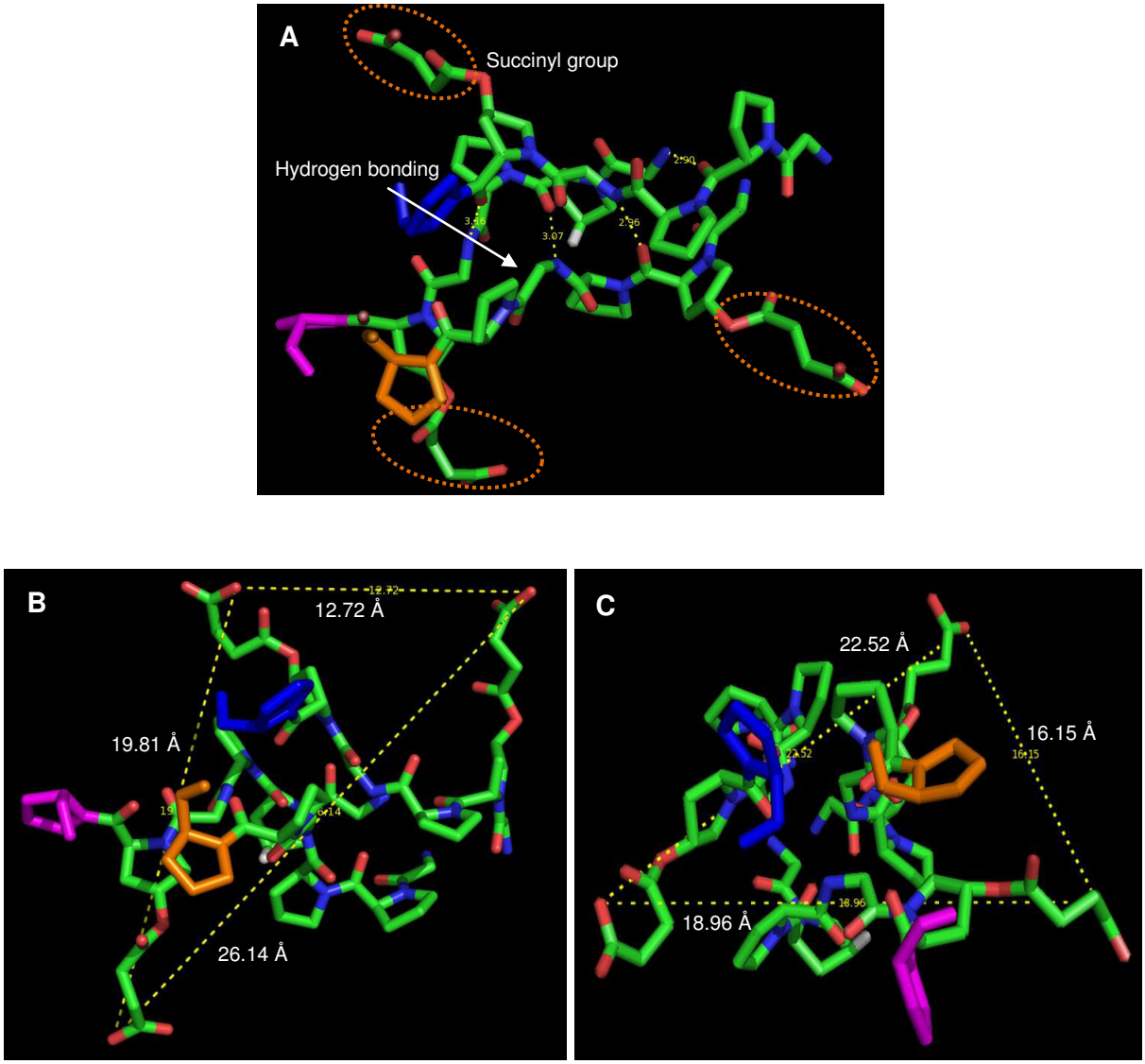

Fig.1-10 The estimated crystal structure of Suc-poly(Pro-Hyp-Gly) with a half of substitution of Hyp residues (A). Arrangement possibilities of carboxy group in triple-helical structure of Suc-poly(Pro-Hyp-Gly) (B,C).

Table 1. Repulsion energy between carboxy group in Suc-poly(Pro-Hyp-Gly).

Possibility 1	
Distance (Å)	**Repulsion energy (kcal/mol)**
12.72	0.33
19.81	0.21
26.14	0.16
Possibility 2	
16.15	0.26
18.96	0.22
22.52	0.18

CHAPTER 2

Polyion Complex Hydrogel Formation and Viability of Rat Bone Marrow Stromal Cells Incorporated in The Polyion Complex Hydrogel

2-1. Introduction

The development of injectable materials is attracting considerable interest in biomedical researchers [1-5]. There are several advantages for injectable biomaterials that form hydrogel *in situ*. This approach minimizes the invasive treatment for the patients [6], fulfills the irregular-shaped tissues defects, and has ability to incorporate cells and bioactive molecules in the solution prior to injection [7]. Through chemical or physical crosslinking, the injectable materials can be formed *in situ* hydrogels.

Photopolymerization [8-10] is one of methods for preparing chemically crosslinked *in situ* hydrogels. The photopolymerized hydrogel can be obtained *in situ* gelation in the presence of photoinitiator upon light irradiation. Smeds and Grinstaff reported *in situ* methacrylated alginate and methacrylated HA hydrogels trough photopolymerization [9]. The methacrylated HA was placed on the corneal perforation of 38 New Zealand rabbit eyes, irradiated with argon ion laser (514 nm), and resulted in sealed corneal perforation. Another photopolymerized hydrogel is obtained from

methacrylated HA/PEG [10]. In tissue engineering, the photopolymerized hydrogel has been investigated for tissue barriers, local drug delivery, cell encapsulation, and scaffold materials [11].

Physically crosslinked hydrogels are formed spontaneously by responding the external stimuli, such as temperature or pH changes. Physical crosslinking, such as ionic bond, hydrogen bond and hydrophobic bond, needs no toxic agent. Therefore, the physically crosslinked hydrogel seems more preferable than chemically crosslinked hydrogel.

Ionic crosslinking is one of the physical crosslinking methods. Kuo et al. has been investigated the ionically crosslinked alginate hydrogel which shows the ability to entrap the osteoblasts homogeneously upon the gelation and has potential to be injected into the body [13]. There is only limited literature described cell incorporation simultaneously during the polyion complex hydrogel formation. Therefore, in this study, I fabricated a hydrogel through multiple ionic interactions between cationic polymer chitosan and anionic synthetic polypeptide, Suc-poly(Pro-Hyp-Gly). This PIC hydrogel enable to incorporate cells simultaneously, can be injected into the specific site of the body, and shows remarkable stability in physiological condition due to multiple bonds formation. To make hydrogel, the soluble precursor,

Suc-poly(Pro-Hyp-Gly) is needed. In addition, the effects of counterions such as sodium, ammonium, acetate and chloride on the hydrogel formation as well as the gelation ratio and swelling ratios, and pore size of the freeze-dried hydrogel were also investigated. Finally, the viability of encapsulated rat bone marrow stromal cells (rBMSCs) into the PIC hydrogel was also investigated.

