Preparation and Characterization of Biomaterials Based on Gelatin

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論　　題

Preparation and Characterization of Biomaterials Based on Gelatin

理工学研究科 ● 総合理工学専攻

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General Introduction

Gelatin has been utilized for wide range of human life such as food, food supplements, drinks, glues and etc. Gelatin is a peptide mixing which approximate higher than 1000 amino acids; produced from collagen by thermal denaturation with acid or base pretreatment. From the Grandview research by Gelatin Manufacturers Association Asia Pacific (GMAP), show the gelatin market volume share by application in year 2013, from production capacity around 350 kilotons, 21% is use in the pharmaceutical applications. It's trend to increase consumption gradually due to growing of population and healthcare awareness. Due to gelatin is made from animal origin [2-3], it's biocompatible when takes in living body and low antigenicity [1]. The characteristic of heat reversibility is come from the compose between 3D gel network of gelatin and microcrystal interconnect with amorphous regions in coiled segment [4-5]. The distinctive property of gelatin is the solution-gelation transition under aqueous condition which can change the gelatin to various forms. It's has been used in many form such as nanofibers, microfiber membranes scaffolds granules, sponges etc. [6]. It has been reported that gelatin composite membrane have so many biomedical applications [7] such as chitin/ gelatin membrane[1], polyvinyl alcohol-gelatin hydrogel membrane [8], chitosan/ hydroxyapatite/ gelatin membrane [9] etc. Electrospinning is one famous method to produce membrane which have nano size of fiber diameter because it's simple and high efficiency [10]. Nano-fiber in form of membrane is the one of gelatin form that used in many applications not only in biomedical applications such as tissue engineering or control release but industrial field also. Gelatin micro-fiber by dry spinning method is very simple and environmental friendly because used only water as a solvent. However only the few amount of research about gelatin micro fiber have been found. Previously, gelatin fibers were prepared by wet spinning method which used a lithium (or calcium) chloride–*N*,*N*-dimethylacetamide (DMAc) as a solvent [11] . In this system, gelatin dissolved in LiCl–DMAc or $CaCl_2$–DMAc at room temperature was solidified by exposure to methanol. However, the mechanical property of the obtained gelatin fibers was not sufficient for practical applications, and long-term immersion in methanol was necessary to remove the salts from the fibers. In addition, ethylene glycol had been used as solvent for produced gelatin fiber by gel-spinning [12]. But after drawing of the fibers, immersed in methanol to extract the ethylene glycol was also necessary. Therefore, the gelatin fibers produced by wet spinning were not suitable

for use in biomedical applications. Gelatin micro-fiber can spun at the high concentrated gelatin aqueous solution on heating excluded into the air through nozzle. Although the tensile strength of the fiber was much stronger than wet spun fiber but the fiber is easy to dissolves in water. So, the cross-linking is necessary to improve the water resistant property. Recently, 3D porous scaffold such as sponge have been used for treat the diseases because it's play important role for cell adhesion, cell re-aggregate [13] and tissue regeneration etc. Various material have been prepared in form of sponge for biomaterial application such as collagen [14], aloe vera [15], hydroxyapatite [16] and gelatin [17] etc. Gelatin sponge can produce by freeze-drying (lyophilization), it have been studied in field of control release by combine with Tri-calcium phosphate (β-TCP) [18], protein delivery by combine with hydroxyapatite [19] and scaffolds for tissue and cell growth by combine with hyaluronic [20] etc. In addition, recently gelatin sponges are increase usage in oral surgery and hemostatic also. Because gelatin alone in each form are not strong enough, in many research always combine, blend with the others materials or crosslink with crosslinking agent in order to improve chemical and physical property. Glutaraldehyde (GTA) is the organic compound which usually use for crosslink with protein because high reactivity and efficiency. Moreover it's also use as crosslinking in the application of sterilize medical, dental equipment, water treatment, preservative etc. GTA is inexpensive and when in the solution of liquid state, it's very high effective for crosslink within short times [21-22]. Cross-linking between GTA and protein such as gelatin is relate to $-NH_2$ of polypeptide react with –CHO of GTA which form N=C structure.

The preparation of chitin/ gelatin membrane with N-acetyl-D-glucosamine (GlcNAc) according to Maillard reaction has been reported. The mechanical property of chitin/ gelatin membrane with GlcNAc was higher than those without GlcNAc. Di-epoxy compounds (Glycol Diglycidyl Ether) have been use for crosslink with collagen and gelatin because low toxicity and good biocompatibity [23]. From all of reason, in this study we focus to prepare gelatin in many form including of fiber, nano sheet and sponge with using several cross-link agents focus on GTA, GlcNAc (reducing sugar) and di-epoxy compound in order to improve the water resistant, mechanical and chemical properties. For the crosslink method, in case of GTA, the cross-linking should be done after each gelatin form was prepared because of the high reactivity while the

others crosslinker can be dissolved directly in gelatin aqueous solution before produce to the others form. The properties may be different due to the different cross-linking method. Expectation results is to expand the usage of gelatin for several fields especially biomedical purpose because gelatin is a safe material and preparation method is a simple and environmental friendly. In addition, gelatin with chitosan composite will be prepared expect to more excellent mechanical property due to the entanglement of polymer chains and antibacterial property. Furthermore, since glycerol act as plasticizer, in the combination with gelatin composite, flexible and elastic property also expected.

In section I, Gelatin micro-fiber have been prepared by dry spinning method. The effect of difference crosslinking agent to mechanism property and water resistance was described.

Chapter 1, gelatin fibers have been prepared by dry spinning using sol-gel property which on heating is solution state and it is in gelation state on cooling. In order to improve fiber water-resistant and mechanical property, the cross-linking is necessary by using reducing sugar, Di-epoxy compounds (denacol) and GTA. The average tensile stress of fiber without crosslink is 120 MPa. Each crosslinker which applied to gelatin fibers results to improved mechanical property indicated from tensile stress of fibers were increased. GlcNAc showed good results in tensile stress and water resistance than the others reducing sugar. Di-epoxy was add to gelatin solution before spin 3, 4 and 5% of gelatin mass, results shown that stress of fiber were increased follow by increased amount of di-epoxy. GTA on the crosslinked gelatin fiber by vapor crosslinked, results shown that stress of fiber was increased follow by increased time of crosslinked. And when apply heat treatment on the fiber stress of fibers was improved. The comparison of each crosslinker by water resistance, GTA and GlcNAc crosslinked showed the good water resistance ability and less swelling up to 90 days.

Chapter 2, time of vapor crosslinking gelatin with GTA was studied to find the optimum point. According to Chapter 1, GTA vapor crosslink is one of crosslinker and results shown that stress of fiber was increased follow by increased time of vapor crosslinked. So the optimum time of stable stress of fiber was studied. It was found that stress of fiber was gradually increased until reach the stable after 7 days. In addition, GTA is recognized as the chemical which may generate health problem by irritating and

corrosive to the skin, eyes and respiratory. In order to avoid unreacted and residual GTA, $NaBH_4$ reducing agent was use for neutralize the fiber. After reducing with $NaBH_4$, the yellowish of fabric changed from bright to pale yellow which refers to reduction of N=C to N-C which may less toxic and more stable to apply in the biomedical application.

In section II, Gelatin nano-fiber has been prepared by electrospinning method. Spinning conditions and effect of difference crosslinking agent was described.

Chapter 3, fabrication of gelatin nano fibers by aqueous method. We focus on the development of non-woven gelatin fabric by electrospinning. Polymeric fibers formed with the simultaneous evaporation of solvent by the action of high voltage to the polymer solution, electrospinning in the form of a non-woven fabric can be achieved. We have carried out electrospinning providing temperature on the basis of dry spinning. Gelatin non-woven fabric has been prepared using GlcNAc and GTA as cross-linker. Non-woven fabrics with 25% gelatin concentration showed fiber diameter in the nanoscale. In terms of mechanical property, the gelatin non-woven fabric with GTA cross-linking showed high mechanical property than the GlcNAc system. The swelling and water uptake ability in water and PBS showed that non-woven fabrics with GTA-cross linking has no little change in morphology. In both the cross-linking methods addition of Glycerol could further overpower the toxicity induced due to cross-linkers. From the cell studies conducted, it is evident that the developed gelatin fibers showed good cytocompatibility and hence would find profound application in various tissue engineering.

Chapter 4, fabrication of gelatin nano-fibers tubular structure. From electrospinning, we successful to prepared gelatin nano-sheet. So we concern on the development of non-woven gelatin fabric to tubular structure, expect that may use as artificial blood vessels in the future. Basis condition of electrospinning with rotating drum collector have been investigated including of spun align fiber. Generally, electrospinning is produce the random direction fiber, with using a rotating drum collector which has rotation speed higher than 1000 rpm, fibers can be oriented circumferentially. In addition, two parallel oppositely charge which set up beside rotating drum can produce more aligned fiber.

In section III, Gelatin sponges have been prepared by freeze-drying. Preparation and characterization of sponge with difference crosslinking agent have been study. Physical, chemical and biological properties were evaluated to use as basic information of gelatin sponge and develop in biomaterials such as bone-tissue engineering. In some part of research, the results of gelatin sponge have been comparing with Chitin/ Poly butylene succinate (PBS) sponge.

Chapter 5, gelatin/ chitosan composite were prepared by using GlcNAc and GTA as cross-linker into the form of a sponge by freeze dried. The results from SEM observation shown that the interconnected porosity of each composite sponge was well demonstrated. Sponge showed porosity lower than 50% for GTA, whereas the GlcNAc sponges showed higher than 60%. Thermogravimetric also measured and indicated that there is no phase change in the composite structures all of sponge. Swelling ratio and degradation rate of composite sponge which prepared with GlcNAc system were higher, due to higher porosity of the composite sponges. The comparison of gelatin composite sponge with chitin/ PBS sponge, chitin/ PBS showed the higher swelling ratio due to high porosity which can observe from SEM image. Greatly in amount of porous structure is advantage property for a tissue engineering material.

Chapter 6, adsorption and desorption was evaluated to use as biomedical application. Protein adsorption is very important in the field of biomedical research. The prepared sponge was used for studies on adsorption and desorption Fluorescein isothiocyanate (FITC) labeling of Bovine Serum Albumin (BSA) as a model instead of growth factor. The effect of FITC-BSA concentration and temperature to adsorption behavior of gelatin/ chitosan sponge were investigated. Langmuir adsorption isotherm model was the assumption that the adsorption behavior occur on a surface by monolayer adsorb, and found that fit with the experiment data. The thermodynamic constants of adsorption phenomena were found, the adsorptions onto sponge were exothermic reactions. Especially Gibbs free energy (ΔG) was negative values in range of 283-343K which demonstrate the spontaneous nature of adsorption reaction. In addition, desorption behavior also evaluated with different concentration and pH of FITC-BSA solution. The high adsorbed amount of FITC-BSA on sponge results to high desorbed up to and 55%. And %desorption decrease follow by decrease pH 7.4, 4 and 2 of buffer solution respectively.

Chapter 7, gelatin/ chitosan composite sponges with GTA and GlcNAc crosslinked were explored the possibility of biomaterial application by *in vivo* and *in vivo* test. Cell seeding after 24 h cultivation were investigated by using mouse osteoblastic MC3T3-E1 cells seed onto the composite sponge, results from SEM showed that the cells could well attached to the based sponge and the elongation was observed in the GlcNAc system than GTA system. *In vivo* test, by applied sponge into rat subcutaneous model for 10 weeks, from observation by light microscopy indicated that extent of ingrowth and biocompatibilities were excellent in GlcNAc system than those in GTA system. In addition, loading of FGF2 against to the gelatin based sponge cross-linked with GlcNAc system stimulated ingrowth of cells. And excellent bone forming ability was also obtained using GlcNAc system sponge loaded with FGF2.

In conclusion, the knowledge and achievements obtained by these studies were summarized. The contribution and signification of these studies for biomaterial application were also described.

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Section I

Gelatin micro-fiber by dry spinning

Chapter 1

Preparation and properties of gelatin micro-fiber with difference cross-linker

I.1.1 Introduction

Gelatin (Gel) has been utilized for wide range of human life such as food, food supplements, drinks, glues and etc. due to sol-gel transformation property of aqueous solution. Gelatin has also been applied for biomedical materials such as capsule for drug delivery system and biomedical membranes. The main reasons is Gel had been accepted by human and animal body due to high biocompatibility, low toxicity and biodegradability in animal body. The low toxicity of Gel in animal body is mainly depended on the non-existent of telopeptides which lead to the immunological response of collagen at moderately high level. Recently, people concerning about healthy live and well-being, so the using of the biomaterial are increasing popularity. Gel is the one of material which able to respond this issue including of medical fiber and medical textile. However only the few amount of research about gelatin micro fiber have been found. The way to prepare gelatin fiber is slightly difficult due to insolubility against general organic solvents and sol-gel transition of gelatin aqueous solution. Previously, gelatin fibers were prepared by wet spinning method which used a lithium (or calcium) chloride–N,N-dimethylacetamide(DMAc) as a solvent [1]. In this system, gelatin dissolved in LiCl–DMAc or CaCl2–DMAc at room temperature was solidified by exposure to methanol. However, the mechanical property of the obtained gelatin fibers was not sufficient for practical applications, and long-term immersion in methanol was necessary to remove the salts from the fibers. Since the high concentrated gelatin aqueous solution on heating is sol state and it is in gel state on cooling, concept of spinning method was investigated. The gelatin fiber is spun when the high concentrated gelatin aqueous solution on heating is excluded into the air through nozzle. The method is very simple and environmental friendly because only water is used. Although the tensile strength of the fiber was much stronger than that of wet spun fiber but easy to dissolves in water. In order to improve the water resistance of fiber, the cross-linking agent is necessary to apply to gelatin fiber system. The cross-linked of sugar (non-

reducing and reducing sugar) with gelatin microspheres and disks has been investigated for pharmaceutical application [2]. They found that cross-linking both non-reducing and reducing sugars can be reduced water dissolution of Gel. Furthermore, sugar cross-linking of gelatin molecules has been shown to increase stiffness [3-4] also. According to Maillard reaction, reducing sugar and amino group can occur crosslink reaction which produces browning compounds which well known in the name of Melanoidine. Due to the interaction between carbonyl group of reducing sugar and amino compound [5], it's create structure which high molecular weight and poor characteristic results to physical changes in gelatin and other protein matrices [6-9]. Product from this reaction have the good properties in antibacterial, antioxidant and antitumor, usually use in the food industry such as beer, bread and miso. Epoxy compounds have been investigate as crosslinker with soft collagen, reported that possess good biocompatibility and enhance biomechanical properties [10-11]. In addition, coated epoxy which crosslinked with gelatin onto inner surface of Polyurethane (PU) for vascular grafts application were evaluated. The cell adhesion, spreading, and proliferation were significantly improved by the smooth epoxy fixed gelatin coating [9]. Glutaraldehyde (GTA) is a bifunctional reagent usually used as chemical modifications of proteins and polymers [12] in various applications because GTA has commercial availability, low cost and high reactivity. It reacts rapidly with amine group around neutral pH [13-14]. Due to all of previous research and reason, the objective of this study was the production and characterization the gelatin fiber by dry spinning crosslink with various crosslinking agents including of sugar, di-epoxy compounds and GTA. The crosslinked gelatin fibers were characterized through tensile test and water resistance to compare each effect of crosslinked. Finally, such gelatin fibers and fiber assembly is expected to be better use for several fields especially in biomaterial application.

I.1.2 Experimental

I.1.2.1 Materials

Gelatin, JS200 (Mw=100,000; 200 bloom; type B) cow skin type in powder was from Koei transformation Ltd. Di-epoxy compounds, Ethylene glycol diglycidyl ether Denacal EX-810 was from Nagase ChemteX Corporation. The others crosslinker

including of Sucrose (Suc), Glucose (Glu), Glucosamine (Gluc), N-Acetylglucosamine (GlcNAc) and GTA solution (25%) were from Wako Pure Chemical Industries, Ltd.

I.1.2.2 Preparation of gelatin solutions and spin gelatin fiber

The solution of gelatin was prepared by dissolving gelatin powder in water 50% by weight. The mixture was covered and put in the electric water bath at temperature 50±2°C for 30 min; stirred every 10 min to obtain homogeneous solution. The homogeneous solution of gelatin was filled up in cylinder (50±2°C) which connected to a nozzle (0.83 mm. inner diameter). Control pressure in the range from 0.10 ± 0.04 MPa. was applied on top to the droplet of injected solution. Collection was rotate with speed 50±10 m/min to an aluminum foil wrapped on a collector. The separating distance between the needle tip and the aluminum foil was set to 1.4 m. (Figure 1). The obtained fibers were kept on collector at room temperature for 24 h. to remove residual moisture.

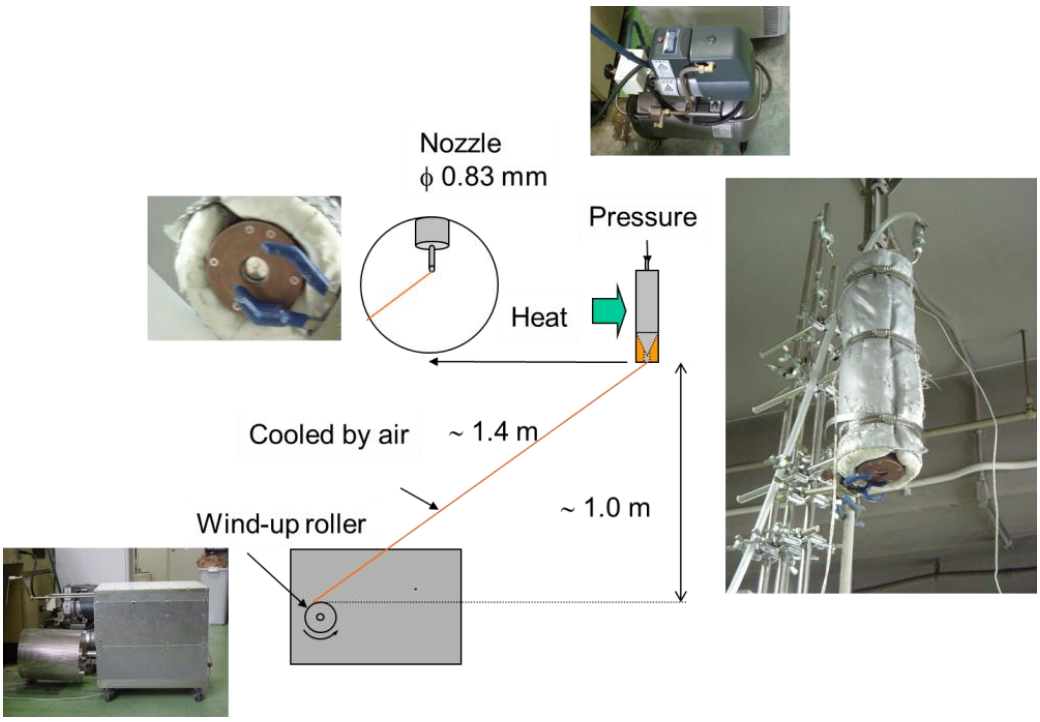

Figure 1. Dry spinning apparatus.

I.2.2.3 Cross-linking method

The treatments of gelatin with each crosslinker were performed as follows.

Sugar cross-linker

Suc, Glu, Gluc and GlcNAc were investigated to compare the effect of each sugar. In the step of prepare gelatin solution, 5% sugar (by gelatin mass) was add to the solution. After spun the fibers, applied heat treatment 120°C up to 24 h. The aldehyde group of reducing sugars can react with the free amino groups of gelatin occur crosslink reaction molecule (Figure 2). According to Amadori rearrangement and Millard reaction, can rapidly forms complex browning at high temperature.

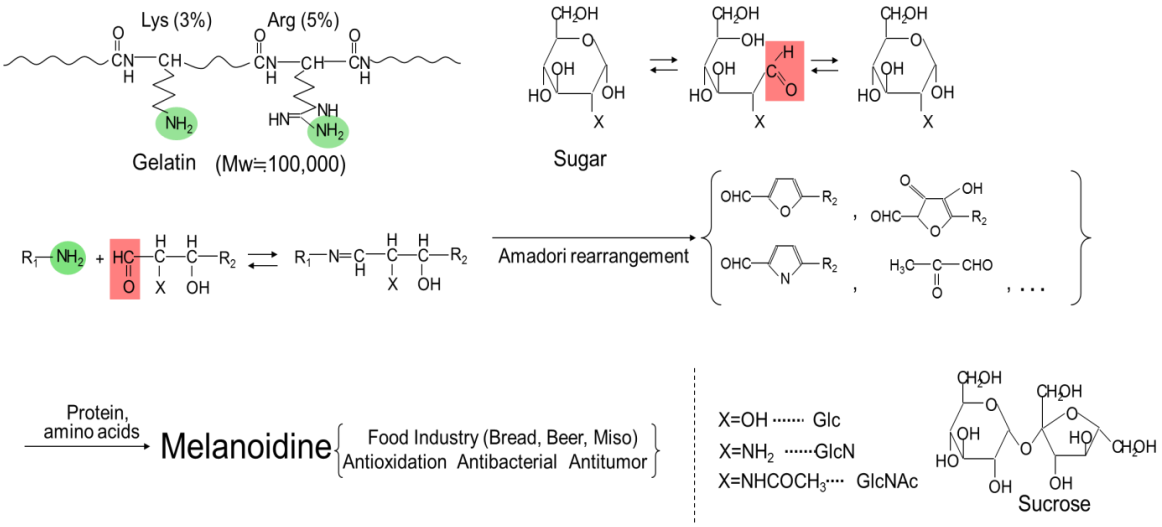

Figure 2. The reaction scheme between gelatin and sugar.

Di-epoxy cross-linker (denacol)

Di-epoxy was add to the Gel solution in the preparation Gel solution step by vary concentration 3, 4 and 5% by mass of gelatin. In addition, heat treatment at 100°C 24 h also collect the data to study mechanism of the system. The hydroxyl group of epoxy can react with the free amino groups of gelatin occur crosslink reaction molecule (Figure 3). Due to this reaction, may effect of increasing viscosity of the gelatin solution before spin so the time of addition di-epoxy in the solution before spin also record.

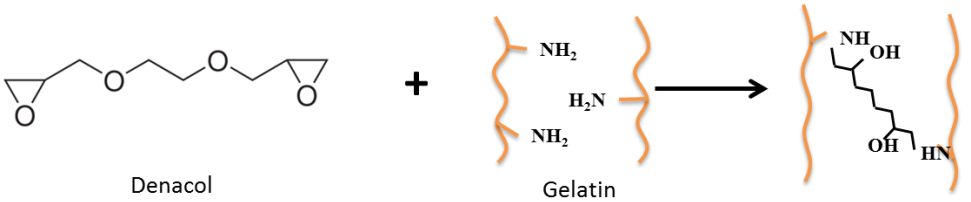

Figure 3. The reaction scheme between gelatin and Di-epoxy Ethylene glycol diglycidyl ether (Denacol).

GTA

After spun gelatin fiber (50% by weight in water), the crosslinking process was carried out by placing the gelatin fiber in desiccator containing GTA aqueous solution and keep at room temperature. After crosslinking, the samples were rinse by immerse in methanol for 15 min x 3 times to remove residual GTA. Time of crosslinking in desiccator and heat treatment has been evaluated to study mechanism of GTA crosslinked system. Figure 4 shown the reaction scheme, the bifunctional compound of GTA links covalently to the amine groups of lysine or hydroxylysine in the gelatin molecules creating a structure more stable [12].

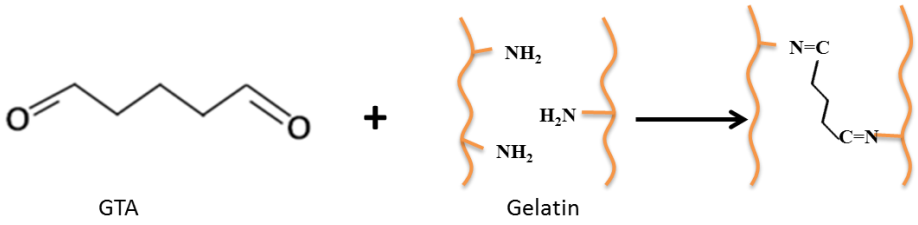

Figure 4. The reaction scheme between gelatin and GTA.

I.2.2.4 Tensile strength

The mechanical property was analyzed using a universal testing machine (STA-1150, A&D company, Ltd., Japan). Figure 5 shown the test condition of tensile and knot strength by apply force. The cross-head loading speed was set at 10 mm/min for 5N loading at 25 ± 2°C, Humidity = 50 ± 2 % and average the value from 15 times/ sample.

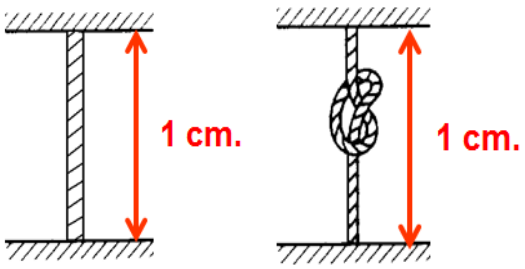

Figure 5. Tensile (left) and Knot (right) strength test condition.

I.1.3 Results and Discussion

I.1.3.1 Appearance

Gelatin fibers was spun by dry spinning and cross-linked using various of crosslinking agent type. Figure 6 shown the gelatin solution after keep in electric water bath 30 min, solution was homogeneous by bubble was float to the top of solution surface. After spun, gelatin with di-epoxy crosslinking obtain the smooth white color fiber, but gelatin fiber which vapor crosslinked with GTA, the fibers became visibly yellowish because the formation of aldimine linkages between the free amine groups of lysine or hydro-lysine amino acid residues of polypeptide chains with the aldehyde group of GTA [15-18]. Fibers with sugar, after applied heat treatment showed the brown color according to Maillard reaction, which produces browning compounds due to the interactions between carbonyl group of sugar and amino compounds of gelatin. Fibers have average diameter in range of 50 ± 5 microns in every crosslink condition.

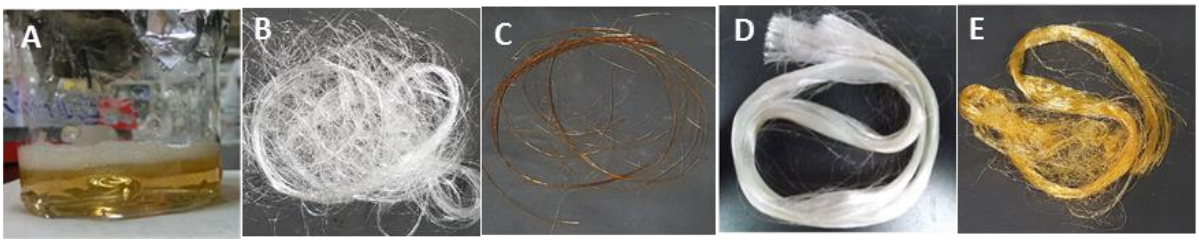

Figure 6. (A) Gel solution before spin, (B) Gel fiber without crosslink, (C) Sugar cross-linked fiber (GlcNAc), (D) Epoxy cross-linked fiber and (E) GTA cross-linked fiber.

From Figure 7, SEM images of a non-crosslinked, three crosslinked fibers and cross-section area of a gelatin fiber without crosslinking (Figure 7E and F), furrows attributed to the nozzle were observed in the longitudinal direction. The gelatin fiber without crosslinking and the denacol-crosslinked fiber exhibited smooth surfaces (Figures 7A and C). The GlcNAc-crosslinked fiber showed a slightly heterogeneous surface due to the complex Maillard reactions (Figure 7B), and the GTA-treated fiber showed a slightly rough surface (Figure 7D). The results indicate that only the surfaces of the fibers were crosslinked by GTA vapor, causing it to swelling slightly owing to moisture.

The mean diameter of the GTA-treated fibers was 60 ± 5 μm, slightly larger than those of the other fibers. An interconnected porous structure was observed in the cross-section of the fiber. The fiber exhibited a high porosity with a pore diameter of less than 1 μm.

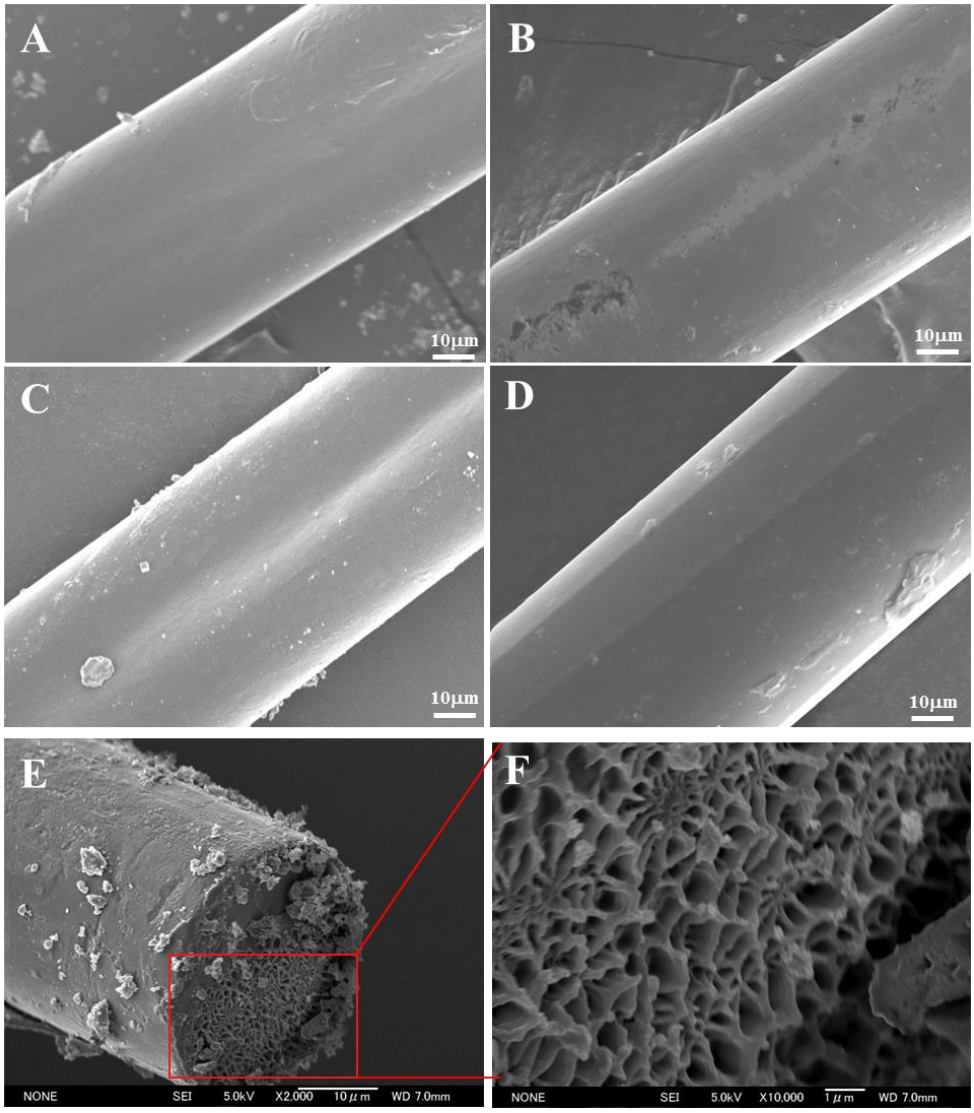

Figure 7. SEM images of (A) gel fiber without crosslinking, (B) GlcNAc-crosslinked fiber, (C) denacol-crosslinked fiber, (D) GTA-treated fiber, (E) cross section of gelatin fiber without crosslinking and (F) high-magnification image of the fiber cross-section.

