Studies on Newly Designed Aliphatic Polyesters for Tissue Engineering Materials

March 2008

MIYASAKO Hiroshi

Contents

Chapter 3:
Preparation, Characterization and Biocompatibility Study of the Scaffold Prototype Derived from Cross-Linked Poly[(ε-caprolactone-*co*-lactide)] for Tissue Engineering Materials

Chapter 4:
Preparation and Characterization of Photo-Cross-Linked Poly[(ε-caprolactone)-*co*-lactide] Having Good Biocompatibility for Tissue Engineering Materials

Chapter 1

General introduction

1-1 Origin of tissue engineering

In 1993, J. Vacanti and R. Langer proposed the concept of tissue engineering.[1] In 1995, hairless nude mouse grafted a human ear on the back of the mouse went around was ran by the BBC in England. The TV clip surprised people all over the world. The nude mouse was delivered by implanting porous material prepared from poly(glycolic acid)(PGA) with chondrocyte to subcutaneously of the nude mouse (Figure 1-1). In this way, J. Vacanti and R. Langer proposed the new method of tissue engineering that artificial organ and tissue were fabricated by combining the cells and the porous materials.

The reason why they advanced these investigations the current implantation procedure was unsatisfactory on several counts. Currently, the people of 65 thousand waited organ donor in America. [2] However, the amount of these people died because they weren't able to acquire implantable tissue and organ. Even if they were able to acquire implantable tissue and organ, they were paid by adverse reaction and tissue disruption for a long term. In there condition, compared to tissue engineering and implantation procedure, the tissue engineering was advantage to be able to design regulated three-dimensional and functional tissue. Consequently, the tissue engineering region proposed by J. Vacanti and R. Langer was investigated by many researchers as a new medical care.

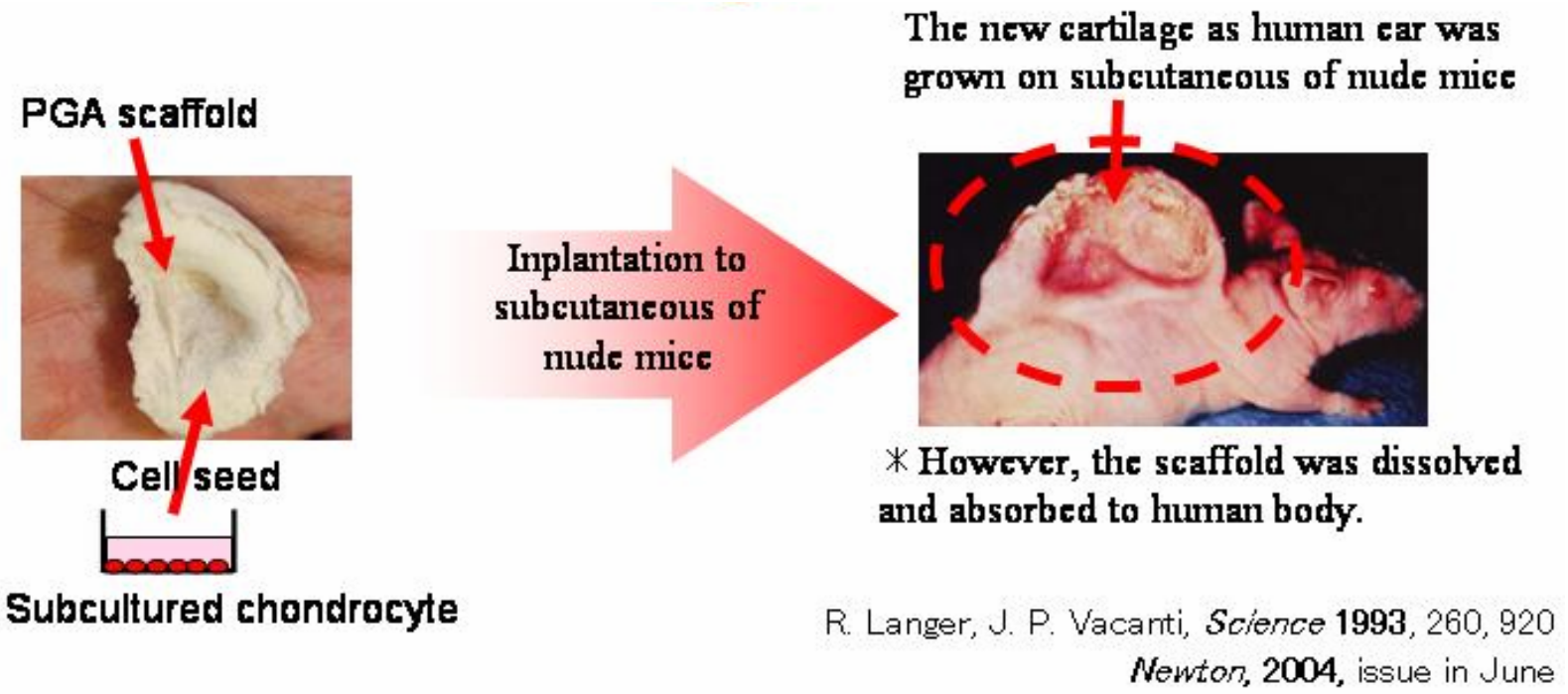


Figure 1-1 The concept of tissue engineering.

1-2 Condition of the scaffold materials for tissue engineering

The scaffold materials were necessity to design three-dimensional tissue. The word of “scaffold materials” means the material provided a foothold for cell growth and differentiation. Therefore, the condition of the formability, biodegradation and biocompatibility etc. were demanded from the scaffold material for construction of three-dimensional tissue.

The human body tissues have various shapes. So, the scaffold for using on an actual medical treatment site as tissue engineering should correspond to those shapes. Consequently, the formability is necessary for the scaffold, because the material having a good formability was able to be prepared the scaffold of the several shapes. The biocompatibility is also necessary for scaffold materials. If the biocompatibility of scaffold material was bad, the cells seeded on the scaffold weren’t able to adhere and grow on the scaffold surface or into the scaffold. Moreover, these materials weren’t matched to the human bodies, and the body caused the inflammatory reaction.[3] The scaffold materials should be a good biocompatibility from such a reason. Additionally, the degradation is necessary for scaffold materials, too. The cells seeded on the scaffold was adhered and grown on the scaffold surface or into the scaffold, and began to form these tissues. So, the scaffold should be degraded with forming the tissue, because the scaffold was replaced to the tissue. Moreover, the material after degradation should be absorbed in human body, because the material that remains in the body might influence the body harmfully. So, the degradation was demanded as the scaffold material for tissue engineering.

As the other condition, the pore size[4,5] and mechanical property[6-9] were demanded. For example, Kuboki *et al*. were prepared artificial extracellular matrix having pore size

of the range of 1-1000 μm, and evaluated about cell growth, cell differentiation and histogenesis.[4] As a result, they reported that the pore size of the range of 300-400 μm was very appropriate in bone regeneration, because the pore size more than 500 μm wasn't maintained dimensional of interior space, and the born were grown at random. Consequently, it suggested result that the pore size was influenced to tissue formation. Additionally, many researchers were investigated to the mechanical property of these scaffold materials. The tissues of the human body were hard tissues as tooth, born, and cartilage etc. and soft tissues as vessel and skin etc. For example, the mechanical property of the scaffold for born tissue engineering should be high, because the scaffold should be held out a surrounding born during forming born. The scaffold should be flexibility in the tissue engineering of the vessel, because the scaffold should be corresponded flexibly like the blood pressure and blood, etc. H. Mizuno *et al.* prepared the cylindrical disks composed of an outer shell of PGA mesh seeded with annulus fibrosus cells with an inner core of nucleus pulposus cells seeded into an alginate gel, and evaluated histological analysis, biochemical analysis and biomechanical analysis of after implanted the material to subcutaneously in athymic mice.[6] As a result of these analyses, they reported that the hybrid material having the mechanical property as the native tissue could be prepared in this study.

Thus the conditions of the formability, biocompatibility, biodegradation, pore size and mechanical property were very important as the scaffold material for tissue engineering.

1-3 Materials for preparation of the scaffold using tissue engineering

1-3.1 Inorganic materials

In these inorganic materials, ceramics and metals as were very useful the scaffold material for born tissue engineering.[10-20] Ceramic implants for born regeneration are based mainly on hydroxyapatite, because the material is inorganic component of vertebrate's bone and tooth.[10-13] In general, the fabrication technique for ceramic implants is sintering of the ceramic powder at high temperatures. Additionally, the hybrid biomaterials are prepared from hydoxyapatite and organic materials as PGA, poly(lactide)(PLA), and the surface of the materials prepared from hydroxyapatite were treated to enhance born regeneration. In this way, many researchers fabricate the implant materials for born tissue engineering by using hydrosyapatite. J. Dong *et al.* prepared the porous material by using polyethleneimine (PEI) and hydroxyapatite (HA) powder, and the pore of material prepared the cross-linked porous gel by PEI.[10] Moreover, they prepared HA/BMO composite material by culturing bone marrow-derived osteoblasts (BMO) in the gel, and the composite material was implanted into the subcutaneous of rat. As a result, alkaline phosphatase activity (ALP) and bone osteocalcin content (OCN) of the composite material was very high in 1 week after implantation. Consequently, they reported that HA/BMO composite material was applicable as the scaffold material for bone tissue engineering. E. Damine *et al.* also prepared the new porous material by absorbing insulin-like growth factor-I (IGF-I) to porous hydroxyapatite (PHA) for bone regeneration, and evaluated the bioactivity of the material *in vivo* in rabbit.[11] These results suggest that PHA with IGF-I was able to expect the scaffold material for born regeneration to form the new born by implanting the material in rabbit.

Stainless steel and titanium or titanium alloys are also very used as metal implants for bone regeneration. The main advantage of metal implants is their excellent mechanical properties, which makes them the most widely applied implant material used in bone surgical repairs. Particularly, since titanium is excellent in mechanical property, corrosion and heat resistance, this is very used as implant materials for born regeneration.[13-17] For example, J. Dolder *et al*. also evaluated the effect of borne marrow stromal cells (BMSCs) cultured in titanium fiber mesh and implanted in a rat cranial defect, and reported about the utility to bone tissue engineering.[15]

In this way, ceramic and metal implants were researched as implant materials for born tissue engineering. However, these materials have some faults, too. For example, the material fabricated from hydroxyapatite is brittleness and slow degradation. The metal implants is the lack of tissue adherence and the low rate of degradation.[5, 21] Consequently, it is necessary to develop a new material to overcome these faults, too.

1-3.2 Organic materials

1-3.2-1 Natural biodegradable polymers

The natural biodegradable polymers, collagen[22-27], fibrin[28-31] and chitosan[32-34] were very used to prepare the scaffold materials for tissue engineering, because these polymers are excellent in biodegradation and biocompatibility. Especially, the cell adhesion and proliferation improves to collagen in dramatic form. Z. Cheng *et al.* prepared poly(ε-caprolactone)(PCL) film, and immobilized collagen to the surface of PCL film introduced acrylic acid (AAc) by UV.[22] They compared the cell adhesion and proliferation of immobilized PCL film and non-immobilized PCL film, and examined it. It suggested result that the cell adhesion and proliferation had improved to the PCL film

immobilized collagen compared with the non-immobilized PCL film in dramatic form. Consequently, it is proved that the collagen is a very useful material as the scaffold material for tissue engineering. The collagen is a main component of the extracellular matrix, and the resistance of the collagen to the tension is very high. However, the materials fabricated from collagen were very brittle. Consequently, the brittleness of these collagen materials has been overcome by doing hybrid the synthesis biodegradable polymer, PGA, poly(lactide)(PLA), and so on. Y. Sumita *el al*. evaluated the performance of collagen sponge with polyglycolic acid fiber mesh as a 3-D scaffold for tooth tissue engineering.[25] As a result of *in vitro* test, cell adhesion and ALP activity of the collagen sponge were high. In *in vivo* analysis at implanting the material into the omentum of immunocompromised rats, cell adhesion and ALP activity of the material was high, and the new tooth generation on the material was confirmed by the routine histological observation by staining with hematoxylin-eosin (H-E) and for immunohistochemistry. Hence, the authors suggested that the collagen sponge was available as the scaffold material for tissue engineering.

Fibrin is also very useful as scaffold materials for tissue engineering. The fibrin is an insoluble body protein largely involved in blood clotting. It is formed through fibrillogenesis of a monomer (called fibrinogen) that flows in the blood. Fibrinogen polymerizes under the action of thrombin to form a mesh in the form of a fibrillar network gel. After healing, fibrin fibrils in humans are broken down through a process called fibrinolysis.[35] In this way, since the fibrin is one of the components included in the body, it can be expected of the materials fabricated from fibrin that the biocompatibility is an excellent material. Consequently, fibrin is being studied well by a many researchers. However, fibrin is mechanically too weak to maintain the desired

shapes and structures when exposed to cells or used in the body. A. Hokugo *et al*. reported that the scaffold material having a good mechanical property and biocompatibility could be fabricated by compounding fibrin and PGA.[29]

In this way, the materials fabricated from the natural biodegradable polymer as collagen and fibrin were lacked the mechanical property, while there materials were had a good biocompatibility.

1-3.2-2 Synthetic biodegradable polymers

Since synthetic biodegradable polymer, such as PCL, PLA, PGA and their copolymers, are excellent in mechanical property, biodegradation and biocompatibility, these polymers were also have been widely studied as the scaffold material for tissue engineering. Especially, PLA in these synthetic biodegradable polymers has good biocompatibility, and can be easily controlled the mechanical property. Additionally, PLA is excellent in safety, because the lactic acid that is degradation product of the material is a product of metabolism of the body. However, the mechanical property of the materials prepared from PLA is very high, because PLA was high crystallinity. Hence, these materials aren't able to match with soft tissue. Consequently, many researchers are done so that a lot of studies may overcome these disadvantages.[36-43] Fumitaka Tasaka *et al*. synthesized comb-type PLA having comb formation by the ring opening polymerization of $_{L}$-lactide (LA) and cyclodepsipeptide consisting of glycolic acid (Glc) and *O*-benxyl-$_{L}$-serine (Ser(OBzl)) with different form the chain length and the branched number.[37] They investigated the thermal property, surface morphology, and biodegradable behavior of these copolymers and copolymer films. According to the paper, the crystallinity of the obtained comb-type PLA is lower than that of the

linear-type PLA. Additionally, the crystallinity and the biodegradable behavior could be controlled by changing the chain length and the branched number. Tatsuro Ouchi *et al*. synthesized PLA-grafted dextran with various lengths and number of graft chains by a trimethylsilyl protection method, and the properties of the cast films prepared from graft-copolymers were investigated through thermal and dynamic mechanical analyses.[39] As a result, the graft-copolymer films exhibited a lower glass transition temperature (Tg), melting temperature (Tm), and crystallinity compared to PLA films.

The biocompatibility of the materials prepared from biodegradable polymers aren't better than that of natural polymers. However, these advantages were able to overcome by introducing the cell activity groups in these molecular constitutions[44-46] or changing the surface of these materials as more activating of the cells.[47-49] E. L. Prime *et al.* succeeds in preparation of the polymeric film conjugated GRGDS.[46] According to this paper, they synthesized a novel PLA-based polymer containing reactive pendent ketone or hydroxyl groups by the copolymerization of $_{L}$-lactide with ε-caprolactone-based monomers. Additionally, they fabricated the thin polymeric film conjugated GRGDS to the film surface, because the cells on the surface of the material were activated. As a result of cell adhesion assay using 3T3 fibroblasts, it is prove that the cells on the polymeric film conjugated GRGDS were more activated. S. Y. Lee *et al.* also prepared the scaffold by solvent-casting method using poly(lactide-co-glycolide) (PLGA) and NaCl, and the pore of the materials were modified by collgen (PLGA/collagen), collagen and hyaluronic acid (PLGA/collagen/HA), collagen and human amniotic membrane (PLGA/collagen/AM), or collagen, human amniotic membrane and hyaluronic acid (PLGA/collagen/AM/HA).[45] Then they evaluated to biocompatibility *in vitro* and restoration ability of the wound *in vivo* of the scaffold materials. According

to this paper, they reported that PLGA/collagen/HA and PLGA/collagen/AM/HA of cell growth *in vitro* were the best highest, and PLGA/collagen/HA for 4 weeks after implanting into wounded eyes of rabbit was recovered the wound of cranberry.

Additionally, as for these materials prepared form biodegradable polymers, a practicable research is done.[6, 8, 48, 50, 51] N. Isogai *et al*. were prepared poly($_L$-lactic acid-ε-caprolactone) copolymer scaffold as the shape of a human ear, and then implanted the material cultured chondrocyte in the dorsum of nude mice.[50] As a result, the utility as scaffold material for tissue engineering was demonstrated that poly($_L$-lactic acid-ε-caprolactone) copolymer seeded with articular chondrocytes supports development and maintenance of cartilage in a human ear shape over periods to 40 weeks in this implantation model. In this way, study on development of scaffold material used biodegradable polymers as PLA was investigated by many researchers well.

1.3-2.3 Inorganic-organic hybrid materials

As shown in the above-mentioned, the mechanical strength of the inorganic materials as ceramic and titanium is high, but these materials lacks flexibility, biodegradable, and biocompatibility. On the other hand, the organic materials as collagen, fibrin, and PLA are excellent in flexibility, biodegradable and biocompatibility, but these materials are a little inferior to mechanical strength. Then, to fabricate the material with these advantages, many researchers came to advance the study on the fabrication of the hybrid material.[52-56] Z. Hong *et al.* are reporting the success in fabricating hybrid material with good biocompatibility, reasonable mechanical strength.[53] They fabricated the hybrid material by using PLA and hydroxyapatite. T. D.

Sargeant *et al.* also succeeded in preparation hybrid bone implant of peptide amphiphile nanofibers and porous titanium.[56] And, they reported that this hybrid material was practical materials for born tissue engineering.

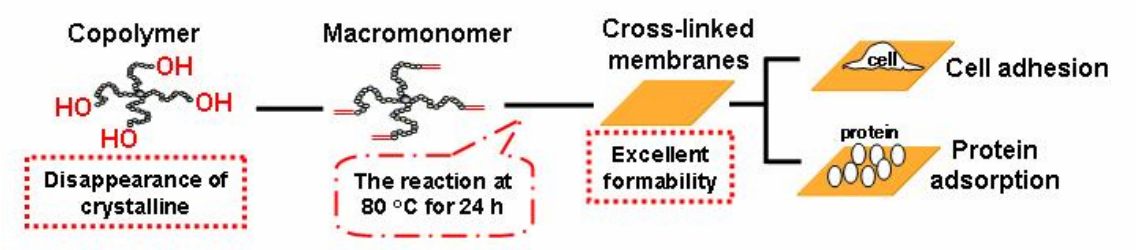
In this way, many researchers have been studying about hybridization of organic and inorganic materials to fabricate the material with good biocompatibility and adequate mechanical property.

1-4 Object of our studies

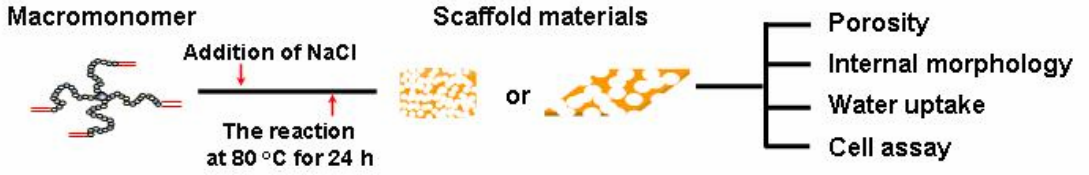
In this way, the research is advanced so that many researchers may have the tissue engineering established as a new medical treatment, and many researchers are developing the study the scaffold material for tissue engineering. However, the soft materials for born and tooth tissue engineering aren't so researched though the hard materials for vascular and skin tissue engineering are researched well. Moreover, the development of the study scaffold materials for the bladder regeneration is being requested to us by Department of Medicine.

In our previous studies,[57, 58] we also succeeded in synthesizing a branched PCL macromonomer with different branch numbers and chain lengths. Using the macromonomers, the cross-linked membrane-type materials were prepared and their permeability control was mainly studied, because the cross-linked materials show a very sensitive thermo-response derived from the sensitive softening behavior based on the crystalline melting of the PCL chains. Our laboratory also evaluated the cell adhesion and cell growth on the cross-linked materials treated the surface of the materials by alkali solution and degradation of the materials. In this way, we expected that these cross-linked materials were able to utilize as the materials for tissue engineering and

(a) Chapter I



(b) Chapter II



(c) Chapter III

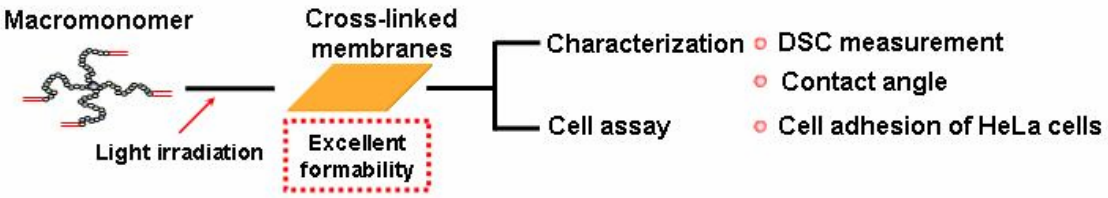


Figure 1-2 The scheme of experimental in this study, (a) Chapter I, (b) Chapter II, and (c) Chapter III.

drug delivery system. Consequently, we paid attention to PCL and PLA researched very well, and developed the material by using a new branched copolymer of PCL and PLA to fabricate a flexible material.

1-5 Composition of this paper

It is entitled, " Studies on Newly Designed Aliphatic Polyesters for Tissue Engineering Materials " and is composed of Chapter 4. In Chapter 2, we synthesized branched copolymer with different CL and LA compositions (Figure 1-2(a)). The corresponding branched macromonomer were then prepared by introducing an acryloyl group at the chain end of the precursor. Additionally, the cross-linked membranes were prepared by reaction at 80 ℃ for 2 h using these branched macromonomers and benzoyl peroxide (BPO) as initiator. These membranes prepared by cross-linked reaction were flexible and high of stability in organic solvent. Additionally, we evaluated about thermal and surface properties by DSC, ESCA and contact angle, respectability. We also evaluated biocompatibility of these cross-linked membranes by SEM observation of HeLa cells morphology and protein adsorption to these membranes.

In Chapter 3, we prepared three-dimensional materials (scaffold), and evaluated about qualification for tissue engineering (Figure 1-2(b)). In Chapter 2, we revealed that CL-LA70/30m (where CL-LA X/Y denotes the macromonomer containing X mol-% of CL and Y mol-% of LA) was a good biocompatibility. Hence, we prepared the porous materials by using CL-LA70/30m. The method of preparation was the same that of Chapter 2, but we prepared by using NaCl as porogen. The particles of NaCl were sieved at four sizes, and prepared the porous materials by heat reaction. The

characterization of these scaffolds were carried out the estimating internal morphology by SEM and water content in PBS(-) at 37 ℃. We also studied the adhesion and proliferation of HBCC to slab-type scaffold prototype by the microscopic observation of cell morphology and alamar Blue® assay.

