Thermosensitive Chitosans as Novel Injectable Biomaterials

Ho Jin Chung,[1] *Jin Woo Bae*,[1] *Hyung Dal Park*,[1] *Jin Woo Lee*,[2] *Ki Dong Park**[1]

[1]Dept. of Molecular Science and Technology, Ajou University, Suwon 443-749, Korea
Fax: (+82) 219 1592; E-mail: kdp@ajou.ac.kr
[2]Dept. of Orthopaedic Surgery, Yonsei University College of Medicine, CPO BOX 8044, Seoul 120-752, Korea

Summary: Two kinds of water soluble thermosensitive chitosan copolymers were prepared by graft polymerization of *N*-isopropylacrylamide (NIPAAm) onto chitosan using cerium ammonium nitrate (CAN) as an initiator (chitosan-g-NIPAAm) and by coupling monocarboxy Pluronic® with chitosan using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as coupling agents (chitosan-Pluronic®). The physicochemical properties of the resulting copolymers were characterized and they formed thermally reversible hydrogel, which exhibits a lower critical solution temperature (LCST) at 34°C in aqueous solutions. The human mesenchymal stem cells (hMSCs) were cultured in the chiotsan-g-NIPAAm *in vitro*. Chondrogenic differentiation was induced in the chitosan-g-NIPAAm gel. Therefore, chondrogenic differentiated cells from MSCs with a thermo-sensitive chitosan-g-NIPAAm could be used as an injectable cell-polymer complex. In summary, chitosan-g-NIPAAm and chitosan-Pluronic® attest to their usefulness as injectable materials because of their thermally reversible property and relatively good biocompatibility.

Keywords: chitosan; hydrogels; *N*-isopropylacrylamide (NIPAAm); Pluronic®; thermo-sensitivity

Introduction

Stimuli-sensitive systems using polymers change their volume and shape reversibly according to the various external physicochemical factors.[1] Chemical signals, such as pH, metabolites, and ionic factors, will alter the molecular interactions between polymer chains or between a polymer chain and solutes present in a system. The physical stimuli, such as temperature or electrical potential, may provide various energy sources for altering molecular interactions. These interactions will change the properties of polymer materials such as solubility, swelling behavior, configurations of conformational change, redox (reduction-oxidation) state, and crystalline/amorphous transition.[8-10]

Temperature is the most widely utilized triggering signal for a variety of modulated or pulsatile drug delivery systems. Thermosensitive polymers have been extensively studied

© 2005 International Union of Pure and Applied Chemistry DOI: 10.1002/masy.200550624

because temperature is an important factor inside the human body. Recently, great attention has been paid to thermosensitive polymer gels because of their potential application for biomedical systems such as drug delivery, tissue engineering, and enzyme or protein modification.[11-12] There are different types of thermal setting gels, for example *N*-isopropylacrylamide derivative and Pluronic®.[2-3] Poly (*N*-isopropylacrylamide) (PNIPAAm) is well known to have a thermally reversible property.[16] It exhibits a lower critical solution temperature (LCST), around 32°C in aqueous solution; that is, it dissolves in water below the LCST and precipitates from aqueous solution above the LCST.[13] Another type of thermosensitive polymer, Pluronic®, consists of more than 30 different non-ionic surface-active agents. These polymers are ABA-type triblock copolymers composed of poly(ethylene oxide) (PEO) (A) and poly(propylene oxide) (PPO) units (B). The Pluronic® series covers a range of liquids, pastes, and solids, with molecular weights and ethylene oxide-propylene oxide weight ratios varying from 1100 to 14,000 and 1:9 to 8:2, respectively. Concentrated aqueous solutions of Pluronic® form thermoreversible gels. Pluronic® F127 was found to gel at a concentration of 20 wt% at 25 °C, which is less than that of the other members of the Pluronic® series. At room temperature (<25 °C), the solution behaves as a mobile liquid, which is transformed into a transparent gel at body temperature.

Chitosan is a positively charged specific polysaccharide, which stimulates cell growth and protein adsorption. It was reported that, besides its good biocompatibility, chitosan has an excellent cell affinity.[17-18] Extracellular molecules such as laminin, fibronectin, and other serum proteins have a good affinity to chitosan.[4] Although there were some differences in the cellular adhesion and proliferation rate, according to the degree of deacetylation on chitosan[14], it has good cellular proliferation and cell attachment properties in chondrogenic human mesenchymal stem cells (MSC).