Moreover, Fourier transform infrared (FT-IR) analysis was studied by using a Varian 670-IR spectrometer (Agilent Tech. Int. Japan, Ltd., Tokyo, Japan). Figure 8, showed the gelatin peaks of the amide I (C=O stretch), amide II (N–H bend and C–H stretch) and amide III (C–N stretch plus N–H in phase bending) were observed at 1636–1640, 1542–1544 and 1240cm−1, respectively. But the results are no significant differences were observed in the spectra of the gelatin fibers with and without crosslinking.

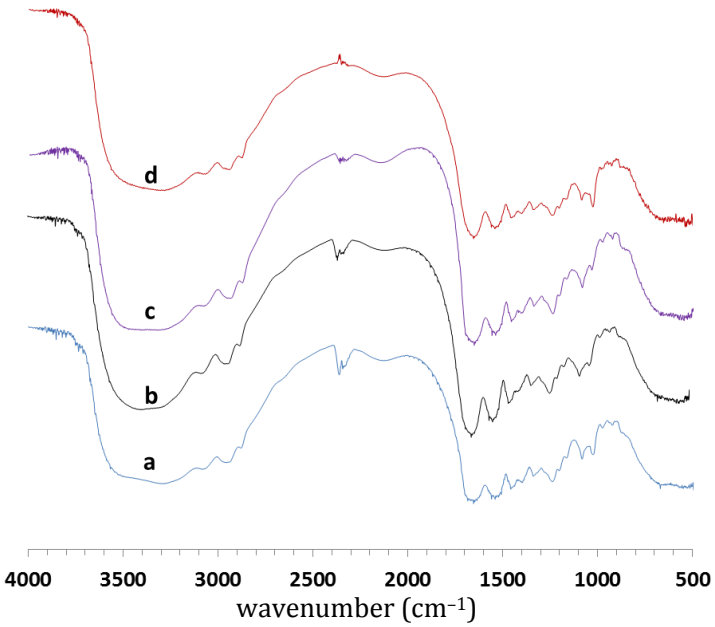

Figure 8. FT-IR spectra of gelatin fibers: (a) gelatin fibers without crosslinking; (b) GlcNAc-crosslinked fibers; (c) denacol-crosslinked fibers; and (d) GTA-treated fibers.

I.1.3.2 Crosslinker effect

Sugar cross-linker

The average tensile stress of gelatin fibers which produced with different sugar crosslinked is indicated in Figure 9. Fibers were sampled directly after the spinning at let it completely dry at room temperature for 24 h. Tensile strength results indicate that stress of fibers, which were cross-linked by either sugars, was improved when compared to original and GlcNAc cross-linked fiber showed stress values higher the others especially when applied heat treatment condition for 120°C 24 h. In addition, the effect of heat treatment time results to increased water resistance ability of fiber and

GlcNAc cross-linked shown the highest water resistance more than 90 days when applied heat treatment for 24 h. GlcN and Glc were not different in results both tensile strength and water resistance. Suc was is weakest crosslinker to Gel fiber in this study about sugar cross linker group, because Suc is non-reducing sugar so it's not form the strong interaction like Millard reaction with amino group in gelatin as same as the others sugar.

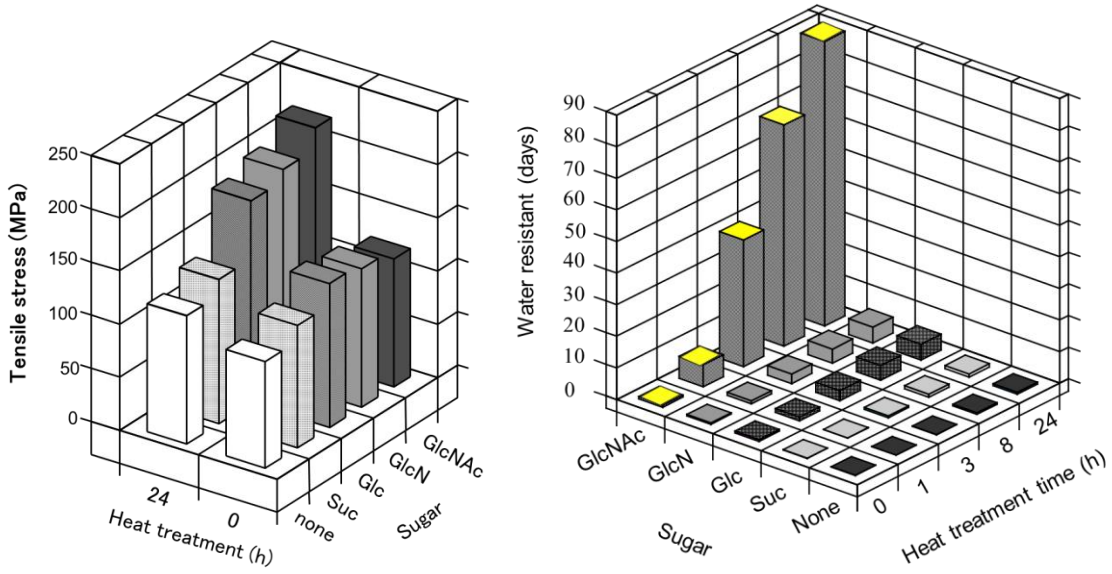

Figure 9. Effect of sugar crosslinked to Tensile strength (left) and water resistance (right) of gelatin fibers.

Di-epoxy cross-linker (Denacol)

Table 1. Shown the results of crosslinking time before spin, after mix the di-epoxy into gelatin solution viscosity of solution gradually increased follow by time and di-epoxy concentration. According to this study, suitable time to crosslinked is between 30-40 min. At the time before 30 min, the gelatin solution was not homogeneous and completely dissolved yet so the fiber was ripped many times during spinning process. From Figure 10, both tensile and knot strength results indicate that stress of fibers which were cross-linked by di-epoxy was improved when compared to original. The average tensile and knot strength of fiber without crosslinker are 120 and 100 MPa respectively. Increasing of crosslinked concentration trend to increase stress of fiber. %Strain (the elongation) shown tendency to increase gradually when increased amount of di-epoxy

compared to original of fiber. The average tensile and knot strain of original fiber are less than 10%. After applied heat treatment, % strains of fiber significantly increase especially tensile strength condition. In the contrary, stress of fibers remained the same or even lower, probably due to thermal decomposition of the gelatin chain [19-20].

Table 1. Effect of crosslink time between di-epoxy and gelatin solution before spin

Di-epoxy (%)	Crosslink time (min)						
	10	20	30	40	50	60	70
0	X	Δ	O	O	O	O	O
3	X	Δ	O	O	Δ	Δ	X
4	X	Δ	O	O	Δ	Δ	X
5	X	Δ	O	O	Δ	X	X

O – well spin, Δ - can spin but fiber rip many time during spin, X-cannot spin

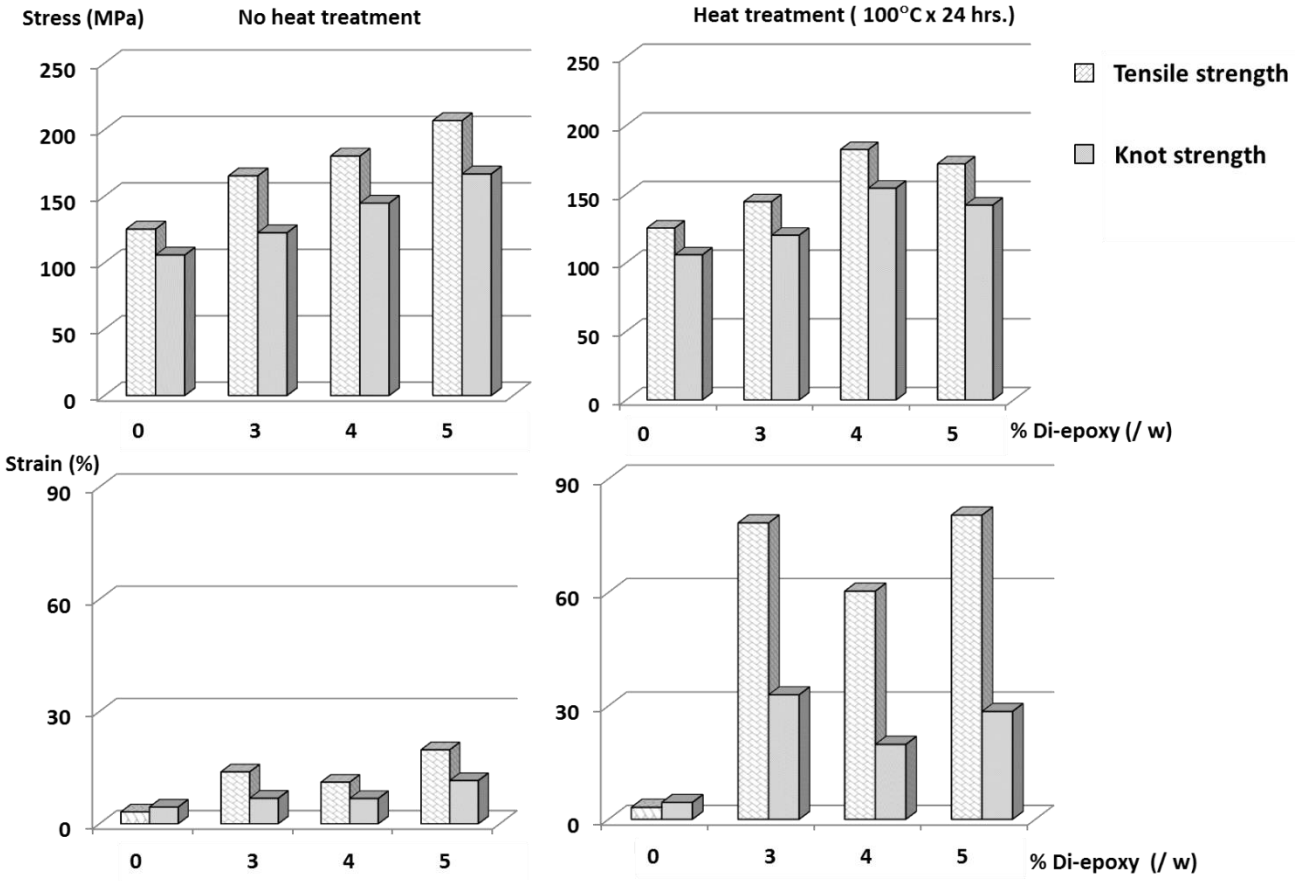

Figure 10. Effect of di-epoxy cross-linked to mechanical strength of gelatin fiber.

Furthermore, tensile strength and % stain of fiber also investigate in wet condition by immerse each fiber into DI water for 2 min. before test. Compare the results by vary % di-epoxy (0, 3, 4, and 5%) and heat treatment temperature (80, 90, 100°C). Results were shown in Figure 11 and Figure 12 for tensile stress and % stain respectively.

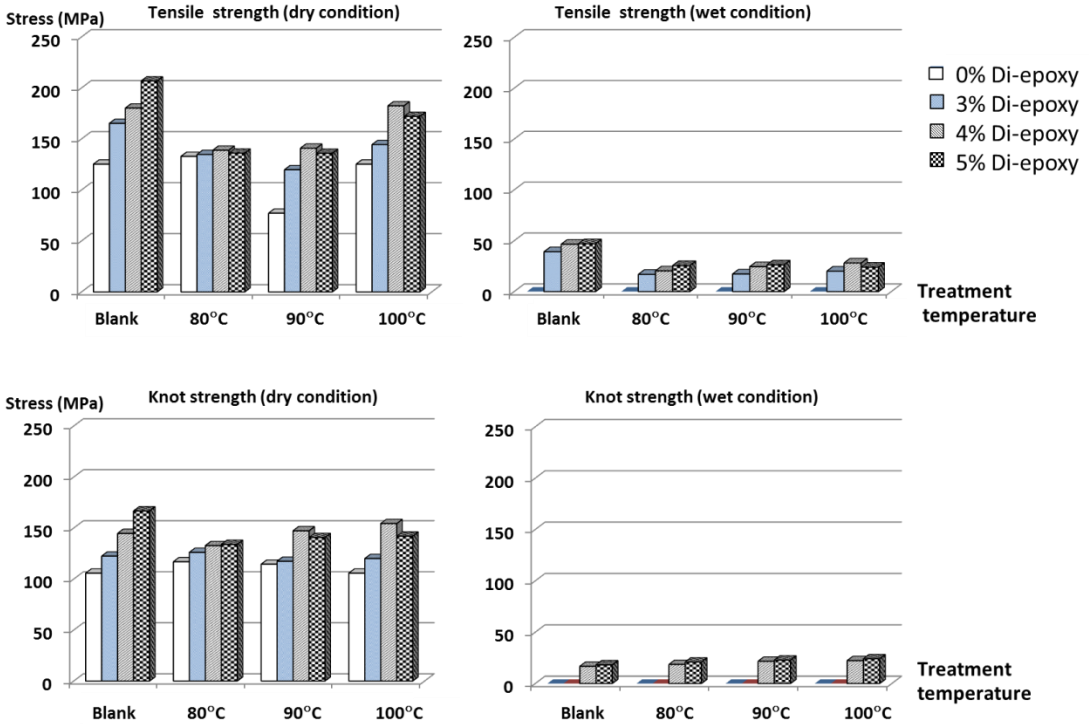

Figure 11. Tensile strength of dry and wet test condition at different % di-epoxy cross-linked.

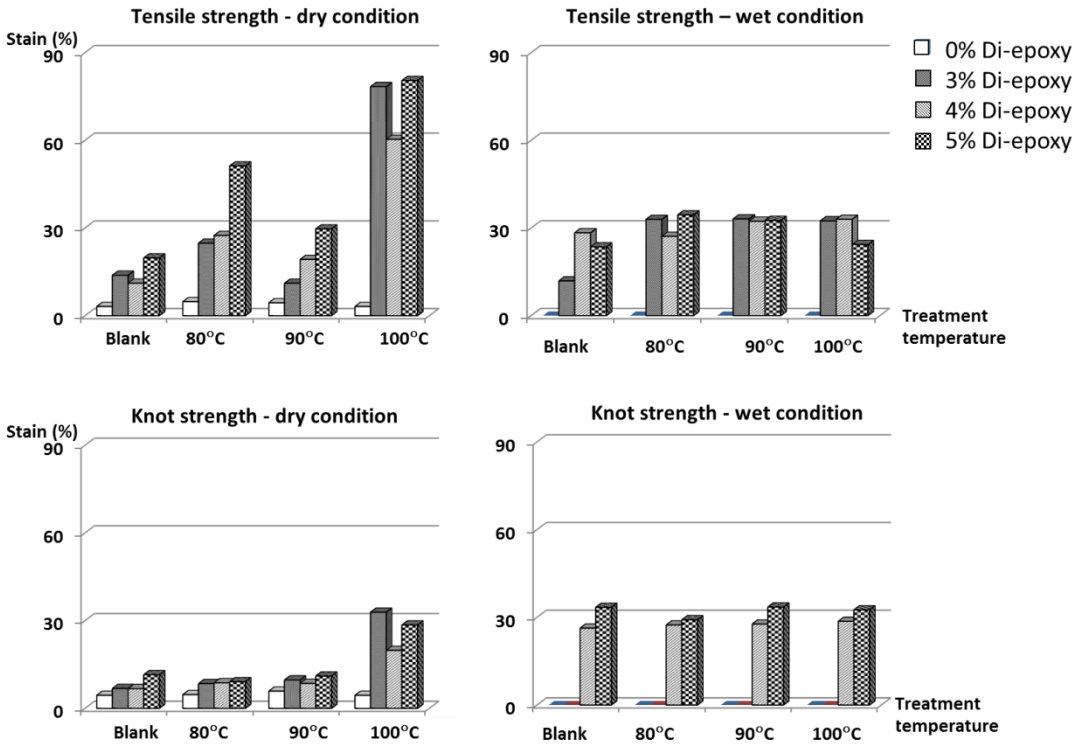

Figure 12. % Strain of dry and wet test condition at different % di-epoxy cross-linked.

GTA

From Figure 13, both tensile and knot strength results indicate that stress of fibers which cross-linked by GTA was improved when compared to original without GTA. Increasing of crosslinked time from 1 day, 2 days and 3 days trend to increase stress of fiber. After applied heat treatment, stress of fibers were improved, indicated that post-treatment can partially enhance the crosslinking [18, 21].

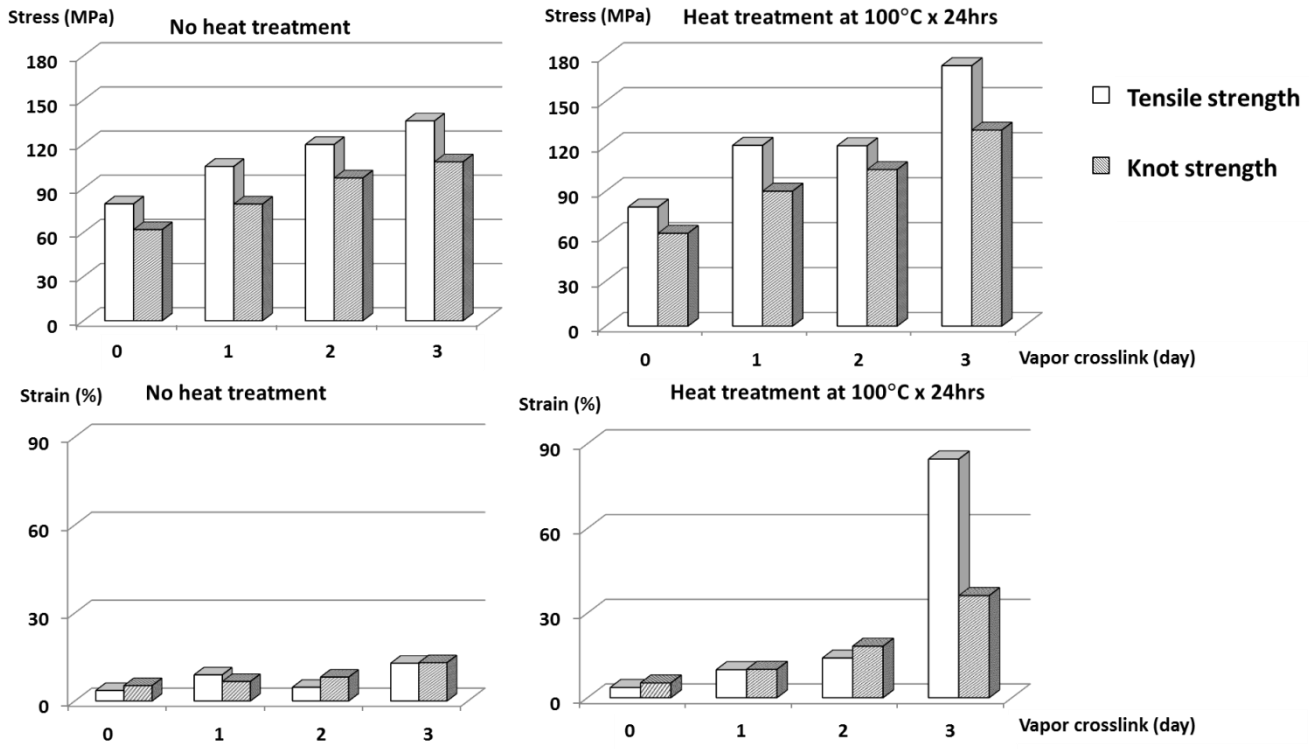

Figure 13. Effect of GTA cross-linked to mechanical strength of gelatin fiber.

I.1.3.3 Water resistance

Furthermore, the comparison of 3 type of crosslink agent has been evaluated by water resistance ability. Fibers have been immersed into DI water and keep at room temperature. GlcNAc which shown the best result from sugar crosslinker was choose for compare with GTA, di-epoxy to original gelatin fiber. Each type of crosslinked fibers were applied heat treatment condition. Observed and compared the difference of each crosslinked fiber to water resistance property in the function of time. The results was shown in Figure 14, after immersed gelatin fiber into DI water, gelatin fiber without crosslink suddenly swelling as same as gelatin fiber crosslinked with epoxy 4%. After 1 day, gelatin fiber was completely dissolved and obtained homogeneous solutions while gelatin fiber with di-epoxy 4% more swelling but not dissolve. Gelatin fiber with epoxy 5% shown a little swelling but can see the fiber shape better than epoxy 4%. Gelatin fiber with GTA and GlcNAc crosslinked shown the good water resistance and good morphology up to 90 days. In addition, swelling ratio after immersed in water for 24h at room temperature of GTA, GlcNAc and di-epoxy 5% are

1.96, 2.40 and 3.77 respectively. Its can confirmed the results of water resistance that GlcNAc and GTA maintained their morphologies than di-epoxy.

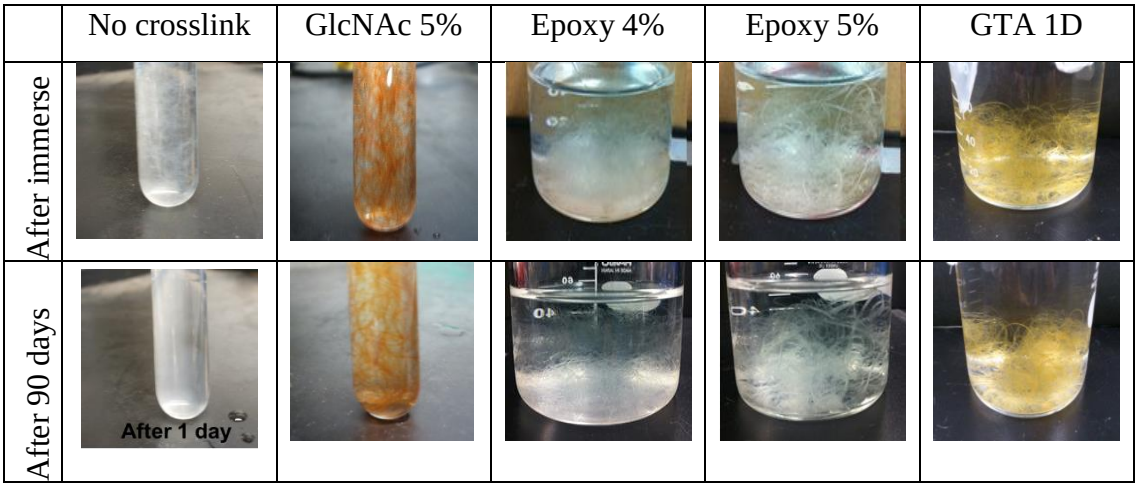

Figure 14. Water resistance ability of each cross-linked fiber.

I.1.4 Conclusions

Gelatin fibers were prepared with sugar, di-epoxy and GTA as cross-linking agents by dry spinning method which used only water as a solvent. The effect of each crossliked type were compared and evaluated. GlcNAc is a reducing sugar which shown good results in tensile stress and water resistance than the others sugar especially when applied heat treatment at 120°C for 24 h. because of Maillard reaction. Di-epoxy compounds can improved the stress of gelatin fiber when add to gelatin solution by body-feed before spin, stress of fiber were increased follow by increased amount of di-epoxy. Due to thermal decomposition of the gelatin chain when apply heat treatment to the fiber, stress of fibers remained the same or even lower. GTA vapour evaporate method, result to increase stress of fiber follow by increased time of crosslinked. And post-treatment can partially enhance the crosslinking results to stress of fibers was improved on heating. The comparison of each crosslinker by water resistance, GTA and GlcNAc crosslinked showed the good water resistance and good morphology up to 90 days by less swelling and same shape of fibers. Finally, such gelatin fibers and fiber assembly is expected to be better use for several fields because gelatin is a safe material and the spinning method is a simple and environmental friendly method.

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Chapter 2

Optimum point of GTA vapour crosslink

I.2.1 Introduction

Glutaraldehyde (GTA) is an organic compound with the formula $CH_2(CH_2CHO)_2$, linear-5-carbon dialdehyde which clear, colorless oily liquid that soluble in all ratio of water and alcohol, as same as in organic solvent [1]. GTA is usually use as a protein crosslinking agent; sterilize medical, dental equipment, water treatment, preservative etc. GTA has had great success because of its commercial availability, low cost and high reactivity. It reacts rapidly with amine groups at neutral pH condition [1-2]. According to its high reactivity, GTA crosslink with gelatin fiber have been done by vapor crosslink. Our assumption is the reaction will start from the surface to core of fiber. The time of crosslinking is the one parameter that we interesting to study and find the optimum time. However, aldehyde is recognize that may cause sick building syndrome (SBS) and the one of the major pollutants of indoor air, sick house syndrome by irritation and corrosive to the skin, eyes and respiratory tract are the major symptoms [3-6]. In order to avoid unreacted and residual GTA after crosslinking with amino group in gelatin, reducing agent could be useful for neutralize the chemical. Sodium borohydride is an inorganic compound with the formula $NaBH_4$ in form of white solid, usually in form of powder, is a general reducing agent that finds wide application in chemistry, both in the laboratory and on a technical scale. Bleaching wood pulp is the application which using large amounts of $NaBH_4$ [7-8]. The compound is soluble in alcohols and some ethers but reacts with water in the acid and neutral condition. $NaBH_4$ is a reagent of choice for the reduction of aldehydes and ketones. Furthermore, it has been also used for the reduction or reductive N-alkylation of amines, heterocyclic which containing nitrogen and oximes can be reduce by $NaBH_4$ as well [9]. The mechanism of the reaction of $NaBH_4$ with aldehydes and ketones can describe in two steps. In the first step, H atom separate from BH_4 and react to the carbon of carbonyl group of aldehyde or ketone. Due to this step, C-H bond will be forms by breaks the double bond between C and O. The second step, new lone pair on the oxygen which remained from the first step will react with proton from water or the others acid

to forms alcohol as the end of reduction mechanism (or an acid such as NH_4Cl) is added to the alkoxide to make the alcohol. This is performed at the end of the reaction, a step referred to as the workup.

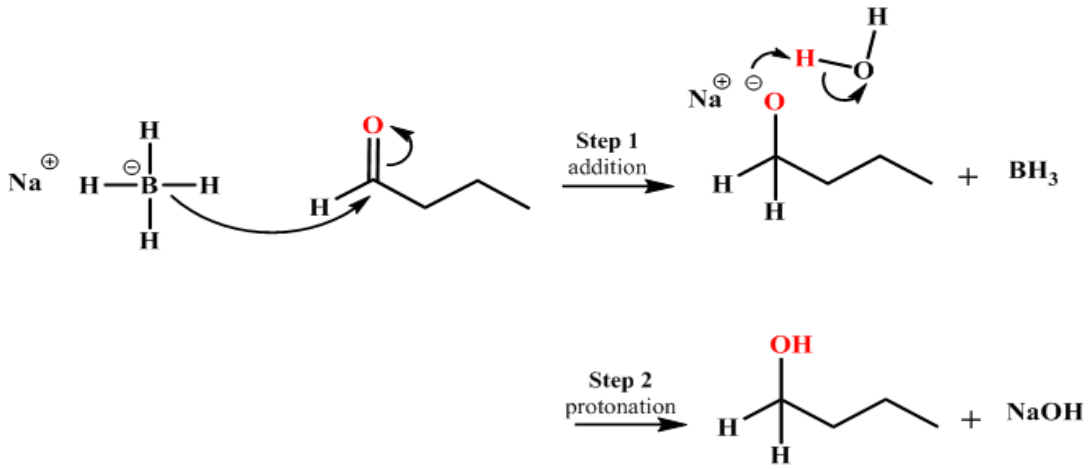

Figure 1. The mechanism of the reaction of sodium borohydride with aldehydes and ketones; Reference:http://www.masterorganicchemistry.com/2011/08/12/reagent-friday-sodium-borohydride-nabh4/

In this lesson, we focus to study the optimum point of GTA crosslinking with gelatin fiber by vapor method including of reducing condition by $NaBH_4$. Tensile strength will be measure to compare in each condition.

I.2.2 Experimental

I.2.2.1 Materials

Gelatin, JS200 (Mw=100,000; 200 bloom; type B) cow skin type in powder was from Koei transformation Ltd. GTA solution (25%) were from Wako Pure Chemical Industries, Ltd.

I.2.2.2 Preparation of gelatin solutions and spin gelatin fiber

The solution of gelatin was prepared by dissolving gelatin powder in water 50% by weight. The mixture was covered and put in the electric water bath at temperature

50±2°C for 30 min; stirred every 10 min to obtain homogeneous solution. The homogeneous solution of gelatin was filled up in cylinder (50±2°C) which connected to a nozzle (0.83 mm. inner diameter). Control pressure in the range from 0.10±0.04 MPa. was applied on top to the droplet of injected solution. Collection was rotate with speed 50±10 m/min to an aluminum foil wrapped on a collector. The separating distance between the needle tip and the aluminum foil was set to 1.4 m. (as same as Figure 1 in Chapter 1). The obtained fibers were kept on collector at room temperature for 24 h to remove residual moisture.

I.2.2.3 Crosslink method

After spun gelatin fiber, the crosslinking process was carried out by placing the gelatin fiber in desiccator containing GTA aqueous solution and keep at room temperature. After crosslinking, the samples were rinse by immerse in methanol for 15 min, 3 times to remove residual GTA. Time of crosslinking in desiccator has been evaluated to study optimum point time of GTA crosslinked system.

I.2.2.4 Reducing with $NaBH_4$

Reducing the crosslinked fiber with $NaBH_4$ was done by immerse fibers after vapor crosslink in desiccator in 0.6 M $NaBH_4$ / 0.05 M HCl solution for 5 hrs. The samples were rinse by immerse in methanol for 15 min, 3 times.

I.2.3 Results and Discussion

After GTA vapor cross-linking, the fabrics became visibly yellowish due to the formation of aldimine linkages between the free amine groups of protein and GTA (Figure 2) [10-13]. And after reducing with $NaBH_4$, the yellowish of fiber changed from bright to pale yellow which refers to reduction of N=C to N-C (Figure 2 and Figure 3).

before crosslink after crosslinked after reduced by $NaBH_4$

Figure 2. Comparison of fiber's color in each step.