2-2. Materials and Methods

2-2-1. Materials

The NH_4Suc-poly(Pro-Hyp-Gly) and NaSuc-poly(Pro-Hyp-Gly) were obtained from esterification of poly(Pro-Hyp-Gly) using succinic anhydride as described in Chapter 1. Chitosan (1000 sugar units; 80% deacetylation ratio) were purchased from Wako Pure Chemical Industries Ltd. A minimum essential medium alpha medium (α-MEM) was purchased from GIBCO Invitrogen Corporation (Grand Island, NY, USA). Fetal calf serum (FCS) was purchased from HyClone (Logan, UT, USA). WST-8 kits was purchased from Dojindo Laboratories (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemicals (Osaka, Japan).

2-2-2. Polyion Complex Hydrogel Formation

Stock solutions (3.8 mg/mL) of chitosan with acetate (ChAcO) and chloride (ChCl) as counterions were prepared by dissolving the chitosan in aqueous solution containing equimolar acetic acid or HCl to the amino group of chitosan, respectively.

The hydrogel was prepared by adding the NaSuc-poly(Pro-Hyp-Gly) or NH_4Suc-poly(Pro-Hyp-Gly) (3.8 mg/mL) to each of the chitosan solutions in the test tube without any mixing or special mold, and then incubated at 37°C for 1 h. After incubation, phase separation occurred and a part of the polymer solution form a gel. The supernatant was removed by MF-filter (Merck Millipore Ltd., Billerica, MA, USA) made from mixed cellulose esters with 0.22 μm of pore size and then the weight of gel can be obtained by subtracting the weight of the wet filter including the wet gel with the weight of the wet filter. The gelation and swelling ratios were obtained by the following equations.

Gelation ratio (%) = 100 × (weight of the obtained hydrogel)/(total weight of two precursor solutions)

Swelling ratio = (weight of the obtained hydrogel)/(weight of the dehydrated gel)

The FTIR of the lyophilized hydrogel was measured using a Spectrum One FTIR spectrometer (PerkinElmer, Wellesley, MA, USA) based on the KBr method with 16 scans and resolution of 2 cm^{-1}. The morphology of the lyophilized hydrogel was observed using a scanning electron microscopy (SEM) S-4800 (Hitachi, Tokyo, Japan) at an acceleration of 15 kV and magnification of 100 × after coating the samples with a gold layer.

2-2-3. Cell Viability

Rat bone marrow stromal cells (rBMSCs) obtained from the femora and tibia of a 6-week-old female Wistar rat as described previously [14] were grown in a α-MEM containing 20% FCS on 80 cm^2 tissue culture flask 153732 (Nalge Nunc International, Roskilde, Denmark) at 37 °C under 5% CO_2. rBMSCs were washed once with PBS and then treated with an aliquot of 0.02% EDTA and 0.25% trypsin. The NH_4 Suc-poly(Pro-Hyp-Gly) was sterilized using Millex-HP filters (Merck Millipore Ltd.) with 0.45 μm of pore size. Fifty thousand cells suspended in 311 μL of NH_4Suc-poly(Pro-Hyp-Gly) solution (3.8 mg/mL) were added to 89 μL of a ChAcO solution (3.8 mg/mL) with 40 μL of 10 × concentrated PBS in each well of 24-well plate. Ten times concentrated PBS was added to ensure that pH and osmotic pressure of PIC gel were close to physiological conditions, which could reduce damage to the encapsulated cells. The molar ratio of the amino group of chitosan to the carboxy group of NH_4Suc-poly(Pro-Hyp-Gly) was 1:1. The mixture was kept at 37° C for 30 min to obtain hydrogel, washed once with 0.5 mL of the medium (α-MEM containing 20% FCS) and then incubated in 1 mL of the medium at 37° C for 7 days under 5% CO_2 in humidified air. The medium was changed at 1 and 4 days.

The viability of the cells in the hydrogel was determined using the WST-8 kits (Dojindo Laboratories, Tokyo, Japan) according to the manufacturer's instructions. One hundred microliter of supernatant of the each well was transferred to the wells of a 96-well plate and the absorbance was measured at 450 nm using a SpectraFluor plus plate reader (Tecan, Männedorf, Switzerland). As controls, 3-D cultures were performed with rBMSCs incorporated into 0.5 mg/mL rat tail tendon type I collagen gel (Trevigen, Gaithersburg, MD, USA).

2-2-4. Statistical Analysis

All collected data were evaluated using the one-way analysis of variance (ANOVA) routine of KaleidaGraph (Version 4.1; Synergy Software, Reading, PA, USA). Tukey's honest significant difference test was used to assess any differences between groups. *P* value less than 0.05 was accepted as statistically significant. All data are expressed as the mean ± standard deviation (SD), with $n = 3$.

2-3. Results and Discussion

2-3-1. Gelation and Swelling Ratios

All combination of the positive precursors (ChAcO (A) and ChCl (B)) and the negative precursors ([NaSuc-poly(Pro-Hyp-Gly)] (C) and [NH_4 Suc-poly(Pro-Hyp-Gly)] (D)) had completed hydrogel formation by 1 h after mixing. The appearances of the PIC hydrogel obtained from all combination are shown in Fig. 2-1. Gel formation and phase separation into gel and supernatant were occurred simultaneously and completed by 1 h after mixing. To remove the supernatant, the obtained gel was isolated on filter. However, it was so soft and fragile that the rheological properties of the hydrogel could not be measured. The mechanism of PIC hydrogel formation was assumed that it follows the percolation model. A physical crosslinking point from interaction of amino group of chitosan and carboxy group of Suc-poly(Pro-Hyp-Gly) is formed as an initial step of gelation. It is followed by assembly of the others crosslinking points that results in more crosslinked polymer chains. Water molecules are entrapped inside the space of crosslinked networks. The continuous assembly results in macroscopic networks that contains a large amount of

water molecules. Therefore, the hydrogel is formed. The stabilization of hydrogel is mainly maintained by ionic bonding that has three times stronger than hydrogen bonding [15]. In addition, all salt formations also occurs at the same time and rate in the gelation system. The illustration of mechanism of hydrogel formation is shown in Fig 2-2.

For all combination of precursors, both the gelation and the swelling ratios were a maximum at a 1:1 molar ratio of the amino group of chitosan and the carboxy group of Suc-poly(Pro-Hyp-Gly) up to approximately 30% of the gelation ratio and the swelling ratio was approximately 130 with a combination of A + D (Figs. 2-3 and 2-4). The increasing number of interaction point between amino group of chitosan and carboxy group of Suc-poly(Pro-Hyp-Gly) tend to increase the gelation ratio. These results suggest that the gelation reached an optimum when the amino group of chitosan reacted with the equimolar carboxy group of Suc-poly(Pro-Hyp-Gly). With higher molecular weight of precursors, the tendency of hydration was higher than low molecular weight. Therefore, it also resulted in higher swelling ratio. Furthermore, the gelation ratio of PIC hydrogel formed by mixing A + D at a molar ratio of 1:1 increased with an increase in precursors concentration (Fig. 2-5). These results suggest that the gelation is mainly caused by polyion complex formation.