In this study, two kinds of water-soluble and thermosensitive chitosan copolymers have been prepared for drug or cell delivery and tissue engineering applications as a novel injectable system. First, PNIPAAm grafted chitosan copolymer (chitosan-g-NIPAAm) was prepared by graft polymerization of NIPAAm onto chitosan using CAN as an initiator. *In vitro* cell study was performed using hMSCs to assess the biocompatibility of the chitosan-g-NIPAAm. Secondly, a Pluronic® grafted chitosan copolymer (chitosan-Pluronic®) was prepared by coupling monocarboxy Pluronic® with chitosan using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as

coupling agents. The physicochemical and biological properties of these copolymers were investigated.

Materials and Methods

Materials. Chitosan (M_r: 1.5×10^5, percentage of deacetylation degree: 85%) was purchased from Fluka (Fluka Chemika, USA). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and succinic anhydride were purchased from Aldrich (Sigma-Aldrich, USA). Pluronic® F127 and 4-dimethylaminopyridine (DMAP) were purchased from BASF (BASF, Korea) and Sigma (Sigma-Aldrich, USA). NIPAAm, cerium ammonium nitrate (CAN) from Acros (Acros Organics, USA) was used. NIPAAm was recrystallized from *n*-hexane. All other reagent grade chemicals and solvents were used as received without further purification.

Preparation of chitosan-g-NPIAAm. Chitosan-g-NIPAAm copolymers were synthesized by the graft polymerization of NIPAAm onto chitosan using CAN as an initiator under a nitrogen atmosphere at 30 °C for 15 hrs (Figure 1).[5] CAN was dissolved in 0.1 *N* HNO_3 and then the solution was used. After the reactions, the product solutions were precipitated in excess acetone and filtered to separate products. To remove the homopolymer of NIPAAm, the products were followed by soxhlet extraction with methanol for 48 hrs. The resulting products were dried under atmosphere for 24 hrs and then under vacuum at 30 °C for 24 hrs.

Preparation of chitosan-Pluronic®. Pluronic® was carboxylated with succinic anhydride to produce monocarboxy Pluronic® (MP).[15] Then, MP was coupled with chitosan by EDC/NHS at R.T. for 24 hrs.[6] The product was dialyzed against distilled water using a membrane (Molecular Weight Cut Off: 20,000) for 3 days and finally lyophilized (Figure 1).

Characterizations. FT-IR (Nicolet Model Magma IR 550) and ^{1}H-NMR (Bruker AMX-500) measurements were used to investigate the chemical structure of modified chitosans. Solubility tests to D. I. water, acetic acid, methanol (MeOH), dimethylsulfoxide (DMSO), *N,N*-dimethylformamide (DMF), tetrahydrofuran (THF), n-Hexane were carried out at room temperature. Each sample (10mg) was dispersed in 10ml of each solvent, and then the mixtures were shaken for 2 days by a vortex mixer (Vortex-Genie 2, Scientific Industries Inc.).

The LCST of chitosan-g-NIPAAm was determined by cloud point measurement. Cloud

point measurement of the chitosan-g-NIPAAm aqueous solutions was performed on a UV-visible spectrophotometer (UV-1601 PC, Shimadzu). The temperature was raised from 10 to 40 °C in 1 °C increments every 30 minutes. The transmittance was measured at 450 nm versus temperature. Cloud point was determined as a temperature when a transmittance was 50% from the initial at 10 °C. The gel point of the copolymer was examined by the vial-tilting method with a 4 ml vial test tube at a temperature interval of 1 °C.

Grafting ratio and efficiency. The percentage and efficiency of grafting (%) were calculated by the difference of weights before and after grafting reaction according to the following formula:

$$\text{Percentage of grafting (\%)} = (W_f - Wc) / Wc \times 100 \quad (1)$$

$$\text{Efficiency of grafting (\%)} = (W_f - Wc) / Wm \times 100 \quad (2)$$

where W_f, Wc and Wm denote the weight of the final product (chitosan-g-NIPAAm or chitosan-Pluronic®), initial chitosan charged, and initial NIPAAm monomer or Pluronic® charged, respectively.