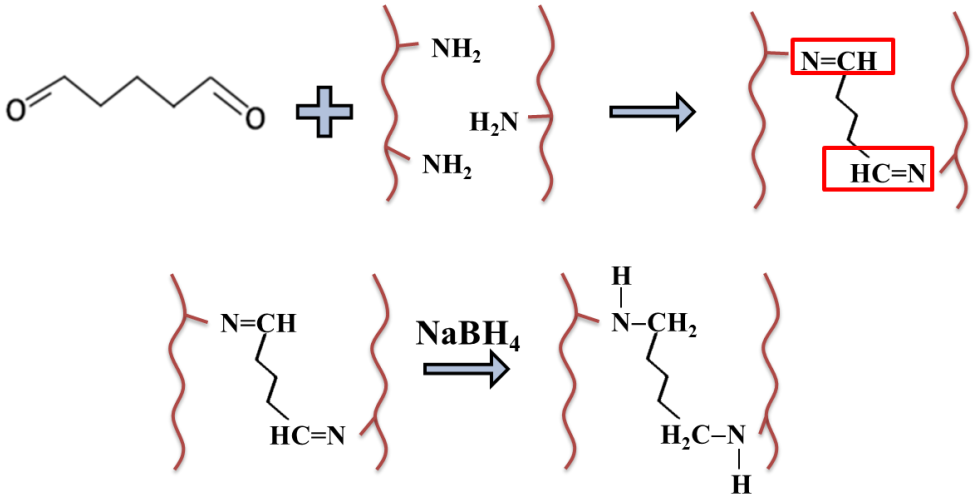

Figure 3. Reaction scheme between gelatin and GTA.

The effect of vapour crosslinking's time to stress of fiber was showed in Figure 4. Stress of fiber in each test condition tensile and knot strength with and without heat treatment at 100°C 24 hrs., show the same trend of results by stress of fiber gradually increase until almost stable after 7 days. These're may indicate that increase time of crosslink seemed able to provide proper crosslinking degree until complete at around 7 days.

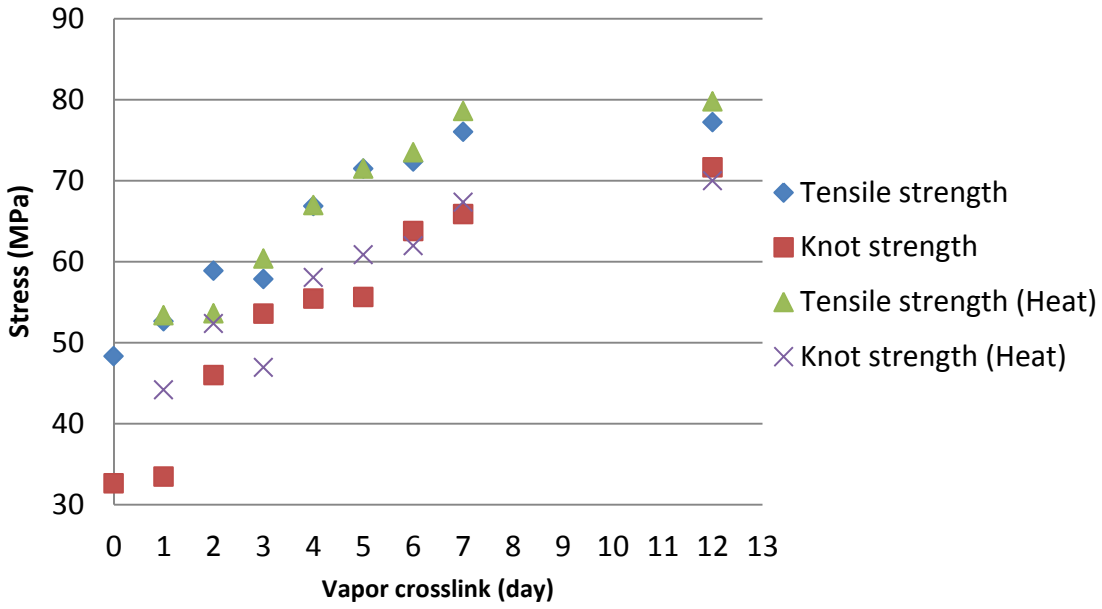

Figure 4. The effect of vapor crosslinking's time to stress of fibers.

Further neutralized condition, fiber which crosslinked for 5 days and applied heat treatment at 120°C for 2 days was choosing to reduce with $NaBH_4$. The mechanical property was showed in the Figure 5. Stress of fiber after reduction, remained the same or little higher while strain was decreased both tensile and knot strength showed the same trend of results. However, when compare with original fiber without crosslink strain of fiber still higher.

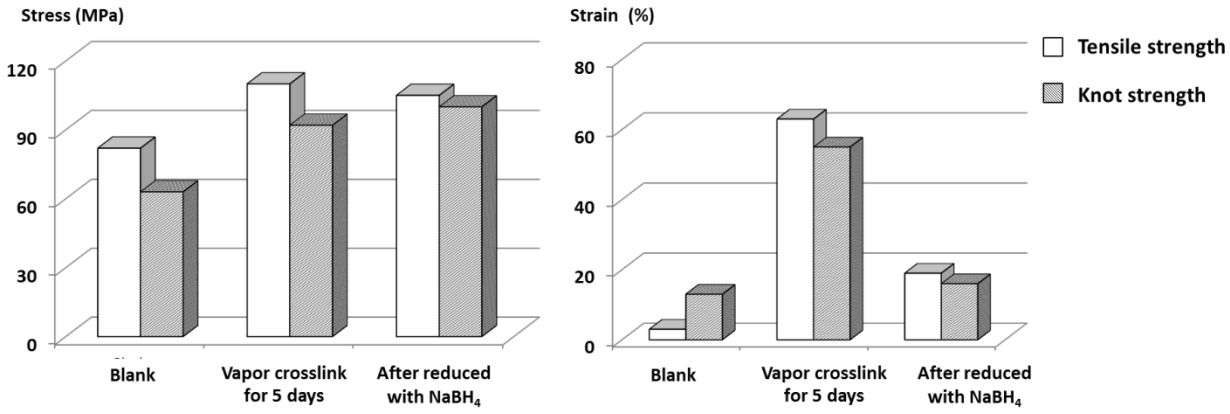

Figure 5. The effect of reduced condition to mechanism property of fiber.

I.2.4 Conclusions

Gelatin micro-fiber was prepared with using GTA as crosslinking agent by vapor method. Mechanical property was evaluated in order to find the optimum crosslinked time, the result was found that stress of fiber reach the stable at 7 days. The toxicity induced by GTA cross-linking was could be controlled by reducing it with $NaBH_4$. In all, the combination of results indicates that the $NaBH_4$ is effective in reduction of the gelatin fiber which crosslinked by GTA. This result may be extended and may help promoting their general use of gelatin fiber in polymer composites.

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Section II

Gelatin nano-fiber by electrospinning

Chapter 3

Fabrication of gelatin nano fibers by electrospinning - aqueous method

II.3.1 Introduction

Gelatin is a natural polymer and a fibrous protein that is present in the skin and bones of animals, synthesized by hydrolysis of the triple helix of collagen. Gelatin is a biocompatible protein, and when it enters living body, shows low anti-genecity and very high bio-absorptivity. The characteristic of heat reversibility is come from the compose between 3D gel network of gelatin and microcrystal interconnect with amorphous regions in coiled [1-2]. The important property of gelatin is the solution-gelation transition under aqueous condition. It has been reported that membranes of chitosan/ gelatin have so many biomedical applications [3]. Morover, the preparation of chitin/ gelatin membrane with N-acetyl-D-glucosamine (GlcNAc) according to Maillard reaction has been reported. The stress and elongation of chitin/ gelatin membrane with GlcNAc was higher than those without GlcNAc. Electrospinning is a method that modified from dry spinning concept for create membrane which consist from fiber in the nano size diameter. Nano fiber has more advantage about surface area and active surface site than micro fiber. Electrospinning technique is simple and cost-effective technique because just 3 apparatus could be required; feeding unit, voltage power supply and collector.

In this work we focus on the development of non-woven gelatin fabric by electrospinning. Polymeric fibers formed with the simultaneous evaporation of solvent by the action of high voltage to the polymer solution, electrospinning in the form of a non-woven fabric can be achieved. We have carried out electrospinning providing temperature on the basis of dry spinning. Fibers can be prepared to have a fiber diameter in the micro nanoscale. Nanofibrous non-woven fabrics have large surface area to volume ratio, and porosity. Gelatin is hydrophilic so we developed a cross-linked non-woven fabric to improve the water resistance. Two cross-linking agent have been compared in this study. First is glutaraldehyde (GTA) bifunctional reagent by vapor cross-linking. Among the chemical cross-linking agents, GTA is the most widely used, due to its high efficiency of collagenous materials stabilization. Cross-linking of

collagenous samples with GTA involves the reaction of free amino groups of lysine or hydroxy lysine amino acid residues of the polypeptide chains with the aldehyde groups of GTA [1]. GTA is easily available, inexpensive and its aqueous solutions can effectively cross-link collagenous tissues in a relatively short period [4-5]. Another is GlcNAc, cross-linking is achieved by Maillard reaction on heat treatment. In this study, we developed a cross-linked non-woven gelatin nanofibrous fabric. Further the developed non-woven fabric was characterized as well as biocompatibility was studied.

II.3.2 Experimental

II.3.2.1 Materials

Gelatin -JS200 cow skin type (Mw=100,000), Koei transformation Ltd.

GlcNAc -Wako Pure Chemical Industries, Ltd.

GTA -Wako Pure Chemical Industries, Ltd.

Glycerine solution (Gly) -99% (mass/mass, Mw 92.09), Wako Pure Chemical Industries, Ltd.

Phosphate Buffer Saline (PBS) -Wako Pure Chemical Industries, Ltd.

II.3.2.2 Preparation of gelatin solutions

Solution was prepared by mixing glycerin 10% in distilled water and dissolving GlcNAc 5% in solution (% by weight of gelatin). After completely dissolved, add gelatin powder in the solution (sample which have no GlcNAc composition, gelatin was dissolved in distilled water directly). The mixture was covered and put in electric water bath at controlled temperature of 60 ± 2°C for 45 min (stirred at 15, 30 and 45 min) after that left in water bath for 45 min to remove entrapped air and obtain homogenous solution. Concentration of Gel was 20, 25, 30 and 35%. Viscosity of Gel solution was measure by Rheometer at 50°C (HAAKE RheoStress 600, EKO instruments, Japan).

II.3.2.3 Electrospinning

The homogeneous solution of gelatin was filled in a 5 ml syringe connected with a needle. Temperature was controlled inside the syringe at 45-60°C. The needle was connected with the high voltage power supplier, and then applied 23 kV to the droplet of injected solution. The distance between collector and capillary tip was set at 7 cm.

and the collector was covered with aluminum foil. The collected fiber mats were left at least 3 hrs. to ensure complete removal of solvent. Details of electrospinning conditions are shown in Table 1-Table 3. Later, the samples were separated for different treatment conditions. Cross-linking of the fibers was done using GTA vapor for 48 hrs, following which the samples were rinsed by dipping in ethanol 20 times. Further neutralization of these fibers was done by immersing in 0.2M $NaBH_4$ for 1 hr. For GlcNAc containing, gelatin fibers heating was provided for 48 h at 120°C in oven.

Table 1. Conditions of electrospinning (gelatin only).

Conc. of gelatin (%)	Flow rate (x 10^{-2} ml/min)	Conductivity (mS/m)	Viscosity at 50°C (cP)	Diameter (nm)
20	19.6	129.2	193.9	254
25	12.2	131.1	498.9	374
30	4.6	136.5	1335.6	3825
35	-	141.6	4345.5	-

Table 2. Conditions of electrospinning (gelatin/ GlcNAc).

Conc. of Gel (%)	Flow rate (x 10^{-2} ml/min)	Conductivity (mS/m)	Viscosity at 50°C (cP)	Diameter (nm)
20	25.0	129.0	146.7	208
25	14.7	134.5	313.5	358
30	5.5	137.9	769.1	2700
35	-	144.1	1947.3	-

Table 3. Conditions of electrospinning (gelatin/ Gly).

Conc. of Gel (%)	Flow rate (x 10^{-2} ml/min)	Conductivity (mS/m)	Viscosity at 50°C (cP)	Diameter (nm)
20	48.8	120.4	168.7	186
25	16.0	129.3	397.9	332
30	6.5	130.1	1319.3	1280
35	-	132.4	3442.5	-

Remark; 35% Gel at all condition cannot spinning because high viscosity.

II.3.2.4 Characterization

The swelling and water uptake ability of the non-woven fabrics were studied in PBS (pH 7.4) and distilled water, respectively. Samples were immersed in PBS and water at room temperature for different time points (30 and 60 min). After the specific time point, samples were taken out and excess water was removed using a filter paper. The structural morphology of non-woven fabrics was characterized using SEM (JSM-6700F (JEOL Ltd.), Tokyo, Japan) at 5 kV acceleration voltages. Composite fibers were analyzed using FTIR spectroscopy (Varian 670-IR Agilent Tech., Japan). Composite fibers were ground and mixed thoroughly with potassium bromide. The IR spectra of the samples were analyzed from 400-4000 cm^{-1}. The mechanical property was analyzed using a universal testing machine (STA-1150, A&D company, Ltd., Japan).The cross-head loading speed was set at 10 mm/min for 5N loading. Thermogravimetric analysis was carried out using TG/DTA instrument (SII TG-DTA6200) at a temperature ranging from 24-600°C.

II.3.2.5 Cell viability

Cell viability of the electrospun gelatin nanofibrous samples were evaluated using Alamar blue assay [6]. Human mesenchymal stem cells (hMSCs) were seeded at a density of 7500 cells/scaffold in a 96 well plate and incubated at different time points. Viability was analyzed by replacing media after 24 and 48 hrs with 200 µl basal medium containing 10% alamar solution. After required incubation the optical density was measured at 570 nm, with 600 nm as the reference wavelength, in a microplate reader (Biotek PowerWave XS, USA). Percentage cell viability was plotted by comparing with the control (cells+media).

II.3.2.6 Cell attachment studies

hMSC attachment on the electrospun scaffolds was evaluated by cytoskeletal staining after 24 hrs of culture period. Cells were seeded at a density of 50,000 cells per scaffold in 24 well plate and maintained for 24 hrs. Further cells were fixed in 4% paraformaldehyde and permeabilized using 0.5% Triton X. Cytoskeletal staining was done using TRITC conjugated phalloidin and imaged in fluorescent microscope (Olympus BX 51).

II.3.3 Results and Discussion

Non-woven gelatin nanofibrous fabric was developed by electrospinning and was cross-linked using GTA vapour or GlcNAc addition followed by heat treatment. From Table 1- Table 3, it was found that 20% and 25% concentration of gelatin showed fiber diameter in the nanometer scale, but at 25% solution wastage was reduced. So we chose 25% condition for further investigation. By placing non-woven fabrics into the desiccators filled with GTA vapor, the gelatin could be reasonably cross-linked. Cross-linking of collagenous materials with GTA involves the reaction of free amino group of lysine or hydrolysine amino acid residues of polypeptide chains with the aldehyde group of GTA [1]. From Figure 1, after GTA vapor cross-linking; the fabrics became visibly yellowish due to the establishment of aldimine linkages between the free amine groups of protein and GTA [7-10]. After reducing with $NaBH_4$, the yellowish of fabric changed from bright to pale yellow which refers to reduction of N=C to N-C. While GlcNAc and amino group can crosslink by Maillard reaction producing brown colored compound due to the interaction between carbonyl group, reducing sugar and amino compound.

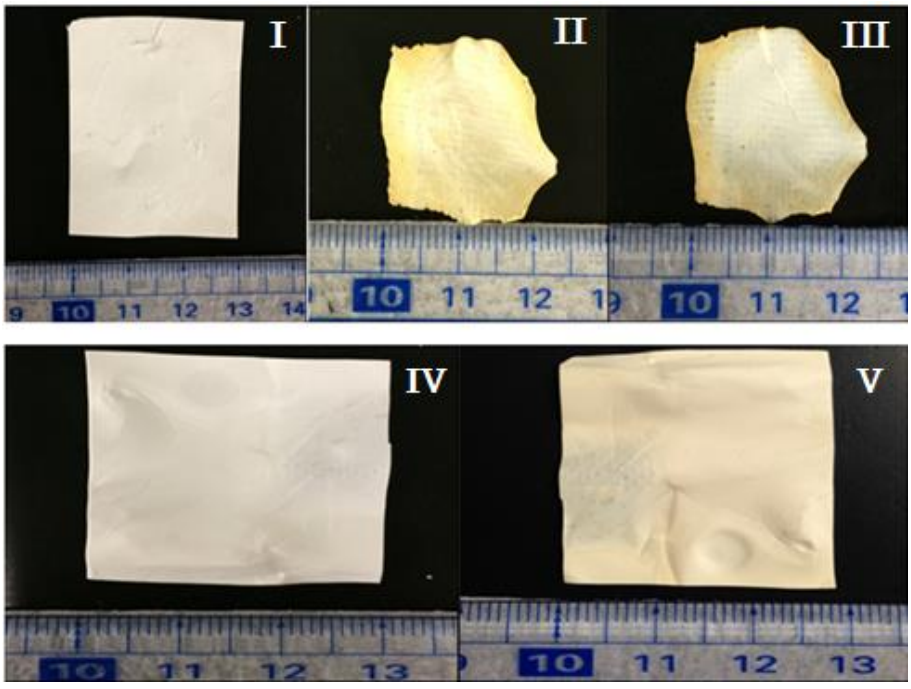

Figure 1. Images of fabrics (I) gelatin, (II) gelatin / GTA, (III) gelatinl/ GTA after reducing with $NaBH_4$, (IV) gelatin/ GlcNAc and (V) gelatin/ GlcNAc after heat treatment.

Figure 2 shows the SEM observation of nanofibrous non-woven fabrics arranged randomly. The smooth and continuous fabrics with no beads could be produced from all compositions of the cross-linking materials at 5 kV applied voltage. From Fig 2A-2D, it is clear that the morphology of post cross-linked (using GTA vapor) non-woven fabrics was found to differ from the original. When comparing with GlcNAc heat treated fibers less interconnectivity than GTA cross-linking was observed. From Figure 3A, it is evident that the regenerated material is pure gelatin powder with split transmittance band, at 1660 cm^{-1} corresponding to the amide. The spectra showed no difference in gelatin powder, gelatin fabrics and gelatin fabrics cross-linked by GTA before and after reduced by $NaBH_4$. This implies that the vapor cross-linking method affect only the surface of the fabrics so it would be difficult to indicate the difference of functional group in the fabrics before and after cross-linking. In Figure 3B, the IR spectra studies of sample have been compared with gelatin powder and GlcNAc powder. The presence of peaks proved that heat treatment did not affect the functional groups of the fibers. From Figure 4, the tensile results indicate that stress of non-woven fabrics, which were cross-linked by either method, was improved when compared to original and GTA cross-linked non-woven fabrics showed stress values higher than the GlcNAc system. In the contrary, % strain (the elongation) of fabrics remained the same or even higher. It is suggested that moisture content could play a greater role than the cross-linking in regulating the elastic and plastic behaviour of this natural biomaterial [4]. In addition, glycerine improved the strain of fabric as we expected and $NaBH_4$ reduction of GTA vapour cross-linked fabrics also didn't affect its mechanical properties.

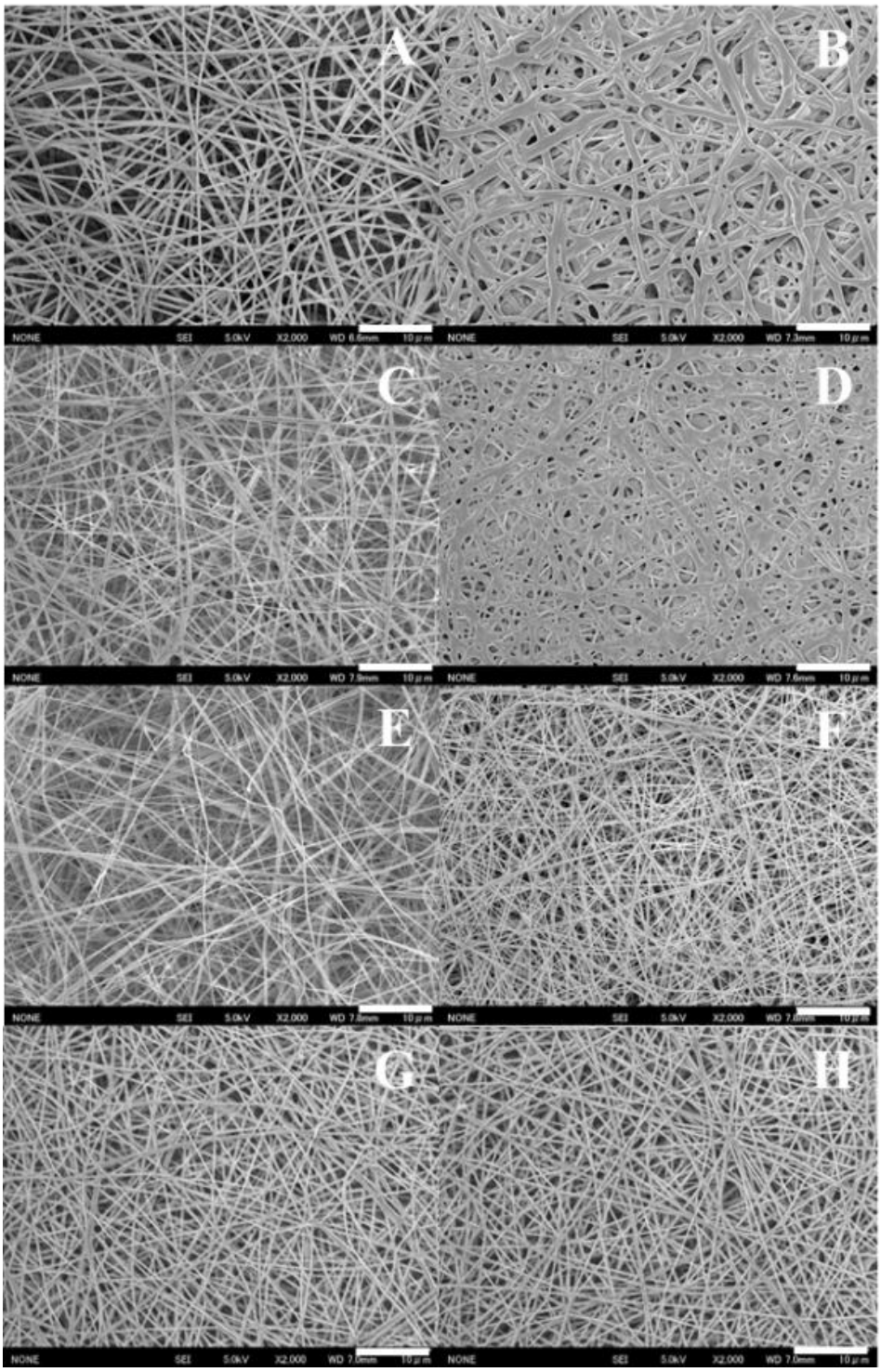

Figure 2. SEM images of gelatin and gelatin/ GlcNAc fabrics; (A, B) gelatin before and after cross-linking with GTA respectively. (C, D) gelatin/ glycerine before and after cross-linking with GTA respectively. (E, F) gelatin/ GlcNAc before and after heat treatment respectively. (G, H) gelatin/ GlcNAc/ glycerine before and after heat treatment respectively; scale bar 10 microns.

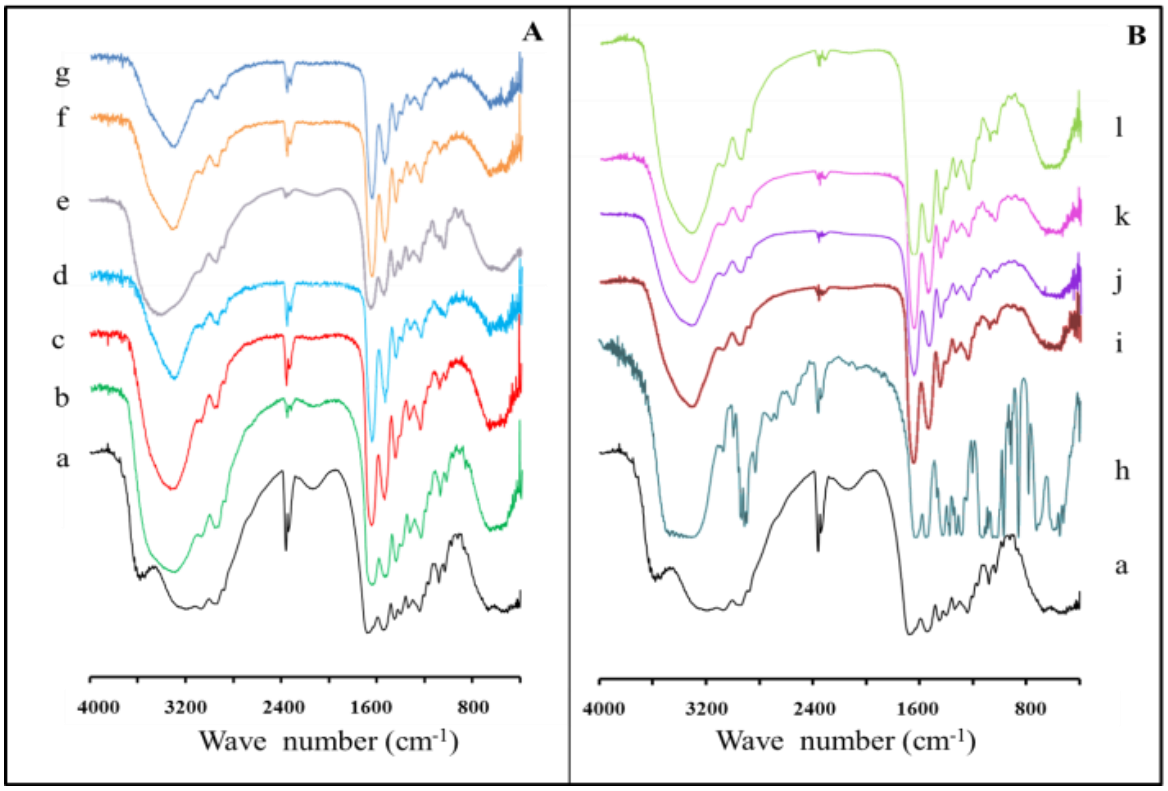

Figure 3. (A) FT-IR spectra of gelatin/ GTA fabrics : a) gelatin powder, b) gelatin fabric, c) gelatin/ GTA fabric, d) gelatin/ GTA/ $NABH_4$ fabric, e) gelatin/ glycerin fabric, f) gelatin/ glycerin/ GTA fabric, g) gelatin/ glycerin/ GTA/ $NABH_4$ fabric ; (B) FT-IR spectra of gelatin/ GlcNAc fabrics : a) gelatin powder, h) GlcNAc powder, i) gelatin/ GlcNAc fabric, j) gelatin/ GlcNAc/ heat treated fabric, k) gelatin/ glycerin/ GlcNAc fabric and l) gelatin/ glycerin/ GlcNAc /heat treated.

The swelling capacity of the fibers was performed to quantify the amount of swelling that occurs when non-woven fabrics were exposed to water and PBS for 30 and 60 min. As shown in Figure 5, higher swelling and water uptake ability was observed in gelatin/ GlcNAc (Figure 5E-H). The gelatin/ GlcNAc fabric showed completely swollen morphology. It may be due to the hydrophilic property of GlcNAc. While the non-woven fabrics after cross-linking with GTA only a slight change in morphology was observed.

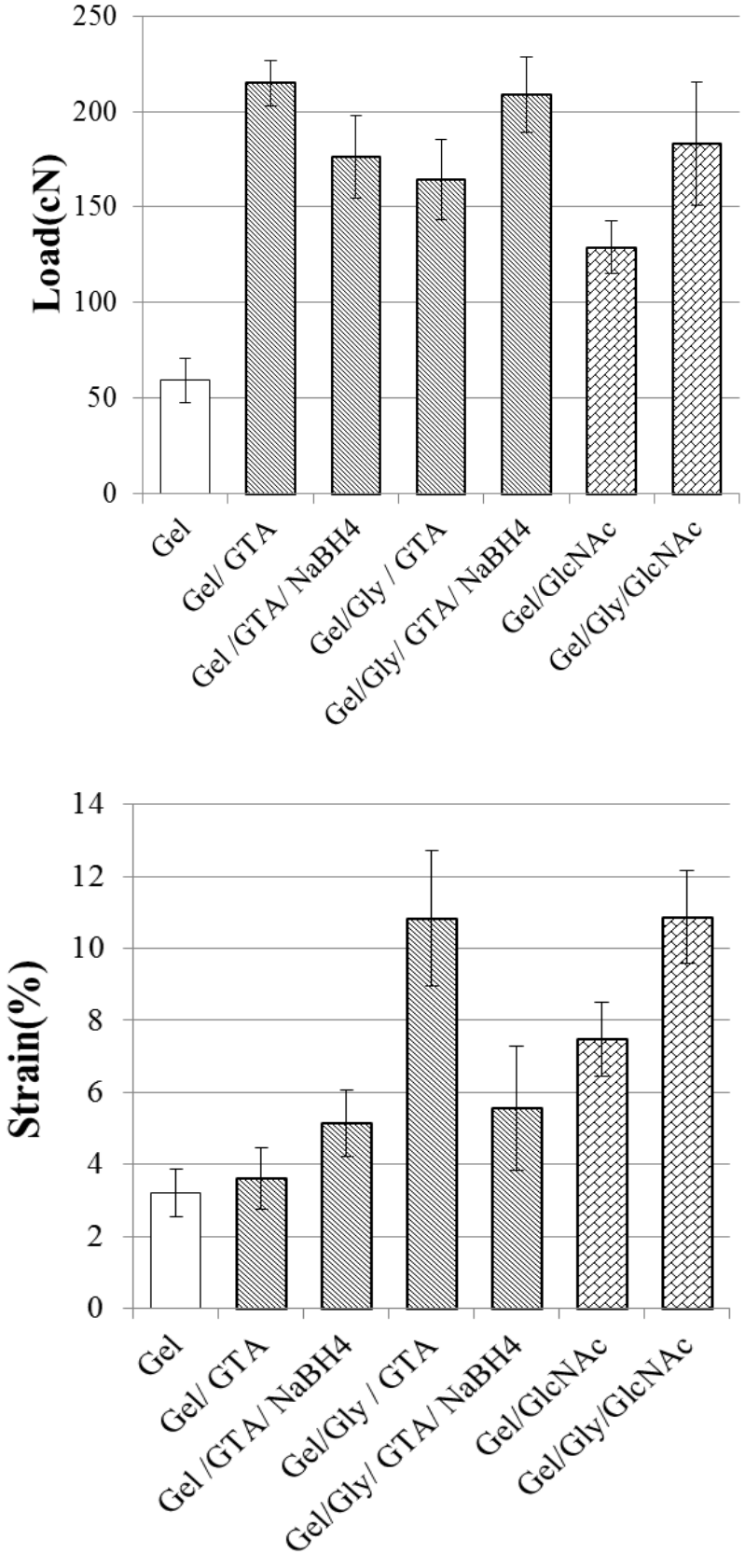

Figure 4. Mechanical property of gelatin/ GTA and gelatin/ GlcNAc fabrics.