This gelation experiments were conducted using Suc-poly(Pro-Hyp-Gly) with 49.4 ± 1.7% (mean ± standard deviation) of succinylation degree to the hydroxy groups at the Hyp residue in poly(Pro-Hyp-Gly). The increasing of the degree succinylation might lead the increasing number of interaction between amino group of chitosan and carboxy group of Suc-poly(Pro-Hyp-Gly), therefore it might increase the gelation and swelling ratios and might influenced the stiffness of the obtained hydrogel.

2-3-2. Effects of Counterions

The amino group of chitosan has a pKa value close to neutral (pKa = 6–6.2) [16] and the pKa of the precursors C and D measured by potentiometric titration were 4.8–4.9. At the molar ratio of the amino group of chitosan and carboxy group of Suc-poly(Pro-Hyp-Gly) of 1:1, the resulting pH was 6.5 after mixing both precursors (A + D). At neutral pH, chitosan, with pKa 6.0 – 6.2, and Suc-poly(Pro-Hyp-Gly) with pKa 4.8 – 4.9, are both in the deprotonated form.

Figs. 2-3 and 2-4 show that ChAcO (A) provided a higher gelation and swelling ratios than ChCl (B) without relation to the negative precursors ([NaSuc-poly(Pro-Hyp-Gly)] (C) and [NH_4Suc-poly(Pro-Hyp-Gly)] (D)). These results

suggest that the gel formation was affected by the types of the counterions of chitosan amino group. The pKa of HCl and acetic acid are approximately –4 and 4.8, respectively; therefore, the interaction of Cl^- ion is stronger than AcO^-, resulting in a weaker polyionic interaction between the two precursors. Therefore, the lower gelation and swelling ratios were exhibited.

Although these phenomena could be explained using the pKa values of chitosan counterions, the explanation was limited when it described by by ion size, hard-soft acid base (HSAB) theory or solvation.

The possible interactions that affect the PIC formation are shown in Fig. 2-6. Based on the ion size, Pauling estimated the ionic radius of Na^+, Cl^-, and O^- to be 0.95 Å, 1.81 Å, and 1.40 Å, respectively [17]. He also estimated the van der Waals radius of the H atom to be 1.2 Å [18]. The covalent bond lengths of C–C (1.5 Å), C–H (1.1 Å), and C–O (1.3 Å) in AcO^- were obtained using the ChemBioDraw Ultra 12.0 Software (PerkinElmer). The ionic radius of AcO^- can be estimated as 2.55 Å from these values and its molecular structure. In the case of Cl^- or AcO^- as a counterion of chitosan, AcO^- has a delocalized negative charge between two oxygen atoms; therefore the coulomb interaction with amino group of chitosan is weaker than Cl^-. It may result in stronger polyionic interaction between the amino group of chitosan and the carboxy

group of Suc-poly(Pro-Hyp-Gly). Therefore, the combination of A + D has higher gelation and swelling ratio than B + D.

The shortest distance between N^+ and Cl^- in the crystal structure of ammonium chloride is 3.34 Å [19]. Therefore, the ionic radius of NH_4^+ was estimated as 1.53 Å by subtraction of ion radius of Cl^-. In the case of NH_4^+ or Na^+ as a counterion of Suc-poly(Pro-Hyp-Gly), the difference of ionic radius between NH_4^+ and Na^+ was not so large, therefore it might not affect the interaction between two precursors. It may result in no significant difference of gelation and swelling ratio between the combination of A + D and A + C.

Quantitatively, interacting force between amino group of chitosan and carboxy group of Suc-poly(Pro-Hyp-Gly) with their counterions were estimated based on Coulomb's law, which is equal to the valence product and the invers of sum of both ions radius. In the case of Cl^- or AcO^- as a counterion of chitosan, the interaction between amino group of chitosan with Cl^- is the strongest (0.299) compare with those between amino group of chitosan and AcO^- (0.245) and between carboxy group of Suc-poly(Pro-Hyp-Gly) and amino group of chitosan (0.245). The strongest interaction of Cl^- with amino group of chitosan may interfere to the interaction of amino group of chitosan and carboxy group of Suc-poly(Pro-Hyp-Gly). In contrast, the AcO^- gave low

interfere to the interaction of amino group of chitosan and carboxy group of Suc-poly(Pro-Hyp-Gly). Therefore, it resulted in higher gelation ratio in the combination of A + D than B + D. However, in the case of Na^+ or NH_4^+ as a counterion of Suc-poly(Pro-Hyp-Gly), the interaction between carboxy group of Suc-poly(Pro-Hyp-Gly) with Na^+ is the strongest (0.286) compare with those between carboxy group of Suc-poly(Pro-Hyp-Gly) and NH_4^+ (0.245) and between carboxy group of Suc-poly(Pro-Hyp-Gly) and amino group of chitosan (0.245). The strongest interaction of Na^+ with carboxy group of Suc-poly(Pro-Hyp-Gly) may interfere to the interaction of amino group of chitosan and carboxy group of Suc-poly(Pro-Hyp-Gly). However, the results showed no significant difference on gelation ratio with Na^+ and NH_4^+ as a counterion of Suc-poly(Pro-Hyp-Gly). Based on that explanation, the quantitative approach cannot explain the effect of counterions in PIC gel formation.

In HSAB approach, hard acids prefer to react with hard base and soft acids prefer to react with soft bases. Through this approach, both Cl^- and AcO^- are classified into hard base group. Therefore, it cannot explain the difference of gelation ratio.

Based on solvation approach, in the case of Cl^- or AcO^- as a counterion of chitosan, Cl^- can interact with one water molecule and AcO^- can interact with two water molecules. Therefore, the PIC gel from the combination of A + D resulted higher

swelling ratio than B + D. In the case of Na^+ or NH_4^+ as a counterion of Suc-poly(Pro-Hyp-Gly), the Na^+ can form a complex with one water molecule and NH_4^+ can interact with four water molecules. Therefore, the PIC gel from the combination of A+D should have higher swelling ration than A+C. This approach cannot describe the result which showed no significant difference on swelling ratio with Na^+ or NH_4^+ as counterion of Suc- poly(Pro-Hyp-Gly).

The existing approaches were not explained well the phenomena because of the polymer chains, random position of counterions in water and the simultaneously salt formation. Using ion-size approach and solvation theory, the effects of chitosan counterions are explainable. However, the effect of Suc-poly(Pro-Hyp-Gly) counterions need further consideration.