In Chapter 4, we prepared the membranes by photo cross-linked reaction of the new method. In general, it is said that photo cross-linked can be preceded rapidly (Figure 1-2(c)). So, we prepared the photo cross-linked membranes by using Cl-LA70/30m and *N, N*-dimethyl-*p*-toluidine and camphorquinone as photo-initiator, and evaluated about thermal property and surface property by DSC, contact angle and SEM observation. We also evaluated biocompatibility of the material by using HeLa cells.

When we advanced such a research, the fabrication the scaffold material corresponding to various shape becomes possible. And, the materials in our study are a promising material for scaffold of tissue engineering.

References

[1] R. Langer, P. Vacanti, *Science* **1993**, *260*, 920.

[2] N. Ohno, M. Aizawa, T. Yoshida "*Tissue engineering: To cutting edge of technology from foundation of tissue engineering*", 1st edition, NTS Co., Japan, 2002, p. 4.

[3] J. E. Babensee, J. M. Anderson, L. V. McIntire, A. G. Mikos, *Advanced Drug Delivery Reviews* **1998**, *33*, 111.

[4] Y. Kuboki, Q. Jin, H. Takita, *J. Bone Joint Surg.* **2001**, *S1-105-114*, 83.

[5] V. Karageorgiou, D. Kaplan, *Biomaterials* **2005**, *26*, 5474.

[6] H. Mizuno, A. K. Roy, V. Zaporojan, C. A. Vacanti, M. Ueda, L. J. Bonassar, *Biomaterials* **2006**, *27*, 362.

[7] M. P. Linnes, B. D. Ratner, C. M. Giachelli, *Biomaterials* **2007**, *28*, 5298.

[8] N. Isogai, T. Morotomi, S. Hayakawa, H. Munakata, Y. Tabata, Y. Ikada, H. Kamiishi, *J. Biomed. Mater. Res.* **2005**, *74A*, 408.

[9] J. Yang, A. R. Webb, S. J. Pickerill, G. Hageman, G. A. Ameer, *Biomaterials* **2006**, *27*, 1889.

[10] J. Dong, H. Kojima, T. Uemura, M. Kikuchi, T. Tateishi, J. Tanaka, *J. Biomed. Mater. Res.* **2001**, *57*, 208.

[11] E. Damien, K. Hing, S. Saeed, P. A. Revell, *J. Biomed. Mater. Res.* **2003**, *66A*, 241.

[12] S. Deville, E. Saiz, A. P. Tomsia, *Biomaterials* **2006**, *27*, 5480.

[13] S. Nishiguchi, H. Kato, M. Neo, M. Oka, H.-M. Kim, T. Kokubo, T. Nakamura, *J. Biomed. Mater. Res.* **2001**, *54*, 198.

[14] J. Dong, H. Kojima, T. Uemura, M. Kikuchi, T. Tateishi, J. Tanaka, *J. Biomed. Mater. Res.* **2001**, *57*, 208.

[15] J. Dolder, E. Farber, P. H. M. Spauwen, J. A. Jansen, *Biomaterials* **2003**, *24*, 1745.

[16] E. A. A. Neel, T. Mizoguchi, M. Ito, M. Bitar, V. Salih, J. C. Knowles, *Biomaterials* **2007**, *28*, 2967.

[17] F. C. Soumetz, L. Pastorino, C. Ruggiero, *J. Biomed. Mater. Res.* **2008**, *84B*, 249.

[18] S. Radice, P. Kern, G. Burki, J. Michler, M. Textor, *J. Biomed. Mater. Res* **2007**, *82A,* 436.

[19] J. R. Jones, O. Tsigkou, E. E. Coates, M. M. Stevens, J. M. Polak, L. L. Hench, *Biomaterials* **2007**, *28*, 1653.

[20] A. R. El-Ghannam, *J. Biomed. Mater. Res.* **2004**, *69A*, 490.

[21] S. F. Hulbert, F. A. Young, R. S. Mathews, J. J. Klawitter, C. D. Talbert, F. H. Stelling, *J. Biomed. Mater. Res.* **1970**, *4*, 433.

[22] Z. Cheng, S.-H. Teoh, *Biomaterials* **2004**, *25*, 1991.

[23] Y. Z. Zhang, J. Venugopal, Z.-M. Huang, C. T. Lim, S. Ramakrishna, *Biomacromolecules* **2005**, *6*, 2583.

[24] S. Zhong, W. E. Teo, X. Zhu, R. Beuerman, S. Ramakrishna, Y. L. Yung, *Biomacromolecules* **2005**, *6*, 2998.

[25] Y. Sumita, M. J. Honda, T. Ohara, S. Tsuchiya, H. Sagara, H. Kagami, M. Ueda, *Biomaterials* **2006**, *27*, 3238.

[26] C. L. Casper, W. Yang, M. C. Farach-Carson, J. F. Rabolt, *Biomacromolecules* **2007**, *8*, 1116.

[27] J. B. Chiu, C. Liu, B. S. Hsiao, B. Chu, M. Hadjiargyrou, *J. Biomed. Mater. Res.* **2007**, *83A*, 1117.

[28] R. Nazarov, H.-J. Jin, D. L. Kaplan, *Biomacromolecules* **2004**, *5*, 718.

[29] A. Hokugo, T. Takamoto, Y. Tabata, *Biomaterials* **2006**, *27*, 61.

[30] D. Eyrich, F. Brandl, B. Appel, H. Wiese, G. Maier, M. Wenzel, R. Staudenmaier, A. Goepferich, T. Blunk, *Biomaterials* **2007**, *28*, 55.

[31] M. P. Linnes, B. D. Ratner, C. M. Giachelli, *Biomaterials* **2007**, *28*, 5298.

[32] K. S. Chow, E. Khor, *Biomacromolecules* **2000**, *1*, 61.

[33] A. R. Sarasam, A. I. Samli, L. Hess, M. A. Ihnat, S. V. Madihally, *Macromol. Biosci.* **2007**, *7*, 1160.

[34] B. Duan, L. Wu, X. Yuna, Z. Hu, X. Li, Y. Zhang, K. Yao, M. Wang, *J. Biomed. Mater. Res.* **2007**, *83A*, 868.

[35] F. Couet, N. Rajan, D. Mantovani, *Macromol. Biosci.* **2007**, *7*, 701.

[36] H. Korhonen, A. Helminen, J. V. Seppälä, *Polymer.* **2001**, *42*, 7541.

[37] F. Tasaka, Y. Ohya, T. Ouchi, *Macromolecules* **2001**, *34*, 5494.

[38] A. O. Helminen, H. Korhonen, J. V. Seppälä, *Macromol. Chem. Phys.* **2002**, *203*, 2630.

[39] T. Ouchi, T. Kontani, Y. Ohya, *Polymer* **2003**, *44*, 3927.

[40] B. G. Amsden, G. Misra, F. Gu, H. M. Younes, *Biomacromolecules* **2004**, *5*, 2479.

[41] B. Amsden, S. Wang, U. Wyss, *Biomacromolecules* **2004**, *5*, 1399.

[42] A. S. Karikari, W. F. Edwards, J. B. Mecham, T. E. Long, *Biomacromolecules* **2005**, *6*, 2866.

[43] K. Nagahama, Y. Nishimura, Y. Ohya, T. Ouchi, *Polymer* **2007**, *48*, 2649.

[44] A. D. Cook, J. S. Hrkach, N. N. Gao, I. M. Johnson, U. B. Pajvani, S. M. Cannizzaro, R. Langer, *J. Biomed. Mater. Res.* **1997**, *35*, 513.

[45] Y. Ohya, H. Matsunami, E. Yamabe, T. Ouchi, *J. Biomed. Mater. Res.* **2003**, *65A*, 79.

[46] E. L. Prime, J. J. Cooper-White, G. G. Qiao, *Macromol. Biosci.* **2007**, *7*, 1272.

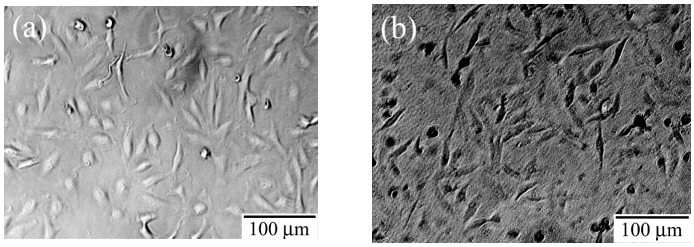
[47] Z. Ma, C. Gao, Y. Gong, J. Ji, J. Shen, *J. Biomed. Mater. Res. (Appl. Biomater.)* **2002**, *63*, 838.
[48] S. Y. Lee, J. H. Oh, J. C. Kim, Y. H. Kim, S. H. Kim, J. W. Choi, *Biomaterials* **2003**, *24*, 5049.
[49] Z. Ma, C. Gao, Y. Gong, J. Shen, *Biomaterials* **2005**, *26*, 1253.
[50] N. Isogai, S. Asamura, T. Higashi, Y. Ikada, S. Morita, J. Hillyer, R. Jacquet, W. J. Landis, *Tissue Engineering* **2004**, *10*, 673.
[51] M. Pattison, T. J. Webster, J. Leslie, M. Kaefer, K. M. Haberstroh, *Macromol. Biosci.* **2007**, *7*, 690.
[52] F. Zhao, Y. Yin, W. W. Lu, C. Leong, W. Zhang, J. Zhang, M. Zhang, K. Yao, *Biomaterials* **2002**, *23*, 3227.
[53] Z. Hong, P. Zhang, C. He, X. Qiu, A. Liu, L. Chen, X. Chen, X. Jing, *Biomaterials* **2005**, *26*, 6296.
[54] D. L. Nihouannen, L. L. Guehennec, T. Rouillon, P. Pilet, M. Bilban, P. Layrolle, G. Daculsi, *Biomaterials* **2006**, *27*, 2716.
[55] A. Sendemir-Urkmez, R. D. Jamison, *J. Biomed. Mater. Res.* **2007**, *81A*, 624.
[56] T. D. Sargeant, M. O. Guler, S. M. Oppenheimer, A. Mata, R. L. Satcher, D. C. Dunand, S. I. Stupp, *Biomaterials* **2008**, *29*, 161.
[57] T. Aoyagi, F. Miyata, Y. Nagase, *J. Controlled Release* **1994**, *32*, 87.
[58] K. Uto, K. Yamamoto, S. Hirase, T. Aoyagi, *J. Controlled Release* **2006**, *110*, 408.

Chapter 2

Preparation of Cross-linked Poly(ε-caprolactone-*co*-lactide) and Biocompatibility Studies for Tissue Engineering Materials

Summary

In Chapter 2, cross-linked materials were prepared using the branched macromonomer with different CL/LA molar ratios, and feasibility studies for tissue engineering were carried out. The thermal and mechanical properties of these materials depended on the CL/LA compositions, however, there was no change in the wettability of each material. The HeLa cells adhesion and growth on the CL-LA70/30c were equal to that on the commercially-available polystyrene dish. The protein absorption experiment using the FBS proteins revealed that the materials with well-grown cells showed better adhesion of the proteins.



Microscopic views of HeLa cells adhered to (a) TCPS and (b) CL-LA70/30c.

2.1 Introduction

Biodegradable polymers, such as poly(ε-caprolactone) (PCL), poly(lactide acid) (PLA), poly(glycolic acid) (PGA) and their copolymers, have been widely studied over the past decade. At present, numerous studies have been extensively continued as a scaffold for the tissue engineering,[1-10] artificial organs, such as artificial-dura-mater,[11-13] or an erosion-type matrix for sustained drug release,[14-16] because of their inherent excellent biodegradability and biocompatibility. Recently, some interesting studies have been reported in terms of polymer designs to improve or modify this functionality, mechanical properties, cell compatibility, aimed at tissue engineering use. For example, Ohya *et al*. synthesized poly(depsipeptide-*co*-lactide) having reactive side chain groups by the ring opening polymerization of $_{L}$-lactide with a depsipeptide consisting of glycolic acid and aspartic acid or lysine.[17] They investigated the biodegradable behavior, cell adhesion and growth on the copolymer films. According to the paper, the crystallinity of the obtained copolymer is lower than that of the $_{L}$-lactide homopolymer and the biodegradable behavior, cell adhesion and growth could be controlled by changing the ratio of the depsipeptides in these copolymers. In terms of the mechanical properties, Kim *et al.* synthesized elastic $_{L}$-lactide and ε-caprolactone copolymers with 1,6-hexanediol. [18] These tubular scaffolds were prepared using the copolymer, and after smooth muscle cells were seeded in them, it was subcutaneously implanted into nude mice. In addition, they estimated the degradation *in vitro* in phosphate buffer solution (pH 7.4) for up to 1 year. To improve the cytocompatibility in order to establish the biointerface for cell engineering, Watanabe et al., reported novel phospholipids polymers with on oligo(lactide).[19]

In our previous studies,[20, 21] we succeeded in synthesizing a branched PCL

macromonomer with different branch numbers and chain lengths. Using the macromonomers, the cross-linked membrane-type materials were prepared and their permeability control was mainly studied, because the cross-linked materials show a very sensitive thermo-response derived from the sensitive softening behavior based on the crystalline melting of the PCL chains. Through the thermal property investigations in these studies, the melting points (the top peaks in the differential scanning carolimetry charts abbreviated as T_m) or softening points in the cross-linked materials (the similar definition of Tm abbreviated as T_s) of the materials were controlled by modulating the branched numbers, the chain length and the copolymer composition.[22] There are some reports in terms of the practical use of CL and LA copolymer-based materials for scaffolds in tissue engineering,[4-10] and usefulness of these materials is now recognized. In Chapter 2, we investigated the feasibility of the CL and LA copolymer-based cross-linked materials mainly as a scaffold for soft tissue.

2.2 Experimental Part

2.2-1 Materials

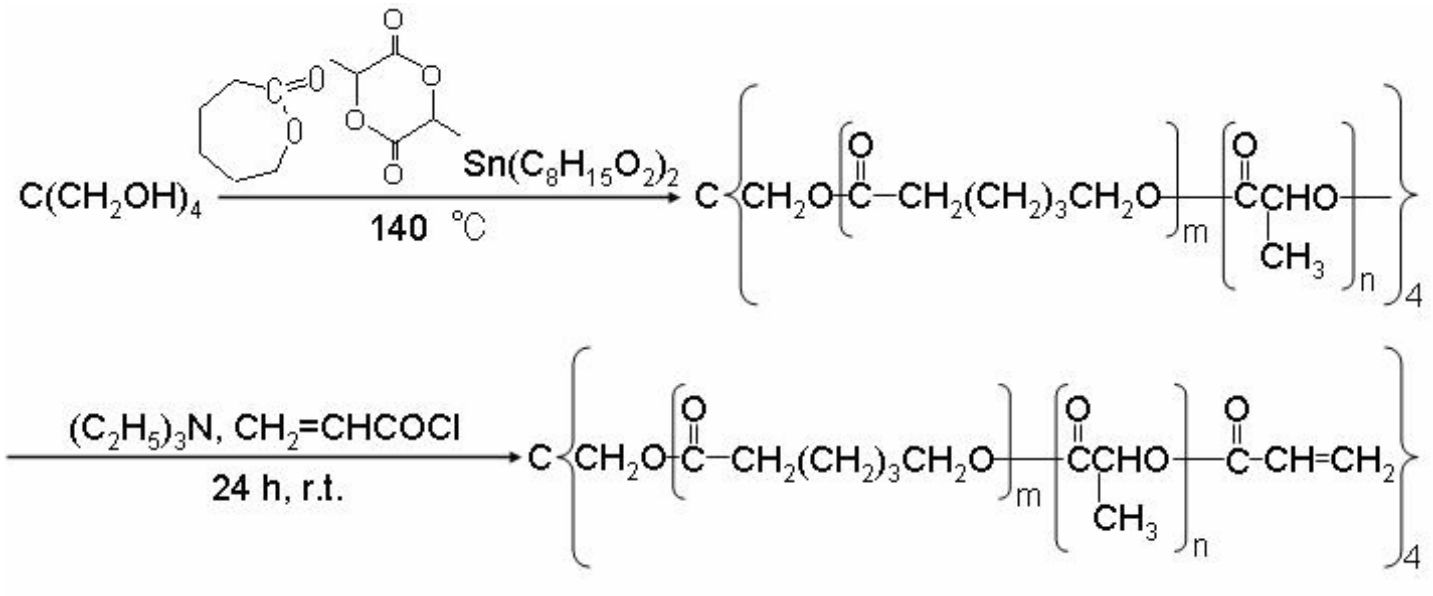
ε-Caprolactone (CL) (from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) was purified by distillation over calcium hydride under reduced pressure. Pentaerythritol and acrylyl chloride were purchased from the same company and used as received. $_{D,L}$-Lactide was kindly supplied by the Musashino Chemical Laboratory (Tokyo, Japan) and recrystallized two times from ethyl acetate before use. Triethylamine (from Wako Pure Chemical Industries Ltd., Osaka, Japan) was dehydrated by distillation over potassium hydroxide. Tin octanoate and the other chemicals also were purchased from Wako pure Chemical Industries, Ltd. (Osaka, Japan). Human uterine cervix epitheloid

carcinoma (HeLa) cells were obtained from the Health Science Research Resources Bank (HSRRB, Japan). Fetal Bovine Serum (FBS) was purchased from Gemeni Bio-Product (USA). Dulbecco's Modified Eagle's Medium, Dulbecco's phosphate buffered saline, Trypsin-EDTA solution, Penicillin-streptomycin solution, bovine serum albumin (BSA) and benzoyl peroxide (BPO) were purchased from Sigma (St. Louis, MO, USA). The NanoOrange® protein quantization kit was purchased from Invitrogen (USA).

2.2-2 Synthesis of branched macromonomer

The branched macromonomers with different CL and LA compositions were synthesized by ring opening polymerization in bulk using tin octanoate as the catalyst, and pentaerythritol as the initiator. The corresponding macromonomers were then prepared by introducing an acryloyl group at the chain ends of the precursor. The synthetic procedure is shown in Scheme 2-1. For example, the copolymer with the CL and LA molar ratio of 70 to 30 was prepared according to the following procedure. Pentaerythritol (0.284 g, 2.09 mmol) and LA (18.0 g, 0.25 mol) were placed in a flask, and dried under reduced pressure for 12 h. Freshly distilled CL (61.6 mL, 0.583 mol) and a catalytic amount of tin octanoate were added to the flask under flowing dry nitrogen. The mixture was stirred for 24 h at 140 °C under a nitrogen atmosphere. After the reaction mixture was diluted with tetrahydrofuran, the solution was poured into a mixture of n-hexane and diethyl ether. The precipitated sample was washed with the same solvent. The branched copolymer was dried under reduced pressure for 24h and a waxy solid (90.8 % yield) was obtained.

The precursor (15.0 g) prepared above was dissolved in 150 mL of dehydrated



Scheme 2-1 Schematic illustrations of branched poly(CL-LA) macromonomer syntheses.
(m/n=100/0, 90/10, 80/20, 70/30, 50/50, 30/70, and 0/100)

tetrahydrofuran containing 1.38 mL of dehydrated triethylamine (0.019 mol), and then 2.58 mL of acryloyl chloride (0.017 mol) was dropwise added. The reaction mixture was stirred for 24 h at room temperature. After concentrating, the sample was diluted with ethyl acetate and the by-product, the precipitated triethylamine hydrochloride salt, was removed by filtration. After the solvent was removed by evaporation and the concentrated sample was dissolved in tetrahydrofuran, the polymer was reprecipitated in hexane/ether. After drying, the macromonomer was obtained as a waxy solid (83.6 % yield).

2.2-3 Preparation of heat cross-linked membrane

The branched macromonomer (0.9 g) was dissolved with 1.02 mL in a xylene solution containing BPO (0.026 g/mL). The mixture was placed in the 0.1 mm space between two glass plates with a 3 × 3 cm^2 Teflon frame spacer. The glass plates were then placed in an oven at 80 °C for 2 h. The membrane-type sample was taken from the glass plates and immersed in a large amount of acetone to remove the unreacted compounds and dried under reduced pressure.

2.2-4 Characterization

The molecular weights of the precursors were estimated by gel permeation chromatography (GPC) (Jasco., Tokyo Japan) TSK α-2500 and α-4000 (Tosoh, Tokyo Japan) gel columns. Poly(ethylene glycol)s were used as the standard for calibration. The chemical structures were confirmed by 400 MHz ^{1}H-NMR (JMN-GSX400, JEOL, Tokyo Japan). The thermal properties of the precursors macromonomers and the cross-linked membranes were measured by differential scanning calorimetry (DSC)

(DSC6100, Seiko Instruments, Chiba Japan). The measurements were run from 0 to 120 °C at the heating rate of 5 °C/min. The surface atomic concentrations of the material were evaluated an by X-ray photoelectron spectroscopy (XPS) analysis. The XPS measurements were carried out using a VG ESCALAB 250 spectrometer (Thermo Electron Co.) employing monochromatic X-ray AlK (1486.6 eV) radiation. Masking of the samples using copper tape with an electron shower was preformed to avoid any charge-up. The static contact angles in water were measured using a goniometer (DropMaster 300, Kyowa Interface Science Co., Ltd., Saitama Japan). The contact angle of the bubble in water were run at 10, 25 and 40 °C.

2.2-5 Culture of HeLa cells

HeLa cells were sub-cultured in D-MEM supplemented with 5 % FBS and penicillin-streptomycin solution containing 100 U/mL penicillin and 0.1 mg/mL streptomycin in humidified environment of 5 % CO_2 at 37 °C. Subsequently, these were rinsed by PBS (-) and harvested by PBS (-) containing trypsin-EDTA solution.

2.2-6 Cell adhesion and growth

The cross-linked membranes were cut in circular pieces (24 mm diameter) and were placed on a 24-well culture plate coated with sterilized grease. The membranes were fixed with stainless rings. 70 % ethanol was added to each well to sterilize the membranes for 1h. After removing the ethanol solution, the membranes were repeatedly washed with PBS(-). The cell suspension was then prepared by D-MEM (include penicillin) with 10 % FBS and added to the 24-well culture plate at the concentration of 1.0×10^4 cells/mL. The culture system was kept in a humidified environment of 5 %

CO_2 at 37 °C. After 0 h, 6 h, 24 h and 48 h culturing periods, the cells was observed by inverted microscopy (ECLIPSE TS100, Nikon, Japan Tokyo) using a digital camera (DS-L1 and DS-5M, Nikon, Japan Tokyo). The cell growth was also estimated by counting the cell numbers using the inverted microscopy.

2.2-7 Evaluation of protein adsorption on the cross-linked membranes

The NanoOrange® was used to determine the amount of adsorbed proteins on the cross-linked membranes. [23, 24] The bovine serum albumin (BSA) was dissolved in PBS at a concentration of 0.45 g/dL. The cross-linked membranes were cut into 5 × 10 mm pieces and immersed in the BSA solution followed by incubation in a water bath at 37 °C for 2 h. These samples were thoroughly rinsed with PBS (-). Subsequently, the samples were immersed in the PBS solution containing 1wt.-% of sodium dodecylsulfate (SDS) to completely detach the adsorbed proteins on the surface and sonicated for 20 min. The SDS solution was added to the diluted NanoOrange® reagent, and briefly mixed by vortex stirring. This solution was heated in the dark at 95 °C for 10 min and cooled to room temperature for 20 min. After a brief vortex mixing, the samples were transferred to a 96-well micro-plate and the fluorescence was measured at 590 nm (excitation, 485 nm) using a plate reader (ARVO MX 120-032, Perkin-Elmer, Japan, Yokohama). The same experiment was carried out using FBS instead of the BSA solution.