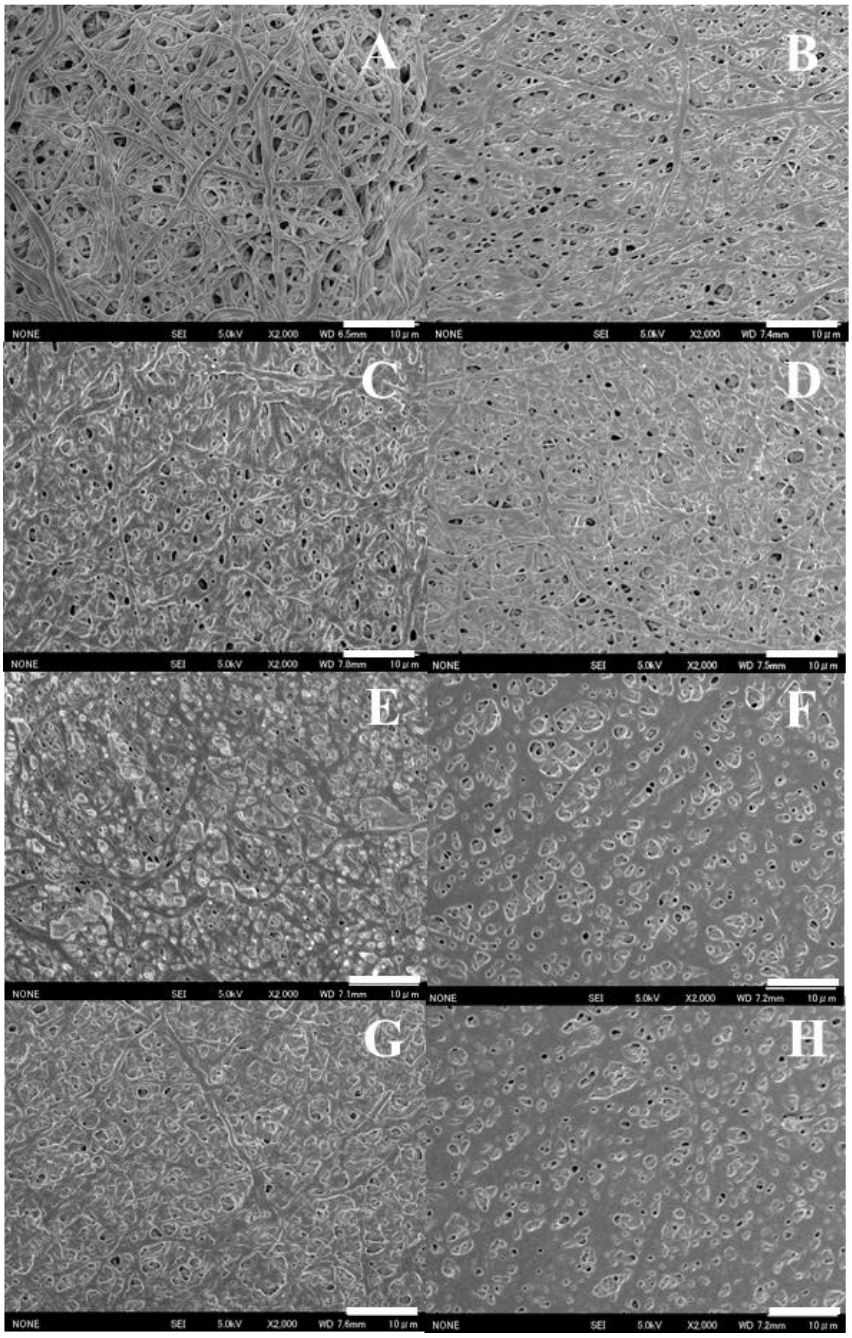

Figure 5. Swelling in PBS and water: (A, B) gelatin/ GTA cross-linked fabrics immersed in water for 30 and PBS for 60 min respectively. (C, D) gelatin/ glycerin/ GTA cross-linked fabrics immersed in water for 30 and PBS for 60 min respectively. (E, F) Heat treated gelatin/ GlcNAc fabrics immersed in water for 30 and PBS for 60 min respectively. (G, H) Heat treated gelatin/ glycerin/ GlcNAc fabrics immersed in water

for 30 and PBS for 60 min respectively; scale bar 10 microns. (*GTA fabrics in neutralized condition).

Figure 6 shows the thermogram of the gelatin at each test condition. The thermogram showed an initial weight loss of 10%, which was attributed to the loss of moisture. The initial dip at 100 °C in the thermogram of composite non-woven fabrics was due to moisture loss and thereafter it got straightened. This indicates that there was no phase change in the composite structure. Gelatin powder, gelatin/ GlcNAc fabrics heat treated and gelatin cross-linked with GTA showed similar results.

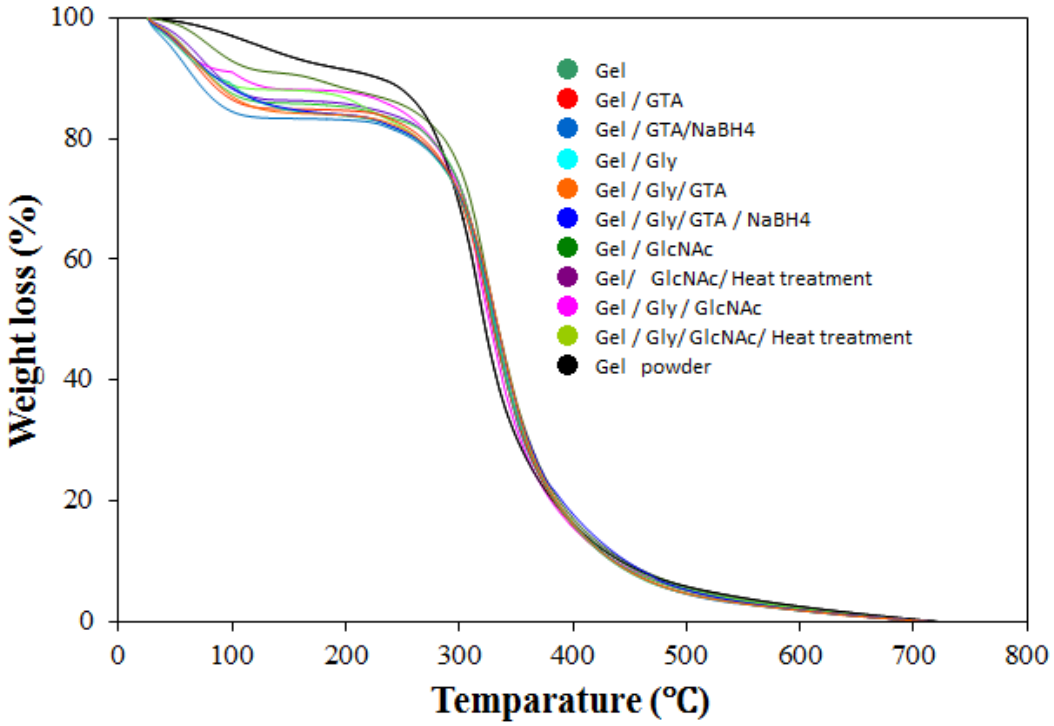

Figure 6. Thermogravimetric analysis of the gelatin non-woven fabrics at each condition.

Figure 7 shows the percentage cell viability observed on the samples after 24 and 48 hours evaluated by Alamar blue assay. All the samples showed more than 80% viability. Viability was slightly more for gelatin/ glycerin sample cross-linked with GTA vapors and further neutralized. Of cross-linking with GlcNAc, sample containing glycerin tends to show better viability. Cells tend to maintain its viability even after 48 hours indicating the cytocompatible nature of the developed scaffold. This study indicates that

all the samples showed a tendency for contributing towards cell proliferation on how much ever cells that had initially attached.

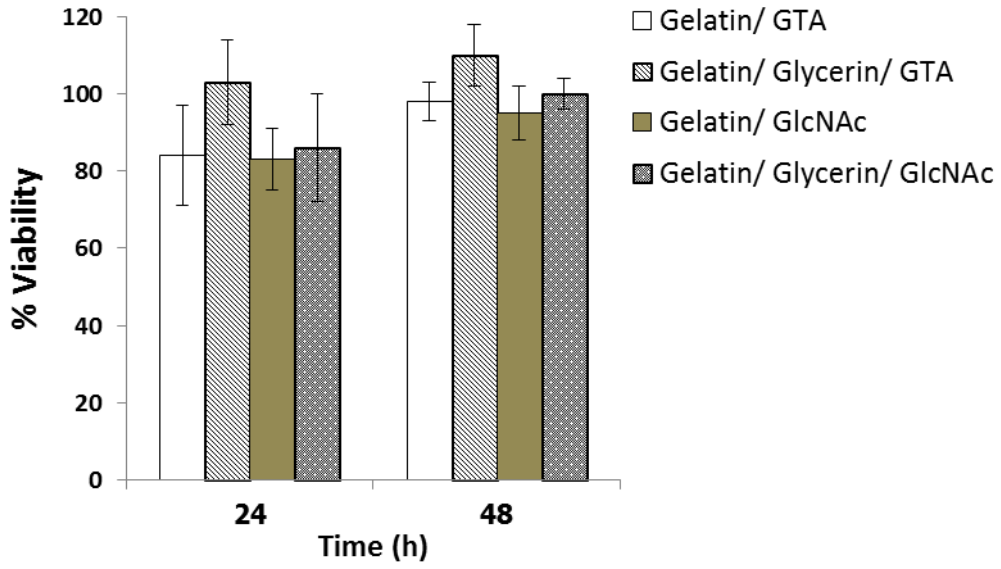

Figure 7. Cell viability determined by Alamar blue assay of cells seeded on gelatin nanofibrous scaffolds in comparison with control; (*GTA fabrics in neutralized condition).

Based on the cell attachment visualized by the actin staining of cells, all the samples showed appreciable cell attachment. hMSCs maintained their spindle shaped morphology in all the samples, but, the number of cell attached was slightly reduced in gelatin/ GlcNAc sample after heat treatment (Figure 8A). The addition of glycerin into this significantly changed the cell responsiveness. Elongation of hMSCs was observed in this sample (Figure 8B). Moreover good cell spreading was observed as cells were seen in most of the frames taken. GTA vapor cross-linking showed cell clumping in the field of vision but the morphology of cell was not affected (Figure 8C). The enhancement in cytocompatibility was evident after addition of glycerin to this scaffold (Figure 8D), where cells tend to spread in more area and elongation of cell was also seen.

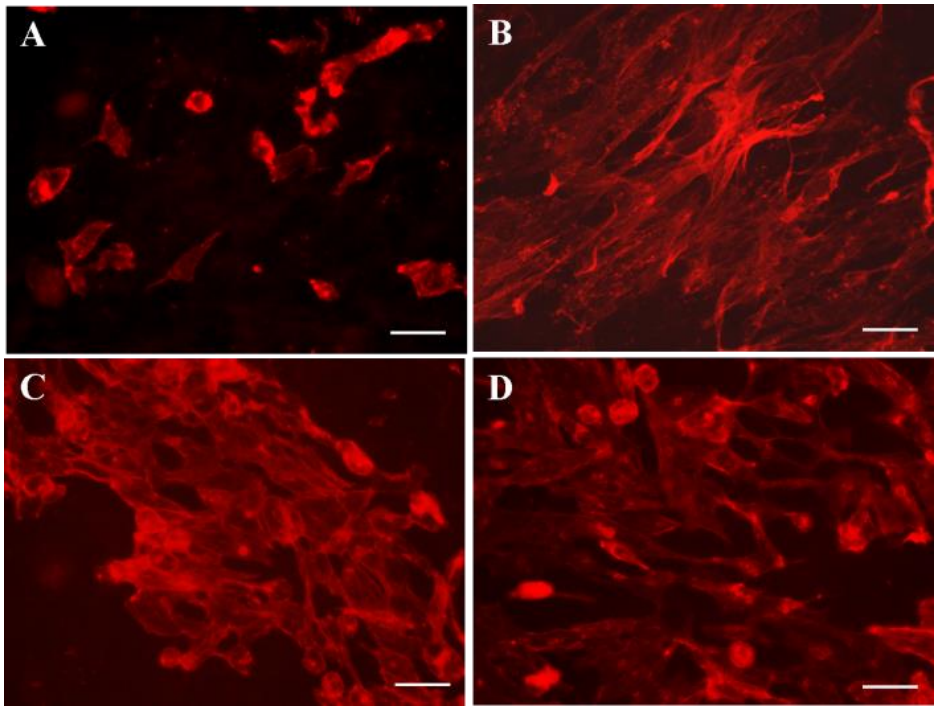

Figure 8. Actin staining of hMSCs depicting the cell attachment on the heat treated samples gelatin/ GlcNAc (A), gelatin/ GlcNAc/ glycerin (B) and GTA vapor cross-linked gelatin (C) and gelatin/ glycerine (D). Scale bar indicates 50 microns. (*GTA fabrics in neutralized condition).

II.3.4 Conclusions

Gelatin non-woven fabric has been prepared using GlcNAc and GTA as cross-linker by electrospinning method. Gelatin non-woven fabrics with 25% gelatin concentration showed fiber diameter in the nanoscale. In terms of mechanical property, the gelatin non-woven fabric with GTA cross-linking showed high mechanical property than the GlcNAc system. The swelling and water uptake ability in water and PBS showed that non-woven fabrics with GTA-cross linking has showed slight change in morphology. The thermogram of composite fibers indicates that there was no phase change in the composite structure. The toxicity induced by GTA cross-linking was could be controlled by neutralizing it with $NaBH_4$. In both the cross-linking methods addition of glycerin could further overpower the toxicity induced due to cross-linkers. From the cell studies conducted, it is evident that the developed gelatin fibers showed

good cytocompatibility and hence would find profound application in various tissue engineering.

II.3.5 References

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Chapter 4

Produce align fiber by electrospinning rotating rod

II.4.1 Introduction

Electrospinning is a simple and cost-effective technique which widely used to fabricate nano-scale fibers. With smaller pores and higher surface area when compare with regular fibers, electrospun fibers have been successfully applied in various fields, such as tissue engineering , filtration industrial, biomedical, pharmaceutical etc.[1]. Set up of electrospinning apparatus basically consists of three major components: syringe with metal needle (feeding unit), voltage power supply and a grounded collector [2]. The electric voltage is applied to the syringe which contain polymer solution, will create electrostatic force at the surface tension of polymer. The polymer jet will spread out in fiber forms from the syringe to the opposite charged collector when electric voltages which apply to the system is stronger than surface tension [3-4]. However continuous single nanofibers or uniaxial fiber are obtained because the unstable of current flow inpolymer jest which flight from the syringe tip to the corrector, effect to bending the direction of fiber jet [5]. Align electrospun polymer fiber shown the good properties than uniaxial fiber for example structural stability, mechanical property, support the formation of tissue of periodontal [6], control cellular organization [7] etc. Several techniques have been developed to create align electrospun nanofibers such as rotating drum, rotating disk, opposite needle, parallel bar etc.(Figure 1) [4] and some technique have been obtained [8]. It has been suggested that by rotating drum collector at very high speed than 1000 rpm, electrospun nanofibers could be aligned circumference [5]. However, many research found that only partial alignment of fibers could be found follow this setup. Figure 2 is one of the setup methods for electrospinning by give a gap between two parallel corrector which can generate aligned nanofiber. Due to electrostatic force effect to the fiber direction that crosses the space between parallel corrector, the aligned fiber was created. Figure 2B is the estimated electric field between the needle and the collector. The arrows refer to the direction of the electrostatic field in the system [9].

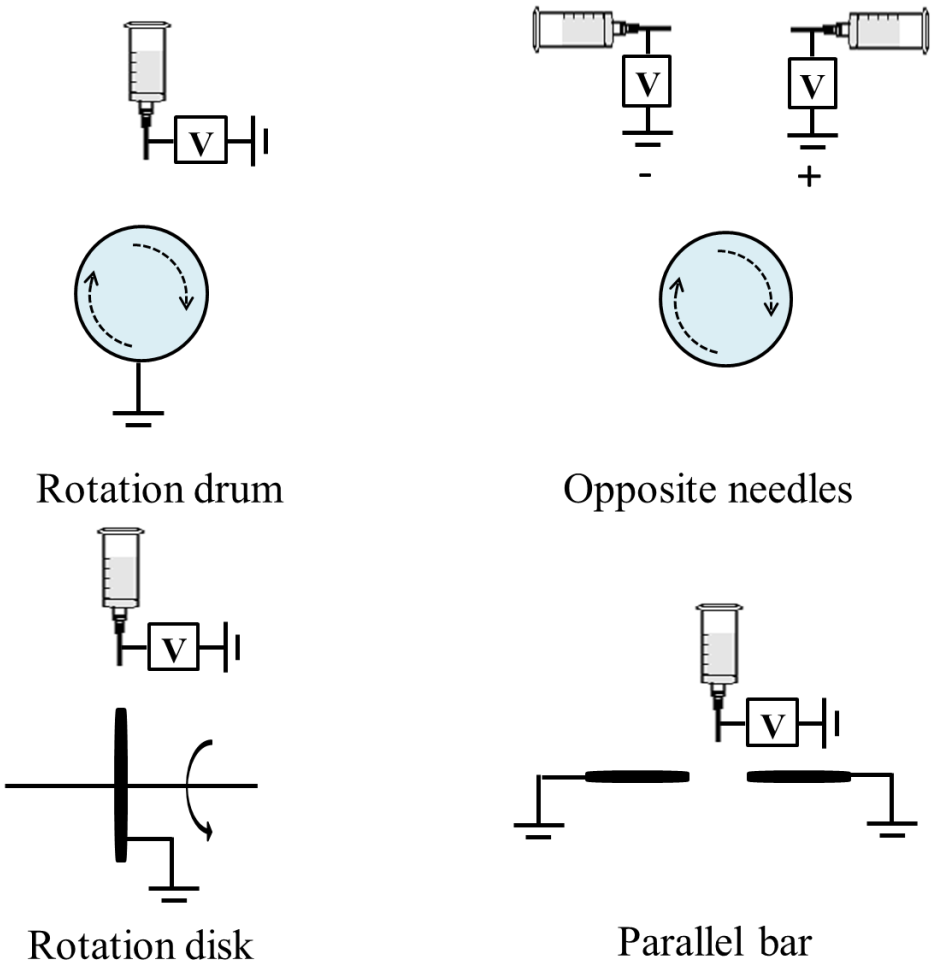

Figure 1. Example of techniques for electrospinning systems to produce aligned fibers [4].

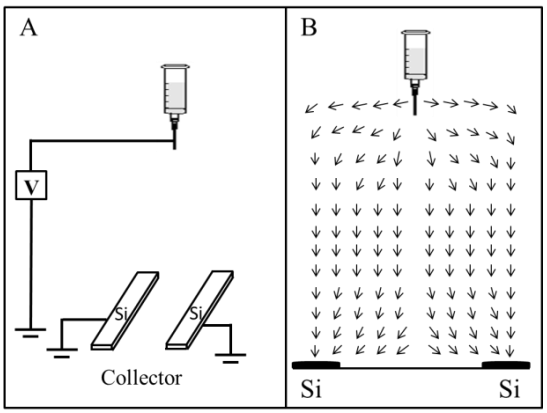

Figure 2. (A) Schematic illustration of the setup for electrospinning to generate uniaxially aligned nanofibers. (B) The collector contained two separated conductive silicon stripes.

Due to all of reason, we focus to develop of non-woven gelatin fabric to tubular structure especially align fiber by use the concept of rotating a drum (cylinder, rod) collector at very high rotating speed and by set up parallel electric bar beside of rotating rod expect to spin align fiber, find basic information and develop gelatin fabric material for use as artificial blood vessels or in the field of tissue engineering in the future.

II.4.2 Experimental

II.4.2.1 Material and instruments

Gelatin -JS200 pork skin type (Mw=100,000), Koei transformation Ltd. Figure 3 show the instrument of rod rotating electrospinning. The rotating rod collector (A) made from stainless diameter 1 cm. Spinneret unit including of syringe and temperature control (B). Motor Oriental Motor, 0-4000 rpm, Japan (C). Electric applied voltage (D).

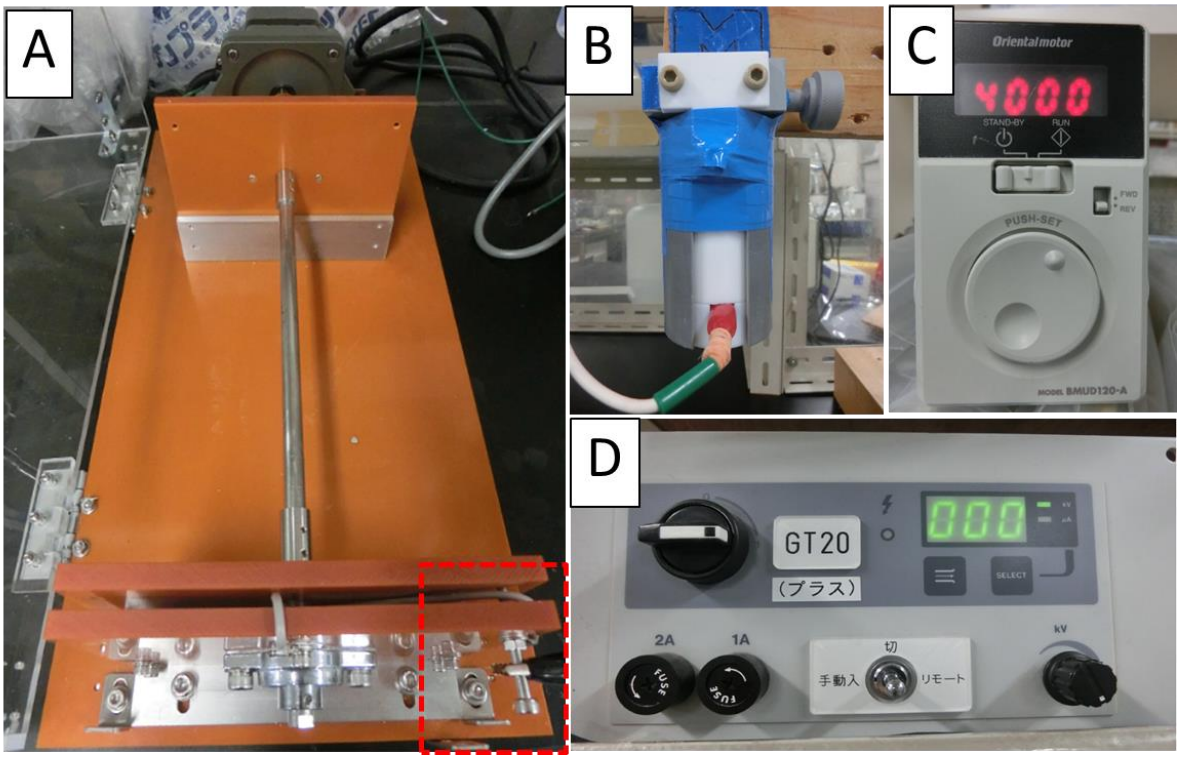

Figure 3. Rod rotating spinning equipment.

II.4.2.2 Preparation of gelatin solutions

Solution was prepared by mixing gelatin powder in distilled water for 25% gelatin solution (by weight). The mixture was covered and put in electric water bath at controlled temperature of 60 ± 2°C for 45 min (stirred at 15, 30 and 45 min) after that left in water bath for 45 min to remove entrapped air and obtain homogenous solution.

II.4.2.3 Electrospinning

II.4.2.3.1 Normal rod rotating setting (random fiber)

The homogeneous solution of gelatin was filled in a 5 ml syringe connected with a needle. Temperature was controlled inside the syringe at 45-60°C. The needle was connected with the high voltage power supply vary in range of 8-23 kV to the droplet of injected solution. The distance between collector and capillary tip was also varying to find the suitable condition. The rotating rod which connects to motor was use as collector. The collected fiber mats were left at least 3 h to ensure complete removal of solvent. Set up of rod rotating spinning is show in Figure 4. First, basic condition will be finding in order to evaluate the spin parameter including of distance between tips and rotating collector, suitable electric voltage and rod rotation speed.

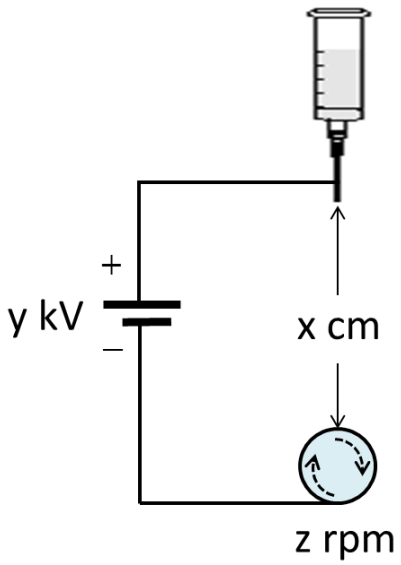

Figure 4. Rod rotating spinning equipment.

II.4.2.3.2 Parallel electric bar setting (align fiber)

Follow concept of introducing a gap into the conventional collector as Figure 2, pararell bar which connect to cathode electric has been set up at both side of rod rotating collector (Figure 5). Distance between both side of cathode bar and suitable electric voltage will vary for investigate the good condition which can create aligned nano fiber.

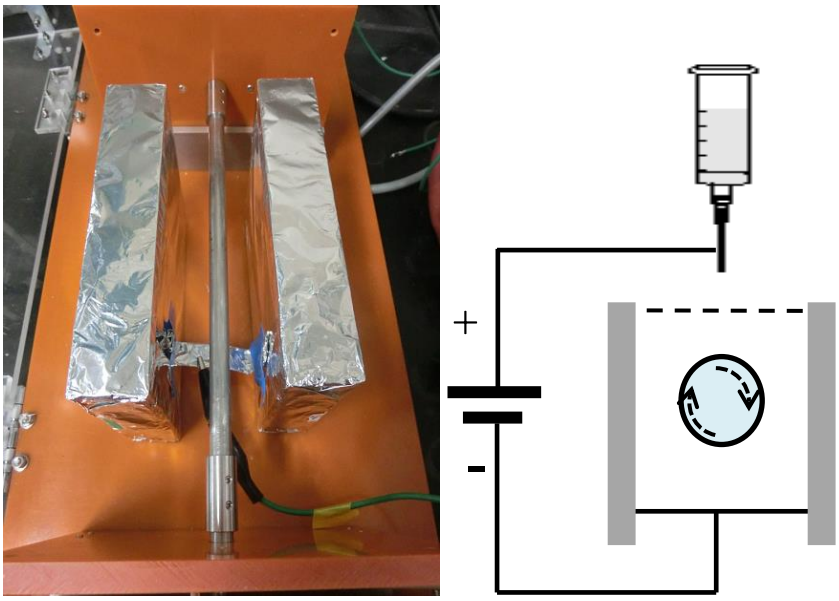

Figure 5. Rod rotating spinning equipment.

II.4.3 Results and Discussion

II.4.3.1 Normal rod rotating setting (random fiber)

Table 1 shows the effect of distance between nozzle tips and rotating rod collector at different applied electric voltage. Higher voltage requires more distance to obtain spun fiber which high productivity and smooth surface. The appropriate distance for spin fiber at 8, 16 and 23 kV is 3, 4 and 5 cm respectively. Because at the short distance, solvent is not completely vaporized results to dry fiber cannot be collected. In addition, High voltages are associated with higher productivity, which in turn draw more material out of nozzle [10].

Table 1. Relation between distance from nozzle tip to rod collector and applied electric voltage.

kV \ x cm	**1**	**2**	**3**	**4**	**5**
8	X	Δ	O	O	O
16	X	X	Δ	O	O
23	X	X	X	Δ	O

X - fiber doesn't dry, solution spread on the rod collector

Δ - fiber doesn't completely dry, low efficiency

O - good spun fiber and efficiency

From Table 1, spinning condition at 23 kV and distance 5 cm from nozzle tips to rod collector has been choose to investigate the effect of rotation speed by vary from 100-4000 rpm. In addition, the directions of rotation (clockwise–anticlockwise) also test to evaluate the diameter and direction of spun gelatin fiber. The results was show in Table 2, increase rotation speed results to little decrease average diameter of fiber and the direction of rotation given the same results. However align fiber cannot create follow this set up; the example of spun fiber was shown in Figure 6. There can confirm that when a conventional rotating mandrel is used, the polymer jet spreads over the entire length of the mandrel, resulting in relatively poor alignment of spun fiber [4].

Table 2. The effect of rotation speed to spun fiber at constant distance from nozzle tips to rod rotating collector at 5 cm. and 23kV applied electric voltage.

Rotation (rpm)	100	500	1000	2000	3000	4000
Diameter (nm)	~200					~180

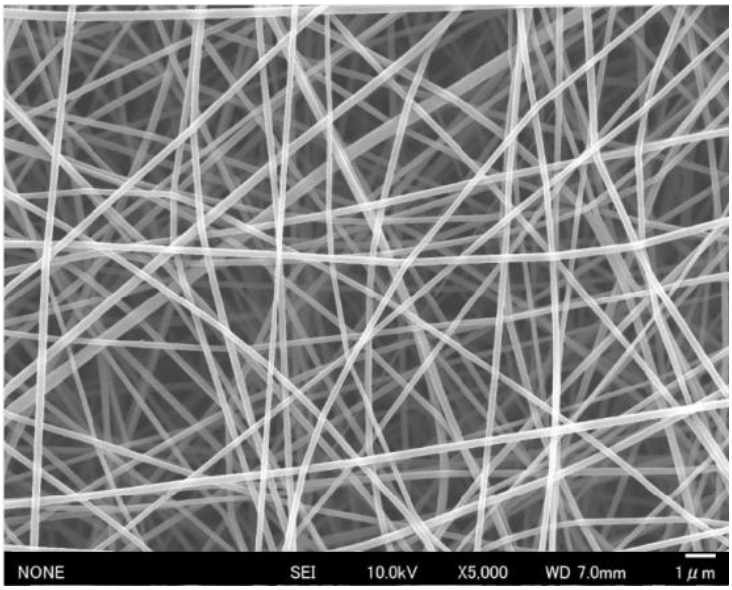

Figure 6. SEM image of gelatin spun nano-fiber at voltage 23 kV, rotation speed 4000 rpm, distance X = 5 cm.