2-3-3. FTIR Analysis of Polyion Complex Hydrogel

Fig. 2-7 shows the FTIR spectra of freeze-dried PIC gel A + D and B + D at a molar ratio of 1:1 of the amino group of chitosan and the carboxy group of Suc-poly(Pro-Hyp-Gly), as well as chitosan and D. The peaks at 1735 cm^{-1}, 1639 cm^{-1} and 1551 cm^{-1} observed in the spectrum of D were assigned as an ester, amide I and

amide II, respectively. The existence of the ester peak in the spectrum of D revealed that the hydroxy group of the hydroxyproline residue in poly(Pro-Hyp-Gly) might be replaced successfully by a succinyl group through ester formation. The relative peak heights of the ester peak (1735 cm^{-1}) to 1085 cm^{-1} (C–O stretching of chitosan) are shown in Fig. 2-8. The relative peak heights of the obtained hydrogels gave higher values than did the precursor D. These results suggest that both precursors were incorporated into the gel and led to the formation of a PIC gel.

2-3-4. Morphology

The morphology of the lyophilized gel only reflects the characteristics of the obtained hydrogels but not their cell-encapsulating nature. The morphology of the lyophilized gels A + D, B + D, A + C and B + C were observed by SEM (Fig. 2-9). All lyophilized gels showed a highly porous structure with a pore size of 10–300 μm. The combination of A + D and A + C gave a smaller pore size than did B + D and B + C. The existence of AcO^- as a counterion of the amino group of chitosan resulted in a stronger interaction between the two precursors than Cl^-, as described in 2-3-2. Therefore this strong interaction might interfere with the mobility of water molecules

trough the hydrogel network and then decrease water crystal growth rate in the freezing process, and then resulted in small pore size. The high interference of Cl^- to amino group of chitosan tend to make weaker interaction between two precursors, resulted in looser structure. The looser structure may induce the chain flexibility and result in bigger macropores. Although the bigger macropore is more preferable for cell movement, the difference on the macropore size cannot alter the penetration of oxygen and nutrient. From the SEM images, the existing of counterions might influence the macropore formation of the lyophilized hydrogel.

2-3-5. Cell Viability

The rBMSCs suspended in NH_4Suc-poly(Pro-Hyp-Gly) were mixed with a ChAcO solution, resulting in a PIC hydrogel incorporated with rBMSCs. After incubation at 37 °C under 5% CO_2 in humidified air for 7 days, the viability of the cells in the PIC hydrogel was assessed. The numbers of live cells at 1, 4, and 7 days were determined using WST-8 kits. Comparing with the two-dimensional (2D) culture control, at day 1, the cell number of encapsulated cells was decreased drastically. However, it proliferated along with the incubation day. After addition of chitosan into

NH_4Suc-poly(Pro-Hyp-Gly) suspended with rBMSCs, the decreasing of pH value (approximately 6.5) was occurred. The low pH value of the mixed solution might cause decreasing of the cell number at initial. This phenomena also seen in the 3-D culture control (rat tail tendon type I collagen gel). However, the encapsulated rBMSCs survived and proliferated. Comparing with the 3D culture control, the proliferation rate of the encapsulated rBMSCs in the PIC hydrogel was lower than the 3D control. An alternative route, for example using a cationic precursor that can be dissolved in solution with neutral pH, such as arginine-modified poly(Pro-Hyp-Gly), instead of chitosan might increase the cell number at initial. The proliferation rate might also be improved by using cell attachment peptide-conjugated poly(Pro-Hyp-Gly) in addition to Suc-poly(Pro-Hyp-Gly).

2-4. Conclusion

All the combination of precursors of chitosan and Suc-poly(Pro-Hyp-Gly) with their counterions tested formed PIC hydrogel, where the concentration of each precursor was as low as 3.0 – 3.8 mg/mL. Both gelation and swelling ratios had a maximum value at an equal molar ratio (1:1) of the anionic and cationic group.

Furthermore, chitosan acetate gave a PIC hydrogel with both a significantly greater gelation and swelling ratios than that of chitosan chloride. Changing counterions altered pore size of the freeze-dried PIC hydrogel. Chitosan acetate gave a PIC hydrogel with a larger pore size than did chitosan chloride. The morphology of all lyophilized gels showed a highly interconnected porous structure with a pore size of 10–300 μm. Cell encapsulation was also performed successfully by mixing rBMSCs with PIC hydrogel simultaneously during its formation. The PIC hydrogel was maintained in culture medium for 7 days at 37 °C, and the encapsulated cells survived and proliferated. Although the cell numbers encapsulated into the PIC hydrogel decreased drastically at day 1 and the rate of proliferation was slow, this PIC hydrogel has the potential for forming 3-D scaffolds capable of encapsulating cells upon further improvement.

2-5. References

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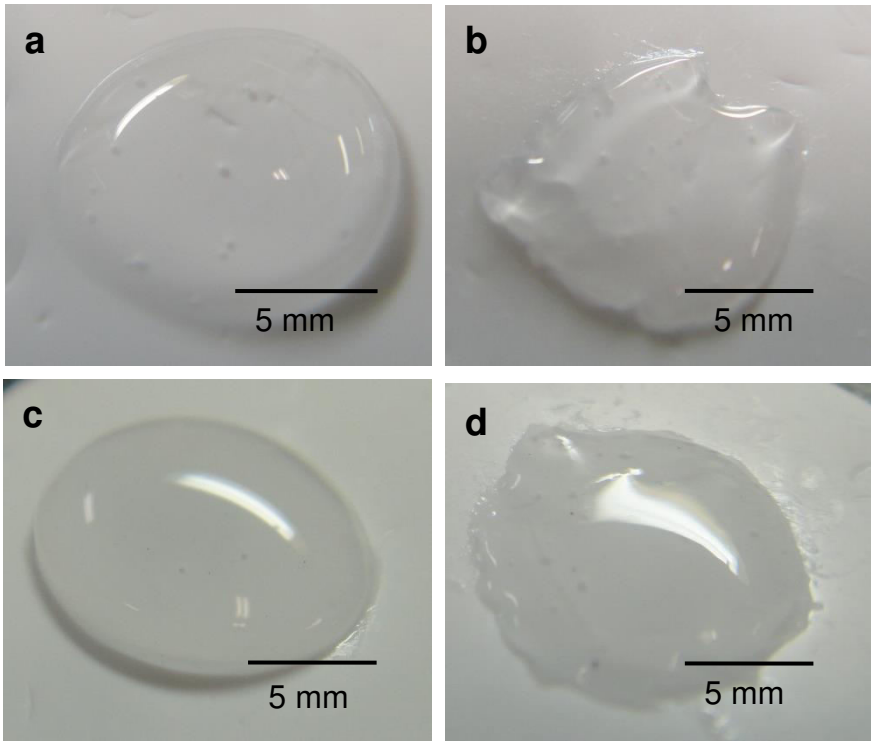

Fig.2-1 Appearance of polyion complex hydrogels formed by mixing A+D (a), B+D (b), A+C (c) and B+C (d) a molar ratio of the amino group of chitosan and to the carboxy group of Suc-poly(Pro-Hyp-Gly) of 1:1.

A, B, C, and D are ChAcO, ChCl, NaSuc-poly(Pro-Hyp-Gly), and NH_4 Suc-poly(Pro-Hyp-Gly), respectively.