2.2-8 Statistical analysis

The data from the cell growth and proteins absorption are presented as the mean ± SEM of three or more experiments and a statistically significant difference between

each result was confirmed by the Student t-test.

2-3 Results and Discussion

2.3-1 Preparation of cross-linked materials

The branched CL and LA copolymers, CL-LA100/0, CL-LA90/10, CL-LA80/20, CL-LA70/30, CL-LA50/50, CL-LA30/70, and CL-LA0/100(where CL-LA X/Y denotes the copolymers containing X mol-% of CL and Y mol-% of LA) were synthesized by the ring-opening polymerization of a mixture of CL and LA in bulk. A preliminary experiment suggested that a higher content of the LA composition in the copolymer produced an unsatisfactory mechanical strength due to the lower glass transition temperature. Therefore, the LA content was varied from 10 to 70 mol-%. As described above, we used the tetra-functional alcoholic compound, pentaerythritol, as the initiator to obtain four branched polymers. In terms of the hydroxyl compounds as initiators, some reports that deal with the multi-branched polyester preparations using naturally-occurred saccharides [25, 26] as well as synthetic poly(vinylalcohol) derivative [11] have been published. In any case, we can conveniently obtain well-designed aliphatic polyesters with the desired branch numbers.

Table 2-1 lists the synthetic conditions, composition of each monomer and estimated molecular weights of the branched CL-LA copolymers. As shown Table 2-1, we synthesized seven kinds of copolymers different with CL-LA compositions. The yield of these copolymers was very high more then 84 %. After it had collected it, CL-LA100/0, CL-LA90/10, and CL-LA80/20 were white solids, and CL-LA70/30 was a material as soft as the sponge, whereas CL-LA50/50 and CL-LA30/70 were viscous materials. CL-LA0/100 also was white powder. The compositions were calculated from the

Table 2-1 Preparation of branched poly(CL-LA) by copolymerization.

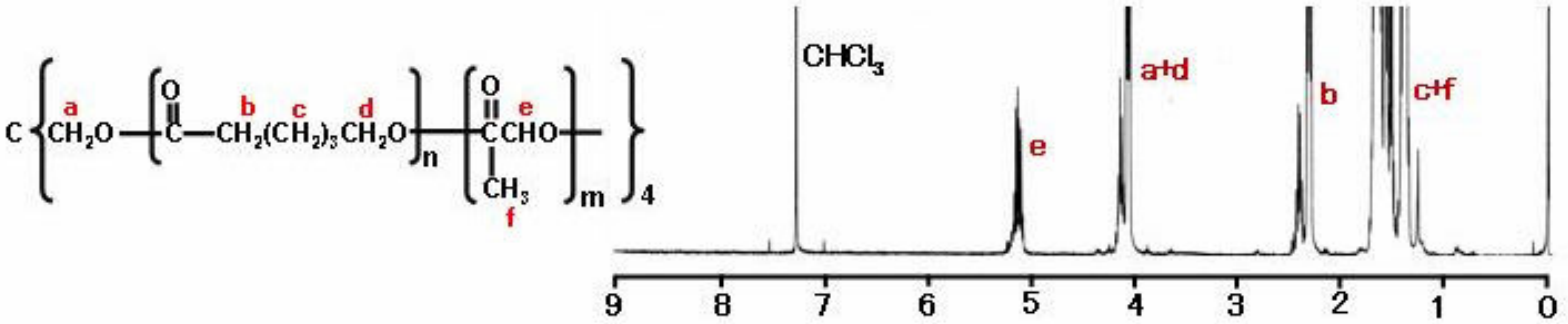
Sample name	in feed (mol-%)		in composition (mol-%)		Mn	Mw	Mw/Mn	Mtheory	Yield
	CL	LA	CL	LA					(%)
CL-LA100/0	100	0	100	0	50700	67200	1.33	45700	91
CL-LA90/10	90	10	91	9	39200	52600	1.34	44000	90
CL-LA80/20	80	20	82	18	20500	31700	1.54	42300	85
CL-LA70/30	70	30	70	30	22500	35900	1.59	40600	91
CL-LA50/50	50	50	53	47	25200	39800	1.58	37200	94
CL-LA30/70	30	70	30	70	21700	29700	1.37	33900	89
CL-LA0/100	0	100	0	100	20900	23800	1.14	28800	84

^{1}H-NMR spectra shown in Figure 2-1(a). Actually, they were estimated by comparing the peak integrals of the ethylene protons of CL (peak b at 2.30 ppm) and that of the methane protons of LA (peak e at 5.10 ppm). The calculated values and ones in the feed were very similar, and moreover, the molecular weights of the copolymers obtained by GPC corresponded to the theoretical values calculated in the feed of the copolymer syntheses.

The cross-linkable moieties were introduced into the chain ends by the reaction with acryloyl chloride. The ^{1}H-NMR spectrum of the macromonomer of CL-LA70/30m (where CL-LA X/Ym denotes the macromonomers containing X mol-% of CL and Y mol-% of LA) is illustrated in Figure 2-1(b). The successful introduction of an acryloyl group at the chain ends of the branched poly(CL-LA) was confirmed by the additional peak signal at 5.7～6.7 ppm and their intensity. The DSC peak of these copolymers and macromoomers were shown in Figure 2-2(a) and (b). As a result of the DSC analyses, the T_m linearly shifted to a lower temperature with the increasing LA content in the copolymers and macromonomers. These results surely indicate the successful syntheses of the precursors with the desired CL and CL composition.

The membrane-type cross-linked materials were prepared using the branched macromonomers by the reaction at 80 ºC for 2 h in the presence of BPO. Needless to say, the acrylyl group is activated by light irradiation or heating. In this experiment, BPO is very effective for accelerating the cross-linking reaction. [27] The yields of the materials obtained by CL-LA90/10m, CL/LA80/20m, and CL-LA70/30m were almost 100 % and the cross-linked materials possessed a good stability against the swelling in some organic solvents. However, molding of the materials obtained by CL-LA50/50m, and CL-LA30/70m were bad, and weren't able to be collected as one beautiful film.

(a) CL-LA70/30



(b) CL-LA70/30m

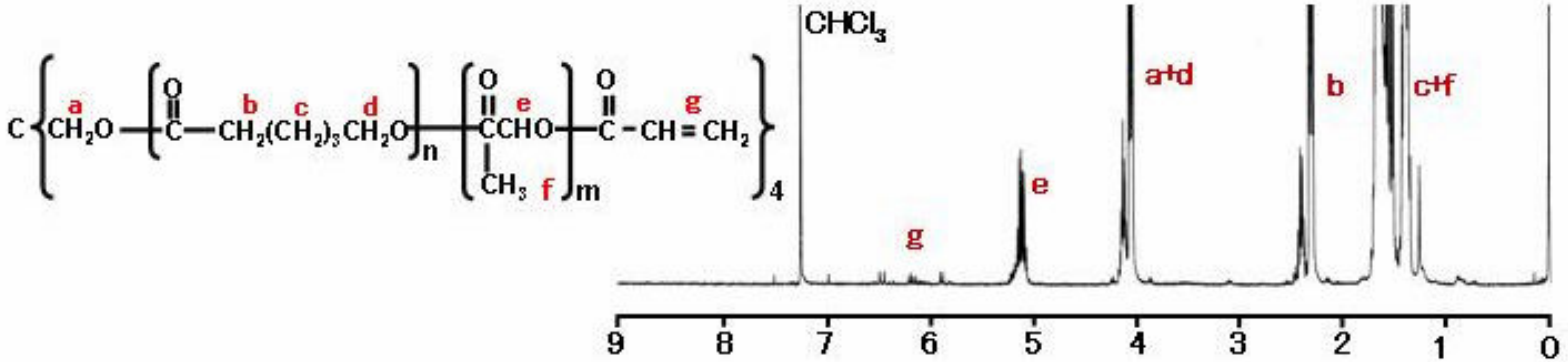


Figure 2-1 1H-NMR spectra of (a) CL-LA7030 and (b) CL-LA7030m.

(a) CL-LA70/30

(b) CL-LA70/30m

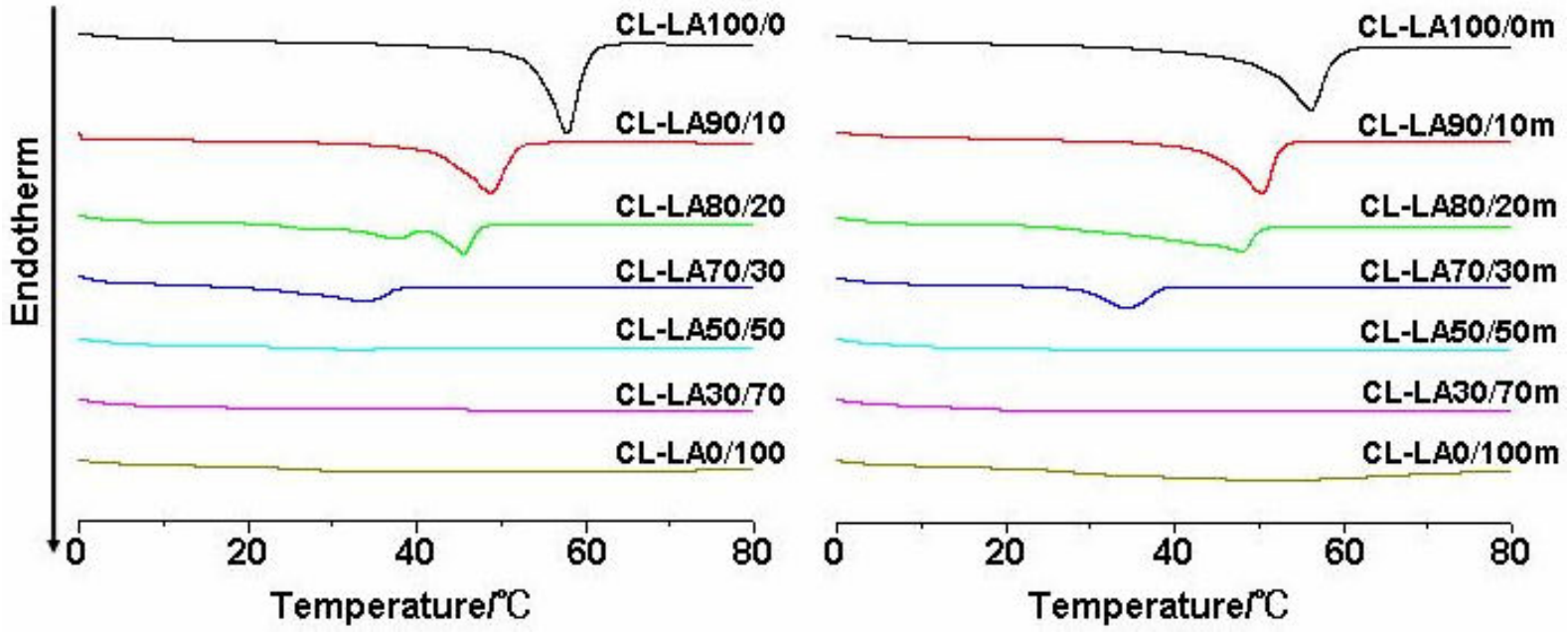


Figure 2-2 DSC measurements of (a) CL-LA7030 and (b) CL-LA7030m.

Hence, we evaluated the characterization and biocompatibility of these cross-linked membranes by using CL-LA90/10m, CL-LA80/20m, and CL-LA70/30m, because it were easy to evaluate it. The cross-linked sample names were abbreviated as CL-LA X/Yc. X and Y with the same meaning as described above.

2.3-2 Surface estimation by wettability and XPS measurement

Generally speaking, the surface properties, such as surface wettability, charge and pre-adsorbed proteins are the dominant factors controlling cell adhesion. [28, 29] We then measured the static contact angles of the air bubble on the cross-linked membrane-type materials in water. The values measured at 10, 25 and 40 °C are shown in Figure 2-3. As discussed later, as the Ts of each material are around these temperatures, we can postulate that the polymer chain mobility would be considerably changed. That is why we measured the surface wettability at each temperature. Unexpectedly, the result showed that the static contact angles are approximately the same among the cross-linked membranes of CL-LA90/10c, CL-LA80/20c, and CL-LA7030c and could not observe the temperature-dependency. It seems that the micro- or nano-level chain mobility change did not influence the macroscopic wettability. For the liquid crystalline component, the wettability change in response to temperature was reported. [30] The phase transition would induce a wettability change. In our study, the materials remained in the solid state even after softening. No phase change in the materials was due to keeping the same surface wettability.

Next, we carried out a surface element analysis using XPS. The chemical contents are summarized in Table 2-2. The presence ratio of the carbon atoms based on both the ether-type and ester-type was almost same among CL-LA90/10c, CL-LA80/20c and

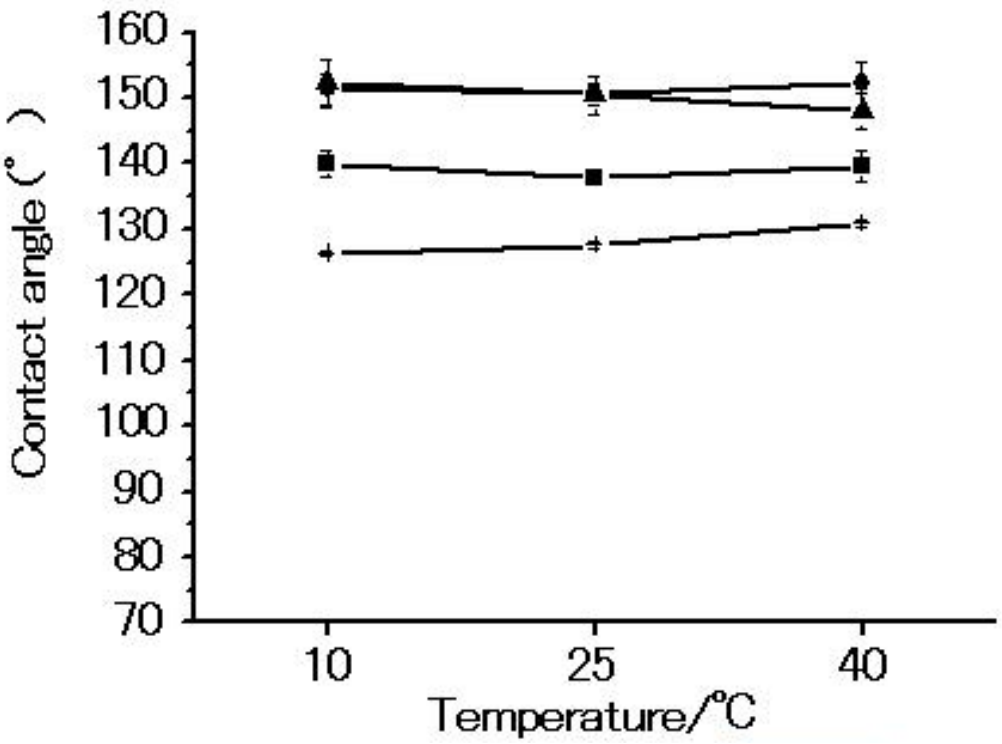


Figure 2-3 Contact angles of membrane (in water). ●: CL-LA7030c (Ts was 25.3 ℃), ▲: CL-LA8020c (Ts was 35.5 ℃), ■: CL-LA9010c (Ts was 46.0 ℃), ┼: TCPS.

Table 2-2 XPS analysis of branched poly(CL-LA)c

	polymer concentration (wt.-%)	BPO (wt.-%)	Theoretical value			ESCA analysis		
			C (%)	O (%)	O/C	C (%)	O (%)	O/C
CL-LA100/0c	**50**	**3**	**63.1**	**28.1**	**0.45**	**-**	**-**	**-**
CL-LA90/10c	**50**	**3**	**62.5**	**29.2**	**0.47**	**71.8**	**24.4**	**0.34**
CL-LA80/20c	**50**	**3**	**61.3**	**30.3**	**0.49**	**71.0**	**26.0**	**0.37**
CL-LA70/30c	**50**	**3**	**60.6**	**31.6**	**0.52**	**71.2**	**26.0**	**0.37**

CL-LA70/30c meaning a similar atom composition among them. This result would be closely related to no wettability change in these material surfaces.

2.3-3 Thermal properties of the CL-LA cross-linked materials

To obtain a flexible CL-LA-based material, investigations of thermal properties, especially the melting or softening behavior, were carried out. To clarify he profiled DSC charts of the precursors, macromonomers and cross-linked materials, CL-LA90/10c, CL-LA80/20c and CL-LA70/30c, are shown in Figure 2-4 and Table 2-3. These results indicate that the T_m or T_s shifted to lower temperatures and the enthalpy changes decreased with an increased LA content in the copolymers and corresponding macromonomers. The crystallinity based on the CL chains would be effectively disrupted with the increasing LA content.

In the cross-linked materials, we could also observe the T_s shifting to a lower temperature and a decrease in the enthalpy change by comparison with the corresponding macromonomers. The cross-linking reaction would prevent the chain mobility and obstruct the chain rearrangement to form a crystalline polymer. From these results, we focused on the CL-LA70/30c as a possible material because the T_s is under body temperature and shows an elastic character. The material seems to be suitable to handle and process for designing a soft scaffold.

2.3-4 Cell adhesion and growth on the cross-linked CL-LA membrane

The cell adhesion and growth on the prepared cross-linked membranes were evaluated by comparing with the tissue culture polystyrene (TCPS), that is commercially available. Figure 2-5, 2-6, 2-7 and 2-8 show the morphology of the cells

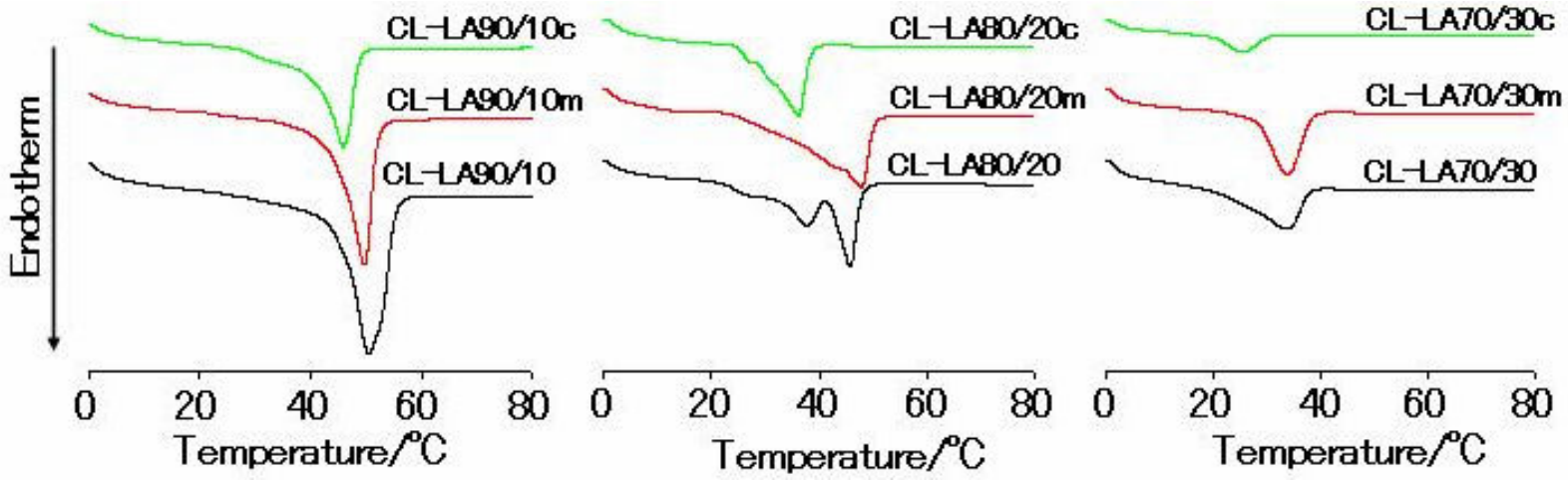


Figure 2-4 DSC thermograms of CL-LA90/10, CL-LA80/20 and CL-LA70/30 materials.

Table 2-3 Thermal analysis of branched poly(CL-LA) copolymer, macromonomer, and heat cross-linked membrane.

	X/Y=90/10		X/Y=80/20		X/Y=70/30	
	Tm (℃)	⊿H (mJ/mg)	Tm (℃)	⊿H (mJ/mg)	Tm (℃)	⊿H (mJ/mg)
CL-LAX/Y	50.0 ± 0.7	87.6 ± 16.6	38.2 ± 0.6 46.2 ± 0.5	60.4 ± 8.0	33.6 ± 0.1	36.6 ± 5.3
CL-LAX/Ym	49.8 ± 0.5	67.2 ± 3.0	46.1 ± 1.6	59.5 ± 0.8	32.1 ± 1.9	30.5 ± 6.2
CL-LAX/Yc	46.0 ± 0.6	56.0 ± 0.2	35.5 ± 0.2	37.3 ± 0.1	25.3 ± 0.2	17.1 ± 5.7

adhered on these samples after 0, 6, 24, and 48 h culturing periods, respectively. In Figure2-5, it was confirmed that the cells homogeneously were able to be seeded on these samples. As seen in Figure 2-6, it is proved that the round cells on these samples after 0 h become long and slender after 6 h, and these cells extended on these samples. Especially, the cells well adhered and extended on the CL-LA70/30c surface, and they were almost comparable to that on the TCPS. These profiles are similar to the results after 48 h that are shown in Figure 2-8. In Figure 2-9, we quantified the cell growth profiles by counting the number of cells that adhered on the CL-LA cross-linked samples. Interestingly, on the CL-LA70/30c, the adhered cell number was more than that on the TCPS after 6h and the adhesion was almost the same between CL-LA70/30 and TCPS after 24 and 48 h. The cell number on CL-LA80/20c and CL-LA90/10c was intentionally less. As indicated above, there is no change in the static contact angles among the samples of each water temperature. We then checked the protein absorption on each surface because the protein adsorption on the material surface is the first event in the contact of cells suspended in a culture medium.[28, 29] It was considered that the polymer chain mobility would be related to the protein absorption.