II.4.3.2 Parallel electric bar with rod rotating setting (align fiber)

Figure 7 shows SEM image of spun gelatin nano fibers at constant electric voltage 23 kV and rotation speed=4000 rpm by varied distance between parallel cathode 2, 3 and 4 cm. All condition had average diameters in range of 200-250 nm**.** These images found that the gelatin were partial aligned at distance between parallel cathode 3 cm (Figure 7c and 7d). Electric voltage that applied to system induce electrostatic and interaction between different charge of fiber and collector, results to force the fiber's direction go straight to the ground collector and obtained aligned fiber [11]. The best alignment of the nanofibers that we found was obtained with 3 cm width of gap between parallel bar and rotating rod collector.

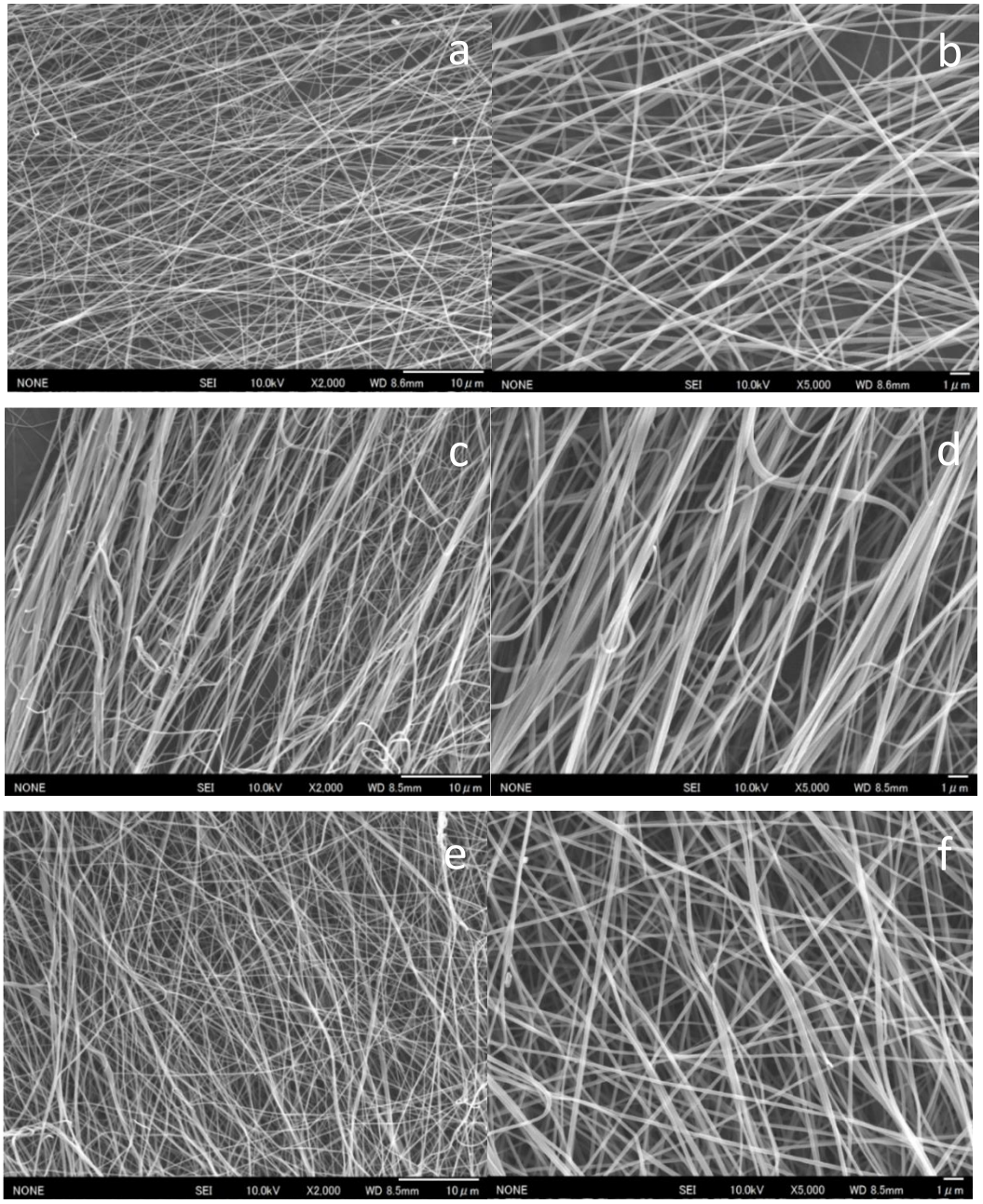

Figure 7. SEM image at difference of distance between rotating rod and cathode at constant electric voltage 23 kV and rotation speed=4000 rpm (a), (b) 2 cm at magnification 2000 and 5000 respectively, (c), (d) 3 cm at magnification 2000 and 5000 respectively (e), (f) 4 cm at magnification 2000 and 5000 respectively

From Figure 8, spinning condition at 4000 rpm and distance 3 cm of the parallel negative charge has been choose to investigate the effect of electric voltage by vary from 8, 16 and 23 kV. The results show in Figure 8, all condition had average diameters

in range of 200-250 nm**.** These images found that at 23 kV is still the good condition. This may came from at lower voltage; electrostatic forces were not strong enough to form continuous jet from the tip of the syringe needle. Then electric field between negative parallel bars was effect to motion of the fiber.

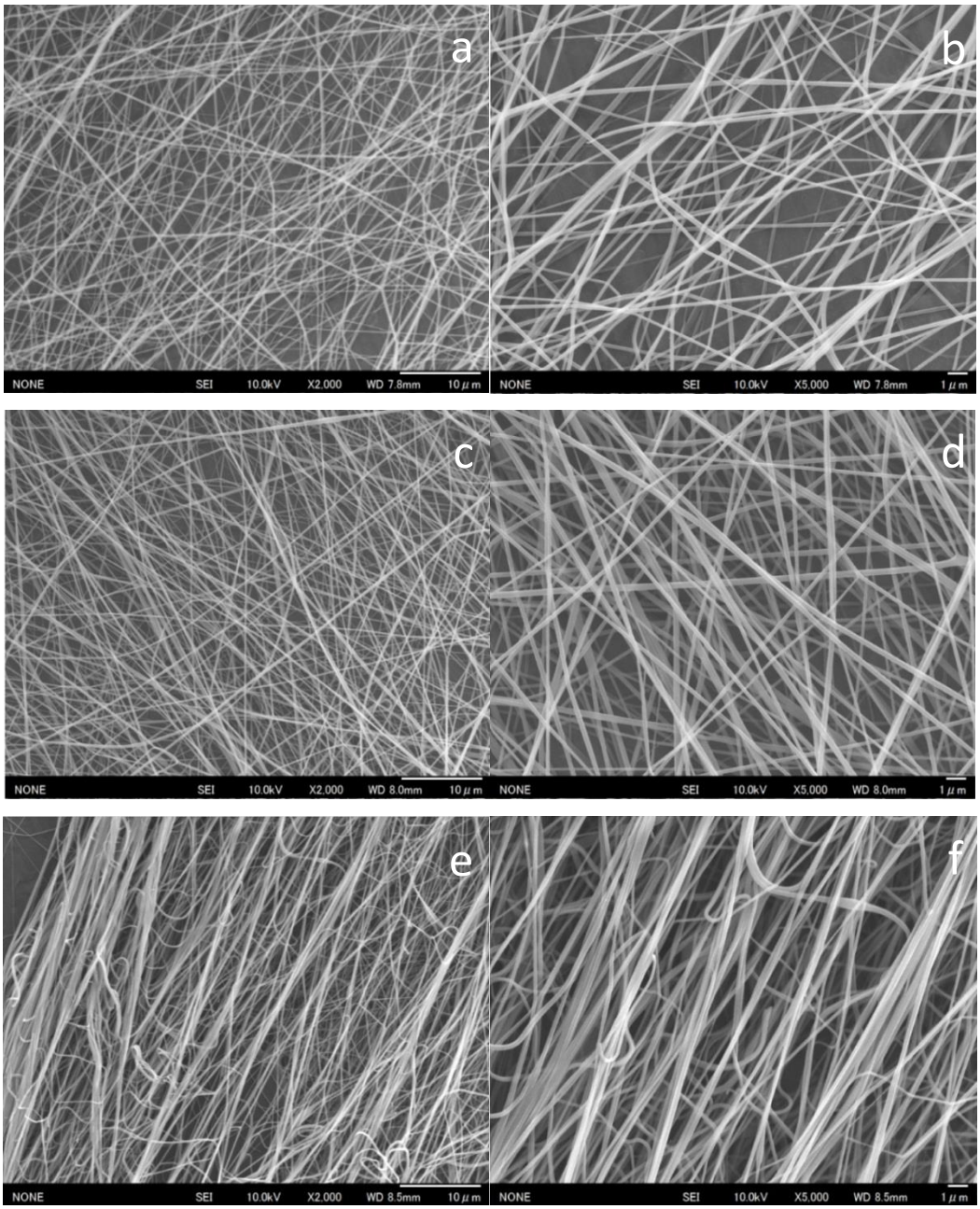

Figure 8. SEM image at difference of electric voltage, constant rotation speed=4000 rpm and distance between rotating rod and cathode = 3 cm (a), (b) 8 kV at magnification 2000 and 5000 respectively, (c), (d) 16 kV at magnification 2000 and 5000 respectively (e), (f) 23 kV at magnification 2000 and 5000 respectively**.**

II.4.4 Conclusions

Fabrication of gelatin nano-fibers tubular structure were prepared by electrospinning. The basic setting to create random fiber (nonaxially) and aligned fiber (uniaxially) had been found. Rotating a drum collector at very high rotating speed, relatively poor alignment of spun fiber. Electrospinning with a rotating drum collector consisting of parallel electric bar separated by gap was effective to prepare aligned fiber. The electrospinning conditions that gave the best alignment of are 4000 rpm and distance between parallel electric bar 3 cm and applied electric voltage 23 kV. The development of non-woven gelatin fabric to tubular structure is possible such as combine with the others polymer, expected to use as artificial blood vessels in the future.

II.4.5 References

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Section III

Gelatin sponge by freeze- drying

Chapter 5

Preparation and characterization of gelatin and composite gelatin sponge for biomaterial application

III.5.1 Introduction

In the field of tissue engineering and bone tissue engineering, the important elements are including of cell, growth factor and three-dimensional cell matrixes or scaffold [1]. The suitable scaffold containing growth factor should proper biodegradability including of discharge own matrix (scaffold and growth factor) and be as establishment for bone osteo-intergration which affects the stability of new implants [2]. Ideally, the completely degradation of scaffold is necessary in order to prevent the other reaction from the implant with original tissue [3]. In addition, size and porosity of scaffold are the important parameters which results to the supply of growth factor mechanism and degradation of scaffold itself ability [4]. Moreover, the morphology of pore size is strongly influent to implanted scaffold ability such as rate of tissue ingrowth [3]. From all of reason, in this research we focus to study in the scaffold element in form of sponge which used gelatin as a main material. The sponge form of gelatin has been well-characterized as a scaffold material for drug delivery systems [5] and as a matrix for osteoconductive calcium phosphate ceramics [6] in bone tissue engineering [7]. However the extraction process of collagen to produced gelatin, destructed natural crosslink in the collagen structure results to poor mechanical property of gelatin [8]. In order to improve the mechanical property and improve stability of gelatin scaffold (sponge) during implantation, gelatin scaffold usually react or stabilized with cross-linking agent [9]. We use Glutaraldehyde (GTA) and N-Acethyl-D-Glucosamine (GlcNAc) for crosslink with gelatin sponge. The objective of this study were the production, characterization and comparison the gelatin composite sponge with different crosslinking in many property by expected to use as the basic information and develop gelatin sponge in the field of biomedical or bone tissue engineering in the future.

III.5.2 Experimental

III.5.2.1 Materials

Gelatin -PGS 250-WIA (Mw~100,000), KOEI CHEMICAL.

Chitosan- FM-80, Koyo Chemical Co. Ltd.

Lysozyme ($5.3x10^4$units/ml)- Fluka company.

Phosphate Buffered Saline (PBS. pH 7.4)- Qualified, Life Technologies.

GlcNAc and GTA 25% solution were purchased from Wako Chemical Industries.

Others chemicals reagent were purchased from Wako Chemical Industries and used without further purification.

III.5.2.2 Preparation of Sponge

The composite sponge was prepared by dissolving gelatin, 5.6 w/w% chitosan solutions in acetic acid and GlcNAc into the DI water. The mixture was covered and put in the electric water bath at temperature 50 ± 2°C for 10 hrs. to remove entrapped air and obtain homogeneous solution. After 10 hrs. let the sample cool down at room temperature. Then, samples were freeze-dried to obtain the sponge. Samples were separate to different condition. First, cross link the sponge with GTA (sponge without GlcNAc) by vapor evaporate method for 48 hrs. After that, rinsing with methanol 30 min for 3 times. Another which have GlcNAc were heat at 100°C x 24 hrs. in the oven. The concentration of mixed samples and test condition which applied to each sample was shown in the Table 1. Thermogravimetric analyses of the scaffolds were carried out using TG/ DTA instrument (SII EXSTAR 6000) at a temperature range of 10-550°C. In addition, the chitin/ poly-butylene succinate (PBS) sponge was also prepared in order to compare the result with gelatin composite sponge in some properties. Chitin (α-Chitin; 85% degree of acetylation which obtained from Koyo Chemical Co. Ltd., Koyo, Japan.) was prepared in methanol saturated with $CaCl_2$ to obtain chitin solution (2% w/v). PBS (powder was purchased from Sigma Aldrich, St. Louis, MO, USA) was dissolved in chloroform (0.5% w/v). Different ratios of chitin and PBS solutions were mixed to form gels of 10%–30% v/v of PBS. The solutions were blended under heating, and stirred continuously until a gel was formed; the latter was dialyzed against distilled water for 3 days, and freeze drying for 24 h.

Table 1. The preparation of chitosan/ gelatin.

Condition	5% gelatin solution			
	gelatin	chitosan	GlcNAc	chitosan + GlcNAc
Heat treatment at 100°C x 24 hrs.	X	X	O	O
GTA vapor cross-link 48 hrs.	O	O	X	X
Chitosan 0.8%*	X	O	X	O
GlcNAc 5%*	X	X	O	O

* In relation to the gelatin mass, X = Not detect and O = Detect

III.5.2.3 Porosity and Pore size

Porosity of the composite sponges was determined using liquid displacement method [10]. Sample dimensions were measured using a vernier calliper and volume (V) was calculated. Briefly, samples of measured weight (Wi) were soaked in a known volume of ethanol for 24 hrs. to allow ethanol to penetrate into the pores of the sample. The final weight of the sample was noted as Wf. Porosity was calculated using the following equation.

$$\% \, porosity = \left[\frac{(Wf - Wi)}{\rho_{\,\mathrm{EtOH}}\, x\, V}\right] x\, 100$$

ρ_{EtOH}*: density of ethanol*

In addition, pore size of composite sponge was obtained from calculation.

III.5.2.4 Swelling

The swelling was studied in Phosphate Buffer Saline (PBS, pH 7.4). Dry weight of the composite sponges was noted as Wi. Samples were immersed in PBS at 37◦C for 24 hrs. and then taken out and wet weight was taken as Wf, after removing the excess water with filter paper. Swelling ratio was determined using the formula:

$$Swelling\ ratio = \frac{Wf - Wi}{Wi}$$

III.5.2.5 Biodegradation

In vitro biodegradation of the composite sponge was studied in PBS with lysozyme at 37◦C. Initial weight (Wi) samples after incubated at different time points were taken out and washed with deionized water to remove the salts and freeze dried. Dry weight of the samples was noted as Wd. Degradation percentage was calculated using the equation:

$$\%Degradation = \left[\frac{(Wi - Wd)}{Wi}\right] x\ 100$$

III.5.3 Results and Discussion

III.5.3.1 Preparation of Sponge

Figure 1 shown the sponge after preparations. From different condition; cross link the sponge after freeze-dried with GTA for 48 hrs. [(Figure 1a) gelatin/ GTA 48 hrs. and (Figure 1b) gelatin/ chitosan/ GTA 48 hrs.] and GlcNAc with heat treatment 100°C x 24 hrs. [(Figure 1c) gelatin/ GlcNAc/ 100°C x 24 hrs. and (Figure 1d) gelatin/ Chitosan/ GlcNAc/ 100°C x 24 hrs.]. The sponge with heat treatment condition showed the color more brown than crosslink with GTA condition according to Maillard reaction, which produces browning compounds due to the interactions between carbonyl group of GlcNAc and amino compounds of chitosan and gelatin. The surface morphology of the composite sponges was shown by SEM image (Figure 1a-1d). In chitin/ PBS system (Figure 1e-1g), increase concentration of PBS results to reduced porosity of the scaffold. However, all of composite sponges showed an interconnected porous structure in the micrometer scale. An optimum porosity is a prerequisite for better gas and nutrient exchange. The macro-porous nature of the scaffold also aid in proper cell infiltration, and a homogeneous cell-laden construct could be achieved. Figure 2 shown thermogram of the composite sponge. The thermogram of all sponge showed an initial weight loss of 10%, which was attributed to the loss of moisture. The initial dip at 100 ◦C in the thermogram of composite scaffolds was due to moisture loss and thereafter it got straightened. This indicates that there is no phase change in the composite structure.

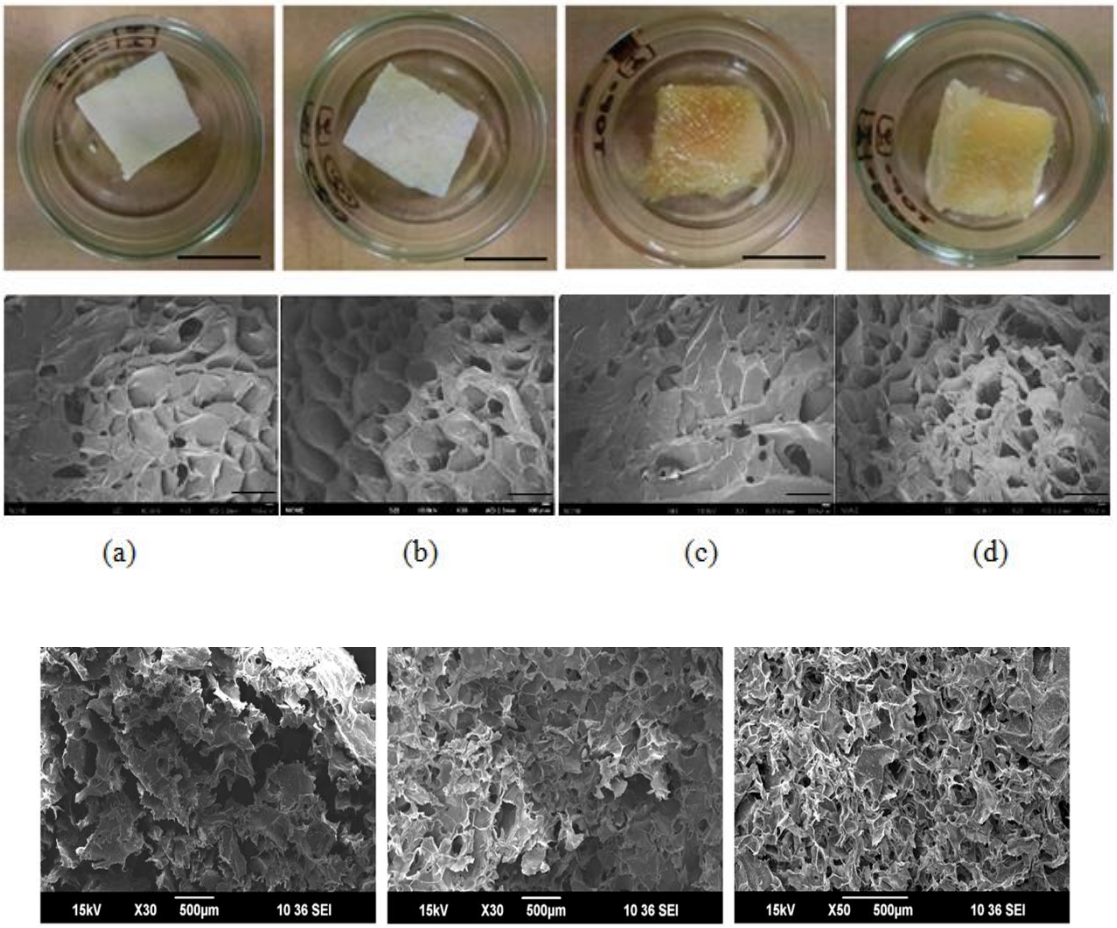


Figure 1. (a) gelatin/ GTA 48 hrs, (b) gelatin/ chitosan/ GTA 48 hrs., (c) gelatin/ GlcNAc/ heat treatment 100°C x 24 hrs. and (d) gelatin/ chitosan/ GlcNAc/ heat treatment 100°C x 24 hrs, (e) chitin / PBS 10%, (f) chitin/ PBS 20% and (g) chitin/ PBS 30%. Scale bar represent 1.5 cm and 500 μm for sponge figure and SEM image respectively.

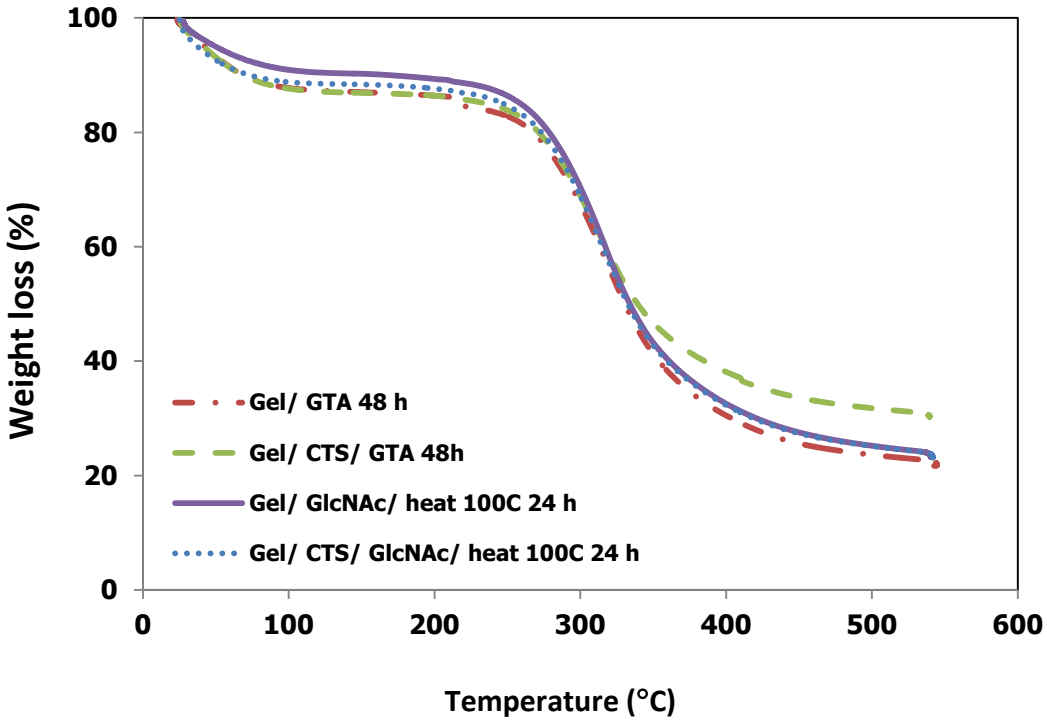


Figure 2. Thermogravimetric analysis of the composite sponge.

III.5.3.2 Porosity and Pore size distribution

The porous nature of GTA and GlcNAc composite sponges were evaluated, GTA group showed porosity lower than 50%. Whereas the GlcNAc sponges showed higher porosity than 60%, especially gelatin/ chitosan/ GlcNAc/ heat treatment 100°C x 24 hrs. sponge showed the highest porosity 82% (Figure 3). A highly porous structure is a very beneficial property for a tissue engineering material. The pore size range of 4 composite sponge was 60 - 700 μm and the mean pore size for was 220, 340, 150 and 210 μm for gelatin/ chitosan/ GTA 48 hrs., gelatin/ GTA 48 hrs., gelatin/ chitosan/ GlcNAc/ heat treatment 100°C x 24 hrs. and gelatin/ GlcNAc/ heat treatment 100°C x 24 hrs. respectively (Figure 4). GTA cross linked group sponge shown the higher pore size than GlcNAc group.

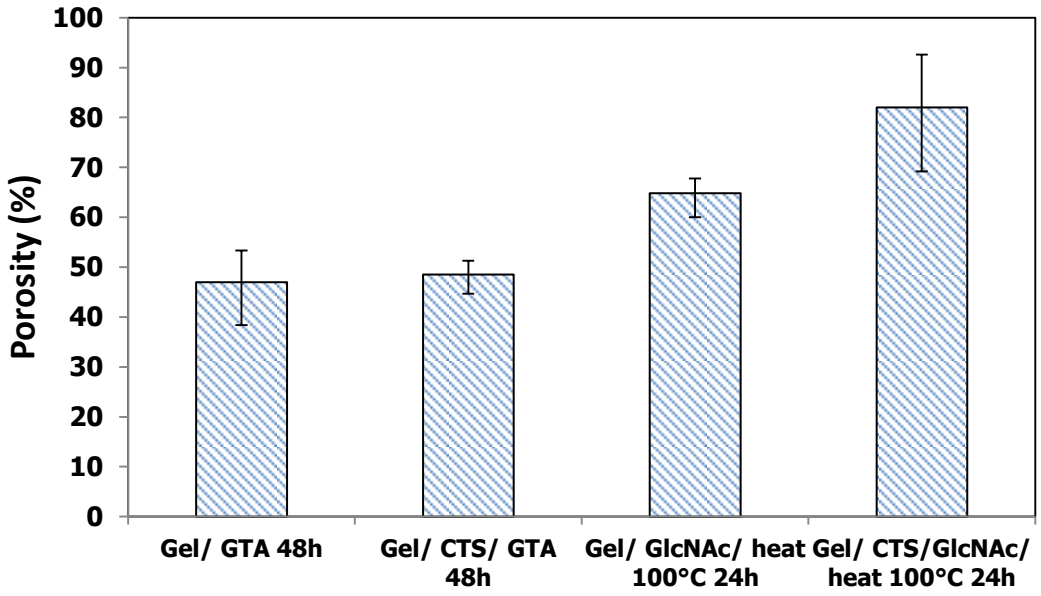
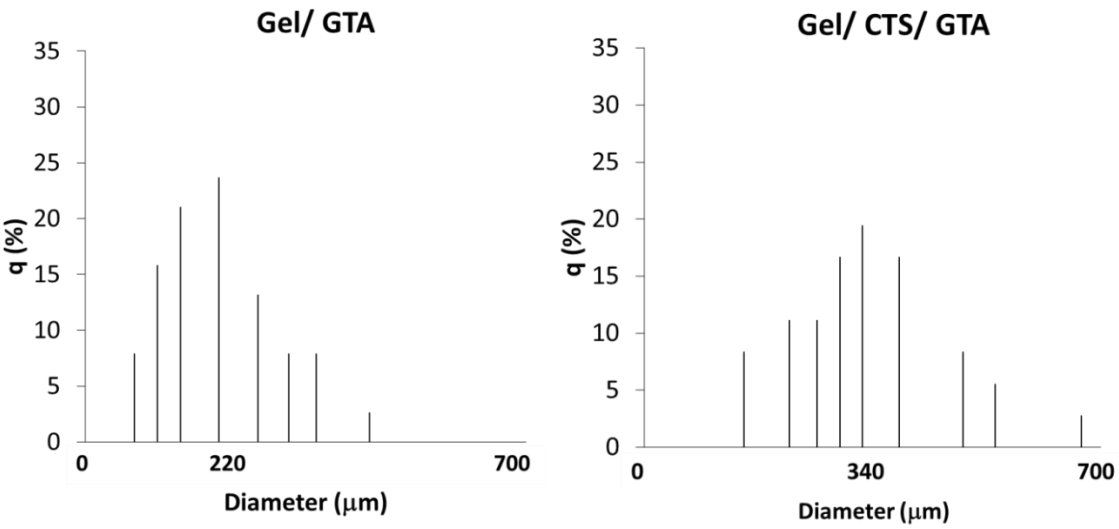


Figure 3. Porosity studies of composite sponge.



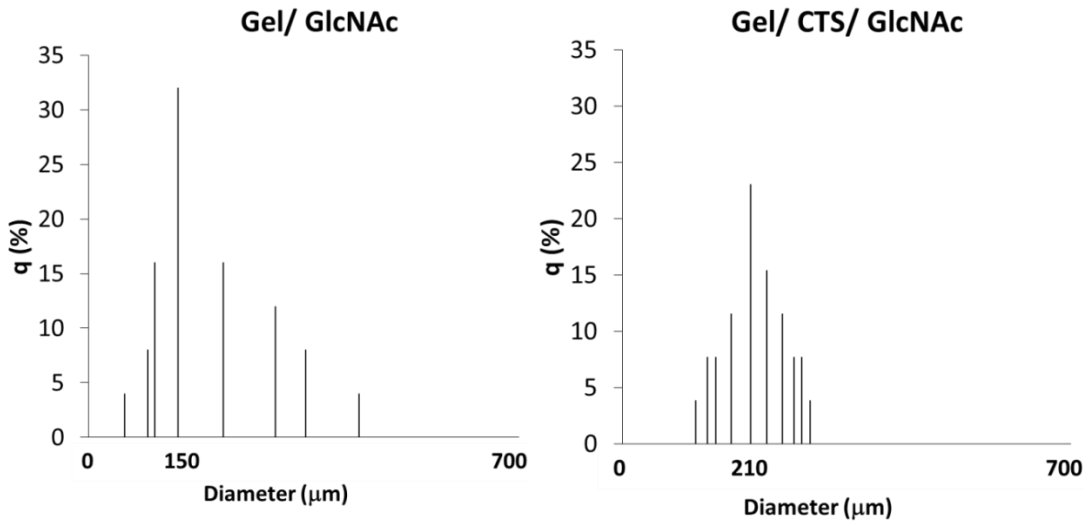


Figure 4. Pore size distribution of composite sponge.

III.5.3.3 Swelling

The swelling capacity of PBS was showed in Figure 5. A higher swelling ability was observed in chitin system and Gel/ GlcNAc than GTA sponges group. This is due to the higher porosity of sponges as compared to the GTA sponges group which confirm the results from Figure 1 and Figure 3. In addition, the swelling behaviors of gelatin, chitin and chitosan are depends on number of hydrophilic groups like hydroxyl, carboxyl and amino groups which effect to swelling ability [11].