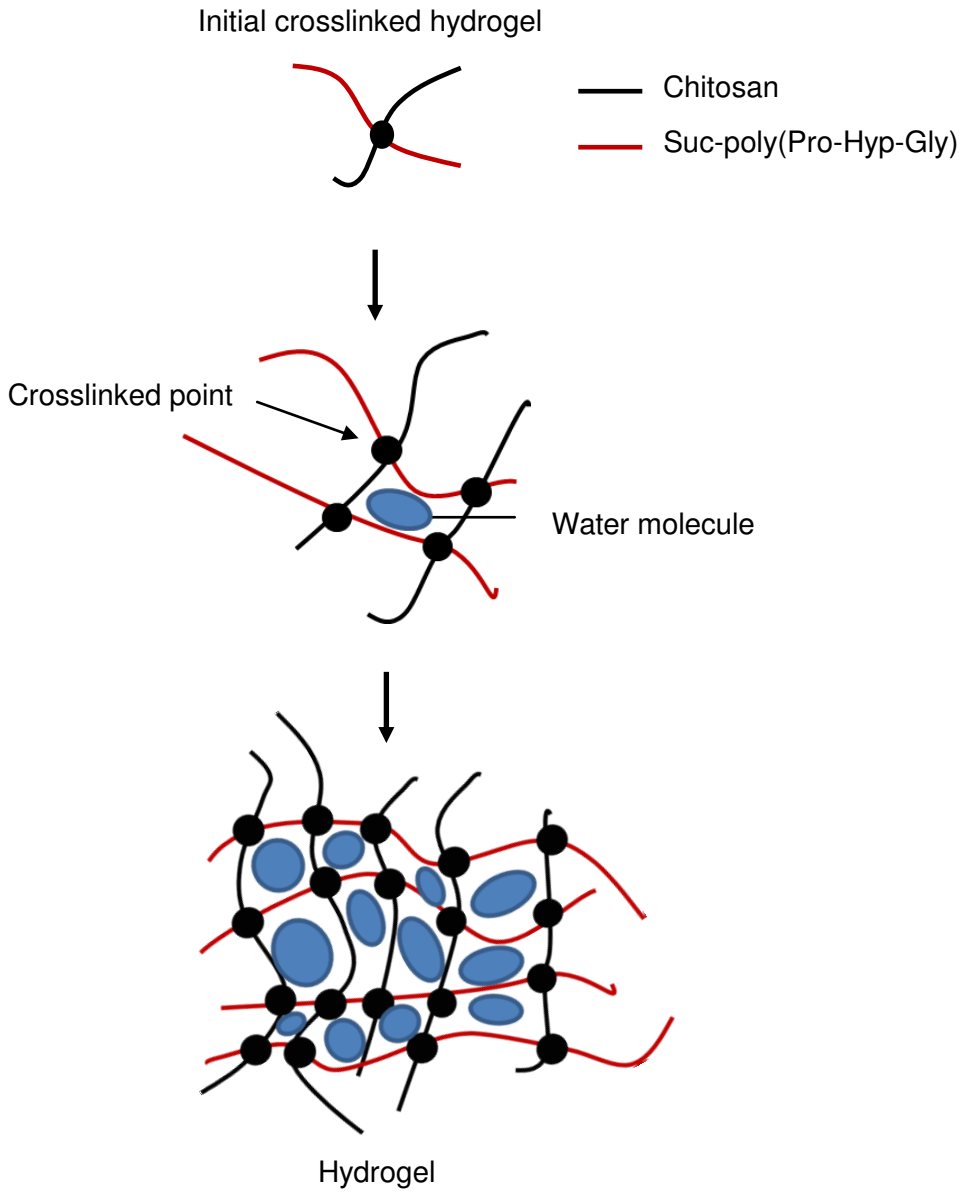

Fig. 2-2 Mechanism approach of polyion complex hydrogel formation

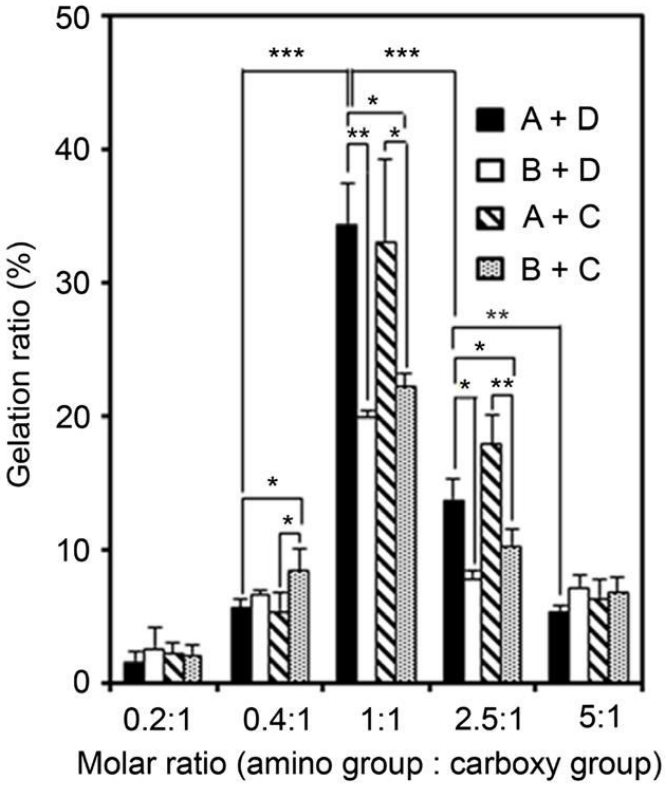

Fig.2-3 Gelation ratio of a hydrogel with various molar ratios of the amino group of chitosan to the carboxy group of Suc-poly(Pro-Hyp-Gly) and counterions.

A, B, C, and D are ChAcO, ChCl, NaSuc-poly(Pro-Hyp-Gly), and NH_4 Suc-poly(Pro-Hyp-Gly), respectively. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

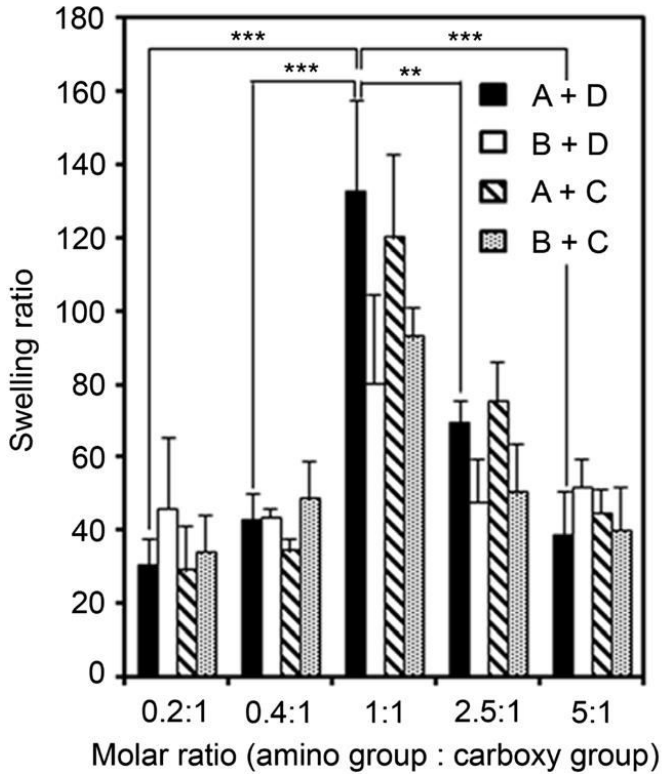

Fig.2-4 Swelling ratio of a hydrogel with various molar ratios of the amino group of chitosan to the carboxy group of Suc-poly(Pro-Hyp-Gly) and counterions.