2.3-5 Protein absorption on the cross-linked CL-LA membrane

First, we investigated the protein adsorption using albumin at 37 ºC. Figure 2-10 shows the amount of albumin adsorbed on these cross-linked membrane-type materials and TCPS as the control. It is very interesting that the CL-LA70/30c is the best in the model protein adsorption and better than the TCPS with a statistically significant difference. The characteristic difference among CL-LA90/10c, CL-LA80/20c and CL-LA70/30c is only their T_s. As seen in the DSC charts of Figure 2-4, the softening

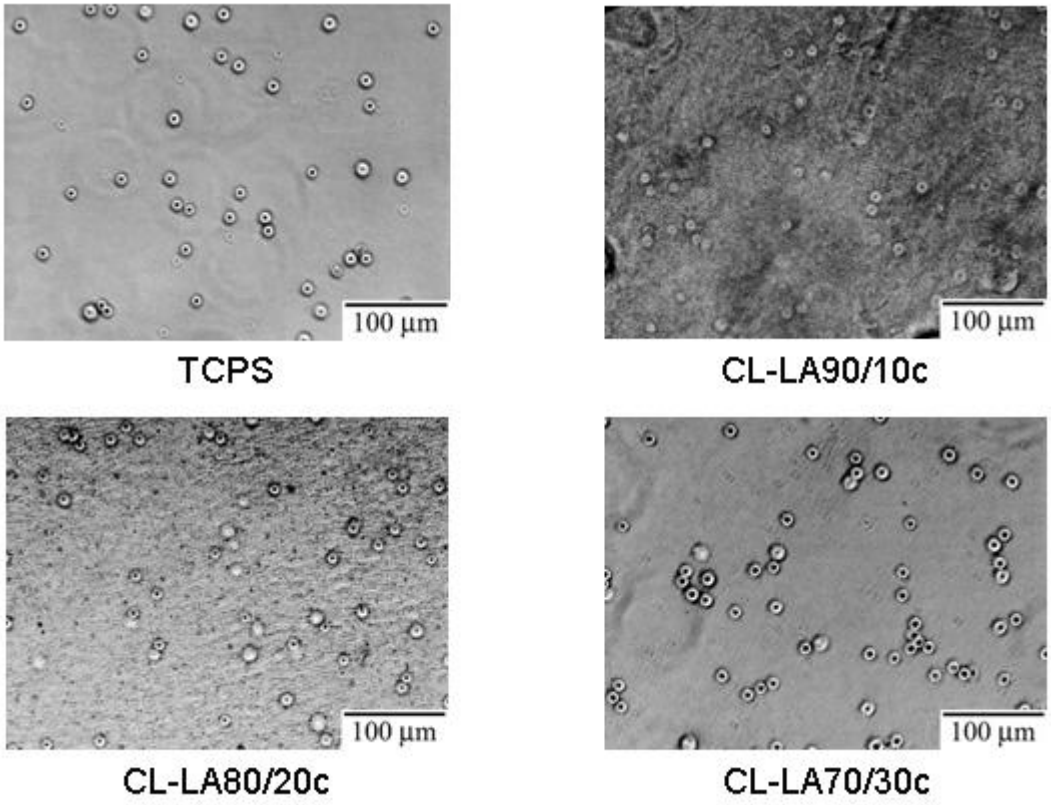


Figure 2-5 Microscopic views of HeLa cells adhered to CL-LA membrane. Cells were cultured for 0 h.

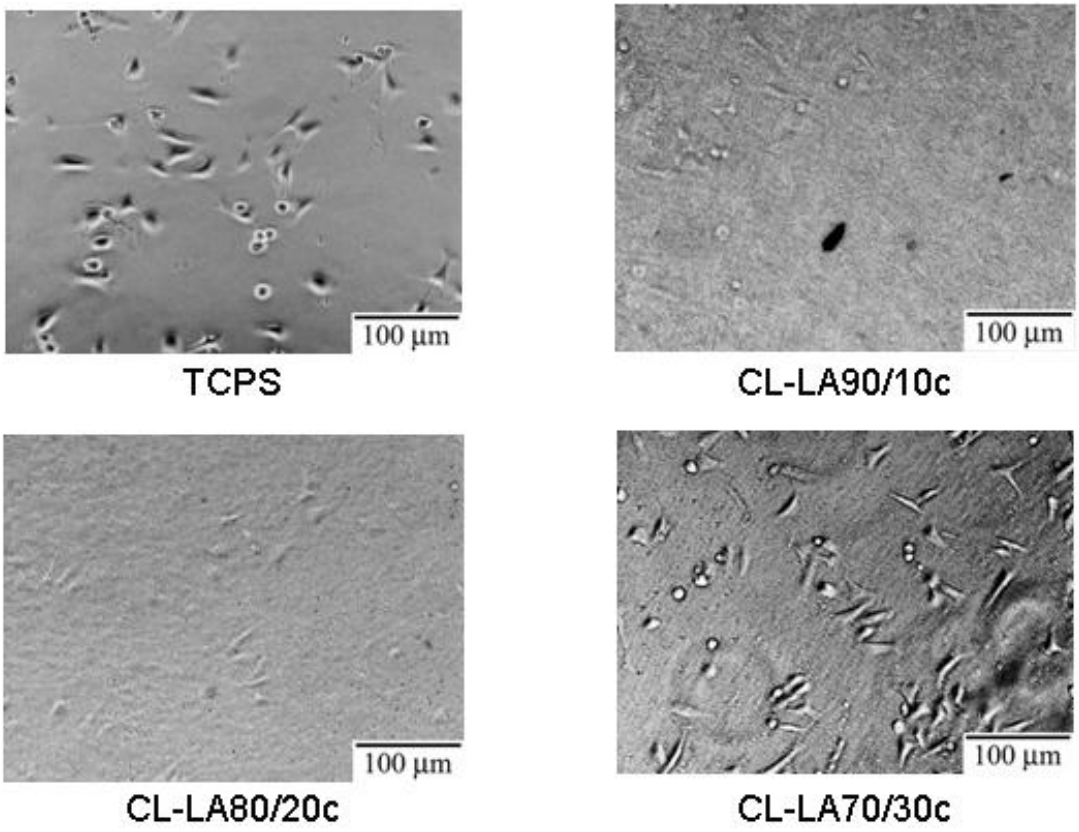


Figure 2-6 Microscopic views of HeLa cells adhered to CL-LA membrane. Cells were cultured for 6 h.

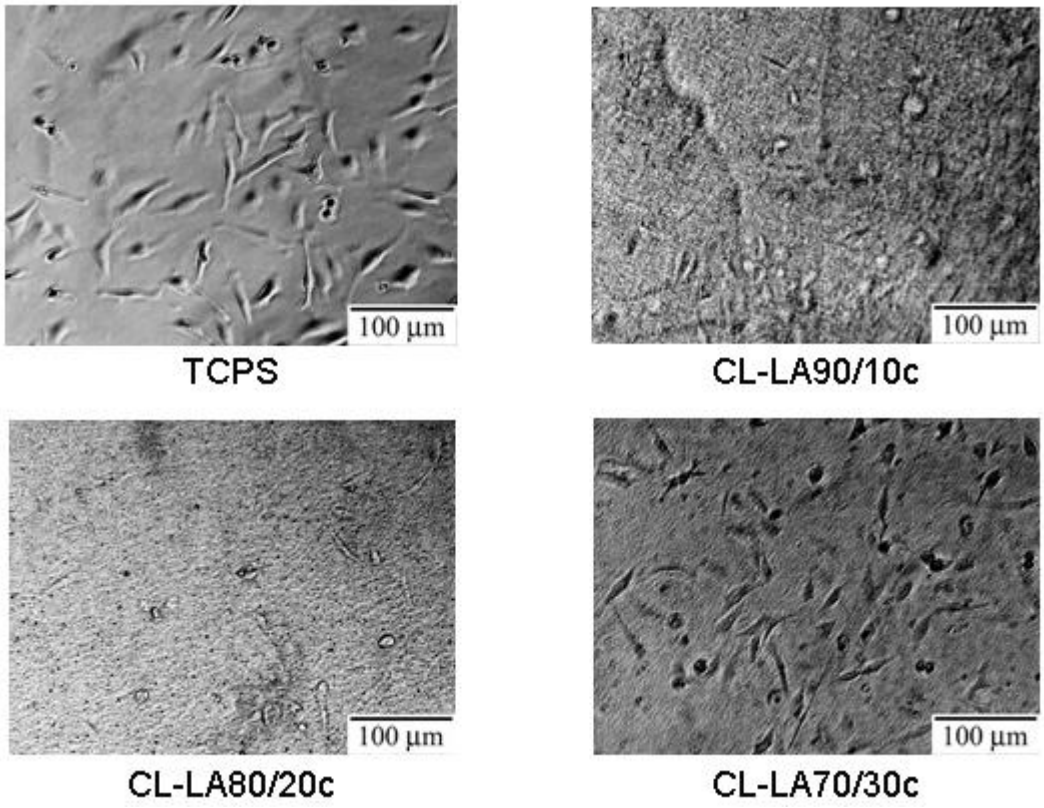


Figure 2-7 Microscopic views of HeLa cells adhered to CL-LA membrane. Cells were cultured for 24 h.

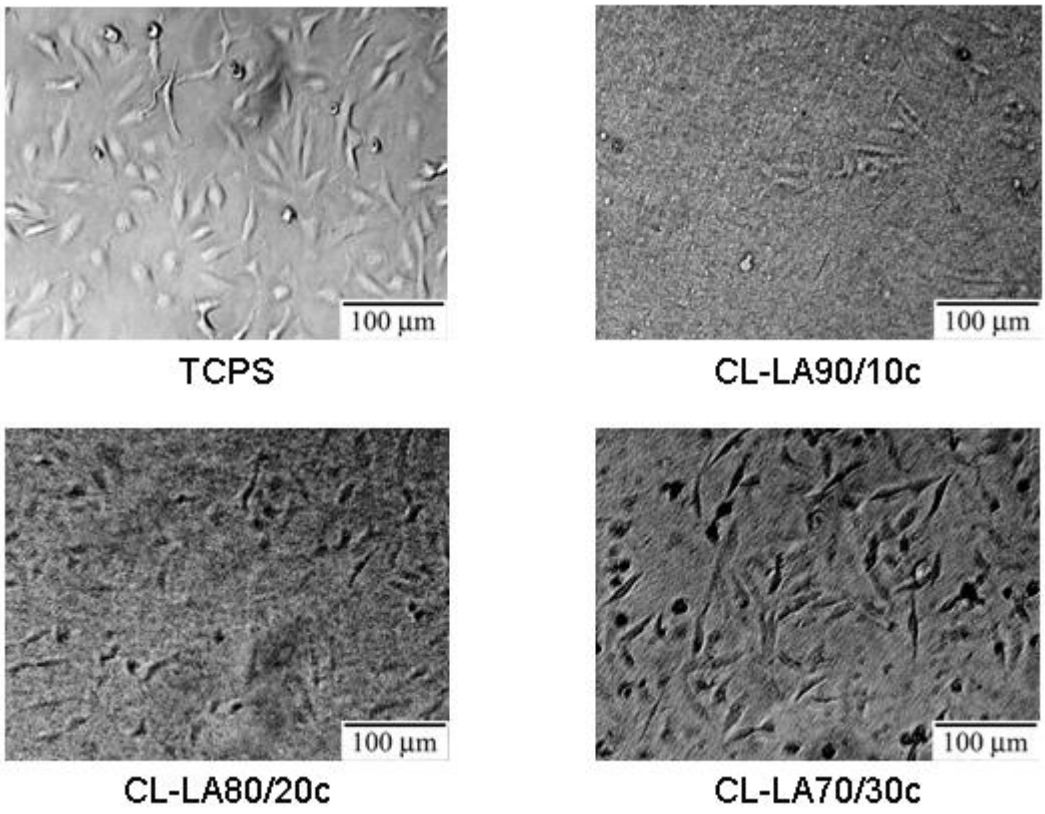


Figure 2-8 Microscopic views of HeLa cells adhered to CL-LA membrane. Cells were cultured for 48 h.

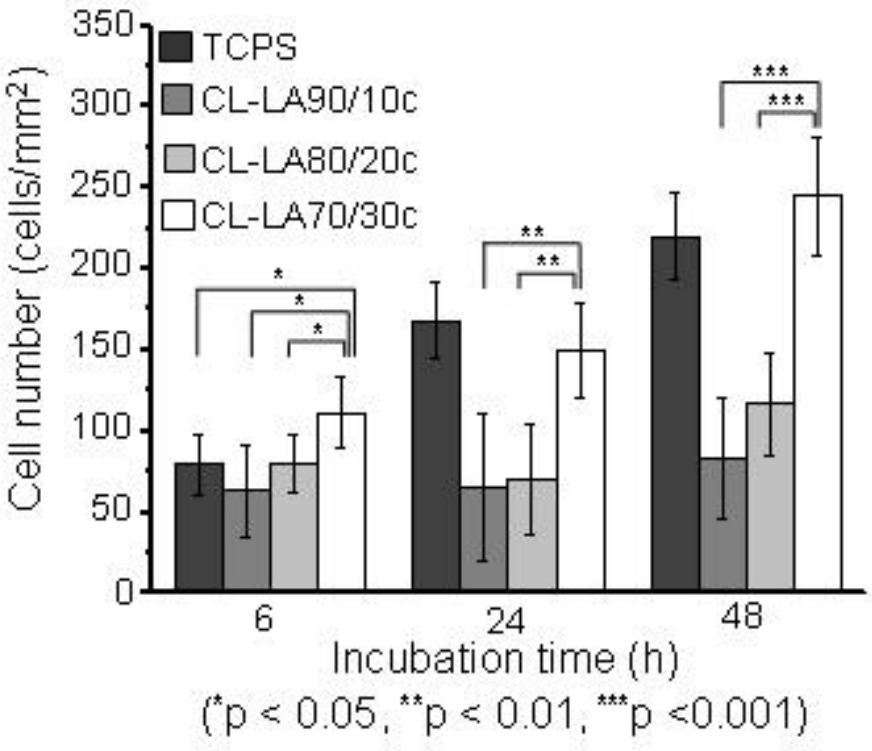


Figure 2-9 Cell growth on these membranes and TCPS. *P < 0.05, **P < 0.01 and ***P < 0.001 indicate statistically significant difference.

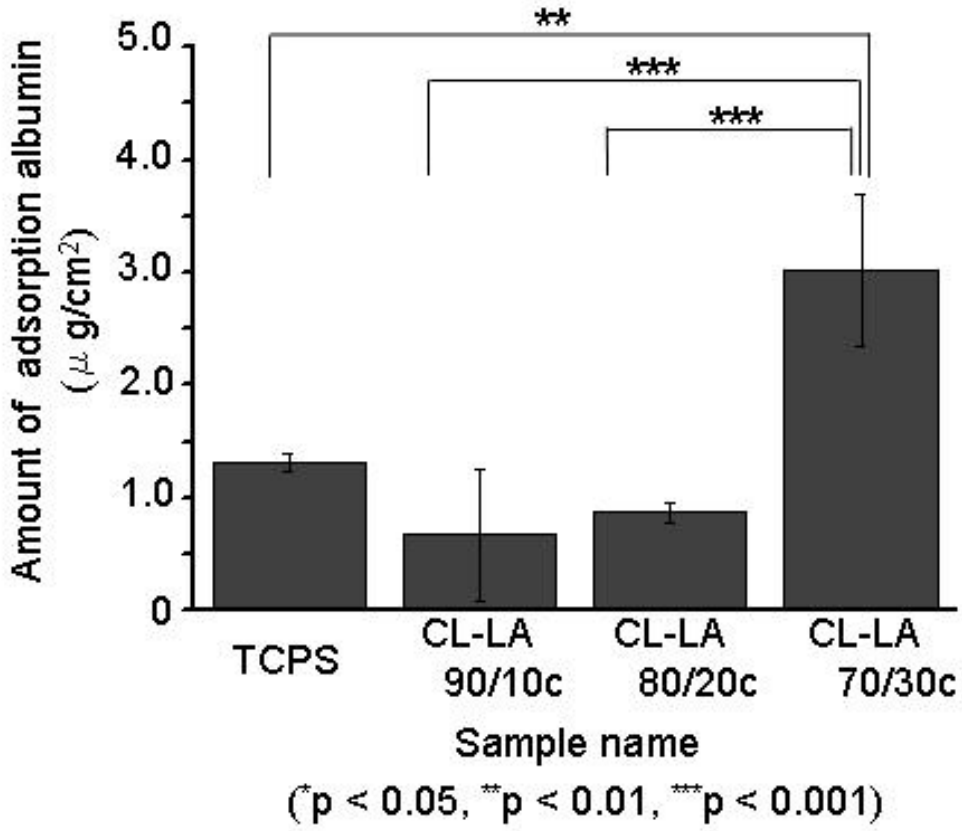


Figure 2-10 Amount of albumin adsorption on these membranes and TCPS (n=3). **p < 0.01 and ***P < 0.001 indicate that between CL-LA7030c and TCPS, CL-LA7030c and CL-LA9010c, and CL-LA7030c and CL-LA8020c. These statistically differ on a significant basis.

behavior proceeded in the temperature range, for example, in CL-LA70/30c, the range is from 15 to 35 ºC. Therefore, at 37 ºC, the behavior of CL-LA70/30c completely finished witch means that the polymer chain would be able to move at the cell culturing temperature. The higher polymer chain mobility on the CL-LA70/30c would enhance the protein absorption. We then intended to quantify the total protein absorption from the culture medium and from FBS. Figure 2-11 shows the absorption ratio versus the one on TCPS and this experiment was carried out at 37 ºC. As expected, the total proteins absorption onto the CL-LA70/30c was more than that onto CL-LA90/10c and CL-LA80/20c. Compared with the initial cell adhesion in Figure 2-9 and the protein absorption in Figure 2-9, we could see a good correlation. To confirm the effect of the T_s on the total protein absorption, the same experiments were carried out at 15 ºC (Figure 2-12). At this temperature, we could observe no difference among the three samples. Apparently, the high cell adhesion during the initial stage might be based on the high protein absorption. The reason why the cell growth on CL-LA70/30c after 24 or 48 h is comparable to that on the TCPS is not clear. Because the T_g of polystyrene is high, on the other hand, the one for CL-LA70/30c is around 25 ºC. On these surfaces, the adhesive proteins, such as fibronectin, would probably be produced and stably adsorbed.

Consequently, we could confirm that the cross-linked CL-LA copolymer-based material was very promising for a flexible scaffold. We have been preparing the porous scaffold using the salt leaching technique.[31-33] These results will be reported in the near future.

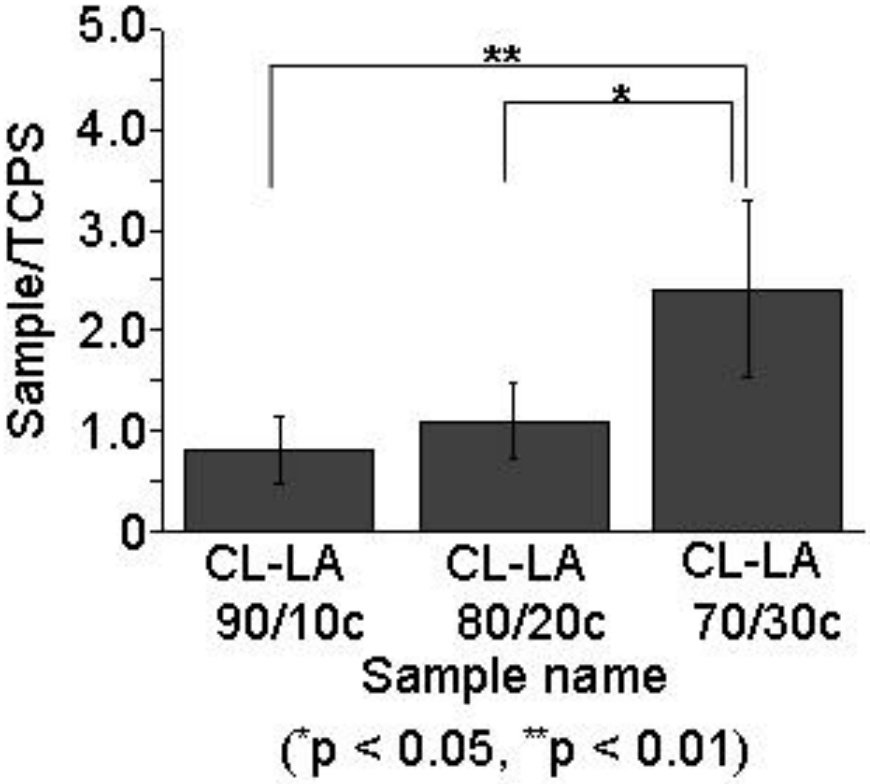


Figure 2-11 Evaluation of FBS protein adsorption on these membranes and TCPS at 37 °C (n = 3). The vertical axis means the ratio against the TCPS. *P < 0.05 and **P < 0.01 indicate that between CL-LA7030c and CL-LA9010c, CL-LA7030c and CL-LA8020c, these statistically differ on a significant basis.

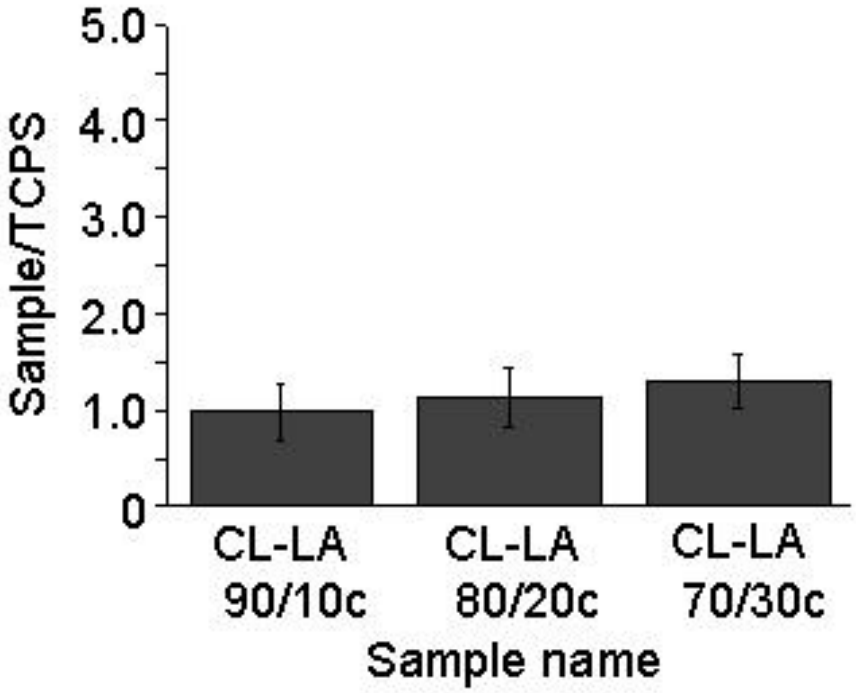


Figure 2-12 Evaluation of FBS protein adsorption on these membranes and TCPS at 15 °C (n = 3). The vertical axis means the ratio against the TCPS. Between CL-LA7030c and CL-LA9010c, CL-LA7030c and CL-LA8020c, there is no statistically significant difference.

2.4 **Conclusion**

The branched poly(CL-LA) with different CL/LA compositions were synthesized by the ring opening polymerization of CL and LA, and the corresponding macromonomers were prepared by introducing an acryloyl group at the chain ends. The cross-linked membranes were prepared by a reaction at 80 ºC for 2h using BPO as the initiator. The DSC measurement indicated that the melting points and softening points were closely related to the CL and LA compositions. Nevertheless, we could find no change in the surface characteristics among the cross-linked materials based on the wettability and XPS measurements. The cross-linked materials showed a typical elastic character and seemed to be suitable for a flexible scaffold. Cell adhesion on these membranes was evaluated using HeLa cells. Among the samples, CL-LA70/30c is comparable to the commercially available TCPS. Moreover, more FBS proteins are preferably absorbed onto the surface. This reason would be based on the high chain mobility due to the lower softening point. These results prove that the cross-linked CL-LA with a suitable composition is a promising material for the flexible scaffold of tissue engineering.

References

[1] R. Langer, P. Vacanti, *Science* **1993**, *260*, 920.

[2] J. J. Marler, J. Upton, R. Langer, J. P. Vacanti, *Advanced Drug Delivery Reviews* **1998**, *33*, 165.

[3] J. M. Moran, D. Pazzano, L. J. Bonassar, *Tissue Engineering* **2003**, *9*, 63.