III.5.3.4 Biodegradation

Enzymatic degradation behavior of the composite sponges was studied by incubating the samples in solution containing lysozyme at 37◦C for 7, 18 and 25 days. Percentage degradation of the composite sponge as a function of time is shown in Figure 6. GlcNAc composite sponges showed the higher degradation rate after 25 days, can be attributed to the comparatively higher swelling and porosity of the composite sponges which makes the GlcNAc group available for the action of lysozyme [12].

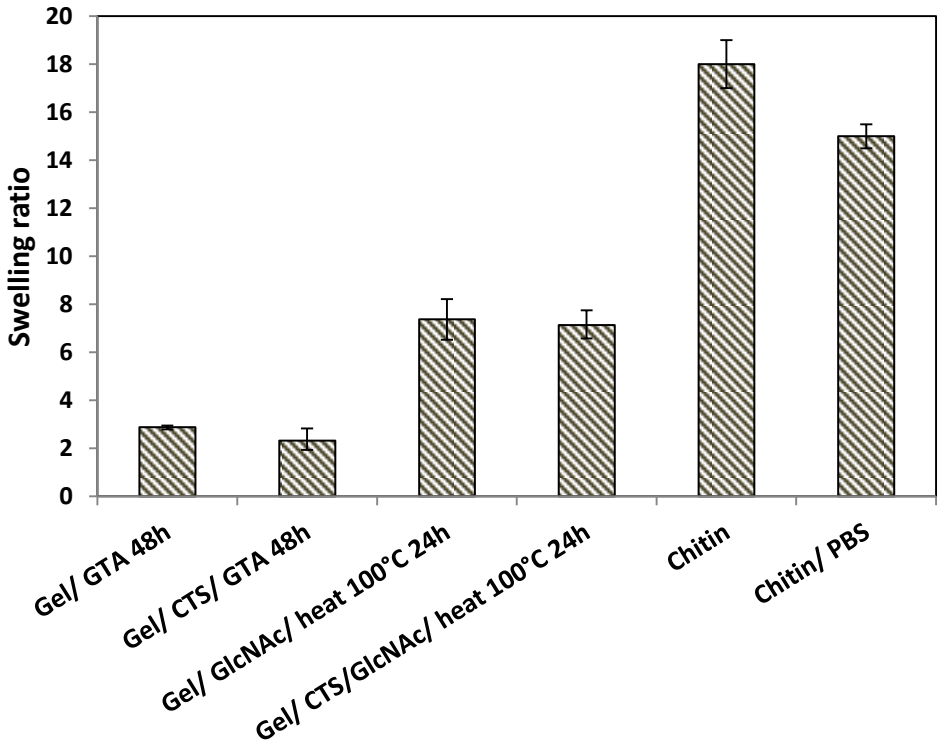


Figure 5. Swelling ratio in PBS.

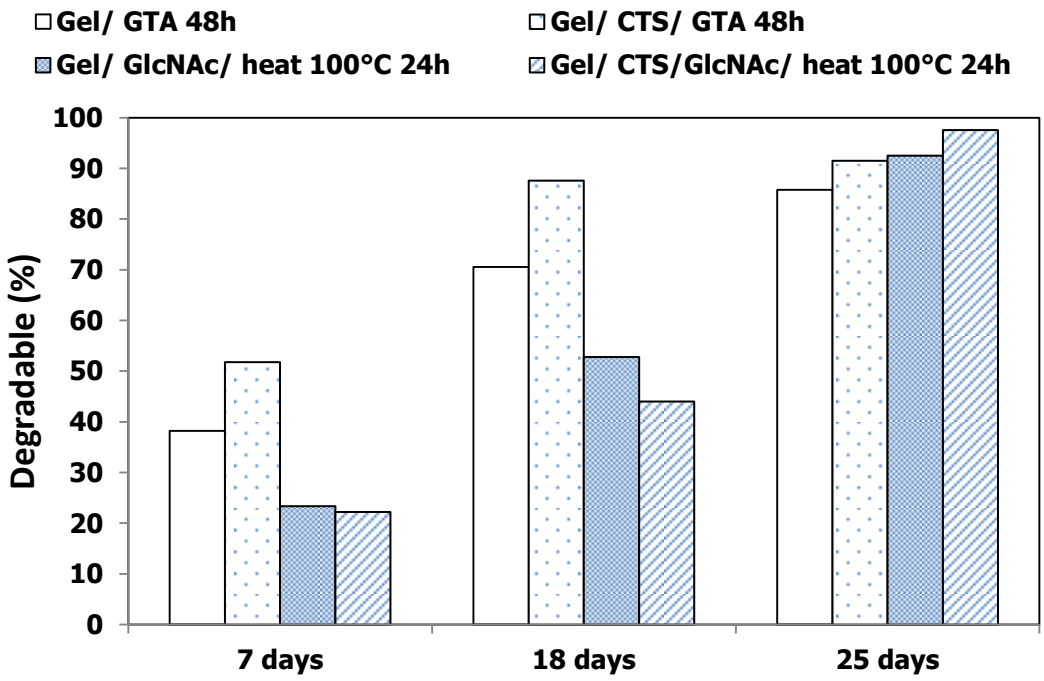


Figure 6. Biodegradation studies of composite sponges in PBS containing lysozyme.

III.5.4 Conclusions

The gelatin/ chitosan composite were prepared by using GlcNAc and GTA as cross-linker into the form of a sponge by freeze dried. The results from SEM observation shown that the interconnected porosity of each composite sponge was well demonstrated. Thermogravimetric indicated that there is no phase change in the composite structures all of sponge. Swelling ratio and degradation rate of composite sponge which prepared with GlcNAc system were higher, due to higher porosity of the composite sponges. The comparison of gelatin composite sponge with chitin/ PBS sponge, chitin / PBS showed the higher swelling ratio due to high porosity which can observe from SEM image. These results may come from the different solvent and preparation method. However the pore size of both sponge system were in the micro scale.

III.5.5 References

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Chapter 6

Adsorption and desorption behavior of BSA on gelatin/ chitosan sponge

III.6.1 Introduction

Gelatin is a mixture of peptides and proteins that derived from collagen. Treatment by acid or base hydrolysis of collagen as known in the commercial name of gelatin type A or B respectively. Gelatin is biocompatible when it takes in human and animal body, it shows low antigenicity [1] in contrast with collagen that antigenicity according to its animal origin [2-3]. In addition, chitosan has been studied as biomaterial, food, and chemical industries because its good biocompatibility, biodegradability, hemostasis etc. [4-9]. It's has been used in form of nanofibers, scaffolds, membranes, sponges etc. [10]. Chitosan/ gelatin sponge with the others materials has been successfully prepare for wound dressing [11], tissue engineering [12], bone tissue engineering [13] and vital organ engineering [14] etc.

In this research, we focus to study about gelatin/ chitosan sponge by cross-link with N-Acetyl-D-glucosamine (GlcNAc) and Glutaraldyhude (GTA). According to Maillard reaction, carbonyl group in GlcNAc (and others reducing suger) can react with amino group in gelatin occur crosslink reaction which create browning compounds [1]. GTA is commercial availability, low cost and its high reactivity. It reacts rapidly with amine groups at around neutral pH condition [15-16]. Protein adsorption is very important in the field of biomedical research. The interaction between protein and surface material that occur for adsorption can determine by a) changes in the hydration of protein molecule and material's surface, b) electrostatic interaction and c) structure rearrangement in the adsorbing protein molecule [17-18]. We used Fluorescein isothiocyanate labeling of Bovine serum albumin (FITC-BSA) for protein adsorption as a model instead of growth factor in this study. FITC is one of the simplest and most commonly used reagents for labeling proteins. The isothiocyanate reactive group (–N=C=S) of FITC can form bonds with amine on proteins (Figure 1).

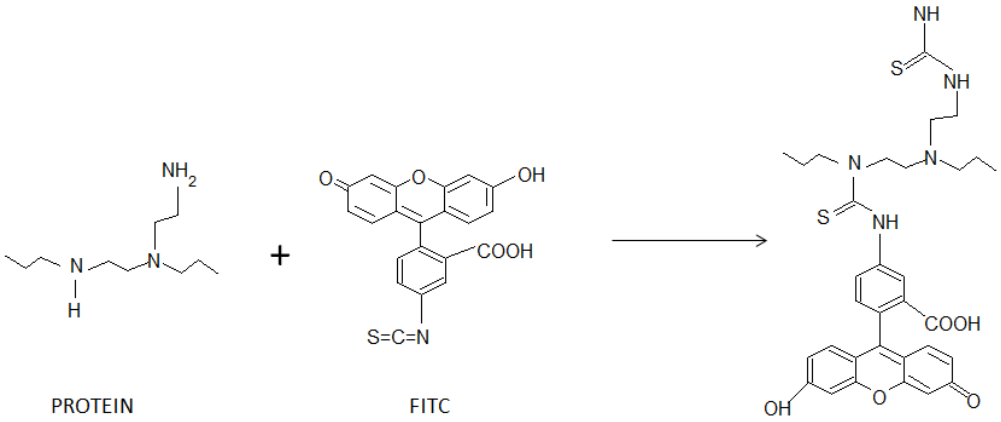

Figure 1. FITC labeling of protein structure.

The effect of concentration, temperature and pH to adsorption and desorption behavior of sponge were investigated. The adsorption constant and thermodynamic parameter was also evaluated in order to use the data as basic information of gelatin/ chitosan sponge to apply or improve property in field of biomaterial.

III.6.2 Experimental

III.6.2.1 Materials

Gelatin: PGS 250-WIA was purchased from KOEI CHEMICAL.

Chitosan : FM-80 was received from Koyo Chemical Co. Ltd.

BSA fraction V, 96% was purchased from Sigma-Aldrich Co.

GlcNAc, Phosphate Buffer Saline (PBS, pH 7.4), FITC (≥ 95%) were purchased from Wako Pure Chemical Industries, Ltd.

III.6.2.2 Preparation of FITC-BSA

BSA was labeled with FITC for used as protein adsorption model in order to avoid the effect of gelatin adsorption at the same wavelength of BSA. First, 3 mg/ml BSA solution was prepared in 0.1 mol/ l carbonate buffer (pH = 9.0). Mixing with 1 mg/ ml FITC solution in Dimethyl sulfoxide (BSA solution 1 mg/ ml: FITC solution 100 μg) keep overnight at 4^{o}C. To remove any uncoupled FITC, use dialysis against water [19]. The light absorption at 494 nm by UV-Vis spectrophotometer was finally below 0.003 for the supernatant. The concentration and F : P ratio were determined according to the

methods described by the manufacturer (Thermo Scientific, Tech tip #31). In every preparation step have to avoid the light.

Calculate molarity of the protein:

$$Protein\ concentration\ (M) = \frac{A280-(Amax \times CF)}{\varepsilon} \times dilution\ factor \text{ ……..(1)}$$

ε = protein molar extinction coefficient (BSA ~43,824 M-1 cm-1@280nm)
A_{max} = Absorbance (A) of a dye solution measured at the wavelength maximum (FITC = 494 nm)
CF = Correction factor; adjusts for the amount of absorbance at 280 nm cause by dye (FITC = A280/ A494nm)

Calculate the degree of labeling:

$$Moles\ dye\ per\ mole\ protein = \frac{Amax\ of\ label\ protein}{\varepsilon' \times protein\ concentration\ (M)} \times dilution\ factor \text{ …(2)}$$

ε′ = molar extinction coefficient of the fluorescent dye (BSA ~68,000 M-1 cm-1@494nm)

III.6.2.3 Adsorption

The adsorption sponge was determined by immersing the sponge ~ 0.1g in FITC-BSA in PBS until reaching adsorption equilibrium, the solution were sampling and measured by using a Spectrofluorometer at excitation and emission wavelength 495 nm and 565 nm respectively. Characterize the absorbance peaks and estimate the BSA concentration by use of a predetermined standard concentration–intensity calibration curve. FITC-BSA was varying at 0.1-4.0 mg/ml in PBS pH 7.4 at room temperature have been investigated to study the effect of solution concentration. And same concentration of FITC-BSA with different temperature at 10, 25 (R.T.), 37, 50 and 70°C, also carried out to study the effect of temperature to adsorption efficiency. Adsorption constant and Thermodynamic parameter was evaluated.

III.6.2.4 Desorption

Similarly, the release study was also carried out for the same FITC-BSA loaded on the sponge after wash in DI water, applied freeze-dried again to sponge. Immerse adsorbed sponge in 25 ml of PBS pH 7.4, 37°C. The solution were sampling and measured by using a spectrofluorometer as the same method with adsorption experiment. The effect of adsorbed concentration in sponge and pH (7.4, 4.0 and 2.0) of solution to %desorption have been investigated.

$$\%\ Desorption = \left[\frac{conc.release\ at\ time}{FITC\ labeling\ BSA\ adsorb\ in\ sponge}\right] x\ 100 \text{.....(3)}$$

III.6.3 Results

III.6.3.1 Preparation of FITC-BSA

A typical UV-Vis spectrum of FITC in PBS is shown in Figure 2. For determined the correction factor of FITC, the maximum peak of FITC at about 494 nm and 280 nm which is absorption wavelength of BSA protein. So, the correction factor for calculate value of FITC-BSA by eliminate amount of absorbance which cause by dye is 0.12014/ 0.34737 = 0.345. Protein concentration and ratio of labeling were $2.965x10^{-5}$ (eq.1) molar and 4.025 (eq.2) respectively.

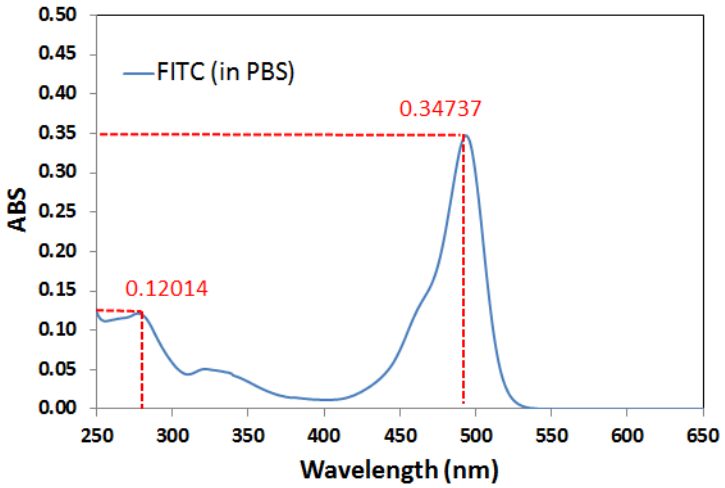

Figure 2. Typical UV-Vis spectrum of FITC in PBS.

III.6.3.2 Absorption

Figure 3 show the measure condition of spectrofluorometer (up) and the calibration curve of FITC-BSA (down) which have R^2=0.9916.

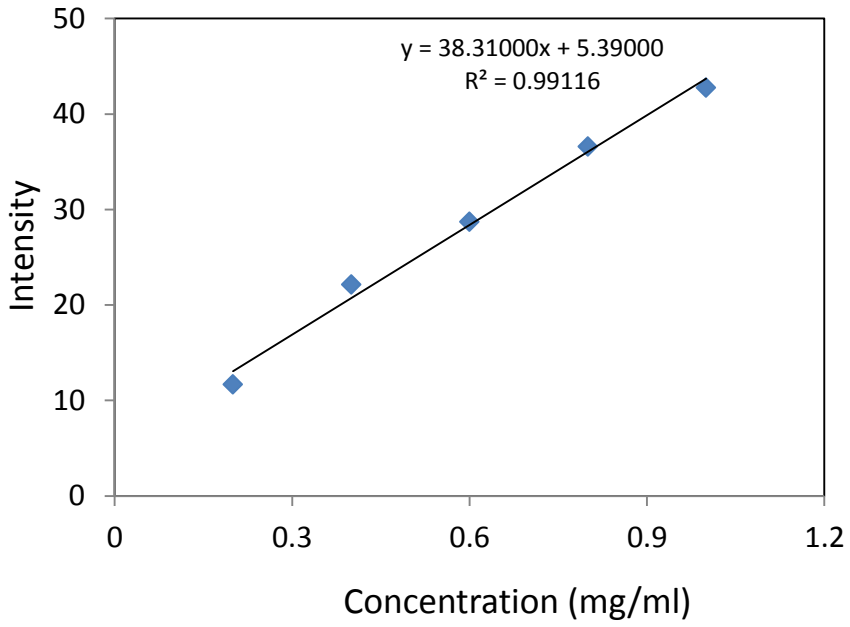

Figure 3. Typical UV-Vis spectrum of FITC in PBS.

III.6.3.2.1 Effect of concentration

Figure 4 show that when concentration of solution increased, the sponge can absorbed amount of FITC-BSA increased until reach the equilibrium around 30 and 12 mg/ g of sponge for GlcNAc and GTA crosslinking group respectively. When compared between gelatin sponge and gelatin/ chitosan sponge in each GTA and GlcNAc crosslinked (composition and preparation sponge were showed in chapter 5), both composite didn't shown too much different in adsorption amount.

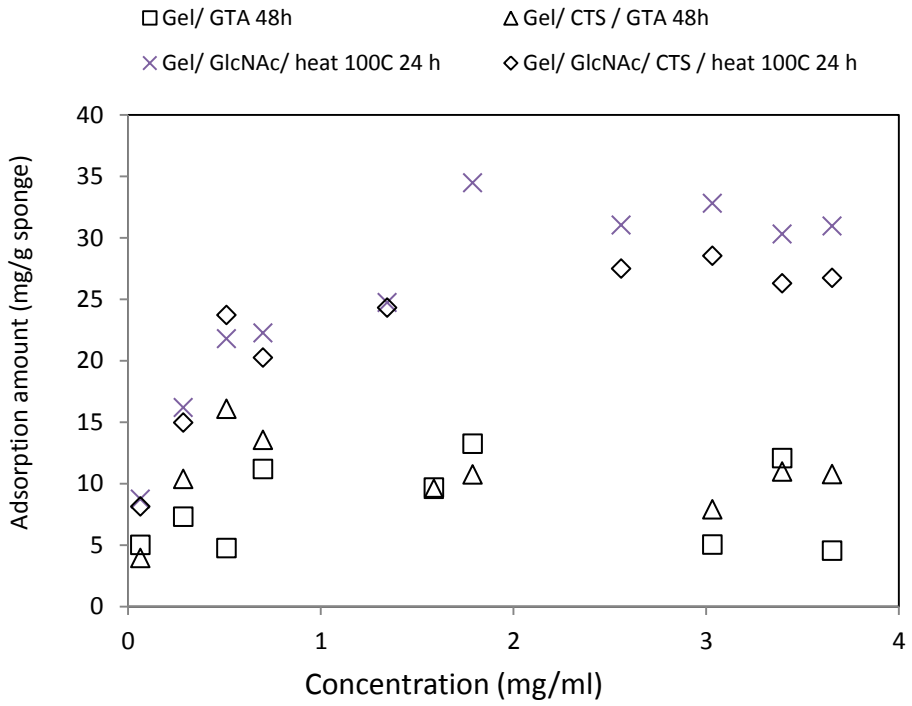

Figure 4. The effect of FITC-BSA's concentration to adsorption amount of sponge.

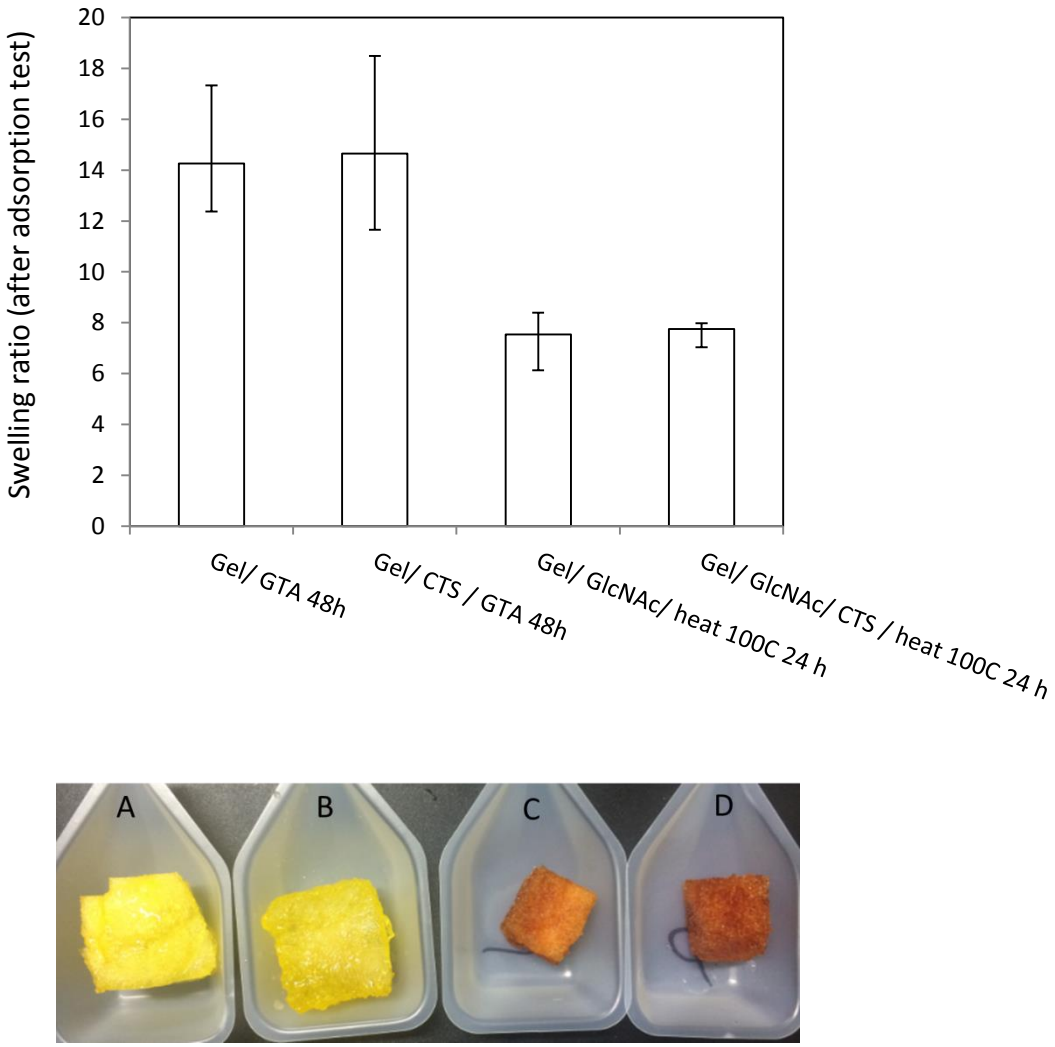

Figure 5. The swelling ratio of sponge after adsorption test (up) and photo of swelling sponge (down); A) gelatin/ GTA 48hrs., B) gelatin/ chitosan/ GTA 48hrs., C) gelatin/ GlcNAc/ heat treatment 100°C x 24 hrs. and D) gelatin/ chitosan/ GlcNAc/ heat treatment 100°C x 24 hrs.

Figure 5 shown the swelling ratio of sponge after adsorption test and photo of swelling sponge, GTA crosslinking group sponge shown high swelling ratio than GlcNAc group for 2 times. This may indicates that GlcNAc crosslinking in these sponge effects to more strong structure and reduced chain flexibility in the sponge. Langmuir and Freundlich equation usually used as model for describe the adsorption system. In this research, the Langmuir adsorption isotherm model was the assumption that the

adsorption behavior occur on a surface by monolayer adsorb. Hanes-Woolf plot is commonly used by several researches because of the minimized deviation from the fitted equation resulting in the best error distribution [20].

Langmuir's Hanes-Woolf plot equation:

$$\frac{C_e}{q_e} = \frac{1}{K_L q_m} + \frac{C_e}{q_m} \text{.....(4)}$$

where q_e (mg/g) is the adsorption amount of adsorbent at equilibrium,
q_m (mg/g) is the maximum adsorption amount of FITC-BSA on gelatin sponge,
C_e (mg/ml) is the equilibrium concentration of adsorbate in solution,
and K_L (ml/mg) is the equilibrium adsorption constant.

Figure 6 shows the straight line obtained plotting C_e/q_e versus C_e from the experiment data follow Langmuir's Hanes-Woolf plot equation. K_L and q_m can calculated from slope of plot; value of each sponge are given in Table 2. Experiment data were good fitting with isotherm especially in GlcNAc crosslinked sponge due to $R^2 \geq 0.99$ in both sponge. The maximum adsorption capacity (q_m) for gelatin and gelatin/ chitosan were 32.679 and 27.932 mg/ g respectively. The equilibrium adsorption constant (K_L) for gelatin and gelatin/ chitosan were 5.773 and 9.675 ml/ mg respectively. For GTA crosslinked sponge, although the plots of Ce/ qe vs. Ce were straight lines but the values of the Langmuir constant were negative (Figure 6 and Table 2). The negative value indicates to Langmuir model did not give a good fit to the sorption process [26]. However, from GTA sponge adsorption, Langmuir model gave the highest R^2 than the others model. And from observed and data, can conclude that GlcNAc crosslinked group shown the better adsorption efficiency and more favorable than GTA which can confirmed from spectrofluorescein microscope in Figure 7. After adsorption experiment, wash and freeze-dried sponge to compare the surface morphology at the same initial concentration of adsorption (0.3 mg/ml). From Figure 7, the amount of FITC that occur on GlcNAc crosslinked sponge were higher than GTA group especially on gelatin/ chitosan/ GlcNAc/ heat 100C 24hrs which highest adsorbed amount.

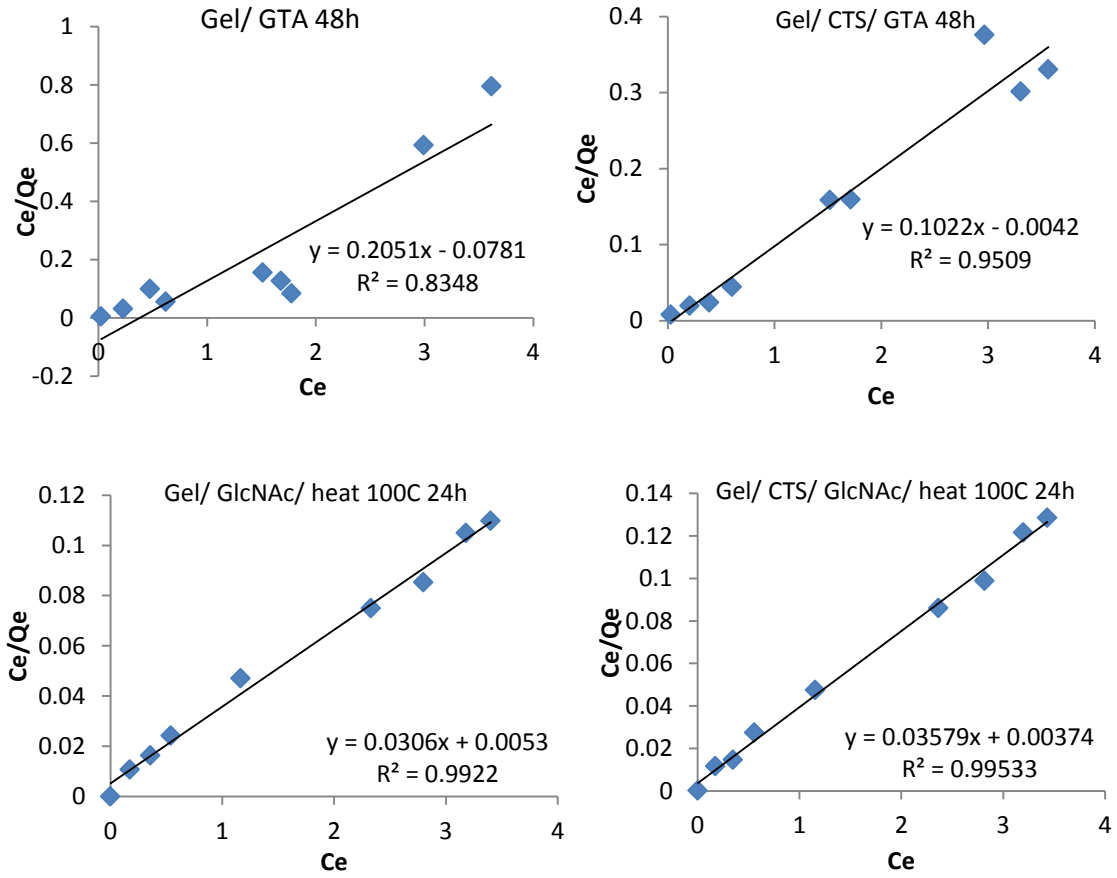

Figure 6. Linearized Langmuir isotherm (eq.4) for FITC-BSA at 25°C.

Table 2. Isotherm parameters from adsorption FITC-BSA by sponge

sample	Gel/ GTA 48h	Gel/ CTS/ GTA48h	Gel/ GlcNAc/ heat 100C24h	Gel/ CTS/ GlcNAc/ heat 100C24h
linear eq.	y= 0.2051x-0.0781	y= 0.1022x-0.0042	y= 0.0306x+0.0053	y=0.0358x+0.0037
R^2	0.8348	0.9509	0.9922	0.9953
q_m (mg/g)	4.875	9.784	32.679	27.932
K_L (ml/mg)	-2.626	-24.333	5.773	9.675

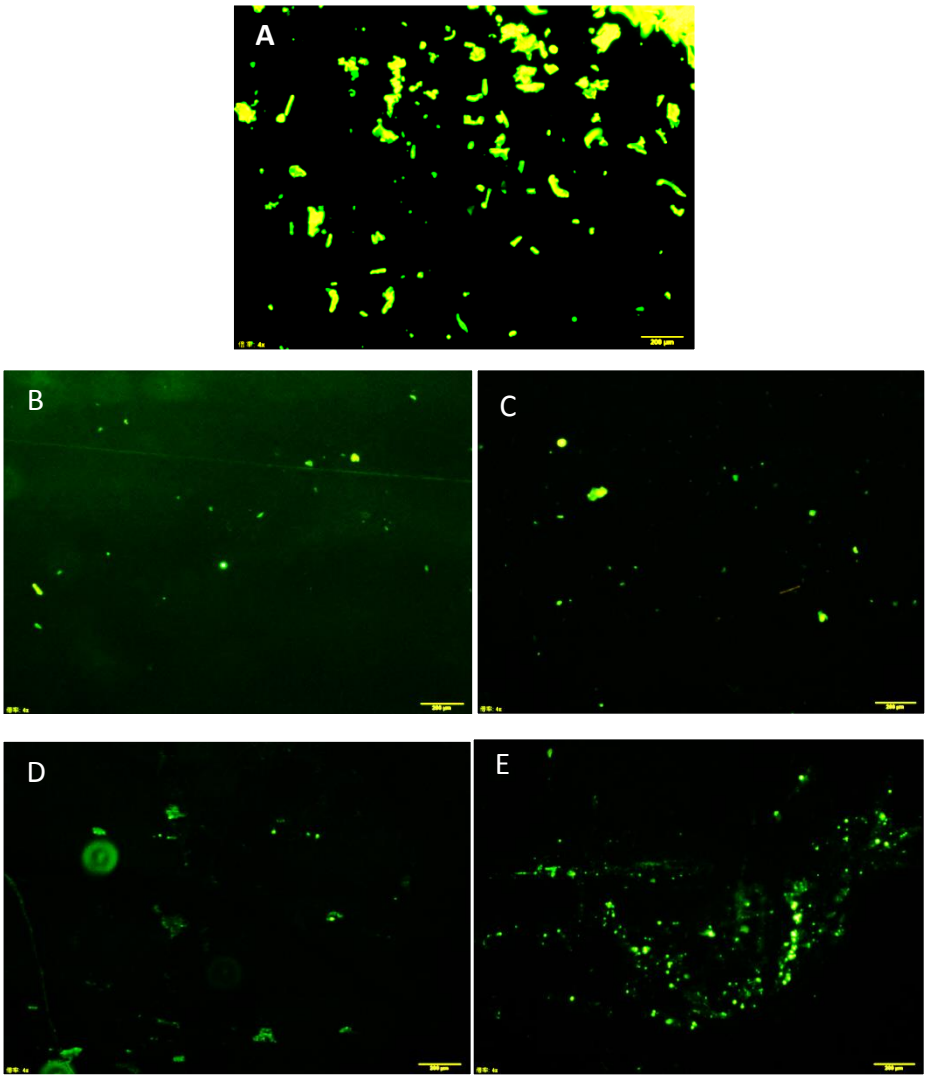

Figure 7. Fluorescein microscope image of FITC-BSA(A), gelatin/ GTA 48h (B), gelatin/ chitosan/ GTA 48h (C), gelatin/ GlcNAc/ heat 100C 24h (D) and gelatin/ chitosan/ GlcNAc/ heat 100C 24h (E); scale bar represent 200 μm.