A, B, C, and D are ChAcO, ChCl, NaSuc-poly(Pro-Hyp-Gly), and NH_4 Suc-poly(Pro-Hyp-Gly), respectively. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

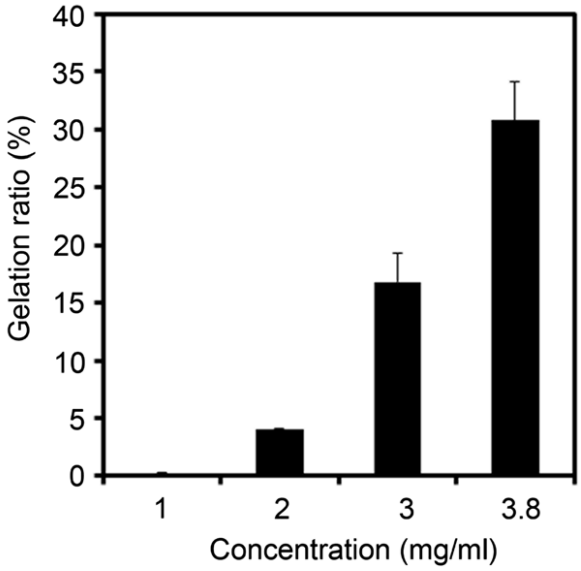

Fig.2-5 The precursors' concentration dependence on the gelation ratio of the PIC hydrogel of A + D at a molar ratio of 1 : 1.

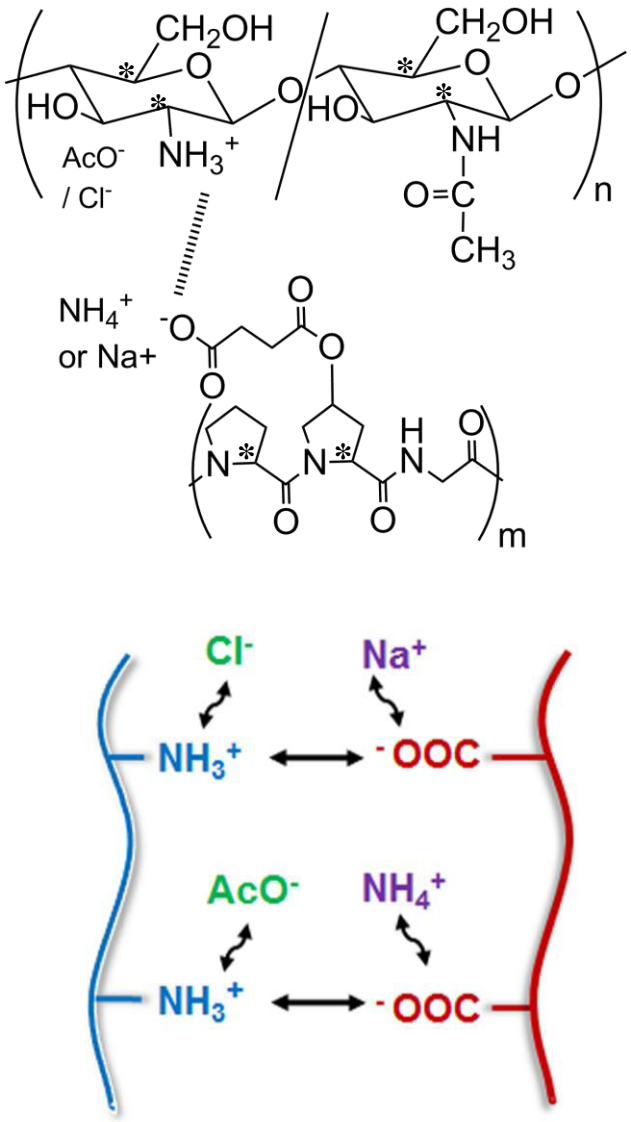

Fig.2-6 Possible interactions that can affect the polyion complec formation. "*" indicates asymmetric carbon.

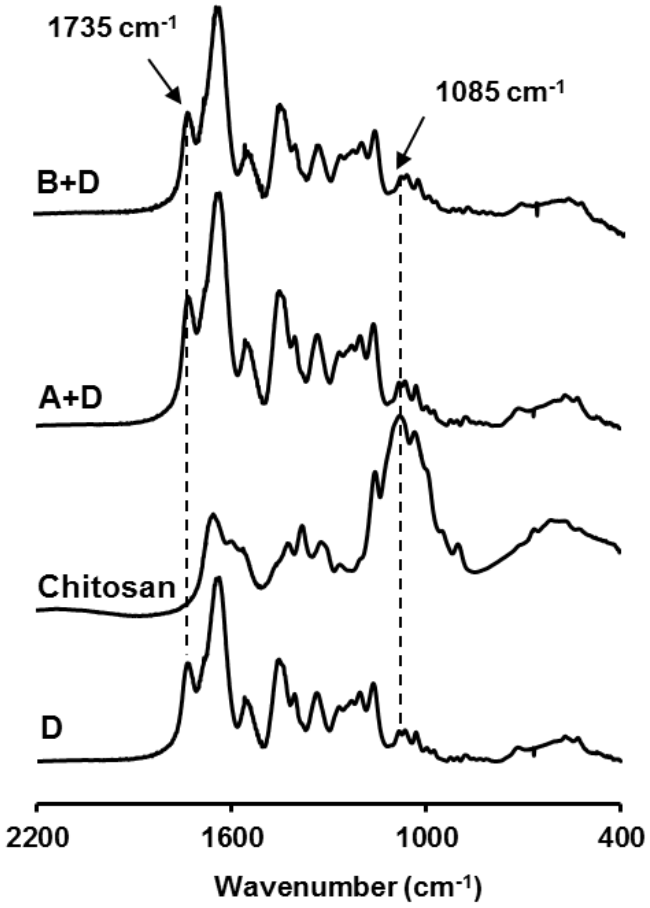

Fig.2-7. FTIR spectra of freeze-dried polyion complex gel formed by mixing A + D and B + D at a molar ratio of the amino group of chitosan and to the carboxy group of Suc-poly(Pro-Hyp-Gly) of 1:1 as well as similar spectra of an equimolar mixture of chitosan and D. Spectra were obtained by the KBr method with resolution 2 cm^{-1} and 16 times of scans at room temperature.