[4] T. Nakamura, Y. Shimizu, Y. Takimoto, T. Tsuda, Y.-H. Li, T. Kiyotani, M. Teramachi, S.-H. Hyon, Y. Ikada, K. Nishiya, *J. Biomed. Mater. Res.* **1998**, *42*, 475.

[5] A. O. Helminen, H. Korhonen, J. B. Seppala, *Macromol. Chem. Phys.* **2002**, *203*, 2630.

[6] O. Jeon, S.-H. Lee, S. H. Kim, Y. M. Lee, Y. H. Kim, *Macromolecules* **2003**, *36*, 5585

[7] K. A. Dabis, J. A. Burdick, K. S. Anseth, *Biomaterials* **2003**, *24*, 2485.

[8] B. Amsden, S. Wang, U. Wyss, *Biomacromolecules* **2004**, *5*, 1399.

[9] N. Isogai, S. Asamura, T. Higashi, Y. Ikada, S. Morita, J. Hillyer, R. Jacquet, W. J. Landis, *Tissue Engineering* **2004**, *10*, 673.

[10] M. Honda, N. Morikawa, K. Hata, T. Yada, S. Morita, M. Ueda, K. Kimata, *Biomaterials* **2003**, *24*, 3511.

[11] Y. Hayashi, T. Yamaki, G. Odake, Y. Hashimoto, S. Ueda, *Child's Nerv Syst* **1997**, *13*, 349.

[12] N. Takahashi, Y. Suzuki, H. Ujiie, T. Hori, M. Iwaki, T. Yamada, *Nuclear Instruments and Methods in Physics Research* **2006**, *B242*, 61.

[13] K. Hida, S. Yamaguchi, T. Seki, S. Yano, M. Akino, S. Terasaka, T. Uchida, Y. Iwasaki, *Surgical Neurology* **2006**, *65*, 136.

[14] Y. Hu, X. Jiang, Y. Ding, L. Zhang, C. Yang, J. Zhang, J. Chen, Y. Yang, *Biomaterials* **2003**, *24*, 2395.

[15] J. H. Lee, A. K. Go, S. H. Oh, K. E. Lee, S. H. Yuk, *Biomaterials* **2005**, *26*, 671.

[16] A. Breitenbach, Y. X. Li, T. Kissel, *J. Controlled Release* **2000**, *64*, 167.

[17] Y. Ohya, H. Matsunami, E. Yamabe, T. Ouchi, *J. Biomed. Mater. Res.* **2003**, *65A*, 79.

[18] S. I. Jeong, B. S. Kim, Y. M. Lee, K. J. Ihn, S. H. Kim, Y. H. Kim, *Biomacromolecules* **2004**, *5*, 1303.

[19] J. Watanabe, K. Ishihara, *Biomacromolecules* **2005**, *6*, 1797.

[20] T. Aoyagi, F. Miyata, Y. Nagase, *J. Controlled Release* **1994**, *32*, 87.

[21] K. Uto, K. Yamamoto, S. Hirase, T. Aoyagi, *J. Controlled Release* **2006**, *110*, 408.

[22] H. Korhonen, A. Helminen, J. V. Seppala, *Polymer* **2001**, 42, 7541.

[23] L. J. Jones, R. P. Haugland, V. L. Singer, *BioTechniques* **2003**, *34*, 850.

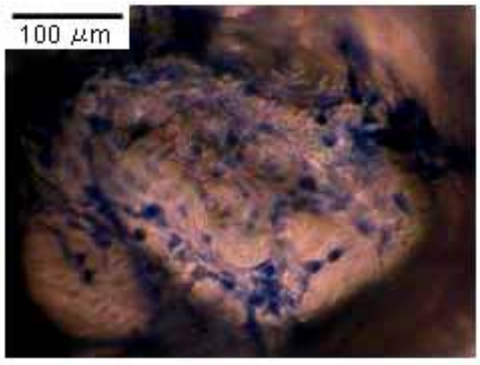
[24] Y. Inoue, J. Watanabe, K. Ishihara, *J. colloid and Interface Science* **2004**, *274*, 465.
[25] T. Ouchi, T. Uchida, H. Arimura, Y. Ohya, *Biomacromolecules* **2003**, *4*, 477.
[26] T. Ouchi, T. Kontani, Y. Ohya, *Polymer* **2003**, *44*, 3927.
[27] M. Roice, K. P. Subhashchandran, A. V. Gean, J. Franklin, V. N. R. Pillai, *Polymer* **2003**, *44*, 911.
[28] M. J. Lydon, T. W. Minett, B. J. Tighe, *Biomaterials* **1985**, *6*, 396.
[29] J. J. Rosen, M. B. Schway, *Polym. Sci. Technol* **1980**, *12B*, 667.
[30] N. T. Correia, J. J. Mouraramos, M. H. C. V. Adao, B. J. V. Saramago, *Mol. Cryst. Liq. Cryst.* **1997**, 300, 45.
[31] Q. Hou, D. W. Grijpma, J. Feijen, *Biomaterials* **2003**, *24*, 1937.
[32] T. M. Freyman, I. V. Yannas, L. J. Gibson, *Progress in Materials Science* **2001**, *46*, 273.
[33] G. Chen, T. Ushida, T. Tateishi, *Biomaterials* **2001**, *C17*, 63.

Chapter 3

Preparation, Characterization and Biocompatibility Study of the Scaffold Prototype Derived from Cross-Linked Poly[(ε-caprolactone)-co-lactide] for Tissue Engineering Materials

Summary

In Chapter 3, we prepared cross-Linked poly[(ε-caprolactone)-*co*-lactide] scaffolds prototype by salt-leaching method using different particle size of NaCl. The characterization of these scaffolds were carried out the estimating internal morphology by SEM and water content in PBS(-) at 37 °C. We also studied the adhesion and proliferation of HBCC to slab-type scaffold prototype by the microscopic observation of cell morphology and alamar Blue® assay. These results suggest that the prototype in this study shows good biocompatibility, because the cells adhered well and penetrated into the internal pore with incubation periods. Consequently, these results indicated that the scaffold prototype could be applied as practical scaffold for tissue engineering.



Microscopic views of Human Bladder Cancer Cells adhere into slab-type scaffold after 7 days incubation periods

3.1 Introduction

Recently, much attention has been attracted to tissue engineering technology as new method for regeneration where the tissues have been lost through trauma or disease.[1-6] As the effective strategy, use of porous scaffold is one of the essential methods for the three-dimensional tissue reconstruction. The requirements of scaffold materials are manifold and extremely challenging. First of all, biocompatibility is imperative, because the material must not elicit an unresolved inflammatory response nor demonstrate immunogenicity or cytotoxicity. Second, the mechanical properties of this material also must be sufficient and not collapse before the regenerated tissue is structurally stabilized.[7-10] Moreover, degradation profiles are also important since the rate may affect many cellular processes including cell growth, tissue regeneration, and host response.[11] In addition, porosity and pore size of the material should be tunable in order to optimize cell seeding, attachment, growth, extracellular matrix production, vascularization, and tissue ingrowth.[12]

In many cases, the scaffolds for tissue engineering are derived from aliphatic polyesters as poly(glycolic acid) (PGA), poly(ε-caprolactone) (PCL), poly(lactic acid) (PLA) and these copolymers because of good biocompatibility and degradation.[2, 3, 6] Therefore, we studied preparation, as well as physicochemical and biological characterization of CL-LA copolymer-based cross-linked materials in Chapter 2.[13] The cell culturing experiments discovered that cells adhesion and growth on the CL-LA70/30c (cross-linked membrane from CL/LA=70/30 composition) were comparable to that on the commercially-available polystyrene dish. Moreover, the protein absorption experiment using the FBS revealed that materials with well-grown cells showed better adhesion of the proteins.[13]

In Chapter 3, we attempted to prepare a scaffold prototype by using the same materials as described above. Since these materials possess the low melting point, they show flexibility at ambient temperature. Considering matching with the soft tissue in the human body, flexible materials are suitable. To afford the porous scaffold prototypes, salt-leaching technique[14, 15] was applied using branched macromonomer with CL/LA (70/30 mol-%) and crashed and sieved NaCl crystals with different particle size. The morphology observation with scattering electron microscopy (SEM) and water content measurement in PBS at 37 °C were carried out to characterize them. We confirmed the adhesion and proliferation of model cell to CL and LA copolymer-based scaffold prototype by the microscopic cell morphology observation adhered on the surface. The cell viability was investigated using alamar Blue® assay.

3.2 Experimental Part

3.2-1 Materials

Xylene, sodium chloride (NaCl), and Trypan Blue were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). The model cell, human bladder cancer cells (HBCC) were established by Medical school of Kagoshima University.[16, 17] Fetal Bovine Serum (FBS) was purchased from Gemini Bio-Product (USA). Dulbecco's Modified Eagle's Medium, Dulbecco's phosphate buffered saline, Trypsin-EDTA solution, Penicillin-streptomycin solution, and benzoyl peroxide (BPO) were purchased from Sigma (St. Louis, MO, USA). The alamar Blue® indicator dye was purchased from TREK Diagnostic Systems, Inc. (USA). The 25 % glutardialdehyde was purchased from MERCK (Tokyo, Japan), and diluted by PBS(-) prior to use.

Branched poly(ε-caprolactone-*co*-$_{D,L}$-lactide) that molar ratio was 70:30 (abbreviated

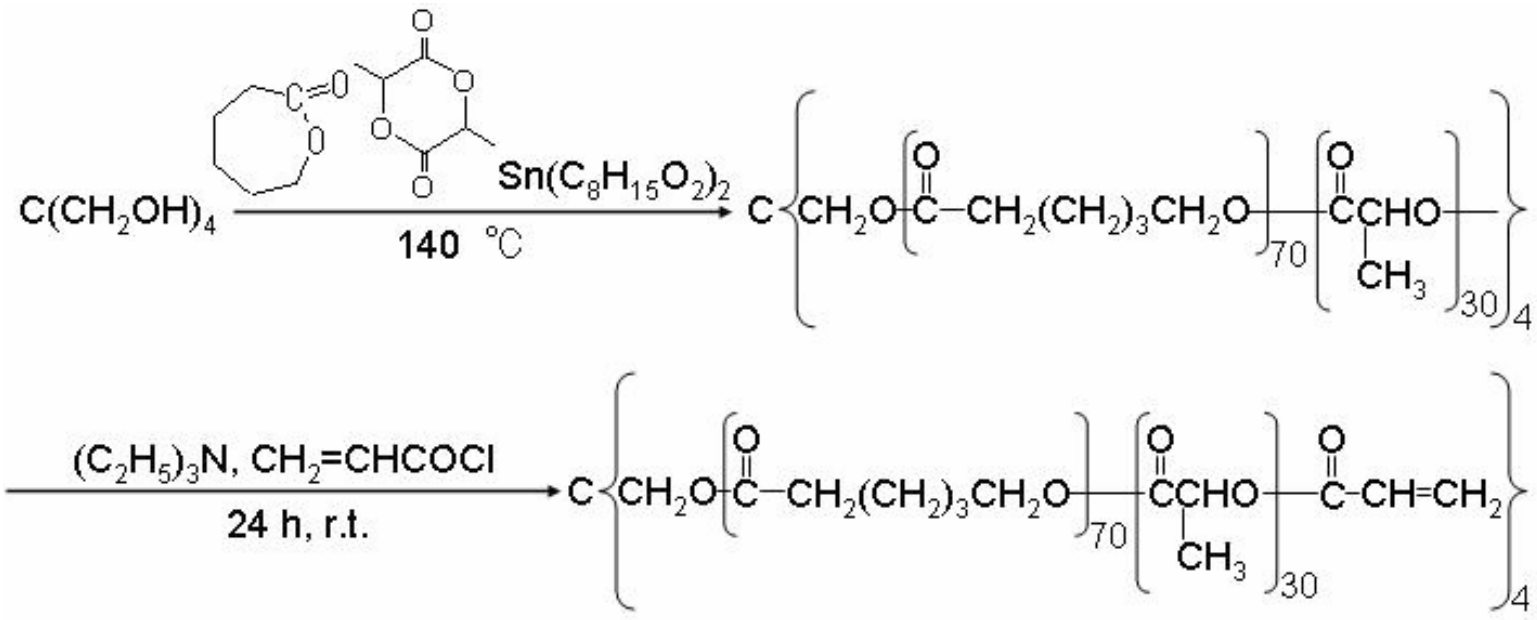
as CL-LA70/30) was synthesized by ring opening polymerization in bulk using tin octanoate as a catalyst and pentaerythritol as an initiator. The number average molecular weight, weight average one and molecular weight distribution were 22500, 35900 and 1.59, respectively. The corresponding macromonomers (abbreviated as CL-LA70/30m) were then prepared by introducing an acryloyl group at the chain ends. The synthetic procedure is shown in Scheme 3-1. The synthesis method of CL-LA70/30m and cross-linked CL-LA70/30c were reported in Chapter 2.[13]

3.2-2 Fabrication of cylindrical type scaffold prototype

The macromonomer, CL-LA70/30m (1.0 g) was dissolved with 3.41 mL of xylene solution containing BPO (0.009 g/mL). Crashed crystals of NaCl (9.0 g) were sieved at size of 53-106 μm, 106-150 μm, 150-250 μm and 250-500 μm and then added to the solution. The mixture was well mixed and placed in the cylindrical sample tube with 3.4 cm by height and 1.0 cm in diameter. These tubes were then placed in an oven at 80 °C for 2 h. The cylindrical-type sample was taken from the tube and immersed in a large amount of acetone to remove an unreacted compound. This sample was then immersed in distilled water to leach out NaCl, and washed thoroughly and dried under reduced pressure. The obtained sponge-type sample was used for scaffold properties evaluation. The sample name was abbreviated as CL-LA70/30sc (CL and LA contents was 70:30 molar ratio).

3.2-3 Preparation of slab- type scaffold sample

The slab-type sample was also prepared using same components as described above for adhered cell study. In this case, NaCl with 250-500 μm in diameter for pore



Scheme 3-1 Schematic illustrations of CL-LA70/30m.

formation, two glass plates with a 4 ×4 cm^2 and 1.0 mm of silicon frame spacer were used to achieve the purpose. Other procedure was the same as described above and the samples are abbreviated as CL-LA70/30ss.

3.2-4 Characterization

The porosity of the prepared samples was estimated by weight loss after NaCl leaching according to the following Equation (1),

$$\text{Porosity } (\%) = 100(1 - \rho/\rho_0) \quad (1)$$

where ρ_0 and ρ are the densities of the non-porous cross-linked material and the obtained sample, respectively. They were calculated by dividing the weight by the volume of non-porous cross-linked materials and these scaffolds.

The thermal properties of the cylindrical- and slab-type samples were investigated by differential scanning calorimeter (DSC) (DSC6100, Seiko Instruments, Chiba Japan). The measurements were carried out from 0 to 120 °C at the heating rate of 5 °C/min. SEM clarified the internal morphology and pore size of these cylindrical type samples. The water content were evaluated in PBS(-) at 37 °C. The percentage of water content was measured by gravimetry according to the Equation (2)

$$\text{Water content } (\%) = (W_t - W_0)/W_0 \quad (2)$$

where W_0 is the weight of the scaffolds itself and W_t is the weight after containing medium at scheduled interval.

We also evaluated the mechanical property of the starting material, CL-LA70/30c. The experiment was carried out on square specimens using a tensile testing machine, Little Senstar LSC-1/30 (Tokyo Testing Machine Co., Japan). The square specimens with 10 mm width and 30 mm length were from the prepared CL-LA70/30c having

0.2-0.3 mm thickness. The both ends of these specimens were fixed, and strained by a crosshead speed of 1 mm/min.

3.2-5 Culture of Human bladder cancer cells

The supplied human bladder cancer cells were sub-cultured in the D-MEM supplemented with 5 % FBS and penicillin (100 U/mL) and streptomycin (0.1 mg/mL) solution in humidified environment of 5 % CO_2 at 37 °C. Subsequently, they were rinsed by PBS(-) and harvested from PBS(-) containing trypsin-EDTA solution.

3.2-6 Cell adhesion and growth

The slab-type scaffold samples were cut into circular pieces (6 mm diameter) and placed on the bottom of 96-well culture plate and fixed with stainless rings. 70 % ethanol was added to each well to sterilize them for 1day. After removing the ethanol solution, they were repeatedly washed with PBS(-) and centrifuged in PBS(-) at 1000 rpm at 25°C for 15min[18] to clean thoroughly. The cell suspension was then prepared by D-MEM (include penicillin) with 10 % FBS and added to the culture plate at the concentration of 3.2×10^3 cells/cm^2. The culture system was kept in a humidified environment of 5 % CO_2 at 37 °C. After 1, 3 and 7 days culturing periods, these scaffold samples were gently washed with PBS(-) to remove an un-adhered cells.

They were then fixed in 2.5 % glutardialdehyde for 4h and dehydrated in 30 %, 50 %, 90 %, 100 % ethanol for every 5 minutes. After drying, they were stained by Trypan Blue and swelled by immersing in ethanol to observe by the inverted microscopy. The cells on the surface were observed by inverted microscopy (ECLIPSE TS100, Nikon, Japan Tokyo) using a digital camera (DS-L1 and DS-5M, Nikon, Japan Tokyo).

In separate experiments, alamar Blue® was used to evaluate the cell growth on the scaffold.[19, 20] After the same periods, 20 vol.-% of the alamar Blue® solution was added to the wells against the total culture medium. Then the plate was incubated for 6 h. After that, 20 μL of the medium was translated to 96-wells black plate, and then diluted with 80 μL of PBS(-). The fluorescence intensities of the mixture solution were measured by a fluorescence plate reader (ARVO MX 120-032, Perkin-Elmer, Japan, Yokohama) set at excitation and emission wavelengths of 544 nm and 590 nm, respectively.

3.2-7 Statistical analysis

The data from the cell growth and protein absorption studies are presented as the mean ± SEM of three or more experiments and a statistically significant difference between each result was confirmed by the Student t-test.

3.3 Results and discussions

3.3-1 The characters of the starting material

In general, native and un-reactive PLA and PLGA are used as a scaffold for hard tissue such as bone, because the glass transition temperature, T_g of these polymers are relatively high and stiff enough to keep the shapes themselves at ambient temperature. Therefore, we intended to design the flexible and soft scaffolds to match with the soft tissues in the human body. We carried out the fundamental studies in terms of synthesis, characterization and protein adhesion as well as cell proliferation as reported in Chapter 2.[13] The branched CL and LA copolymers and the corresponding macromonomers were prepared by a ring-opening copolymerization using tin octanoate as a catalyst and pentaerythritol as an initiator, followed by introducing acryloyl group at the chain ends.

By the cross-linking reaction, we could control the shape according to the desired mold, even though the materials would possess the low T_g. That is why such macromonomer is surely useful to fabricate the scaffold. In that experiment, the membrane-type sample was supplied to the cell adhesion study. This flat material composes of CL and LA random copolymer that composition was 70/30 molar ratio. It is rubbery at the ambient temperature. The mechanical property of CL-LA70/30c was shown in Figure 3-1 and Table 3-1. The tensile strength and strain at break of the CL-LA70/30c were 5.5±0.7 MPa and 437.0±54.0 %, respectively (table 3-1). As seen Figure 3-1, the S-S curve of CL-LA70/30c also showed the same behavior as that of the typical rubber at room temperature. The results suggested that the CL-LA70/30c was the flexible material as a rubber. The model cell, HeLa cells adhesion and growth on the material are comparable to that on the commercially-available polystyrene dish. The protein absorption experiment using the FBS proteins revealed that the materials with well-grown cells showed better adhesion of the proteins. Therefore, we attempted to prepare porous and soft scaffold prototype using the same macromonomer. Now, we adopted salt-leaching technique[14, 15] because of an easiness and practicality in preparation process. Moreover, many researchers also prepared the scaffold by salt-leaching technique, and evaluated to the practicality for tissue engineering material. C.-T. Lee *et al.* reported that the sponge-like scaffold could be prepared by salt leaching and solvent casting methods using grafted copolymer of chondroitin sulfate-*g*-$_L$-lactide, and served as a potential candidate for cartilage tissue engineering by resulting of the biocompatibility test as MTT assay, histological examination, RT-PCR analysis and so on.[14] R. K. Srivastava *et al.* also synthesized by ring ring-opening polymerization of ε-caprolactone and 1,5-dioxepan-2-one using enzyme as catalysis, and evaluate to the usefulness for tissue

Table 3-1 The mechanical property of starting material, CLLA70/30c

	Tensile strength (MPa)	Strain at break (%)
CLLA70/30c	5.5±0.7	437.0±54.0

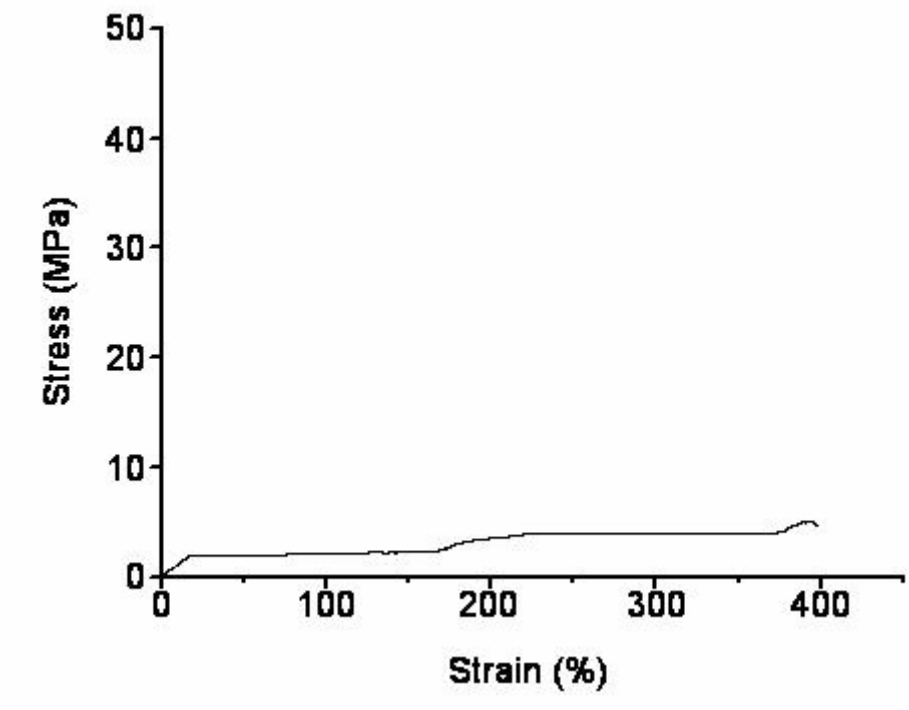


Figure 3-1 The S-S curve of CL-LA70/30c

engineering of the porous scaffold prepared by salt-leaching technique.[15] So, we prepared the scaffold prototype by salt-leaching technique, and evaluated to the porosity, internal morphology, water content, and *in vitro* test of the cells.