III.6.3.2.2 Effect of temperature

FITC-BSA adsorption on sponge was also studies at different temperature of 10, 25, 37 50 and 70°C, an under the suitable condition of pH 7.4 and initial concentration of FITC-BSA is 1.8 mg/ml in each case. The effect of temperature to adsorption behavior of FITC-BSA on sponge was shown in Figure 8, each sponge adsorbed amount decrease when temperature increased especially at 70°C. GTA crosslinked sponge didn't shown the adsorption reaction, It's may come from at 70°C, sponge

dissolved to the solution (Figure 9) results to high % weight loss up to 70% when compare with GlcNAc crosslinked sponge which have % weight loss less than 10%. For GlcNAc group, gelatin shown a little lower adsorption amount than gelatin/ chitosan sponge.

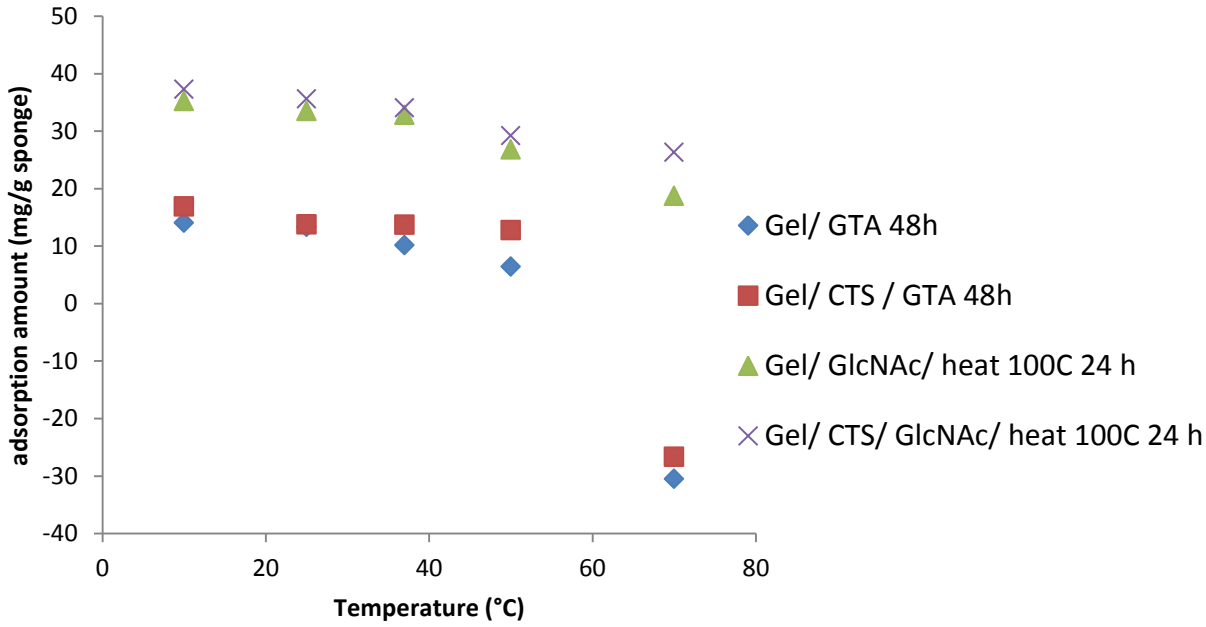

Figure 8. The effect of temperature to adsorption amount of FITC-BSA on gelatin sponge.

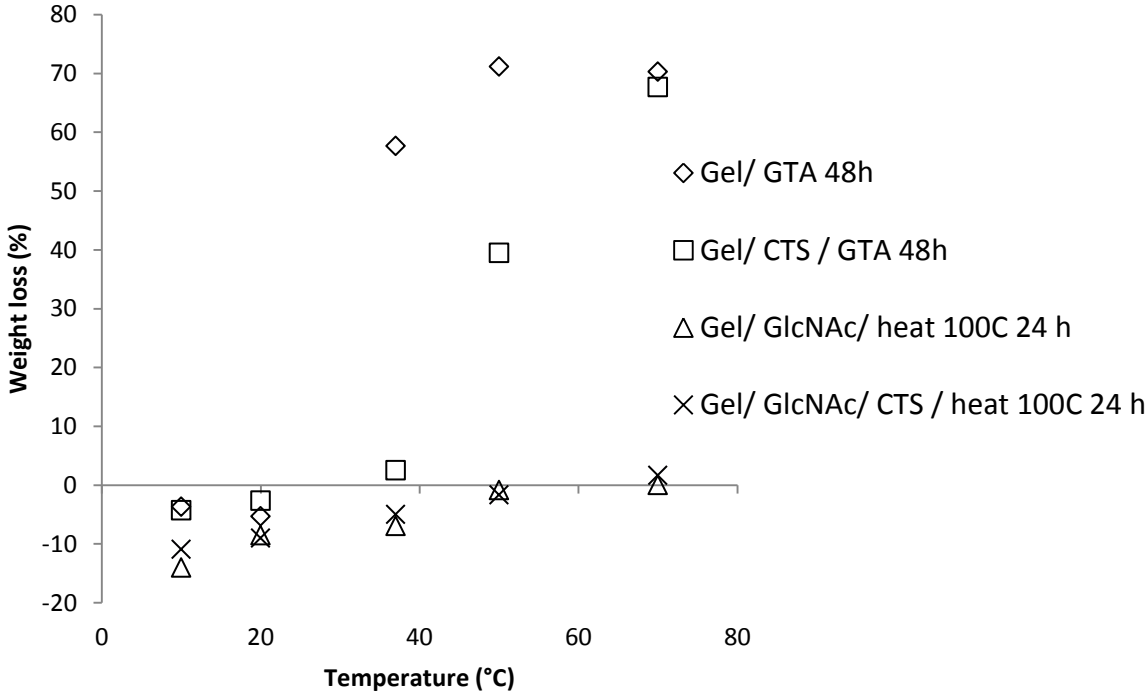

Figure 9. % weight loss of sponge after adsorption test.

From the experiment results, thermodynamic parameter can calculate by using the follow relation [21-22].

$$lnK_d = \frac{\Delta S^o}{R} - \frac{\Delta H^o}{RT} \text{.....(5)}$$

where

$$K_d = \frac{amount\ of\ FITC{-}BSA\ in\ adsorbent}{amount\ of\ FITC{-}BSA\ in\ solution}\ x\ \frac{V}{m}\ \text{(ml/ g) .....(6)}$$

V is the volume of the solution (ml) and m is the weight of the sponge (g).

ΔS°, ΔH°, R and T were entropy (J/ mol.K), enthalpy (KJ/ mol), gas constant (8.314 J/ mol.K) and temperature (K), respectively. ΔH° and ΔS° can obtained from slope and Y-intercept of plots between lnK_d and 1/T (Figure 10). Gibbs free energy (ΔG) was calculated by using the well-known equation:

$$\Delta G = \Delta H - T\Delta S \text{.....(7)}$$

The thermodynamic parameter which calculated from experiment data were collected in Table 3. The negative values of ΔG of each sponge under all temperature conditions indicate the spontaneous nature of adsorption reaction. For each sponge except gelatin/ chitosan/ GlcNac/ heat 100C 24h, ΔH° and ΔS° were negative values, refer to exothermic reaction and at low temperatures make the reaction more favorable. gelatin/ chitosan/ GlcNac/ heat 100C 24h sponge shown the value of ΔH° in negative and ΔS° in positive value, indicated the exothermic reaction whose entropy increases will be spontaneous at all temperatures.

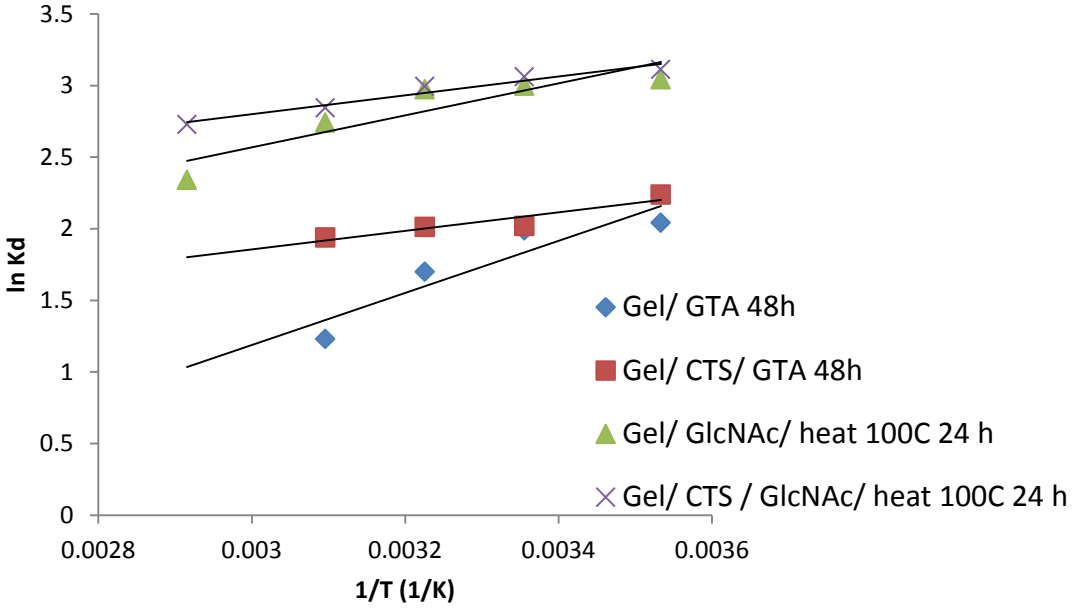

Figure 10. Effect of temperature on the thermodynamic behavior of adsorption of FITC-BSA on gelatin sponge.

Table 3. Thermodynamic parameter for adsorption FITC-BSA on gelatin sponge.

sample	Gel/ GTA 48h	Gel/ CTS/ GTA 48h	Gel/ GlcNAc/ heat 100C 24h	Gel/ CTS/ GlcNAc/ heat 100C 24h
linear eq.	y = 1821.5x - 4.2766	y = 647.7x-0.0866	y = 1115.2x - 0.7771	y = 655.67x+0.8334
R^2	0.8433	0.8730	0.8207	0.9513
ΔS° (J/molK)	-35.55	-0.72	-6.46	6.93
ΔH° (kJ/mol)	-15.144	-5.385	-9.272	-5.451
ΔG (kJ/mol)				
283K (10°C)	-5.081	-5.181	-7.443	-7.412
298K (25°C)	-4.548	-5.170	-7.346	-7.516
310K (37°C)	-4.121	-5.161	-7.269	-7.599
323K (50°C)	-3.659	-5.152	-7.185	-7.689
343K (70°C)	-2.948	-5.138	-7.056	-7.828

III.6.3.3 Desorption

According to adsorption experiment, the results clear that sponge which crosslinked by GlcNAc have better adsorption efficiency than GTA crosslinked. So in the desorption experiment, We choose only GlcNAc crosslinked sponge for study about desorption behavior of sponge.

III.6.3.3.1 Effect of concentration

Figure 11 showed the results from desorption FITC-BSA in PBS from spectrofluorometer at the same wavelength with adsorption test. Results shown the initial rate of FITC-BSA release from composite is rapid from 0 h to 6 h, and same release remains at a steady state up to 12 h. The high adsorbed amount of FITC-BSA on sponge results to high % desorption (in PBS at 37°C), around 25% from 0.3 mg/ ml (~0.5 mg from adsorbed amount ~2 mg) and 55% from 3.5 mg/ml (~1.5 mg from adsorbed amount ~3 mg adsorbed concentration in sponge. Gel/ chitosan have just a few higher %desorption than Gel sponge.

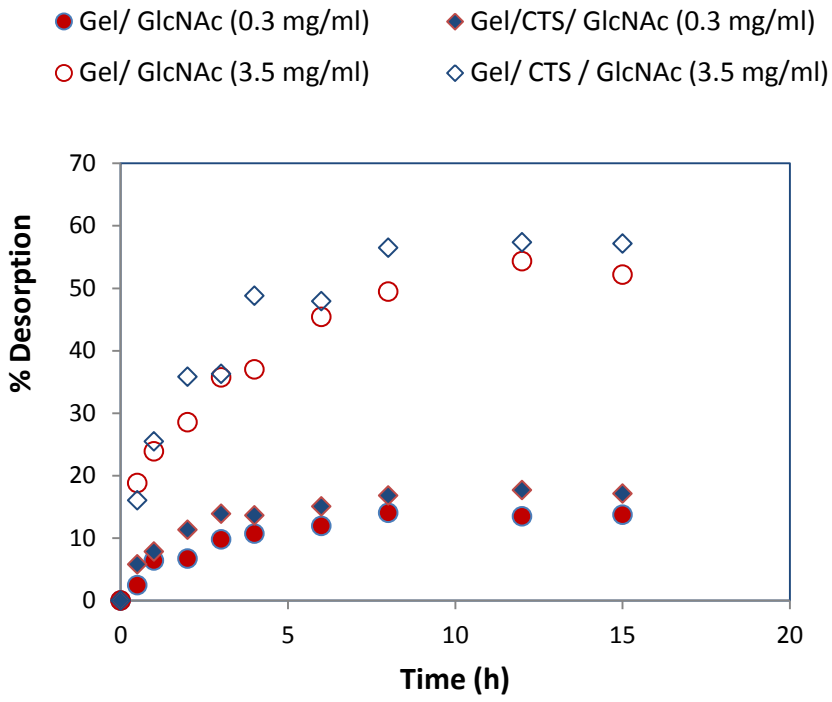

Figure 11. The effect of FITC-BSA at different concentration to % desorption of gelatin sponge in PBS at 37 °C.

III.6.3.3.2 Effect of pH

Figure 12 shown the results of pH to % desorption FITC-BSA in PBS. With the same adsorbed amount at 1.6 mg/ml, sponge were separated to test desorption in different buffer pH solution (2.0, 4.0 and 7.4) at 37°C, after 12 h sampling the sample and measured by using a spectrofluorometer. Results shown % desorption decrease

follow by decrease pH 7.4, 4.0 and 2.0 of buffer solution respectively, by gelatin and gelatin/ chitosan sponge shown the same trend of data. Figure 13 was the example of fluorescein microscopy image after desorption of gelatin/ chitosan sponge, Results can confirm the surface morphology of sponge that remained amount of FITC-BSA increased at lower pH of desorption solution.

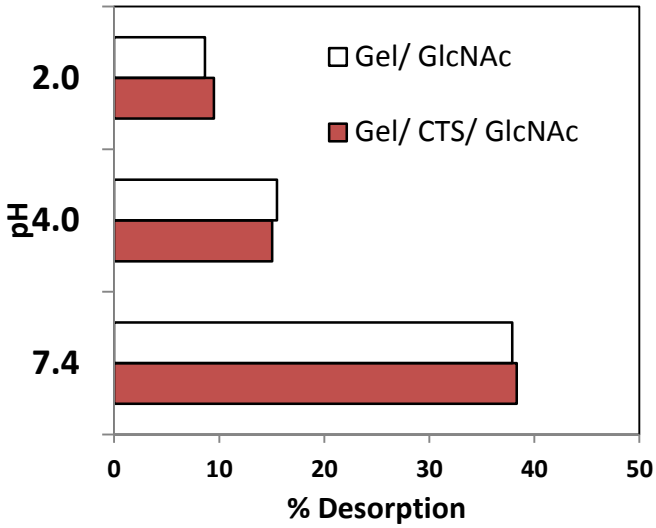

Figure 12. The effect of pH to % desorption of FITC-BSA on gelatin sponge in PBS at 37 °C.

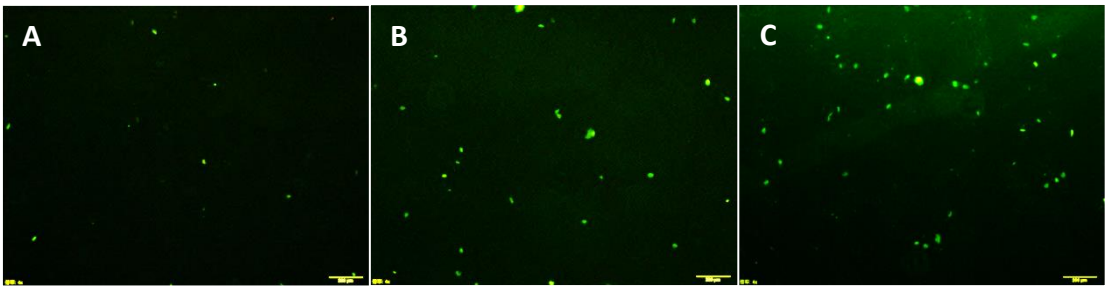

Figure 13. Fluorescein microscope image after desorption at different pH of gelatin/ chitosan/ sponge; pH 7.4(A), pH 4.0(B) and pH 2.0(C); scale bar represent 200 μm.

III.6.4 Discussion

The focus of this study was to understand the protein adsorption and desorption mechanism of sponge which have gelatin as a base material with GlcNAc cross-linked. The reason for added chitosan in the scaffold was to increase interaction between NH_2^+

in gelatin and chitosan and CHO^- of GlcNAc. From all of the result shown the slightly different between gelatin sponge and gelatin/ chitosan sponge, might be came from the amount of chitosan which add to scaffold just only 0.8% of gelatin mass. So, this's may effect to physical property such as tensile or compression property [21] of sponge but slightly effect to protein adsorption and desorption which controlled by hydrophobic interaction, hydrogen bonding, and electrostatic interaction, especially the hydrophilicity and surface charge of materials [22-23].

III.6.5 Conclusions

The gelatin/ chitosan composite sponges were prepared by using GlcNAc and GTA as cross-linker into the form of a sponge by freeze dried. The present study focuses on adsorption and desorption of FITC-BSA as a protein model on sponge. Adsorption of FITC-BSA on sponge were found to increased adsorbed amount with increased concentration on FITC-BSA in solution until reach the equilibrium around 30 mg/g (for this studies). Langmuir isotherm model was fitted with the experiment data with $R^2 \geq 0.99$ for GlcNAc group. Thermodynamic parameter were calculated, results indicated the exothermic adsorption reaction with spontaneous nature. Adsorption reaction was effective in every test temperature for gelatin/ chitosan/ GlcNAc/ heat treatment 100°C 24hrs. Desorption behavior which evaluated only GlcNAc crosslinked sponge by vary concentration and pH of FITC-BSA solution, the results shown the high adsorbed amount of FITC-BSA on sponge effect to high desorbed amount up to and 55% from 3.5 mg/ml adsorbed concentration (around 1.5 mg from adsorb amount 3 mg) in sponge. Sponge were released FITC-BSA at pH 7.4 more rapidly than low pH. The association might be influenced by net charge of protein and participated between sponge and FITC-BSA. However, gelatin and gelatin/ chitosan sponge shown the same trend of results.

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Chapter 7

In vivo and *In vitro* test of chitosan-gelatin based sponge

III.7.1 Introduction

Chitosan (CTS) is derived from chitin, natural polysaccharide that can found in animal which has fun-gal cell walls such as shell of crustaceans. CTS have been studied as biomedical materials due to biocompatible, biodegradable, nontoxic, anti-microbial and hydrating agents. CTS is easily processed into gels [1], membranes [2-6], nanofibers [7-8], beads [9], microparticles [10], nanoparticles [11], scaffolds [12-13] and sponges [14-15] forms. Gelatin (Gel) is also a biocompatible protein because it shows low antigenicity and very high bioabsorption ability when it is applied in the human or animal body. The superior property of gelatin aqueous solution is the solution-gelation transition state based on heat reversibility [16-17]. So many biomedical applications of the membranes between CTS and Gel have been reported [18]. We have already reported the chitin/ Gel and CTS/ Gel membranes, which were successfully prepared for tissue-engineering application due to its biodegradability and biocompatibility [19-20]. Cell adhesion studies of both membranes were carried out using human MG-63 osteoblast-like cells. The cells incubated had attached and completely covered the membrane. Thus, these membranes were bioactive and capable of forming cell adhesion. In addition, the preparation of CTS/ Gel membrane with N-acetyl-D-(+)-glucosamine (GlcNAc) according to Maillard reaction have been reported [21-22]. The stress and elongation of chitin/ Gel membrane cross-linked with GlcNAc showed higher than those without GlcNAc. It is due to the cross-linking effect of GlcNAc by the Maillard reaction. Furthermore, this membrane showed excellent growth of NIH/ 3T3 fibroblast cell [23]. Due to the excellent proliferation rate of cells on the membrane, the membrane could be applied as a skin tissue regeneration template [24].

Fibroblast Growth Factor-2 (FGF2) stimulates cell proliferation, migration, and differentiation associated with wound healing [24]. FGF2 also control proliferation of osteogenic cells, such as osteoblasts and bone marrow stromal cells, resulting in augmentation of bone [25-28] and regeneration of periodontal [29]. Based on *in vivo* test, evaluated of multiwall defects in periodontitis patients have been done [30]. Thus,

preparation of Gel/ CTS scaffold in combination with FGF2 might accelerate bone tissue healing. However, the relation of Gel/ CTS scaffold and FGF2 use has not yet been investigated.

Therefore, the aim of the present study is to evaluate cross-linking effect of Gel/ CTS system using GlcNAc and Glutaraldehyde (GTA). In addition, the effect of implantation of FGF2 was also evaluated *in vitro* and *in vivo*.

III.7.2 Experimental

III.7.2.1 Materials and Preparation of Sponge

The composite sponge was prepared by dissolving Gel (PGS 250-WIA was purchased from KOEI CHEMICAL), CTS (FM-80 was received from Koyo Chemical Co. Ltd.) in acetic acid and GlcNAc (purchased from Wako Chemical Co.) in the water. The composition of solution is shown in Table 1. The mixture was covered and put in the water bath at 50±2°C for 10 h to remove entrapped air and obtain homogeneous solution. After 10 h, solution was cooled down at room temperature and was freeze-dried to obtain the sponge. After that, the sponges were subject to two different cross-linking treatments. First, the sponge was cross-linked with GTA (purchased from Wako Chemical Co.) for 48 h, in which GTA vapor is filled in the desiccator. After the reaction, the sponge was rinsed with methanol 30 min for 3 times. Another cross-linking was performed using GlcNAc. In this case, the sponge was heat treated at 100°C for 24 h in the oven.

Table 1. The preparation of chitosan/ gelatin. **(*** In relation to the gelatin mass**)**

Condition	Gel	Gel/ CTS	Gel/ GlcNAc	Gel/ CTS/ GlcNAc
GTA vapor cross-linking	O	O	X	X
Heat 100°C 24 h	X	X	O	O
CTS 0.8%*	X	O	X	O
GlcNAc 5%*	X	X	O	O

III.7.2.2 In Vitro (cell attachment)

In order to evaluate the cytocompatibility, Gel/ CTS sponge (size 8 x 8 x 6 mm) was seeded with 1×10^4 mouse osteoblastic MC3T3-E1 cells and cultured in humidified 5% CO_2 at 37°C, using MEM medium (alpha-GlutaMAX-I, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Qualified, Life Technologies) and 1% antibiotics (Pen Strep, Life Technologies). After 24 h cultivation, sponge was fixed in 2.5% GTA in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min, rinsed in cacodylate buffer solution and dehydrated in increasing on concentrations of ethanol. Following supercritical drying, sample was analyzed by a scanning electron microscope (SEM, S-4000, Hitachi, Tokyo, Japan) at an accelerating voltage of 10kV after coating with a thin layer of Pt-PD.

III.7.2.3 In Vivo

III.7.2.3.1 Preparation of FGF2 loaded sponge

FGF2 (Fiblast spray 500, Kaken Pharmaceutical, Tokyo, Japan) was diluted with distilled water (Otsuka distilled water, Otsuka Pharmaceutical, Tokyo, Japan) to produce stock solution of 0.5 μg/ μl. In the FGF2-loading groups (Gel/ GlcNAc and Gel/ CTS/ GlcNAc), each scaffold (size 6 × 6 × 3 mm) received 100 μL FGF2 solution (loading dose; 50 μg) under vacuum. Without loaded FGF2, sponge was immersed into distilled water alone.

III.7.2.3.2 Surgical procedure

III.7.2.3.2.1 Rat subcutaneous

The experimental protocol followed the institutional animal use and care regulations of Hokkaido University (Animal Research Committee of Hokkaido University, Approval No. 13-76). Twelve 10-week-old male Wistar rats weighing from 190 to 210 g were given general anesthesia by intraperitoneal injections of 0.6 ml/ kg sodium pentobarbital (Somnopenthyl, Kyoritsu Seiyaku, Tokyo, Japan), as well as a local injection of 2% lidocaine hydrochloride with 1:80,000 epinephrine (Xylocaine Cartridge for Dental Use, Dentsply-Sankin K.K. Tokyo, Japan). After a skin incision was made, four types of sponges (Gel/ GTA, Gel/ CTS/ GTA, Gel/ GlcNAc and Gel/ CTS/ GlcNAc) were implanted into the subcutaneous tissue of the back of rats. Skin

flaps were sutured (Softretch 4-0, GC, Tokyo, Japan) and tetracycline hydrochloride ointment (Achromycin Ointment, POLA Pharma, Tokyo, Japan) was applied to the wound. Ten weeks postsurgery, the rats were euthanized using an overdose of sodium pentobarbital. Implants were excised with surrounding tissues, fixed in 10% buffered formalin and embedded in paraffin according to standard procedures. Six micrometer-thick sections were prepared and stained with hematoxylin and eosin (HE). Sectional observation of stained sample was measured using light microscopy.

III.7.2.3.2.2 Rat bone forming

The experimental protocol followed the institutional animal use and care regulations of Hokkaido University (Animal Research Committee of Hokkaido University, Approval No. 10-42). Twelve Wistar rats were used. Following a skin incision, a flap was made in the scalp. Decortication of a 4 mm^2 area was performed in front of the coronal suture in the cranial bone using a rotating round bur under water irrigation. Subsequently, one of two types of sponges (FGF2/ Gel/ GlcNAc and FGF2/ Gel/ CTS/ GlcNAc) of 8 x 8 x 6 mm was placed on the cranial bone with decortication. Scaffolds were loaded with FGF2 (50 µg). As a control, no implantation was performed. Skin flaps were sutured and tetracycline hydrochloride ointment (Achromycin Ointment, POLA Pharma, Tokyo, Japan) was applied to the wound. Rats were euthanized 10 days after surgery using an overdose of sodium pentobarbital and specimens were collected from the wound. Six µm sections located every 300 µm, including the cranial bone and surrounding soft tissue, were prepared. Sections were stained with HE and examined using light microscopy. The newly formed bone area was measured in each stained section collected 10 days post-surgery using software package (Image J 1.41, National Institute of Health, Bethesda, MD, USA). All statistical procedures were performed using a software package (SPSS Japan, DR. SPSS 11.0).