A, B, and D are ChAcO, ChCl, and NH_4Suc-poly(Pro-Hyp-Gly), respectively.

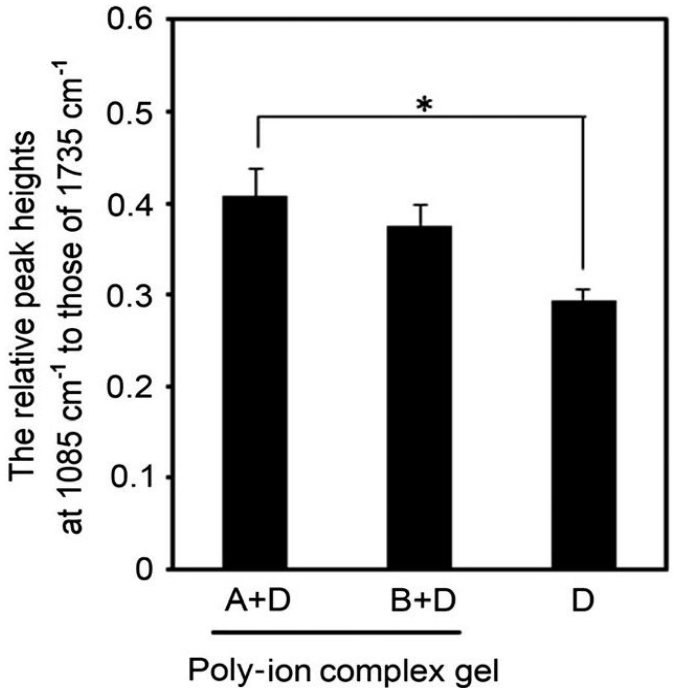

Fig. 2-8 Relative heights of the peak at 1085 cm^{-1} to the ester peak (1735 cm^{-1}) freeze-dried PIC gels formed by mixing A + D and B + D at a molar ratio of the amino group of chitosan and the carboxy group of Suc-poly(Pro-Hyp-Gly) of 1:1.

** $P < 0.05$.*

A, B, and D are ChAcO, ChCl, and NH_4Suc-poly(Pro-Hyp-Gly), respectively.

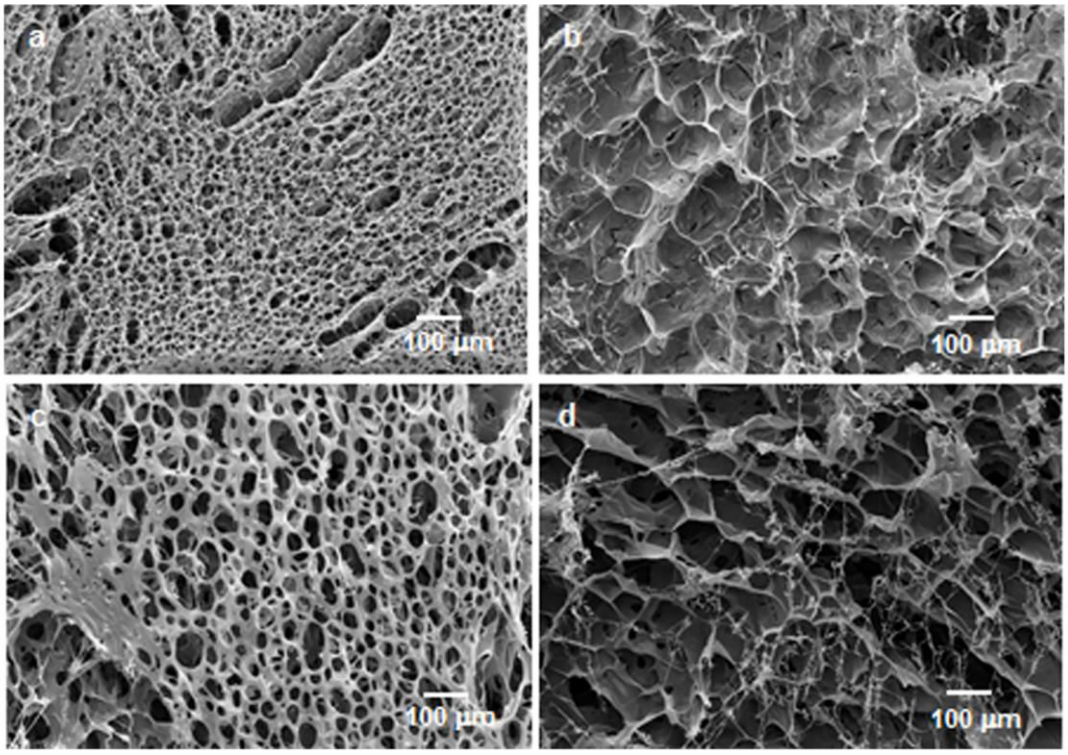

Fig.2-9 SEM images of lyophilized gels A + D (a), B + D (b), A + C (c) and B + C (d). All the images are recorded at × 100 magnification.

A, B, C, and D are ChAcO, ChCl, NaSuc-poly(Pro-Hyp-Gly), and NH_4Suc-poly(Pro-Hyp-Gly), respectively.