3.3-2 Preparation of scaffold prototype

Table 3-2 shows the preparative condition and characterized results of the scaffold prototype. First, we optimized that macromonomer concentration at 25 wt.-% in the solution was the best for the successful preparation, because of a good dispersion of the crashed NaCl particles for pore formation. The prototype from the present condition is flexible sponge as seen in Figure 3-2. The thermal properties were listed in Table 3-1 and Figure 3-3 indicated the DSC curve. As a result, the melting point (T_m) and enthalpy change (ΔH) were determined to be about 37 ℃ and 42 mJ/mg, respectively. In this case, the temperature was higher and enthalpy change was larger compared with the previous sample from the same macromonomer. It might due to the macromonomer concentration. As reported, the concentration was closely related the thermal properties.[22] We also evaluated the porosity of the prototype. The porosity and size control are very important for the practical scaffold fabrication, because of nutrition supply and gas exchange enough to maintain the cell viability. To estimate the porosity, many researchers have reported a gravimetry[22, 23] or a liquid displacement method.[24-26] Other researchers also applied mercury intrusion porosimetry[22, 27-29] and various computer analysis software of SEM.[30, 31] For example, Hu *et al.* evaluated the porosity of the scaffold prepared from PLA/PLGA by gravimetry at room temperature.[23] Zhang *et al.* adopted the liquid displacement method to estimate the porosity of the composite foams prepared from polymer/hydroxyapatite/dioxine

Table 3-2 Preparation and characterization of CL/LA scaffold materials.

	Shape of material	Polymer concentration (wt.-%)	BPO (wt.-%)	Salt particle size (µm)	Salt content (wt.-%)	Porosity (%)	Tm (°C)	⊿H (mJ/mg)
CL-LA70/30sc53	**cylinder**	**25**	**3**	**53 - 106**	**90**	**77.6 ± 5.3**	**38.7**	**43.0**
CL-LA70/30sc106	**cylinder**	**25**	**3**	**106 - 150**	**90**	**80.0 ± 4.8**	**39.0**	**36.2**
CL-LA70/30sc150	**cylinder**	**25**	**3**	**150 - 250**	**90**	**86.0 ± 4.1**	**38.9**	**43.4**
CL-LA70/30sc250	**cylinder**	**25**	**3**	**250 - 500**	**90**	**86.0 ± 3.2**	**38.4**	**44.2**
CL-LA70/30cc	**cylinder**	**25**	**3**	**-**	**-**	**-**	**38.1**	**42.8**
CL-LA70/30sm250	**membrane**	**25**	**3**	**250 - 500**	**90**	**85.2 ± 2.6**	**37.9**	**31.0**

Figure 3-2 Photograph of CL-LA70/30sc250 (a) and CL-LA70/30ss250 (b).

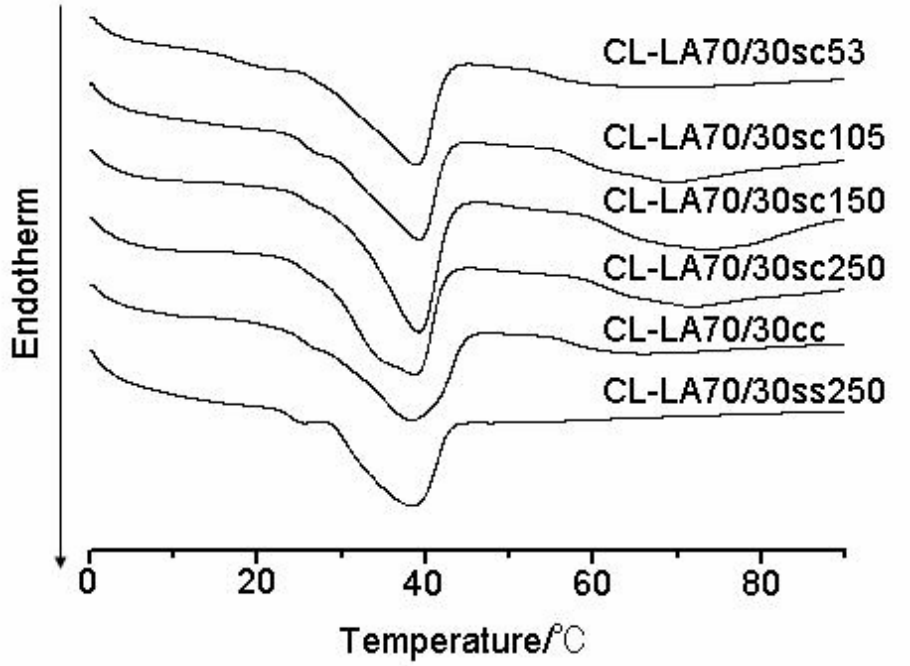


Figure 3-3 DSC thermograms of the cylindrical- and slab-type samples.

mixtures by using ethanol.[25] Hu *et al.* used the density value (1.25 g/cm^3) of the 670 kDa PLA for calculation of the polymer. For correct estimation, we measured the actual density of the materials from the non-porous cross-linked materials. The estimated results were shown in Table 3-2. As shown, the porosity closely depended on the particle size of crashed NaCl. The smaller NaCl particles we use, the smaller pores were. It is suggested that the scaffold prepared by using small particle size of NaCl could be constricted by purification process and reduced pressure dry. Anyway, it is proved that these scaffolds were highly porous structure, because the porosity of these scaffolds was a range from 77.6 ± 5.3 % to 86.0 ± 3.2 %.

3.3-3 Structure of scaffold prototype

Figure 3-4 shows SEM views of the cylindrical scaffold prototype. We can see the well-developed porous structure, consisting of open pore channel and inter connected framework. CL-LA70/30cc (Figure 3-4(e)) (without the salt) did not show any pores. Consequently, it is suggested that CL-LA70/30sc53, CL-LA70/30sc106, CL-LA70/30sc150 and CL-LA70/30sc250 prepared were the porous materials by using very effective salt-leaching technique. The pores of these scaffolds also slightly contracted, but the sizes were controlled by the particle size of the salts. Especially, Figure 3-4(f) shows SEM photograph of CL-LA70/30ss250 (slab-type sample). The internal morphology of slab- type material showed a porous structure and internal connected framework as well as that of cylindrical type scaffold.

The water uptake in a scaffold is important aspects because of the supply cell growth environment for tissue engineering.[32-35] Therefore, we evaluated the water uptake of scaffold by gravimetry in PBS(-) at 37 ℃. The water content of these

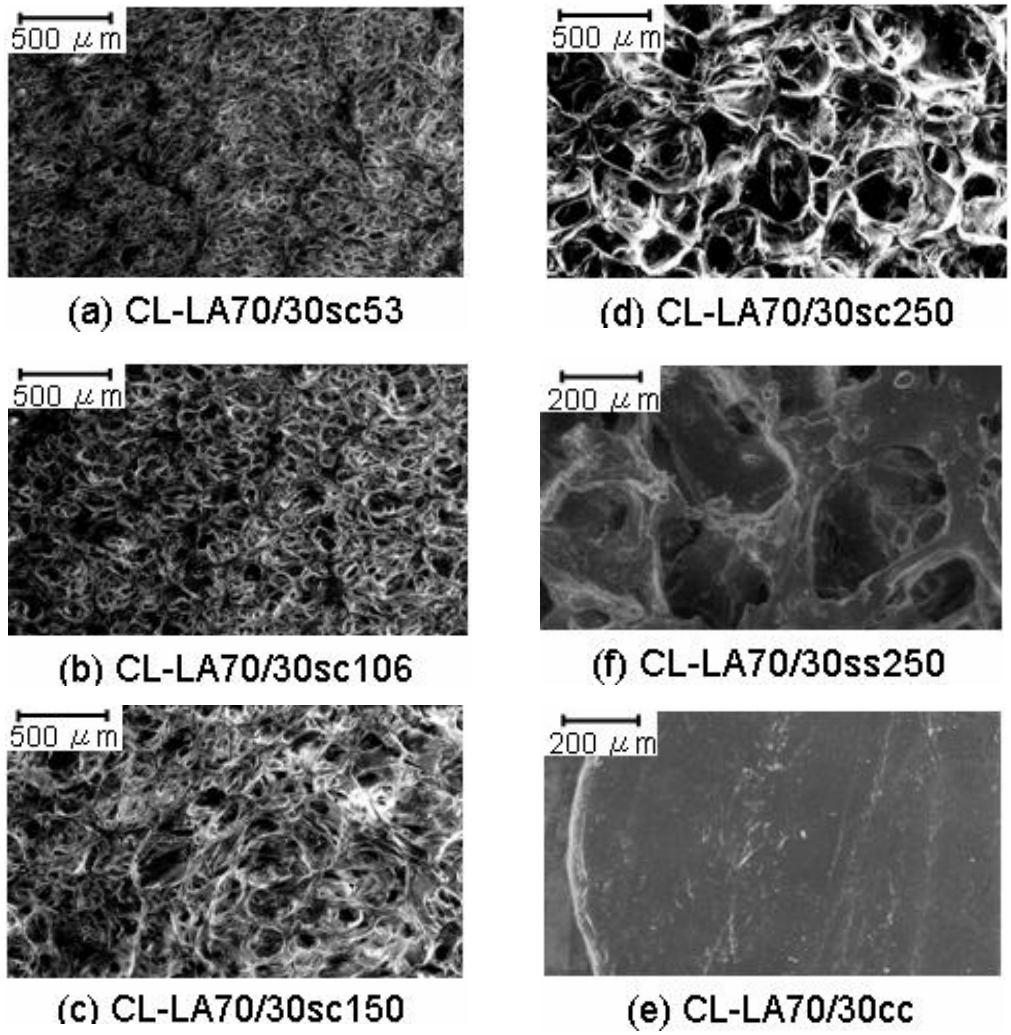


Figure 3-4 Morphological observation by SEM of cylindrical- and slab-type samples.

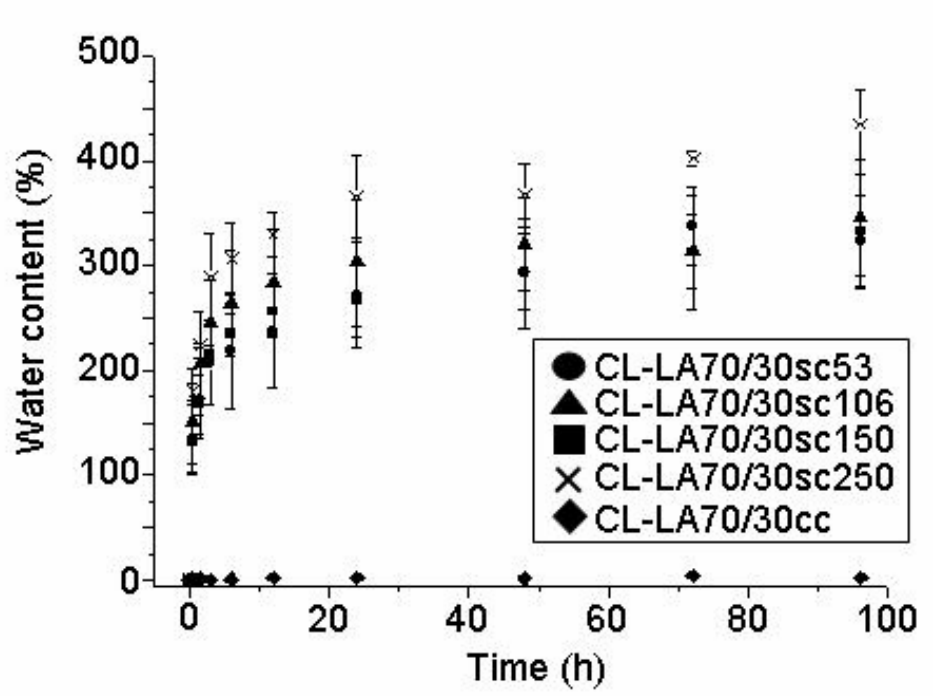


Figure 3-5 Water uptake at 37 ℃ in PBS(-) of cylindrical-type samples.

cylindrical scaffolds was shown in Figure 3-5. CL-LA70/30sc53, CL-LA70/30sc106, CL-LA70/30sc150 and CL-LA70/30sc250 absorbed water very rapidly with about 15 min, and then gradually reached equilibrium for about 24 h. The ratio of water uptake was very high at 300 %, when water uptake of these scaffolds was reached equilibrium. Additionally, each sample could hold the water in the porous structure, because the water weight in these materials did not decrease even after 24 h, whereas CL-LA70/30cc (non-porous sample) did not absorb water. These results suggest that the prototype prepared in this study could uptake and preserve the large amount of water in its pores enough to provide the cell growth environment.

3.3-4 Cell proliferation in the scaffold prototype

In Chapter 3, human bladder cancer cells (abbreviated as HBCC) were used to study the feasibility as the scaffold. Generally, lactide and glycolide-based copolymers are relatively stiff because of their higher T_g. Therefore, they are suitable for such bone and cartilage regeneration.[3, 4, 25, 27, 28] On the other hand, in terms of the scaffold for soft tissues or organs such bladder that require retractility, soft and flex scaffold would be appropriate. Hence, we intended to design the soft scaffold prototype with rubbery character. Hydrogel are also studied aiming at extra-cellular matrix, however, the mechanical strength seem to be not satisfied.[36-38]

Figure 3-6 shows the cell morphology of the HBCC adhered on the CL-LA70/30sm250 after 1, 3 and 7days culturing periods. The low magnification of the microscope was applied for survey the cell morphology on the surfaces of the prototype. As seen in the figure, the HBCC are well adhered and extended on the CL-LA70/30sm250 surface. Moreover, Figure 3-7 indicated highly magnified picture on

the same prototype. The cells go into the pores and adhere and spread on the inner surfaces. In Figure 3-8, we confirmed the cells growth profiles by the alamar Blue® assay. The alamar Blue® was used as the cell viability assay, and many researchers also evaluated to the cell proliferation. Y. Fukuhira *et al.* evaluated to the proliferation of NIH3T3 cells on honeycomb-patterned film prepared by poly(lactide) and dioleoylphosphatidylethanolamine as adducing.[20] Moreover, E. Tsiridis *et al.*[39, 40] and L. Disilvio *et al.*[41] also evaluated to cell proliferation by alamar Blue assay®. The cell number actually increased with incubating time and the cells surely proliferated in the porous structure of the scaffold prototype. As reported in our previous study, the starting materials for the prototype show good cell adhesion, spreading and proliferation. These characters are taken over in the scaffold prototype. In terms of biodegradation of the materials, we have confirmed the enzyme degradation using a lipase preliminarily (data not shown). For the practical regeneration of the bladder, we need more precise studies such as tissue compatibility, permeability mechanical strength and so on.

Consequently, we could confirm the feasibility of the new type of scaffold with retractility such a rubber. Moreover, since the starting materials are macromonomer type they are capable of introducing other functional groups by copolymerization. The groups would contribute to proper modification or immobilization of bioactive molecules on the scaffold surfaces. We will report the new studies of more functional scaffold materials in the future.

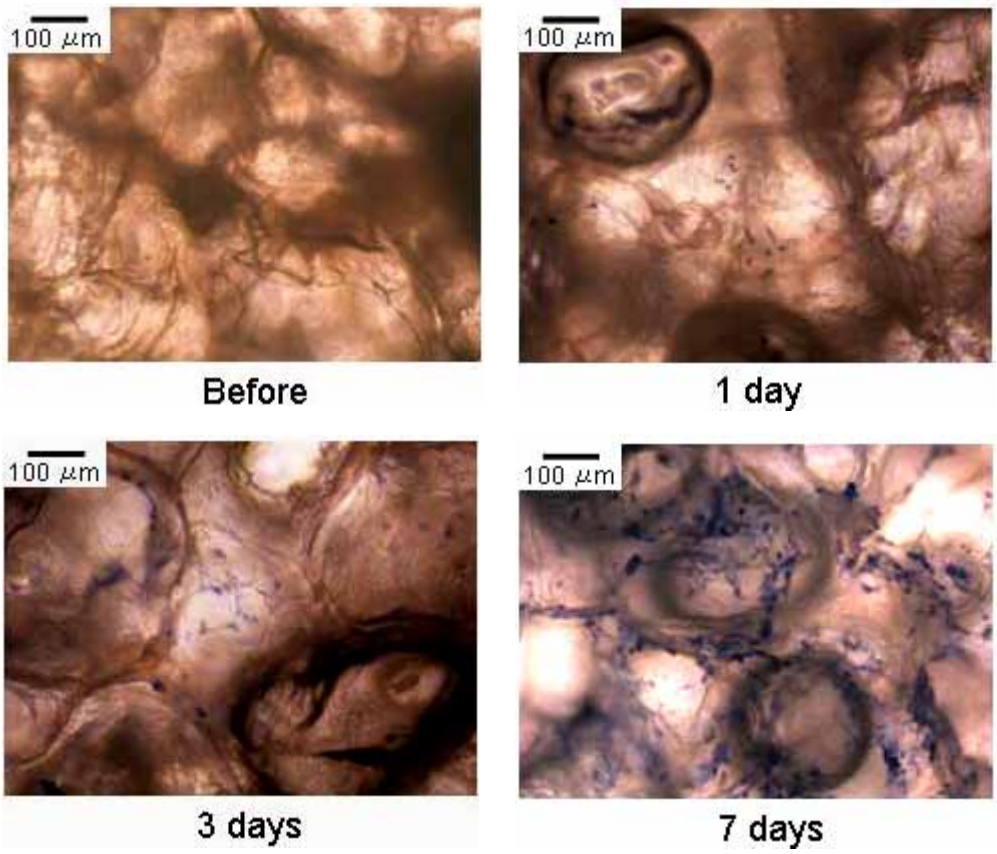


Figure 3-6 Microscopic views of HBCC adhered to CL-LA70/30ss250 surface with incubation periods.

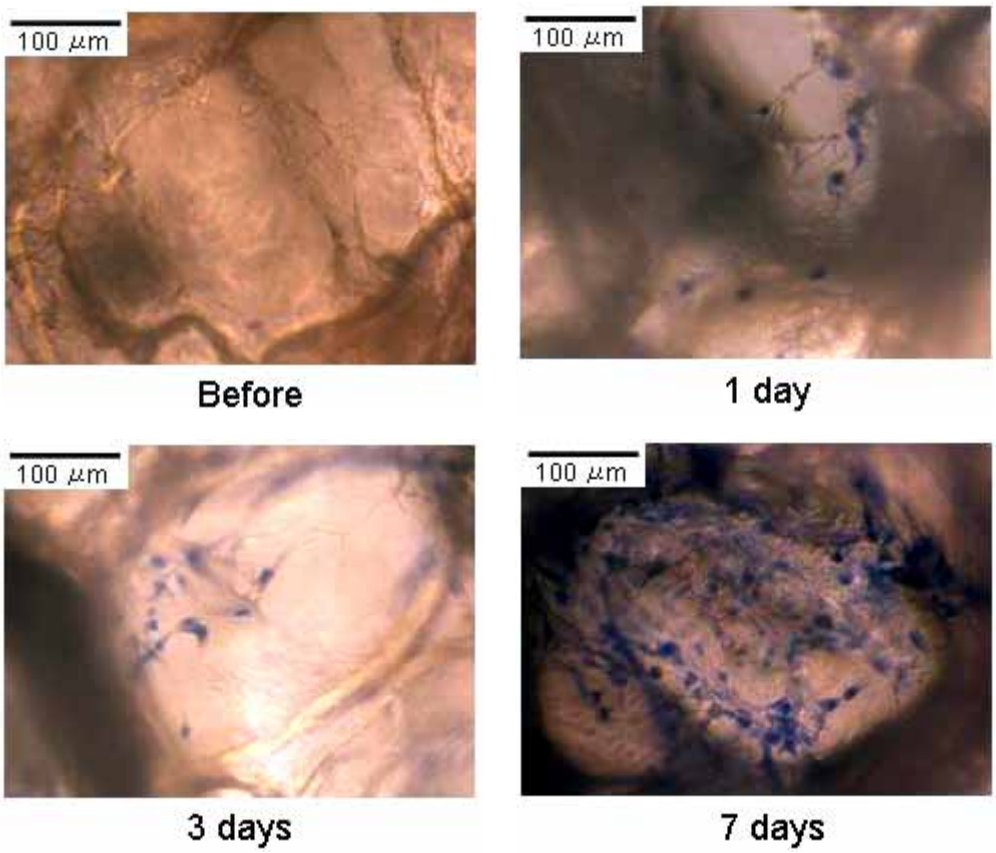


Figure 3-7 Microscopic views of HBCC adhered to CL-LA70/30ss250 inside with incubation periods.

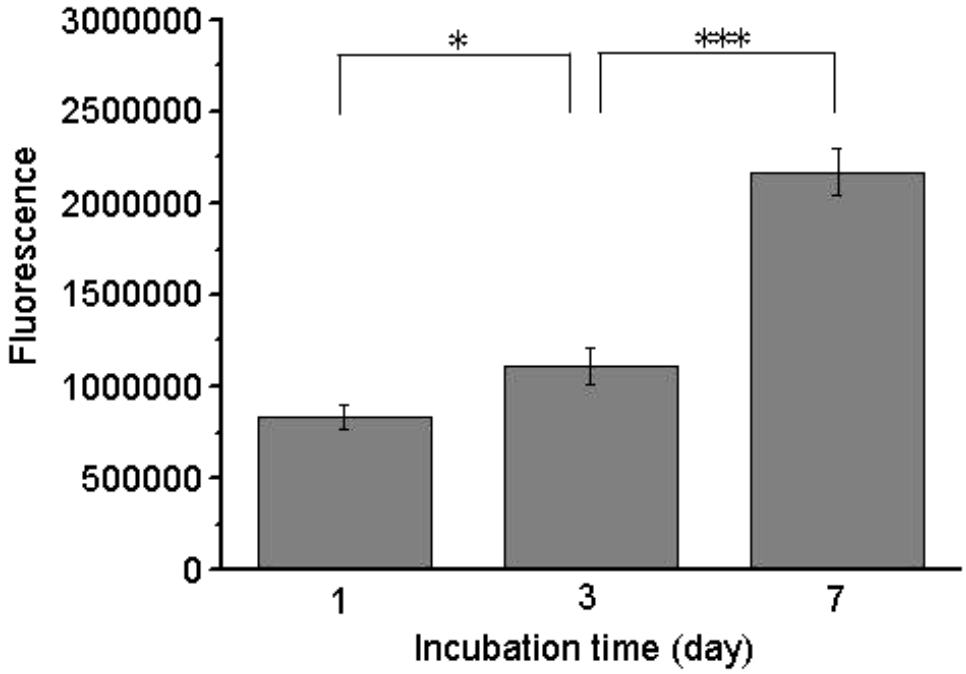


Figure 3-8 Cell growth of HBCC cultured to CL-LA70/30ss250 by alamar Blue® assay (n=3). *p < 0.05 and ***P < 0.001 indicate that between 1 day and 3days, 3days and 7 days cell culture, these statistically differ on a significant basis.

3.4 Conclusion

We succeeded in preparation of flexible scaffold prototype by using branched macromonomer with CL/LA (70/30 mol-%) and salt-leaching method with NaCl particles with different size. From results of porosity estimation by gravimetry, internal morphology observation by SEM, and water uptake measurement in PBS(-) at 37 ℃, it is proved that these scaffold prototypes could be supplied the good cell growth environment. Moreover, it is suggested that these materials were a good biocompatibility by cell growth assay of HBCC. Consequently, we expected that the prototype prepared in this study would be utilized for scaffold materials in practical tissue engineering application.

Reference

[1] J. J. Marler, J. Upton, R. Langer, J. P. Vacanti, *Advanced Drug Delivery Reviews* **1998**, *33*, 165.

[2] S. Y. Lee, J. H. Oh, J. C. Kim, Y. H. Kim, S. H. Kim, J. W. Choi, *Biomaterials* **2003**, *24*, 5049.