III.7.3 Results and Discussion

III.7.3.1 In Vitro (cell attachment)

For a material to be used as an implant within the body, it is necessary to satisfy biocompatibility assessment. Culturing cells directly on the surface of composites may also indicate synergistic interaction of cells with the scaffold. Figure 1 shows cell

attachment experimental results for Gel/ GTA and Gel/ CTS/ GlcNAc systems using osteoblastic MC3T3-E1 cell line. The interaction between sponge surface and the cells has been visible. In addition, cell spreading with cell process elongation was indicated, the osteoblastic MC3T3-E1 cell becomes flat and its plasma membrane spreads over the scaffold. Facilitating cellular adhesion, growth and differentiation onto a surface can aid in wound healing and tissue growth. In comparison the results with Chitin and Chitin/ PBS sponge, SEM image depicting the human dermal fibroblasts (HDF) attachment on the prepared scaffolds, HDF attachment on the chitin scaffold showed clumping or cell aggregations (Figure 7A, D). Compared to chitin, chitin/ PBS scaffold showed better cell attachment (Figure 7B, E). More uniform spreading was evident in the blend. Although cell type which studied are different in the gelatin and chitin system sponge, but the results indicating that both Gel based sponge with GTA or GlcNAc crosslinked and chitin base sponge possess excellent cyto-compatibility

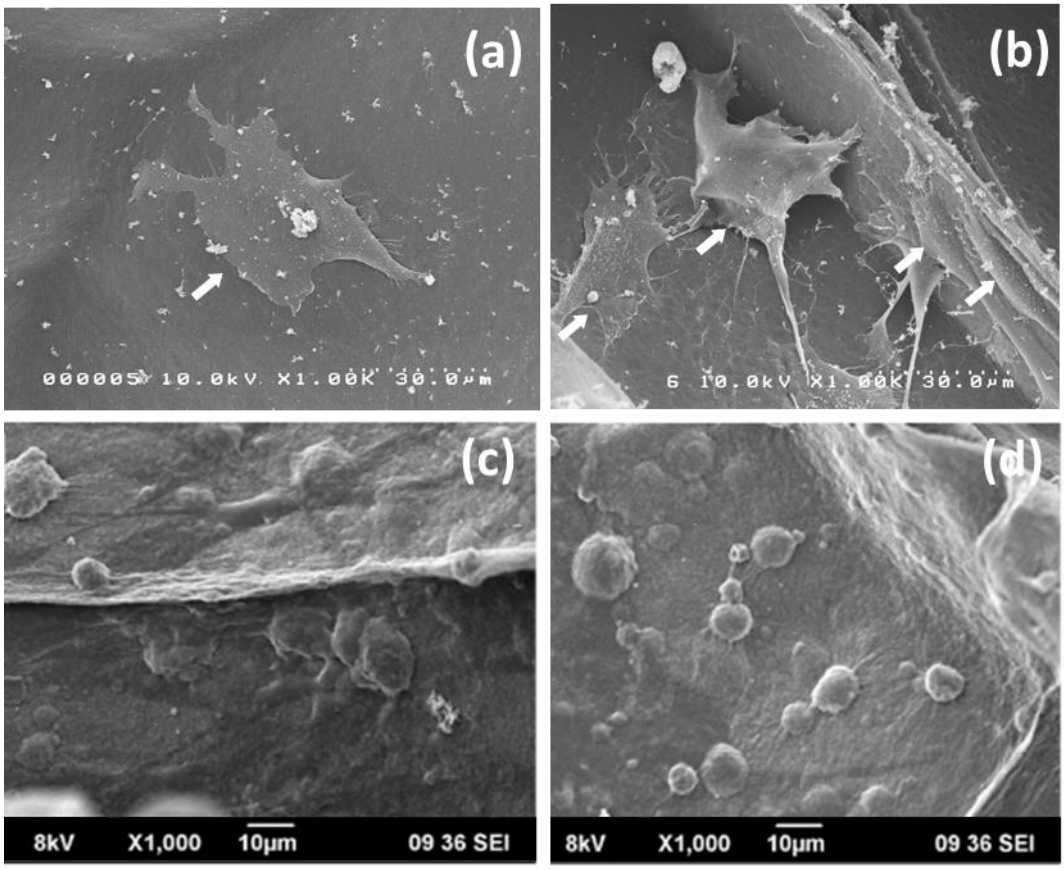


Figure 1. SEM micrograph of cell seeding in cellular affinity; (a) Gel/ GTA, (b) Gel/ CTS/ GlcNAc, (c) Chitin and (d) Chitin/ PBS.

III.7.3.2 In Vivo

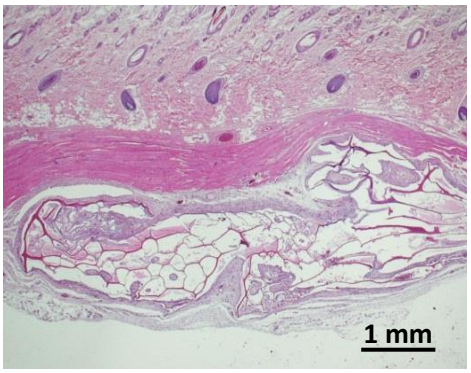
Biocompatibility of gelatin based sponges were evaluated by two different *in vivo* test.

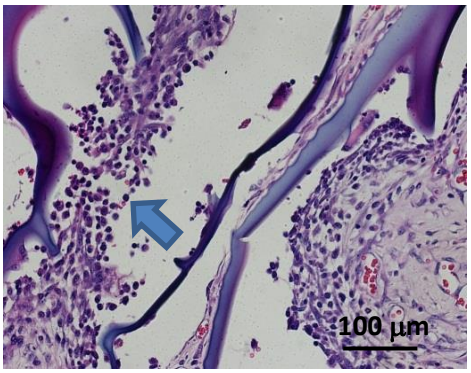
III.7.3.2.1 Rat subcutaneous

Sponges were implanted into the subcutaneous tissue of the back of Wistar rats. FGF2 loaded sponges were also implanted. Histological results after 10 days implantation are shown in Figure 2. All of the sponges showed ingrowth of fibroblastic cells and extent of ingrowth was in the following order; GTA cross-linked system (a)~(d) < GlcNAc cross-linked system (e)~(h) < FGF2 loaded GlcNAc cross-linked system (i)~(l). In the case of GTA cross-linked system (a)~(d), the permeation of inflammatory cells (lymphocytes) is accepted along the implanted sponge. Thus, GTA system showed low biocompatibility. In contrast, GlcNAc system (e)~(h) showed excellent biocompatibility because permeation of inflammatory cells was rarely observed. In addition, phagocytosis by macrophage was also observed in GlcNAc system. In the case of FGF2 loaded system (i)~(l), neutrophil was observed at all. In addition, both the ingrowth of fibroblast cell and phagocytosis by macrophage were prominently stimulated. Therefore, GlcNAc cross-linked system seems to be a favorable material for loading several growth factors.

The present Gel based sponges containing CTS (g)(h)(k)(l) were cross-linked with GlcNAc. Since Gel contained amino acid with amino group as side chain in some extent (10~20%), Gel reacts with several reactive species produced during the complex reaction triggered with the reaction of carbonyl group of GlcNAc and amino group of Gel, during the cross-linking reaction at 100°C. CTS also reacts with GlcNAc on the same way with Gel. Overall reactions are called Millard reaction and forms melanoidins which is a complex mixtures of polymer compounds [21-22]. It is well known that biomaterials containing CTS brings about an inflammatory reaction, probably existence of amino group participates. Low inflammatory action in the present systems that contains CTS (g)(h)(k)(l) may be attributed with modification of CTS by Millard reaction.

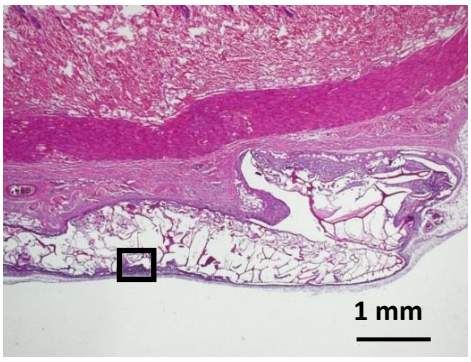
Gel/ GTA

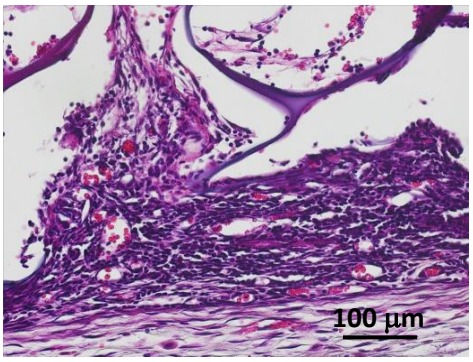




(a) (b)

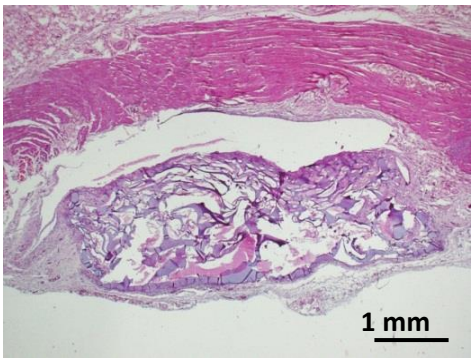
Gel/ CTS/ GTA

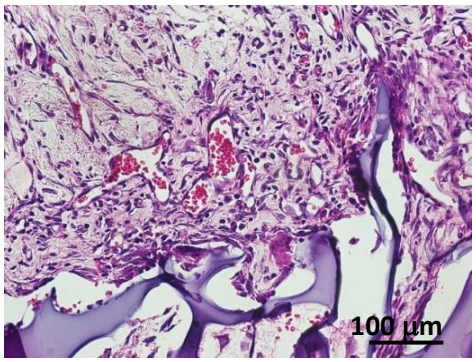




(c) (d)

Gel/ GlcNAc





(e) (f)

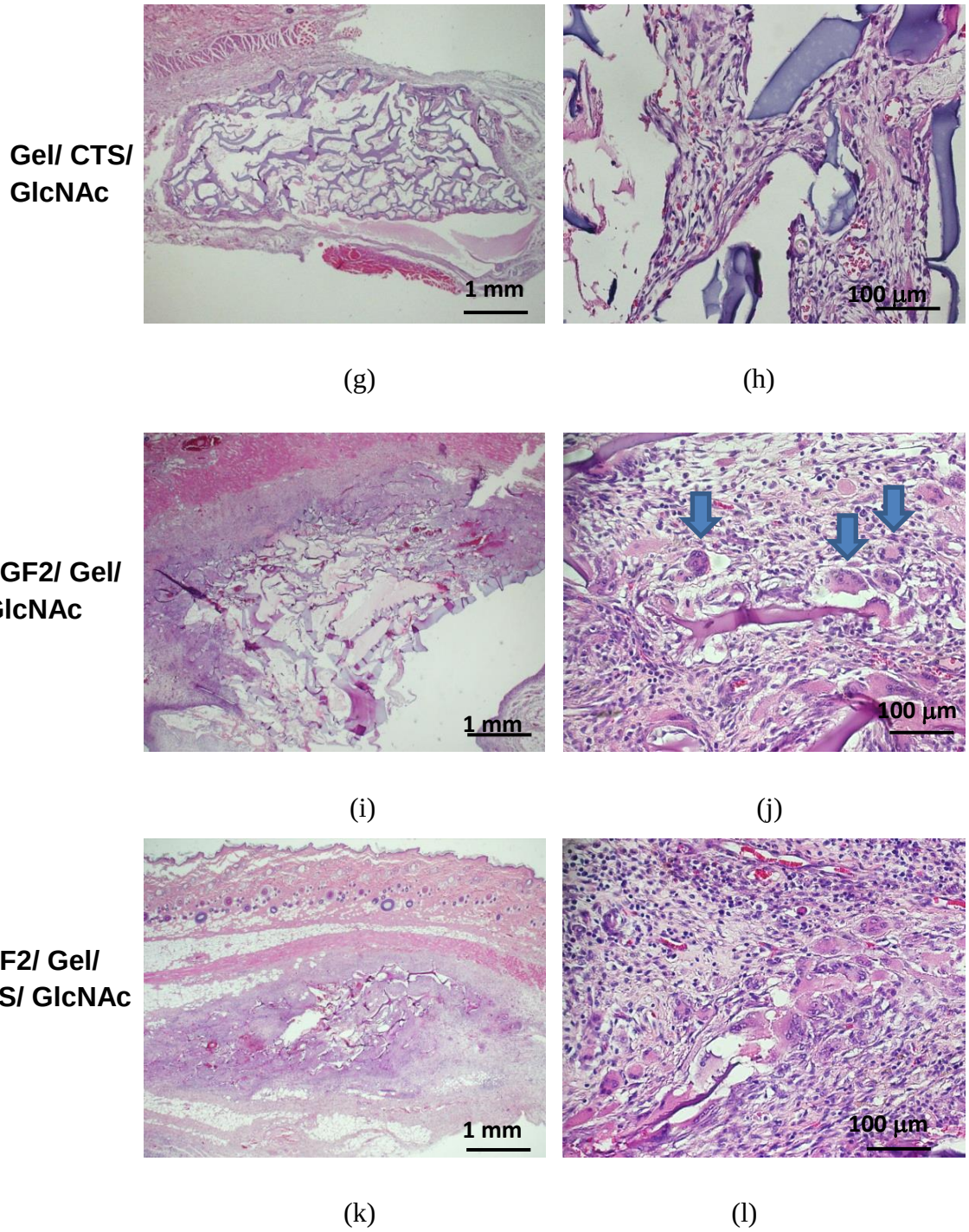


Figure 2. Histological appearance of subcutaneous tissue of back of rat implanted with several sponge after 10 days. (a), (b); Gel/ GTA, (c), (d); Gel/ CTS/ GTA, (e), (f) Gel/ GlcNAc, (g), (h); Gel/ CTS/ GlcNAc, (i), (j); FGF2/ Gel/ GlcNAc, (k), (l); FGF2/Gel/ CTS/GlcNAc. Arrow in (b) and (j) indicate to Lymphocyte and Macrophage respectively.

III.7.3.2.2 Rat bone forming test

In order to examine the bone forming activity of the Gel based sponges, two different types of sponges loaded with FGF2 were applied for head of Wistar rats. Histological images for FGF2/ Gel/ GlcNAc are shown in Figure 3. Implantation of FGF2/ Gel/ GlcNAc scaffold frequently promoted bone augmentation; in which newly formed bone are osteoblastic cells, osteocyto-like cells and bone marrow. Such ingrowth of bone cells were significant and residual scaffold was degraded by phagocytosis with macrophage. These findings are the same level as a collagen sponge [27]. However, inflammatory response was exhibited after implantation of FGF2/ Gel/ CTS/ GlcNAc scaffold (Figure 4). Although the scaffold was degraded and replaced by connective tissue, newly formed bone was slightly observed.

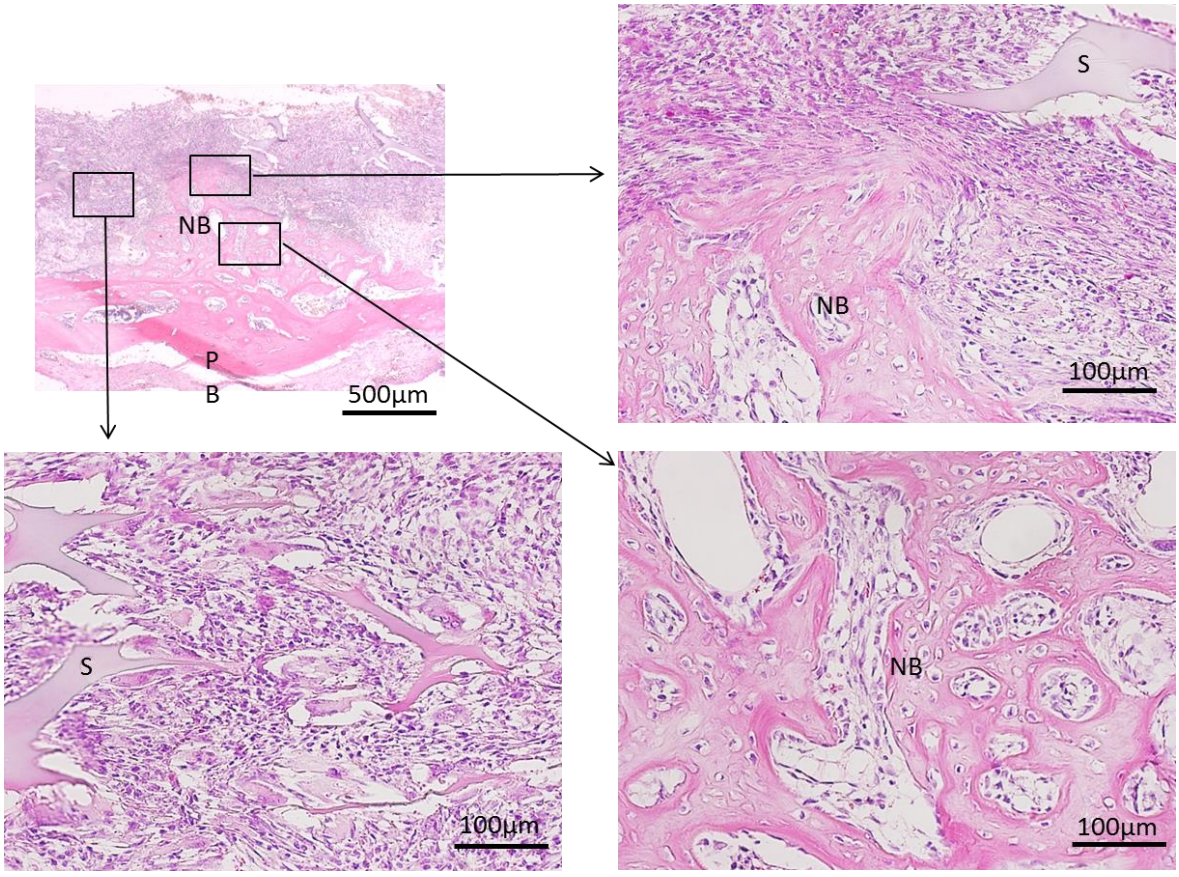


Figure 3. Histological appearance of bone tissue of head of Wistar rat implanted with FGF2/ Gel/ GlcNAc sponge after 10 days. (NB; new bone, S; scaffold, PB; preexisting bone)

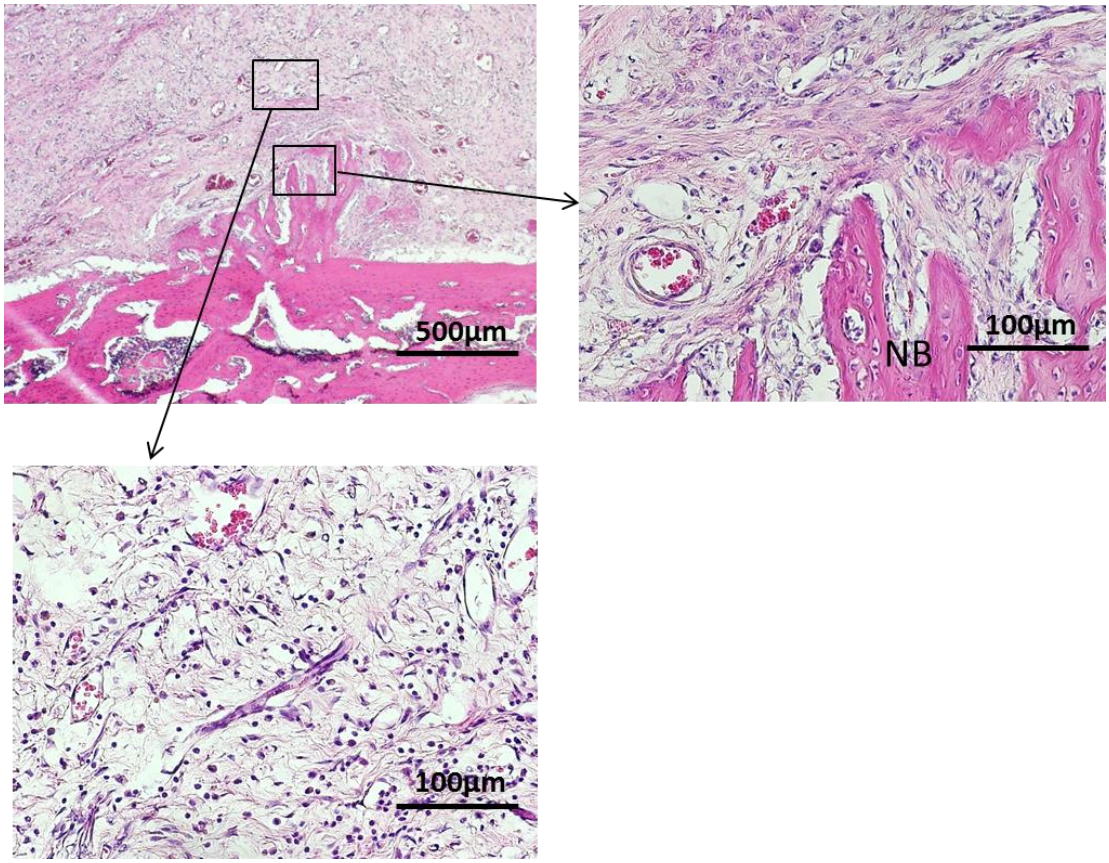


Figure 4. Histological appearance of bone tissue of head of Wistar rat implanted with FGF2/ Gel/ CTS/ GlcNAc sponge after 10 days. (NB; new bone, S; scaffold)

The newly formed bone area was calculated from the histomorphometric measurements in each stained section collected 10 days post-surgery Wistar rats. The result is shown in Figure 5. The means and standard deviations were calculated for each group. Bone area of the FGF2/ Gel/ GlcNAc scaffold was significantly greater than that of FGF2/ Gel/ CTS/ GlcNAc and control. The difference was statistically significant based on the statistical analysis using the Scheffé test. Therefore, the present Gel/ GlcNAc system with FGF2 is bioactive and suitable for tissue engineering application, especially in bone reproductive capability.

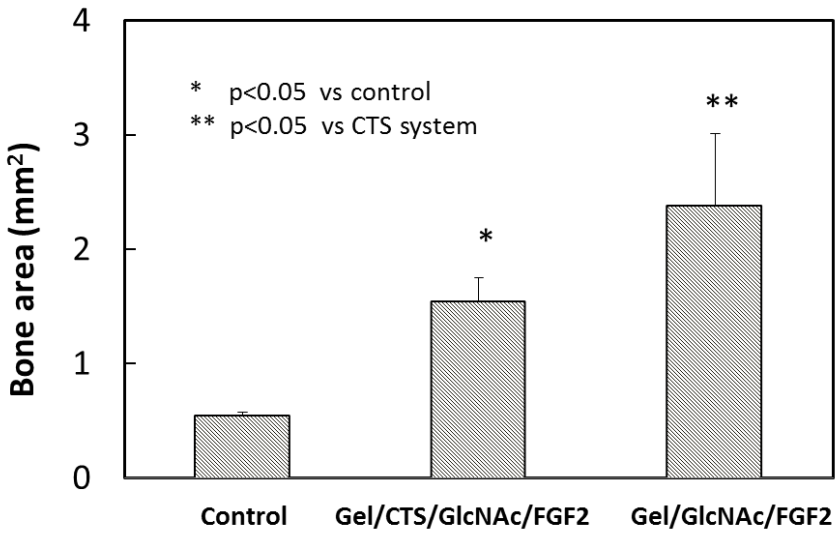


Figure 5. Histological appearance of bone tissue of head of Wistar rat implanted with FGF2/ Gel/ GlcNAc and FGF2/ Gel/ CTS/ GlcNAc sponge after 10 days.

III.7.4 Conclusions

The Gel/ CTS composite were prepared by using GlcNAc and GTA as cross-linker into the form of a sponge by freeze drying. Cell seeding investigation using mouse osteoblastic MC3T3-E1 cells confirmed that the cells could well attached to the based sponge and the elongation was observed in the GlcNAc system better than GTA system. In comparison of cell attachment by the human dermal fibroblasts (HDF) on Chitin and Chitin/ PBS sponge, the results found that HDF attachment on the chitin/ PBS scaffold better than chitin alone sponge. Although cell type which studied are different in the gelatin and chitin system sponge, but the results indicating that both Gel based sponge and chitin base sponge possess excellent cyto-compatibility. *In vivo* test with the rat subcutaneous model indicated that extent of ingrowth and biocompatibility was excellent in GlcNAc system than those in GTA system. The loading of FGF2 against to the Gel based sponge cross-linked with GlcNAc system stimulated ingrowth of cells. In addition, excellent bone forming ability was also obtained using GlcNAc system sponge loaded with FGF2. Thus, the present Gel based GlcNAc system is bioactive and suitable for tissue-engineering application, especially in bone reproductive capability.

III.7.5 References

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Concluding Remarks

This thesis summarizes the results of preparation gelatin in various forms consisting of micro-fiber, nano-fiber and sponge. Characteristic and properties of each form have been studied.

In section I, the effect of various crosslinking agents to gelatin fiber was described.

In chapter 1, produced gelatin fibers have average diameter in range of 50±5 microns with every crosslinked agent. The average tensile stress of fiber without crosslink is 120 MPa. Each crosslinker which applied to gelatin fibers results to improved mechanical property indicated from tensile stress of fibers were increased. GlcNAc shown good results in tensile stress and water resistance than the others sugar especially when applied heat treatment at 120°C for 24 h. Di-epoxy was add to gelatin solution before spin 3,4 and 5% of gelatin mass, results shown that stress of fiber were increased follow by amount of di-epoxy. But when apply heat treatment to fiber, stress of fibers remained the same or even lower, probably due to thermal decomposition of the gelatin chain. GTA on the crosslinked gelatin fiber for 1, 2 and 3 days by vapor crosslinked, results shown that stress of fiber were increased follow by increased time of crosslinked. And when apply heat treatment on the fiber stress of fibers was improved. The comparison of each crosslinker by water resistance, GTA and GlcNAc crosslinked showed the good water resistance ability and less swelling up to 90 days.

In chapter 2, the effect of GTA vapor crosslinked to gelatin micro-fiber was studied. Mechanical property was evaluated in order to find the optimum crosslinked time, the result was found that stress of fiber reach the stable at 7 days. The toxicity induced by GTA cross-linking was could be controlled by reducing it with $NaBH_4$. In all, the combination of results indicates that the $NaBH_4$ is effective in reduction of the gelatin fiber which crosslinked by GTA.

In section II, electrospiining conditions and effect of difference crosslinking agent to gelatin nano-fiber was described to apply the materials as biomaterial application.

In chapter 3, diameter of fibers, viscosity and flow rate of solution were increased depending on the concentration of gelatin. Non-woven fabrics which were

spun with 25% gelatin concentration only showed a fiber diameter in the nanoscale. In order to improve the properties of the non-woven fabrics, they were either cross-linked with GTA vapor after spinning or by the addition of GlcNAc in the gelatin solution prior to spinning and further cross-linked by applying heat. The developed non-woven fabrics were characterized using scanning electron microscopy (SEM), rheometer, FT-IR, TGA and mechanical tensile testing. In terms of mechanical property, cross-linking of non-woven fabrics by GTA vapor showed improved properties when compared to without cross-linking as well as with GlcNAc cross-linking. The swelling and water uptake ability showed that the non-woven fabrics with GTA cross-linking had no morphological changes. TGA thermogram confirmed no phase change in the composite structure.

In chapter 4, the basic setting to create random fiber (nonaxially) and aligned fiber (uniaxially) had been found. Rotating a drum collector at very high rotating speed, relatively poor alignment of spun fiber. Electrospinning with a rotating drum collector consisting of parallel electric bar separated by gap was effective to prepare aligned fiber. The electrospinning conditions that gave the best alignment of are 4000 rpm and distance between parallel electric bar 3 cm and applied electric voltage 23 kV. The development of non-woven gelatin fabric to tubular structure is possible such as combine with the others polymer, expected to use as artificial blood vessels in the future.

In section III, preparation and characterization of sponge with difference crosslinking agent have been study. Physical, chemical and biological properties were evaluated to use as basic information of gelatin sponge and develop in biomaterials such as bone-tissue engineering.

In chapter 5, cross-linking effect of chitosan/ gelatin sponge was evaluated using GlcNAc and GTA as crosslinker. The composite material was made into the form of a sponge by freeze dried. The results from SEM observation shown that the interconnected porosity of each composite sponge was well demonstrated. Thermogravimetric indicated that there is no phase change in the composite structures all of sponge. Water uptake, PBS swelling ratio and degradation rate of composite sponge which prepared with GlcNAc system were higher, due to higher porosity of the composite sponges. The comparison of gelatin composite sponge with chitin/ PBS

sponge, chitin/ PBS showed the higher swelling ratio due to high porosity which can observe from SEM image. This result may come from the different solvent and preparation method. However the pore size of both sponge systems was in the micro scale. In the future, the focus on comparison of chitin and gelatin system would be expectation.

In chapter 6, adsorption and desorption behavior of FITC-BSA on gelatin/ chitosan sponge which crosslinked with GlcNAc and GTA were evaluated. Adsorption of FITC-BSA on sponge were found to increased adsorbed amount with increased concentration on FITC-BSA in solution until reach the equilibrium around 30 mg/ g (for this studies). Langmuir isotherm model was fitted with the experiment data with $R^2 \geq 0.99$ for GlcNAc group. Thermodynamic parameter were calculated, results indicated the exothermic adsorption reaction with spontaneous nature. Adsorption reaction was effective in every test temperature for gelatin/ chitosan/ GlcNAc/ heat treatment 100°C 24hrs. Desorption behavior which evaluated only GlcNAc crosslinked sponge by vary concentration and pH of FITC-BSA solution, the results shown the high adsorbed amount of FITC-BSA on sponge effect to high desorbed amount up to 55% from 3.5 mg/ ml adsorbed concentration (around 1.5 mg from adsorb amount 3 mg) in sponge. Sponge were released FITC-BSA at pH 7.4 more rapidly than low pH. The association might be influenced by net charge of protein and participated between sponge and FITC-BSA. However, gelatin and gelatin/ chitosan sponge shown the same trend of results.

In chapter 7, gelatin/ chitosan composite sponge with GTA and GlcNAc crosslinked were explored the possibility of biomaterial application by *in vivo* and *in vivo* test. Cell seeding investigation using mouse osteoblastic MC3T3-E1 cells confirmed that the cells could well attached to the based sponge and the elongation was observed in the GlcNAc system better than GTA system. In comparison of cell attachment by the human dermal fibroblasts (HDF) on Chitin and Chitin/ PBS sponge, the results found that HDF attachment on the chitin/ PBS scaffold better than chitin alone sponge. Although cell type which studied are different in the gelatin and chitin system sponge, but the results indicating that both Gel based sponge and chitin base sponge possess excellent cyto-compatibility. *In vivo* test with the rat subcutaneous

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List of Publications

1. Sankar Deepthi, Chandrika Viswanathan Sidhy Viha, Thitirat Chaochai , Tetsuya Furuike, Hiroshi Tamura and Rangasamy Jayakumar, "Fabrication of Chitin/ Poly(butylene succinate)/ Chondroitin Sulfate Nanoparticles Ternary Composite Hydrogel Scaffold for Skin Tissue Engineering", Polymers, 6 (12), 2974-2984 (2014).
2. Thitirat Chaochai, Hirofumi Miyaji, Takashi Yoshida, Erika Nishida, Tetsuya Furuike and Hiroshi Tamura, "Preparation of Chitosan-Gelatin Based Sponge Cross-linked with GlcNAc for Bone Tissue Engineering", Journal of Chitin and Chitosan Science, 3 (2), 1-8 (2015).
3. Tetsuya Furuike, Hideaki Nagahama, Thitirat Chaochai and Hiroshi Tamura, "Preparation and Characterization of Chitosan-Coated Poly(L-Lactic Acid) Fibers and Their Braided Rope", Fibers, 3, 380-393 (2015).
4. Thitirat Chaochai, Yusuke Imai, Tetsuya Furuike and Hiroshi Tamura, "High Performance Gelatin Fiber by Dry Spinning", Accepted to Fiber (2016.01.14).
5. Thitirat Chaochai, Tsubasa Okubo, Sankar Deepthi, Rangasamy Jayakumara, Tetsuya Furuike and Hiroshi Tamura, "Fabrication of gelatin nano fibers by aqueous method", Submitted to International Journal of Biomedical Materials Research : Part B- Applied Biomaterials (2015.9.21).
6. Thitirat Chaochai, Tetsuya Furuike and Hiroshi Tamura, "Adsorption and Desorption Behavior of BSA on Gelatin/ Chitosan Sponge", in preparation for Journal of Chitin and Chitosan Science.

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