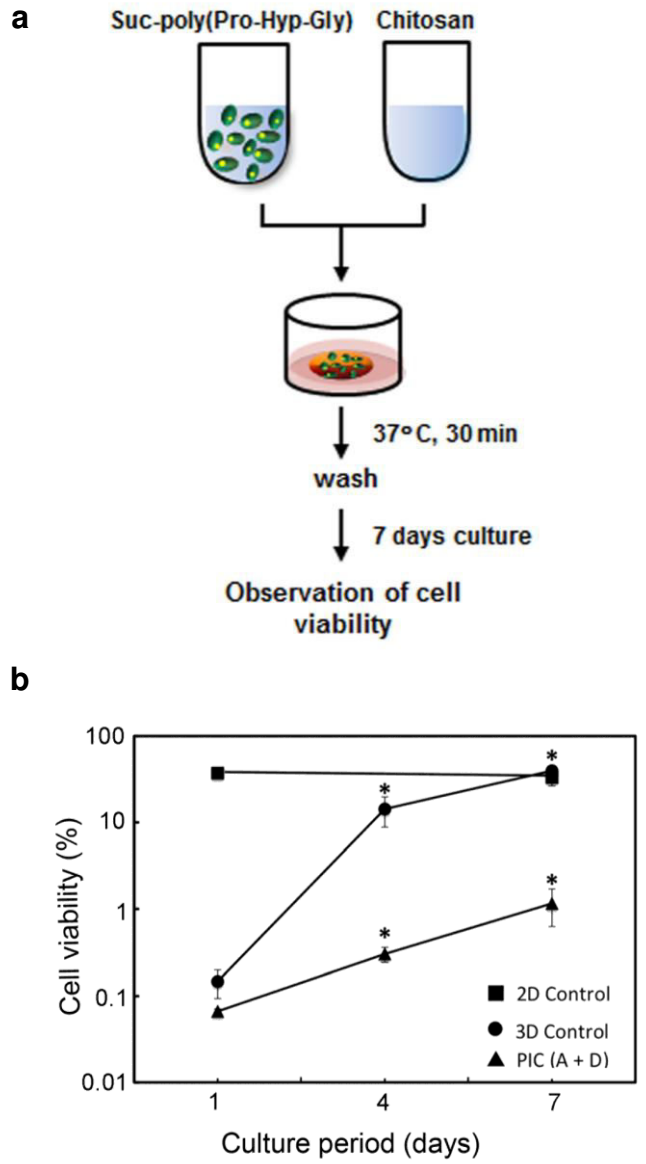

Fig. 2-10 Schematic method of the rBMSCs (5×10^4 cells/well) encapsulated into PIC hydrogel; (b) cell viability of encapsulated rBMSC into the PIC hydrogel, ChAcO+NH_4Suc-poly(Pro-Hyp-Gly) at 1, 4, and 7 days. The 2D control was culture of the rBMSCs on a well of a 24-well microplate. The 3D control was a culture of rBMSCs incorporated into rat tail tendon type I collagen gel. *$p < 0.01$*

Table 1. Water content and yield of the polyion complex hydrogel according to the nature and molar ratio of the constituents (n = 3)

Precursors	Molar ratio (amino group : carboxy group)	Water content (mg)	Dried gel weight (mg)
A + D	0.2:1	6.82 ± 2.85	0.23 ± 0.05
	0.4:1	22.59 ± 2.04	0.55 ± 0.13
	1:1	137.06 ± 12.17	1.06 ± 0.11
	2.5:1	54.92 ± 6.07	0.81 ± 0.09
	5:1	21.41 ± 1.20	0.62 ± 0.24
B + D	0.2:1	10.76 ± 6.08	0.23 ± 0.08
	0.4:1	26.55 ± 0.88	0.62 ± 0.05
	1:1	79.51 ± 10.22	1.06 ± 0.27
	2.5:1	31.23 ± 2.01	0.70 ± 0.15
	5: 1	28.65 ± 3.51	0.57 ± 0.03
A + C	0.2:1	8.94 ± 3.15	0.33 ± 0.06
	0.4:1	21.53 ± 5.08	0.66 ± 0.21
	1:1	131.96 ± 23.92	1.11 ± 0.06
	2.5:1	71.27 ± 8.12	0.92 ± 0.08
	5:1	25.51 ± 5.45	0.59 ± 0.10
B + C	0.2:1	8.85 ± 2.85	0.27 ± 0.01
	0.4:1	33.63 ± 6.32	0.71 ± 0.02
	1:1	88.42 ± 3.36	1.01 ± 0.09
	2.5:1	40.72 ± 5.06	0.85 ± 0.16
	5:1	27.02 ± 4.23	0.59 ± 0.12

A, B, C, and D are ChAcO, ChCl, NaSuc-poly(Pro-Hyp-Gly), and NH_4Suc-poly(Pro-Hyp-Gly), respectively.

GENERAL CONCLUSION

The aim of this thesis is to describe the development of a functional 3D scaffold from synthetic collagen-like polypeptide, Suc-poly(Pro-Hyp-Gly), and chitosan by polyion complex formation that can support the proliferation and differentiation of stem cells. To obtain an uniform cell encapsulation, the cells were encapsulated simultaneously during the hydrogel formation. The effects of counterions such as sodium, ammonium, acetate and chloride ions on the PIC gel formation and cell viability of encapsulated rat bone marrow stromal cells in the hydrogel were also discussed. Chapter 1 shows the synthesis and characterization of Suc-poly(Pro-Hyp-Gly). The NaSuc-poly(Pro-Hyp-Gly) and NH_4 Suc-poly(Pro-Hyp-Gly) showed peak MW at over 120 kD similar to that of poly(Pro-Hyp-Gly). Both polypeptides showed a positive Cotton effect at 225 nm and a negative Cotton effect at near 197 nm, showing the formation of a triple-helical structure like poly(Pro-Hyp-Gly). It also suggests that succinylation and counterions had almost no effect on the molecular weight or triple-helical structure of the obtained polypeptide. The mean residual molar ellipticity at 225 nm of Suc-poly(Pro-Hyp-Gly) was constant below 40°C, however, it slightly decreased when increasing temperature

over 40°C to 60°C;, suggesting that triple-helical structures of these polypeptides were thermally stable up to 60°C and the stability of the triple-helical structure was not affected by the counterions. For stability in various pH, the mean residual molar ellipticity at 225 nm of Suc-poly(Pro-Hyp-Gly) showed no significant change even at pH 12.

Chapter 2 shows the characterization of polyion complex hydrogel and assessment of the viability of encapsulated rBMSCs in the hydrogel. PIC hydrogel was obtained for any precursor combination where the concentration of each precursor was as low as 3.0-3.8 mg/mL. It showed a maximum gelation and swelling ratios at an equal (1:1) molar ratio of the amino group of chitosan to the carboxy group of Suc-poly(Pro-Hyp-Gly) up to approximately 30% of the gelation ratio and 130 for swelling ratio with a combination of ChAcO + NH_4Suc-poly(Pro-Hyp-Gly) . Chitosan acetate gave a PIC hydrogel with gelation and swelling ratio both significantly greater than that of chitosan chloride. The pore size of freeze-dried PIC hydrogel was influenced by the type of counterions. The assessment of cell viability of encapsulated rBMSCs showed that the encapsulated cells survived and proliferated. Although the cell numbers encapsulated into the PIC hydrogel decreased drastically at day 1 and the proliferation rate was slow, this PIC hydrogel has the potential as 3D scaffold.

LIST OF PUBLICATIONS

Yuni Kusumastuti, Yoshiaki Shibasaki, Shiho Hirohara, Mime Kobayashi, Kayo Terada, Tsuyoshi Ando and Masao Tanihara. Encapsulation of rat bone marrow stromal cells using a poly-ion complex gel of chitosan and succinylated poly(Pro-Hyp-Gly). *J Tissue Eng Regen Med (2015)*. Published online in Wiley Online Library (*wileyonlinelibrary.com*) DOI: 10.1002/term.1987.

Conferences (Oral presentation)

Yuni Kusumastuti, Yoshiaki Shibasaki, Shiho Hirohara, Kayo Terada, Tsuyoshi Ando and Masao Tanihara. Preliminary study on gelation of succinylated Poly(Pro-Hyp-Gly) and chitosan by polyion complex interaction for cartilage repair, *5th European Conference of the International federation for Medical and Biological Engineering (IFMBE)*, September 14-18 2011, IFMBE Proceedings 37, pp. 1070–1073.

Yuni Kusumastuti, Yoshiaki Shibasaki, Shiho Hirohara, Kayo Terada, Tsuyoshi Ando and Masao Tanihara. Polyion complex gel of succinylated Poly(Pro-Hyp-Gly)/Chitosan for injectable and cell-implantable scaffold. JSRM Proceedings, The 11th Congress of Japanese Society for Regenerative Medicine (JSRM), 12-14 June 2012, Yokohama, Japan.

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