[3] N. Isogai, S. Asamura, T. Higashi, Y. Ikada, S. Morita, J. Hillyer, R. Jacquet, W. J. Landis, *Tissue Engineering* **2004**, *10*, 673.

[4] Y. Sumita, M. J. Honda, T. Ohara, S. Tsuchiya, H. Sagara, H. Kagami, M. Ueda, *Biomaterials* **2006**, *27*, 3238.

[5] F. Couet, N. Rajan, D. Mantovani, *Macromol. Biosci.* **2007**, *7*, 701

[6] K. W. Ng, D. W. Hutmacher, *Biomaterials* **2006**, *27*, 4591.

[7] L. Lu, S. J. Peter, M. D. Lyman, H.-L. Lai, S. M. Leite, J. A. Tamada, S. Uyama, J. P. Vacanti, R. Langer, A. G. Mikos, *Biomaterials* **2000**, *21*, 1837.

[8] L. Guan, J. E. Davies, *J. Biomed. Mater. Res.* **2004**, *71A*, 480.

[9] L. Wu, J. Ding, *Biomaterials* **2004**, *25*, 5821.

[10] L. Wu, J. Zhang, D. Jing, J. Ding, *J. Biomed. Mater. Res*. **2006**, *76A*, 264.

[11] J. E. Babensee, J. M. Anderson, L. V. McIntire, A. G. Mikos, *Advanced Drug Delivery Reviews* **1998**, *33*, 111.

[12] V. Karageorgiou, D. Kaplan, *Biomaterials* **2005**, *26*, 5474.

[13] H. Miyasako, K. Yamamoto, A. Nakao, T. Aoyagi, *Macromol. Biosci.* **2007**, *7*, 76

[14] C.-T. Lee, C.-P. Huang, Y.-D. Lee, *Biomacromolecules* **2006**, *7*, 2200.

[15] R. K. Srivastava, A.-C. Albertsson, *Biomacromolecules* **2006**, *7*, 2531.

[16] M. Takemoto, T. Shirahama, T. Miyauchi, T. Matsusako, N. Kaneda, H. Muramatsu, M. Ozawa, Y. Ohi, T. Muramatsu, *Int. J. Cancer (Pred. Oncol.)* **1997**, *74*, 7.

[17] T. Kayajima, T. Shirahama, I. Yanase, Y. Ohi, *Jap. J. Urol. Surg.* **1989**, *2*, 577.

[18] D. W. Hutmacher, T. Schantz, I. Zein, K. W. Ng, S. H. Teoh, K. C. Tan, *J. Biomed. Mater. Res.* **2001**, *55A*, 203.

[19] J. Watanabe, K. Ishihara, *Biomacromolecules* **2005**, *6*, 1797.

[20] Y. Fukuhira, E. Kitazono, T. Hayashi, H. Kaneko, M. Tanaka, M. Shimomura, Y. Sumi, *Biomaterials* **2006**, *27*, 1797.

[21] K. Uto, K. Yamamoto, S. Hirase, T. Aoyagi, *J. Controlled Release* **2006**, *110*, 408.

[22] F. A. Maspero, K. Ruffieux, B. Müller, E. Wintermantel, *J. Biomed. Mater. Res.* **2002**, *62A*, 89.

[23] Y. Hu, D. W. Grainger, S. R. Winn, J. O. Hollinger, *J. Biomed. Mater. Res.* **2002**, *59A*, 563.

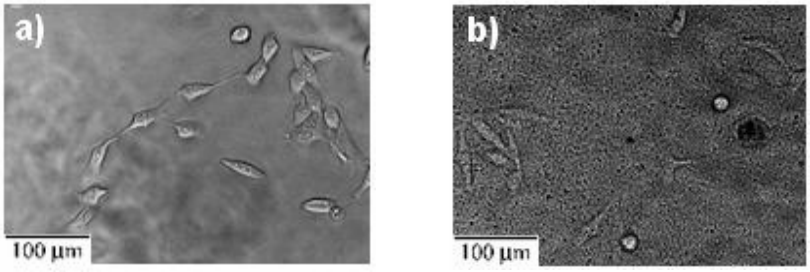
[24] Y.-Y. Hsu, J. D. Gresser, D. J. Trantolo, C. M. Lyons, P. R. J. Gangadharam, D. L. Wise, *J. Biomed. Mater. Res.* **1997**, *35A*, 107.
[25] R. Zhang, P. X. Ma, *J. Biomed. Mater. Res.* **1999**, *44A*, 446.
[26] R. Nazarov, H.-J. Jin, D. L. Kaplan, *Biomacromolecules* **2004**, *5*, 718.
[27] E. A. Botchwey, S. R. Pollack, E. M. Levine, C. T. Laurencin, *J. Biomed. Mater. Res.* **2001**, *55A*, 242.
[28] G. Chen, T. Ushida, T. Tateishi, *J. Biomed. Mater. Res.* **2001**, *57A*, 8.
[29] S. H. Oh, S. G. Kang, E. S. Kim, S. H. Cho, J. H. Lee, *Biomaterials* **2003**, *24*, 4011.
[30] S. Nishiguchi, H. Kato, M. Neo, M. Oka, H.-M. Kim, T. Kokubo, T. Nakamura, *J. Biomed. Mater. Res.* **2001**, 54, 198.
[31] S.-N. Park, J.-C. Park, H.O. Kim, M. J. Song, H. Suh, *Biomaterials* **2002**, *23*, 1205.
[32] J. Mao, L. Zhao, K. de Yao, Q. Shang, G. Yang, Y. Cao, *J. Biomed. Mater. Res.* **2003**, *64A*, 301.
[33] T. K. Kim, J. J. Yoon, D. S. Lee, T. G. Park, *Biomaterials* **2006**, *27*, 152.
[34] J. M. Oliveira, M. T. Rodrigues, S. S. Silva, P. B. Malafaya, M. E. Gomes, C. A. Viegas, I. R. Dias, J. T. Azevedo, J. F. Mano, R. L. Reis, *Biomaterials* **2006**, *27*, 6123.
[35] D.-J. Yang, L.-F. Zhang, L. Xu, C.-D. Xiong, J. Ding, Y.-Z. Wang, *J. Biomed. Mater. Res.* **2007**, *82A*, 680
[36] R. A. Stile, K. E. Healy, *Biomacromolecules* **2001**, *2*, 185.
[37] R. A. Stile, K. E. Healy, *Biomacromolecules* **2002**, *3*, 591.
[38] S. Kim, K. E. Healy, *Biomacromolecules* **2003**, *4*, 1214.
[39] E. Tsiridis, Z. Ali, A. Bhalla, M. Heliotis, N. Gurav, S. Deb, L. DiSilvio, *J. Orthopaedic Research Society* **2007**, *7*, 1425.
[40] E. Tsiridis, A. Bhalla, Z. Ali, N. Gurav, M. Heliotis, S. Deb, L. Disilvio, *Injury, Int. J. Care Injured* **2006**, *37S*, S25.
[41] L. DiSilvio, J. Jameson, Z. Gamie, P. V. Giannoudis, E. Tsiridis, *Injury, Int. J. Care Injured* **2006**, *37S*, S33.

Chapter 4

Preparation and Characterization of Photo-Cross-Linked Poly[(ε-caprolactone)-*co*-lactide] Having Good Biocompatibility for Tissue Engineering Materials

Summary

In this study, we prepared CL and LA copolymer-based cross-linked membrane by the photo-cross-linking reaction using CL-LA70/30m (branched macromonomer with CL/LA=70/30 mol-%) and *N, N*-dimethyl-*p*-toluidine and camphorquinone as photosensitizer. The characterization of the photo-cross-linked membrane was carried out the estimating thermal property by DSC measurement, surface property by contact angle, and surface morphology by SEM observation. The cell adhesion and proliferation on the membrane was evaluated by using HeLa cells. These results suggest that the membrane prepared in this study shows excellent biocompatibility, because the cell adhesion and proliferation of HeLa cells on the membrane was equivalent to that of the tissue culture polystyrene (TCPS). Consequently, the membrane can be expected to be used as the biomaterial for tissue engineering and drug delivery system and so on.



Microscopic views of HeLa cells adhered to a) TCPS and b) CL-LA70/30p after 24 h incubation periods.

4.1 Introduction

Biodegradable polymers as poly(ε-caprolactone)(PCL), poly(lactic acid)(PLA), poly(glycolic acid)(PGA) and these copolymers were used very well in an environmental field and the medical treatment field. Especially, these polymers were used as biomaterials,[1, 2] the scaffold materials for tissue engineering,[3-8] protein and drug delivery system[9-14] in the medical treatment field, since these polymers were excellent biodegradability and biocompatibility.[14-21] In these biodegradable polymers, PLA was especially researched because of having these advantages that the safety of lactic acid produced at metabolism was high, the biocompatibility of PLA was excellent, and the mechanical strength of PLA was able to set high because of having high crystalline.

In Chapter 2, we prepared the flexible materials by cross-linked reaction using the branched macromonomer with CL/LA molar ratio. The thermal properties of these materials depended on the CL/LA compositions, however, these was no change in the wettability of each material. The cell culturing experiments by using HeLa cells proved that the adhesion and growth of these cells on the CL-LA70/30c (cross-linked membrane from CL/LA=70/30 composition) were comparable to that on the commercially-available polystyrene dish. Moreover, the protein absorption experiment using the FBS revealed that the amount of protein adsorption of this membrane in FBS was more than that of other membranes. Consequently, we suggest result that the biocompatibility of cross-linked membrane in Chapter 2 due to crystalline melting of the surface of the membrane. In this way, we proved that this material was able to be very useful as biomaterial and the scaffold for tissue engineering materials.[22] Additionally, we reported that CL-LA70/30m (macromonomer from CL/LA=70/30

composition) scaffold prototype by salt-leaching method using different particle size of NaCl in Chapter 3, and the prototype prepared in Chapter 3 could uptake and preserve the large amount of water in its pores enough to provide the cell growth environment by estimating scatting electron microscopy (SEM) observation, water content in PBS at 37 °C, and biocompatibility of human bladder cancer cells (HBCC) to these materials. Consequently, we revealed that the scaffold prototype in chapter 3 was practical material as scaffold for tissue engineering. However, it is a little difficult to prepare the material of more complex shape because reactive time of the heat-cross-linking reaction is long. So, we paid attention to photo-cross-linking techniques as a new different making method to rise forming, and to prepare the material of more complex shape.

Generally, the photo-cross-linked techniques were known that the polymerization rate is sufficiently rapid under physiologic condition.[23-30] Additionally, these techniques have the advantage of greater temporal and spatial control of polymerization and greater flexibility during scaffold implantation than chemical cross-linking methods. These materials prepared with photo-cross-linked techniques are researched as injectable materials and scaffold materials for tissue engineering,[24, 25, 26, 29, 30] materials for protein delivery and cell delivery.[23, 24,26, 27]

In Chapter 4, we prepared photo-cross-linked membrane (CL-LA70/30pc) by using CL-LA70/30m, *N, N*-dimethyl-*p*-toluidine and camphorquinone as photosensitizer. The characterization of Cl-LA70/30pc were carried out the estimating thermal property by differential scanning calorimeter (DSC), surface property by contact angle, and surface morphology by scattering electron microscopy (SEM). We also studied the adhesion and proliferation of HeLa cells to CL-LA70/30pc by the microscopic observation of cell morphology.

4.2 Experimental Part

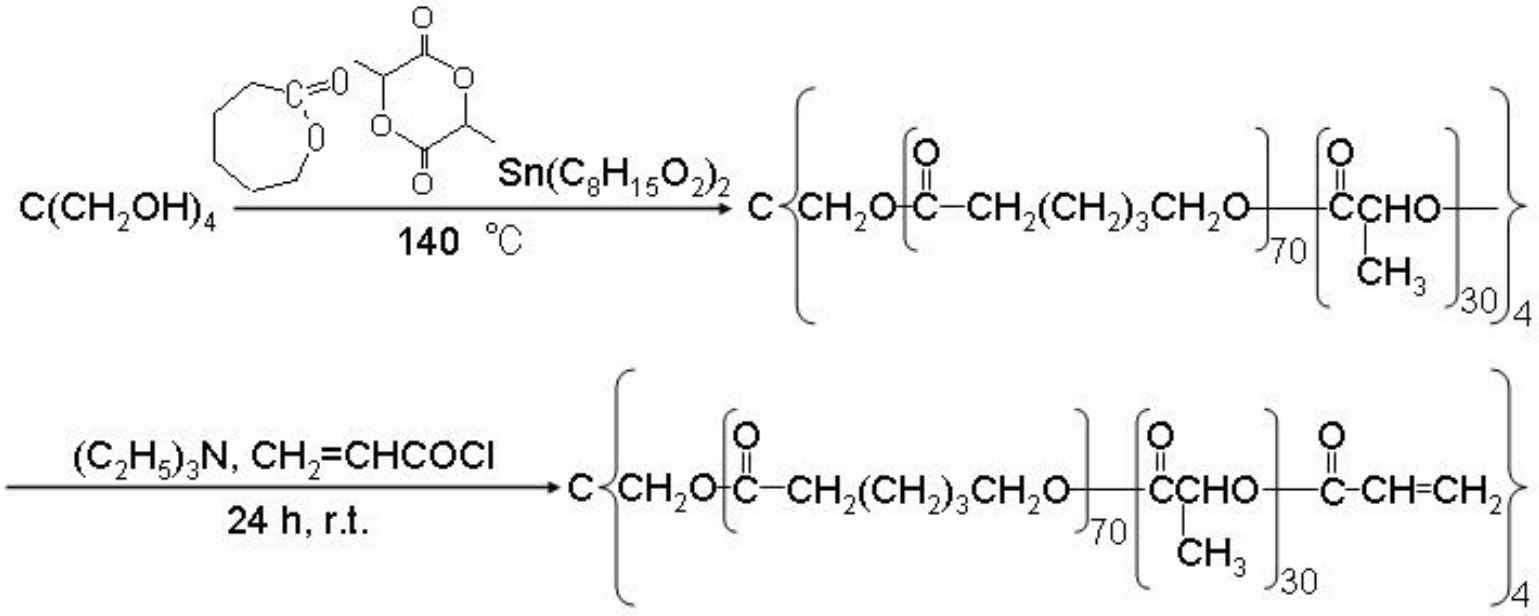
4.2-1 Materials

Xylene, *N, N*-Dimethyl-*p*-toluidine and (±)-Camphorquinone were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Human uterine cervix epitheloid carcinoma (HeLa) cells were obtained from the Health Science Research Resources Bank (HSRRB, Japan). Fetal Bovine Serum (FBS) was purchased from Gemeni Bio-Product (USA). Dulbecco's Modified Eagle's Medium, Dulbecco's phosphate buffered saline, Trypsin-EDTA solution, Penicillin-streptomycin solution, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA).

Branched poly(ε-caprolactone-*co*-D,L-lactide) that molar ratio was 70:30 (abbreviated as CL-LA70/30) was synthesized by ring opening polymerization in bulk using tin octanoate as a catalyst and pentaerythritol as an initiator. The number average molecular weight, weight average one and molecular weight distribution were 22500, 35900 and 1.59, respectively. The corresponding macromonomers (abbreviated as CL-LA70/30m) were then prepared by introducing an acryloyl group at the chain ends. The synthetic procedure is shown in Scheme 4-1. The synthesis method of CL-LA70/30m and cross-linked CL-LA-7030c were reported by Chapter 2.

4.2-2 Preparation of photo-cross-linked membrane

The branched macromonomer (0.9 g) was dissolved with 1.02 mL in a xylene solution containing *N, N*-dimethyl-*p*-toluidine and camphorquinone (0.026 g/mL).[23] The mixture was placed in the 0.1 mm space between two glass plates with a 3 ×3 cm^2 Teflon frame spacer, and photo-crosslinking reaction was ran by irradiating light to the both side of the glass plates for 5 minutes. The membrane-type sample was taken from



Scheme 4-1 Schematic illustrations of CL-LA70/30m.

the glass plates and immersed in a large amount of acetone to remove the unreacted compounds and dried under reduced pressure.

4.2-3 Characterization

The thermal properties of the CL-LA70/30pc were measured by differential scanning calorimetry (DSC) (DSC6100, Seiko Instruments, Chiba Japan). The measurements were run from 0 to 120 °C at the heating rate of 5 °C/min. The static contact angles were measured using a goniometer (DropMaster 300, Kyowa Interface Science Co., Ltd., Saitama Japan) at room temperature. The surface morphology of the membrane was observed by SEM.

4.2-4 Culture of HeLa cells

HeLa cells were sub-cultured in D-MEM supplemented with 5 % FBS and penicillin-streptomycin solution containing 100 U/mL penicillin and 0.1 mg/mL streptomycin in humidified environment of 5 % CO_2 at 37 °C. Subsequently, these were rinsed by PBS (-) and harvested by PBS (-) containing trypsin-EDTA solution.

4.2-5 Cell adhesion and growth

The cross-linked membranes were cut in circular pieces (6 mm diameter) and were placed on a 96-well culture plate coated with sterilized grease. The membranes were fixed with stainless rings. 70 % ethanol was added to each well to sterilize the membranes for 1h. After removing the ethanol solution, the membranes were repeatedly washed with PBS(-). The cell suspension was then prepared by D-MEM (include penicillin) with 10 % FBS and added to the 96-well culture plate. The culture system

was kept in a humidified environment of 5 % CO_2 at 37 °C. After 0 h, 6 h, and 24 h culturing periods, the cells was observed by inverted microscopy (ECLIPSE TS100, Nikon, Japan Tokyo) using a digital camera (DS-L1 and DS-5M, Nikon, Japan Tokyo).

4.3 Results and discussions

4.3-1 The characters of the starting materials

As described in Chapter 1, 2, and 3, PLA and PLGA are used as a scaffold for tissue engineering. Especially, the scaffold materials prepared from PLA and PLGA were match hard tissue such as bone, because the glass transition temperature, T_g of these polymers are relatively high and stiff enough to keep the shapes themselves at ambient temperature. However, these materials lack compatibility to the soft tissue because crystalline of the material is very high. [15, 17, 18] Consequently, we synthesized the branched CL and LA copolymers by a ring-opening copolymerization using tin octanoate as a catalyst and pentaerythritol as an initiator in Chapter 2, because we fabricate flexible material for matching to the soft tissue. These cross-linked membranes were prepared by using these macromonomers introduced acrylyl group at the chain end of these branched copolymers, and estimated to the characterization and biocompatibility of these materials. These suggested result that the cross-linked membrane from CL-LA70/30m was a flexible, excellent in molding and the biocompatibility. Additionally, these scaffold materials having flexibility were able to prepare by using CL-LA70/30m and different particle size of NaCl in Chapter 3. As a result of the characterization and biocompatibility test, we proved that the scaffold from CL-LA70/30m was a practical material as tissue engineering material. In this way, it is suggested result that the material having good biocompatibility is able to be prepared by

using CL-LA70/30m. Consequently, we prepared the CL-LA70/30pc by the photo-cross-linked reaction using CL-LA70/30m to prepare the material having flexible and good biocompatibility.

4.3-2 Characterization of the photo-cross-linked membrane

In general, many researchers report that the photo-cross-linked reaction proceeds rapidly, and the polymerization condition was very easily controlled. [24-29] B. G. Amsden *et al.* also prepared the materials by photo-cross-linked reaction using acrylated star-poly(ε-acprolactone-*co*-$_{D, L}$-lactide) and 2, 2-dimethosy-2-phenyl-acetophenone as photo-initiator.[27] As a result of mechanical and biodegradation test, they have demonstrated that the feasibility of preparing an elastomeric material, composed of biodegradable linkage, via a photocuring process. Additionally, M. Dadsetan *et al.* prepared photo-cross-linkable oligo[poly(ethylene glycol) gumarate] (OPE) hydrogels by altering the ratio of cross-linker/polymer in precursor solution.[30] As a result of mechanical, biodegradable and biocompatibility test, these materials having desirable characterization were able to be prepared by altering the cross-linking density. Consequently, they reported that photo-cross-linkable OPF hydrogels may be useful for cartilage tissue engineering and cell delivery application. Hence, we prepared the CL-LA70/30pc by photo-cross-linking reaction using CL-LA70/30m and *N, N*-dimethyl-*p*-toluidine, camphorquinone as photosensitizer in Chapter 4(Table 4-1). As a result of the reaction, we were able to collect flexible, excellent in mold CL-LA70/30pc. Although we prepared by using materials, for instance, the CL-LA50/50m and Cl-LA30/70m other than CL-LA70/30m, we weren't able to collect it as one film because the molding of these materials was bad. However, the material

Table 4-1 Preparation and characterization of Cl-LA70/30pc

	Polymer concentration (wt.-%)	N,N-dimethyl-p-toluidine (wt.-%)	camphorquinone (wt.-%)	Tm (℃)	⊿H (mJ/mg)
CL-LA70/30pc	**50**	**1**	**1**	**23**	**3**

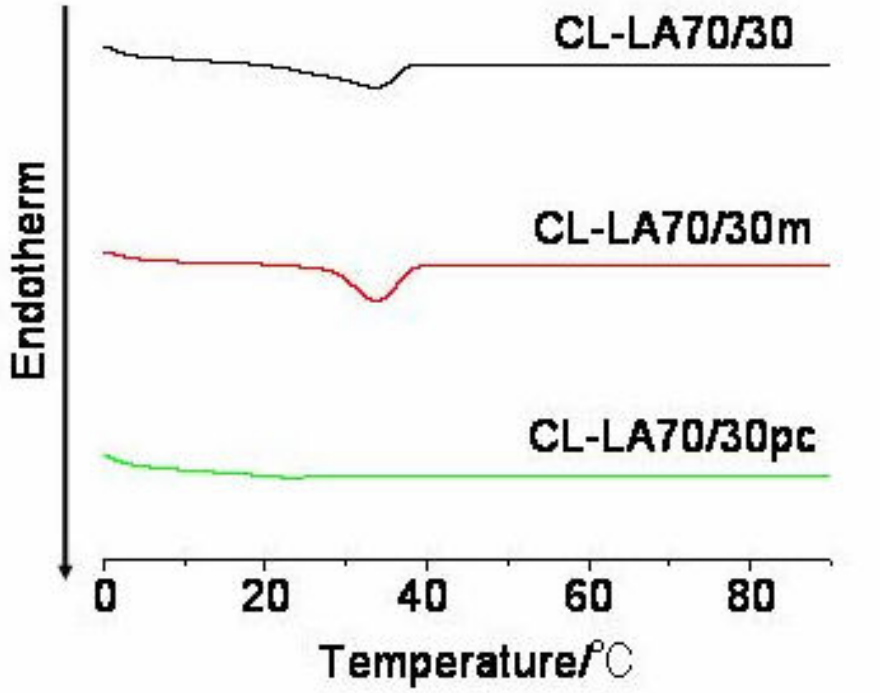


Figure 4-1 DSC thermograms of the CL-LA70/30, CL-LA70/30m, CL-LA70/30c.

Table 4-2 Wettability of CL-LA70/30pc by contact angle.

	Contact angle (°)
TCPS	77.8 ± 0.7
CL-LA70/30pc	81.8 ± 3.7

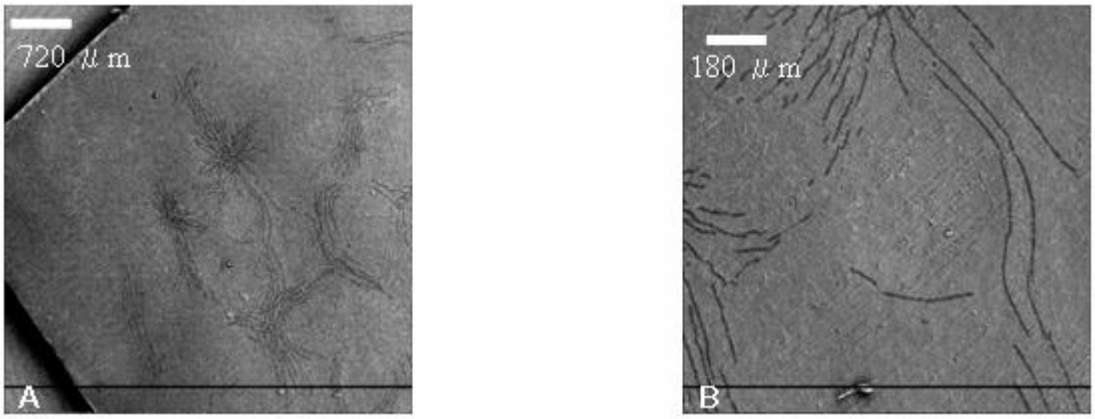


Figure 4-2 Surface morphology of CL-LA70/30pc by SEM.

prepared by CL-LA70/30m, CL-LA70/30pc, has excellent moldability, and we were able to collect it as one film. Consequently, we evaluated the characterization and biocompatibility by using only CL-LA70/30pc that was able to be collected as one beautiful film. Table 4-1 and Figure 4-1 shows about the thermal property of CL-LA70/30pc by DSC measurement. Figure 4-1 also shows the thermal property of branched copolymer with the CL and LA molar ratio of 70 to 30 (CL-LA70/30) and CL-LA70/30m by DSC measurement. As a result, the melting point (T_m) and enthalpy change (ΔH) of CL-LA70/30pc shifted to the low temperature degree side compared with CL-LA70/30 and CL-LA70/30m. These results suggest that the new amorphous region was formed by photo-cross-linking reaction. Table 4-2 shows the wettability of CL-LA70/30pc surface by contact angle at room temperature. As a result, the value of the contact angle of CL-LA70/30pc was about 81.8 ^{o}, and as well as that of TCPS (about 77.8 ^{o}). Additionally, Figure 4-2 shows the surface morphology of CL-LA70/30pc by SEM. As a result, the surface of CL-LA70/30pc was very beautiful, but some cracks were observed by a magnified photograph of SEM. These results suggest that some cracks of the CL-LA70/30pc surface were generated by purification process and drying under reduced pressure. However, it is suggest that some cracks of the CL-LA70/30pc surface don't influenced to the adhesion and proliferation of these cells because the size of these cracks size are smaller than that of these cells.

4.3-3 Biocompatibility of the photo-cross-linked membrane

We aimed at the application development with the biomaterials, and examined the biocompatibility to the CL-LA70/30pc of HeLa cells. The cell adhesion and growth on the membrane was evaluated by comparing with the tissue culture polystyrene (TCPS),

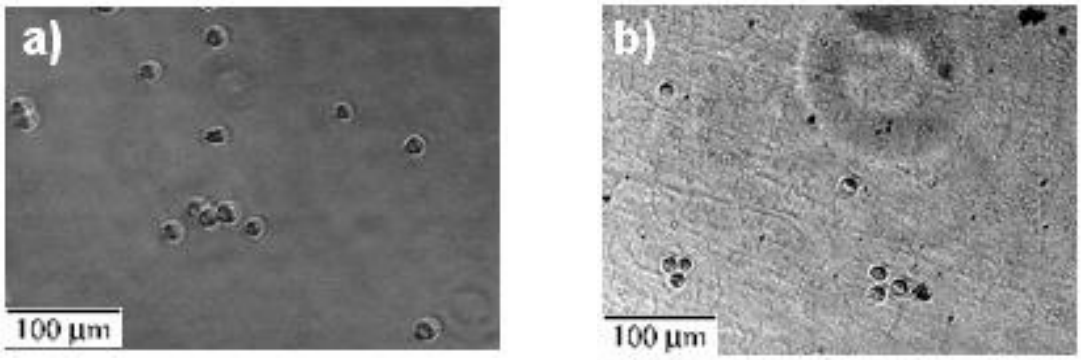


Figure 4-3 Microscopic views of HeLa cells adhered to TCPS a) and CL-LA70/30pc b). Cells were cultured for 0 h.

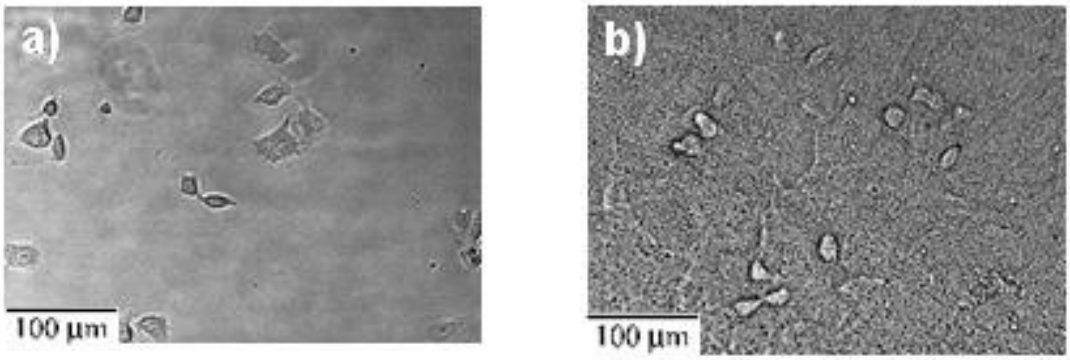


Figure 4-4 Microscopic views of HeLa cells adhered to TCPS a) and CL-LA70/30pc b). Cells were cultured for 6 h.

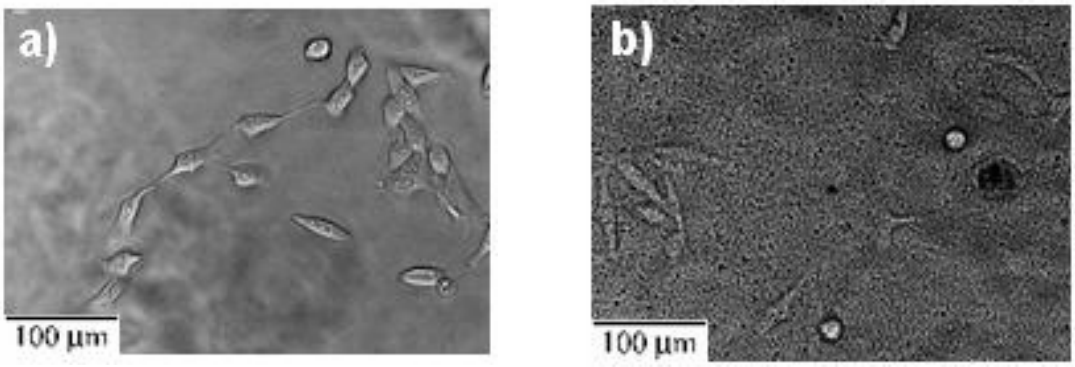


Figure 4-5 Microscopic views of HeLa cells adhered to TCPS a) and CL-LA70/30pc b). Cells were cultured for 24 h.

cells adhered on the membrane after 0, 6, and 24 h culturing periods, respectively. As that is commercially available. Figure 4-3, 4-4 and 4-5 shows the morphology of the seen in Figure 4-3 and 4-4, the cells were homogeneously seeded to the TCPS and the membrane after 0 h, and adhered, extended on the TCPS and the membrane surface after 6 h. In addition to Figure 4-5, the cells were grown on the TCPS and membrane surface after 24 h. The growth ability of the cell on the membrane surface was also equaled to that of TCPS. It is considered that the membrane in this study was an excellent material of the biocompatibility.

Consequently, we could confirm that CL-LA70/30pc prepared by the photo-cross-linking reaction was very promising for a flexible material for biomedical field as tissue engineering and drug delivery system and so on.

4-5 Conclusion

In this study, we succeeded in preparation of flexible membrane by the photo-cross-linking reaction using CL-LA70/30m (the branched macromonomer with CL/LA=70/30 mol-%) and *N, N*-dimethyl-*p*-toluidine and camphorquinone as photosensitizer. The characterization of CL-LA70/30pc was estimated by DSC, contact angle, and SEM observation. The DSC measurement indicated that the melting point and enthalpy were shifted to low-temperature side because the new amorphous region was formed by cross-linking reaction. The measurement of contact angle also found that the wettability of CL-LA70/30pc was equaled to that of TCPS. Additionally, the morphology of the CL-LA70/30pc surface by SEM was showed that the surface of the membrane was very beautiful. The biocompatibility of the CL-LA70/30pc evaluated using HeLa cells. As a result, cell adhesion and growth of HeLa cells on CL-LA70/30pc

was equivalent to that of the commercially available TCPS.

These results prove that the CL-LA70/30pc prepared by the cross-linking reaction is a promising flexible material for biomedical field as tissue engineering and drug delivery system and so on.

References

[1] A. Lendlein, R. Langer, *Science* **2002**, *1673*.

[2] R. Langer, D. A. Tirrell, *Nature* **2004**, *33*, 165.

[3] G. Chen, T. Ushida, T. Tateishi, *Material Science and Engineering C* **2001**, *17*, 63.

[4] Q. Hou, D. W. Grijpma, J. Feijen, *Biomaterials* **2003**, *24*, 1937.

[5] F. Yang, R. Murugan, S. Ramakrishna, X. Wang, Y.-X. Ma, S. Wang, *Biomaterials* **2004**, *25*, 1891.

[6] Q. P. Pham, U. Sharma, A. G. Mikos, *Biomacromolecules* **2006**, *7*, 2796.

[7] T. K. Kim, J. J.Yoon, D. S. Lee, T. G. Park, *Biomaterials* **2006**, *27*, 152.

[8] H. Mizuno, A. K. Roy, V. Zaporojan, C. A. Vacanti, M. Ueda, L. J. Bonassar, *Biomaterials* **2006**, *27*, 362.

[9] T. Ouchi, T. Saito, T. Kontani, Y. Ohya, *Macromol. Biosci.* **2004**, *4*, 458.

[10] H. Hyun, Y. H. Kim, I. B. Song, J. W. Lee, M. S. Kim, G. Khang, K. Park, H. B. Lee, *Biomacromolecules* **2007**, *8*, 1093.

[11] J. H. Lee, A. K. Go, S. H. Oh, K. E. Lee, S. H. Yuk, *Biomaterials* **2005**, *26*, 671.

[12] H. Arimura, Y. Ohya, T. Ouchi, *Biomacromolecules* **2005**, *6*, 720.

[13] B. Rai, S. H. Teoh, D. W. Hutmacher, T. Cao, K. H. Ho, *Biomaterials* **2005**, *26*, 3739.

[14] K. Uto, K. Yamamoto, S. Hirase, T. Aoyagi, *J. Controlled Release* **2006**, *110*, 408.

[15] F. Tasaka, Y. Ohya, T. Ouchi, *Macromolecules* **2001**, *34*, 5494.

[16] Y. Wang, G. A. Ameer, B. J. Sheppard, R. Langer, *Nature Biotechnology* **2002**, *20*, 602.

[17] Y. Ohya, H. Matsunami, E. Yamabe, T. Ouchi, *J. Biomed. Mater, Res.* **2003**, *65A*, 79.

[18] T. Ouchi, S. Ichimura, Y. Ohya, *Polymer* **2006**, *47*, 429.

[19] S. Li, A. Girard, H. Garreau, M. Vert, *Polymer Degradation and Stability* **2001**, *71*, 61.

[20] H. Tsuji, *Polymer* **2002**, *43*, 1789.

[21] H. Tsuji, M. Ogiwara, S. K. Saha, T. Sakaki, *Biomacromolecules* **2006**, *7*, 380.

[22] H. Miyasako, K. Yamamoto, A. Nakao, T. Aoyagi, *Macromol. Biosci.* **2007**, *7*, 76

[23] T. Aoyagi, F. Miyata, Y. Nagase, *Journal of Controlled Release* **1994**, *32*, 87.

[24] K. S. Anseth, V. R. Shastri, R. Langer, *Nature Biotechnology* **1999**, *17*, 156.

[25] W. T. Brinkman, K. Nagapudi, B. S. Thomas, E. L. Chaikof, *Biomacromolecules* **2003**, *4*, 890.

[26] J. P. Fisher, T. A. Holland, D. Dean, A. G. Mikos, *Biomacromolecules* **2003**, *4*, 1335.

[27] B. G. Amsden, G. Misra, F. Gu, H. M. Younes, *Biomacromolecules* **2004**, *5*, 2479.
[28] F. Gu, R. Neufeld, B. Amsden, *European Jounal of Pharmaceutics and Biopharmaceutics* **2007**, *66*, 21.
[29] J. Y. Shen, X. Y. Pan, C. H. Lim, M. B. Chan-Park, X. Zhu, R. W. Beuerman, *Biomacromolecules* **2007**, *8*, 376.
[30] M. Dadsetan, J. P. Szatkowski, M. J. Yaszemski, L. Lu, *Biomacromolecules* **2007**, *8*, 1702.

Concluding Remarks

In this paper, we researched about development and qualification of bio-materials. This time, we were focused on the two keywords of “flexibility” and “biocompatibility”. Consequently, we synthesized polymer of raw materials, and prepared material of shape as membrane type and cylindrical type using application. Additionally, we evaluated about thermal and surface properties, biocompatibility using cells and proteins.

In Chapter 2, we synthesized branched copolymer with different CL and LA compositions. The corresponding branched macromonomer were then prepared by introducing an acryloyl group at the chain end of the precursor. Additionally, the cross-linked membranes were prepared by reaction at 80 ℃ for 2 h using these branched macromonomers and benzoyl peroxide (BPO) as initiator. These membranes prepared by cross-linked reaction were flexible and high of stability in organic solvent. Additionally, we evaluated about thermal and surface properties, biocompatibility of these cross-linked membranes. Consequently, the thermal properties of these materials depended on the CL/LA compositions, however, there was no change in the wettability of each cross-linked membranes. Additionally, these cross-linked membranes were evaluated about biocompatibility, because we objected to application to bio-materials. At first, cell adhesion and cell growth were evaluated by using HeLa in vitro. As a results, nevertheless these cross-linked membranes didn’t change of wettability, the cross-linked membrane prepared CL-LA70/30c (where CL-LA X/Y denotes the cross-linked membrane containing X mol-% of CL and Y mol-% of LA) were equal to

that on the commercially-available polystyrene dish. The protein adsorption experiment using the FBS protein revealed that the materials with well-grown cells showed better adhesion of the protein. These results prove that the cross-linked CL-LA with a suitable composition is a promising material for the flexible scaffold of tissue engineering.

In Chapter 3, we prepared three-dimensional materials (scaffold), and evaluated about qualification for tissue engineering. In Chapter 2, we revealed that CL-LA70/30m (where CL-LA X/Y denotes the macromonomer containing X mol-% of CL and Y mol-% of LA) was a good biocompatibility. Hence, we prepared the porous materials by using CL-LA70/30m. The method of preparation was the same that of Chapter 2, but we prepared by using NaCl as porogen. The particles of NaCl were sieved at four sizes, and prepared the porous materials by heat reaction. The materials was a flexible, and the porosity of these scaffolds by gravimetry, water uptake at 37 ℃ in PBS, internal morphology by SEM of these materials was revealed to have a porous structure and internal connected framework. Additionally, the ratio of water was very high, when water uptake of these materials was reached equilibrium. Consequently, these materials could be gave a good environmental for ingrowth of the cells to retain the medium and nutrients in these materials. The cell adhesion and cell growth of human bladder cancer cells (HBCC) in the material were evaluated by microscope observation and alamar Blue®. As a result, the cells were proliferated in the pore structure of the material. Consequently, we are able to expect that these scaffolds are utilized for scaffold material in tissue engineering application.

In Chapter 4, we prepared the membranes by photo cross-linked reaction of the new method. In general, it is said that photo cross-linked can be preceded rapidly. So,

we prepared the photo cross-linked membranes by using Cl-LA70/30m and photo-initiator, and evaluated about thermal property, surface property, and biocompatibility. As a result, we succeeded in preparation of flexible and a good biocompatibility membrane.

In this way, we have advanced the research by the method of approaching four kinds of. As a result, we succeeded in the preparation of an excellent material of flexibility and biocompatibility. It is proved that these materials prepared in this study were very useful as the scaffold material for tissue engineering.

List of Publication

Chapter 2:

Hiroshi Miyasako, Kazuya Yamamoto, Aiko Nakao, Takao Aoyagi, “Preparation of Cross-linked Poly[(ε-caprolactone)-*co*-lactide] and Biocompatibility Studies for Tissue Engineering Materials”, *Macromolecular Bioscience*, **2007**, 17, 76.

Chapter 3

Hiroshi Miyasako, Kazuya Yamamoto, Takao Aoyagi, “Preparation, Characterization and Biocompatibility Study of the Scaffold Prototype Derived from Cross-Linked Poly[(ε-caprolactone)-*co*-lactide] for Tissue Engineering Materials”, Submitting to *Polymer Journal* in **2008**.

Chapter 4

Hiroshi Miyasako, Kazuya Yamamoto, Takao Aoyagi, “Preparation and Characterization of Photo-Cross-Linked Poly[(e-caprolactone)-co-lactide] Having Good Biocompatibility for Tissue Engineering Materials”, in preparation.

List of Presentations

1. Hiroshi Miyasako, Sachiyo Mitsunaga, Kazuya Yamamoto, Takao Aoyagi, *"Preparation of Branched Poly(CL-LA) Copolymer Cross-Linked Material for Tissue Engineering"*, **Society of Polymer Science, Japan, Kyushu**, August 21, 2004, Fukuoka, Japan.

2. Hiroshi Miyasako, Kazuya Yamamoto, Takao Aoyagi, *"Basic Research into Application to Material for Reproduction Medicine of Divergent Aliphatic Polyester (I)"*, **53th SPSJ Symposium on Macromolecules**, September 15-17, 2004, Hokkaido, Japan.

3. Takanari Muroya, Hiroshi Miyasako, Kazuya Yamamoto, Takao Aoyagi, *"Enzymatic Degradation of Cross-Linked Poly (ε-caprolactone-co-lactide) with Branch Structure"*, **54th SPSJ Annual Meeting**, May 25-27, 2005, Yokohama, Japan.

4. Hiroshi Miyasako, Kazuya Yamamoto, Takao Aoyagi, *"Cell Adhesion of Multi-Blanched Aliphatic Polyesters Prepared by Cross-Linked Formation"*, **54th SPSJ Symposium on Macromolecules**, September 20-22, 2005, Yamagata, Japan.

5. Hiroshi Miyasako, Kazuya Yamamoto, Takao Aoyagi, *"Preparacion of Poly(CL-LA) Cross-Linked Materials and Cell Adhesion Assay"*, **The Nippon Chemical Industrial Co., Ltd. Association West Japan Rally in 2005**, October 22-23, 2005, Yamaguchi, Japan.

6. Takanari Muroya, Hiroshi Miyasako, Kazuya Yamamoto, Takao Aoyagi, *"Degradation of Cross-Linked Poly(ε-caprolactone-co-lactide) with Branch Structure"*, **Society of Polymer Science, Japan, Kyushu**, November 17, 2005, Miyazaki, Japan.

7. Hiroshi Miyasako, Kazuya Yamamoto, Takao Aoyagi, *"Cell Adhesion Assay of Multi-Branched Poly(ε-caprolactone-co-$_{D, L}$-lactide) Cross-linked Materials"*, **Society of Polymer Science, Japan, Kyushu**, August 11, 2006, Kumamoto, Japan.

8. Hiroshi Miyasako, Kazuya Yamamoto, Takao Aoyagi, *"Cell Adhesion on Cross-Linked Materials Prepared by Multi-Branched Poly(CL-LA)"*, **55th SPSJ Symposium on Macromolecules**, September 20-22, 2006, Toyama, Japan.

9. Hiroshi Miyasako, Kazuya Yamamoto, Takao Aoyagi, *"Architectonics of Materials for Tissue Engineering Consist of Biodegradable Polymer"*, **The 15th Society of Polymer Science,** November 16-17, 2006, Osaka, Japan.

10. Hiroshi Miyasako, Kazuya Yamamoto, Takao Aoyagi, *"Sponge-Like Porous Materials Prepared by Multi-Branched Biodegradable Polymer"*, **The 28th Annual Meeting of Japanese Society for Biomaterials**, November 27-28, 2006, Tokyo, Japan.

11. Hiroshi Miyasako, Kazuya Yamamoto, Takao Aoyagi, *"Biocompatibility Studies of Cross-Linked Materials Consisted of Multi-Branched Biodegradable Polymer"*, **87th The Nippon Chemical Industrial Co., Ltd. Association Spring**, March 25-28, Osaka, Japan.

12. Hiroshi Miyasako, Kazuya Yamamoto, Takao Aoyagi, “Design and Qualification of Aliphatic Polyester Cross-Linked Materials for Tissue Engineering”, **56th SPSJ Symposium on Macromolecules**, September 19-21, 2007, Nagoya, Japan.

Acknowledgements

This work was performed at Department of Nanostructure and Advanced Materials, Graduate School of Science and Engineering, Kagoshima University from 2003 to 2008.

I thank Professor Takao Aoyagi for his continuous guidance and encouragement through the course of his study.

Especially, I thank Assistant Professor Kazuya Yamamoto for his continuous guidance, helpful discussions and skilled technical assistance during the study.

Additionally, I thank Professor Jun-ichi Kadokawa, Associate Professor So-ichiro Sameshima, and Assistant Professor Yoshiro Kaneko (the Kagoshima University) for their valuable comments and suggestions.

I thank the Musashino Chemical Laboratory (Tokyo Japan) for kindly providing $_{D,L}$-lactide; Dr. Hideki Enokida (Department of Urology of Graduate School of Medical and Dental Science in Kagoshima University) for supplying the human bladder cancer cells.

I thank Mr. Yoshihisa Ozono and Mr. Terumi Kakoi (Division of Instrumental Analysis, Frontier Science Research Center, Kagoshima University) for their skilled technical assistance with ESCA and SEM and so on; Dr. Aiko Nakao (Beam Application Team, Advanced Development and Supporting Center) for her advice and assistance with ESCA analysis, when I contribute the report; Mr. Takao Endo for his skilled technical assistance and helpful discussions with biochemistry evaluation.

I thank Ms. Yumiko Nanameki for her discussions and encouragement during the

early days of the study; Mr. Takanari Muroya, Mr. Syohei Hirase, Mr. Ko-ichiro Uto, Mr. Yuki Taruno, Mr. Shigeta Handa, and Syohei Miyauchi for their assistance, discussion and encouragement as the member of aliphatic polyester group during the study.

I thank Ms. Yumi Kuboshima, Mr. Tomohiro Kanda, Dr. Daisuke Matsukuma, Ms. Fumiko Yano, as well as members of the Aoyagi Laboratory for their hearty support and kind help.

Finally, I thank Toshimitsu Miyasako, Yoshiko Miyasako and Nozomi Miyasako for their thoughtful attention and continuous encouragement.

March 2008

Hiroshi Miyasako