Cell viability. For cell viability, an aqueous polymer solution (5 wt%, 1ml) of the chitosan-g-NIPAAm copolymer was mixed with the cells (1×10^5 cells/ml). The cells were placed in a 24-well plate, and cultured in Dulbecco's modified Eagle's medium (DMEM) in 5% CO_2 at 37 °C for 5 and 10 days. The number of cells entrapped in the copolymer was counted with hemacytometer using trypan blue. The viability of the copolymer was compared with the alginate bead culture system, which is a representative method to differentiate chondrocytes from MSCs.

Chondrogenic differentiation of human MSCs in thermosensitive chitosan. Chondrogenic differentiation was evaluated by incubation with serum-free DMEM supplemented with 10 ng/ml of transforming growth factor-β3 (R&D Systems, Minneapolis, MN), 100 nM dexamethasone, 30 μg/ml ascorbic acid and 1X insulin-transferrin-selenious (GIBCO, Grand Island, NY). Cells were encapsulated in a 1.2% alginate solution and into 5 wt% chitosan-g-NIPAAm at a density of $2x10^6$ cells/ml. The alginate-cell suspension was gelatinized in a 102 mM $CaCl_2$ solution in 24 well plates (1ml/well). Chitosan-g-NIPAAm-cell suspension was incubated at 37 °C for 30 min and then was supplied with a chondrogenic medium. The cells were cultured for 3, 5, 7 and 14 days. The semi-quantitative reverse transcription–polymerase chain reactions (RT-PCR) were performed to evaluate the changes in aggrecan, collagen type I, II, and X. Each of the PCR products was analyzed by separating the amplicon in a 1.5% agarose gel at 60

V/cm in Tris-Borate-EDTA buffer, and then staining them with ethidium bromide. Each density value for the genes was then normalized to the GAPDH values to yield a semi-quantitative value.[7]

Results and Discussion

Preparation of graft copolymers. Chitosan-g-NIPAAm copolymers were synthesized by the graft polymerization of NIPAAm onto chitosan using CAN as an initiator. The preparation procedure of chitosan-g-NIPAAm are summarized in Figure 1 (a). The contents of the grafted PNIPAAm moiety were controlled by varying the concentration of CAN initiator and NIPAAm monomer, respectively, and the maximum grafted chitosan copolymer was obtained at the 0.4 *M* NIPAAm concentration and 6×10^{-3} *M* CAN concentration.

Chitosan-Pluronic® was prepared by coupling MP with a chitosan backbone at room temperature for 24 hrs by the EDC/NHS method. The synthesis scheme of chitosan-Pluronic® is illustrated in Figure 1 (b). The amide bonds were formed through the reaction between the amino groups of chitosan and the carboxyl groups of MP.

Characterizations. FT-IR and ^{1}H-NMR spectroscopy measurements were carried out to confirm the change of chemical structure of chitosan-g-NIPAAm and chitosan-Pluronic®. In the FT-IR spectrum of chitosan-g-NIPAAm, characteristic absorption bands of PNIPAAm and chitosan were overlapped in the 1600~1300 cm^{-1} region. However, the broad C-O absorption bands of chitosan are clearly observable at around 1120 cm^{-1} in the grafted copolymer. These results indicate that NIPAAm was introduced onto chitosan. The FT-IR spectrum of chitosan-Pluronic® indicated that peaks that appeared at 1635cm^{-1} and 1530cm^{-1} could be assigned to carbonyl stretching vibration (amide I) and N-H bending vibration (amide II) of a primary amino group. An increase of the characteristic peaks of amide II was observed when it was compared with chitosan itself. This result suggests that the amide bonds between carboxylic groups of monocarboxy Pluronic® and the amine ones of chitosan are formed (data not shown).

In ^{1}H-NMR results, the spectrum of chitosan (Figure 2, a) exhibits the typical peaks including the proton on the anomeric carbon (at 4.90 ppm), the methyl protons from partially acetylated chitosan (at 2.12 ppm), and the proton on the carbon bearing amino (partially acetamido) groups (at 3.11 ppm). The spectrum of PNIPAAm (Figure 2, b) exhibits two broad peaks (-CH-CH_2) at 1.40-1.90 ppm, a peak (-NH-CH<) at 3.19 ppm,

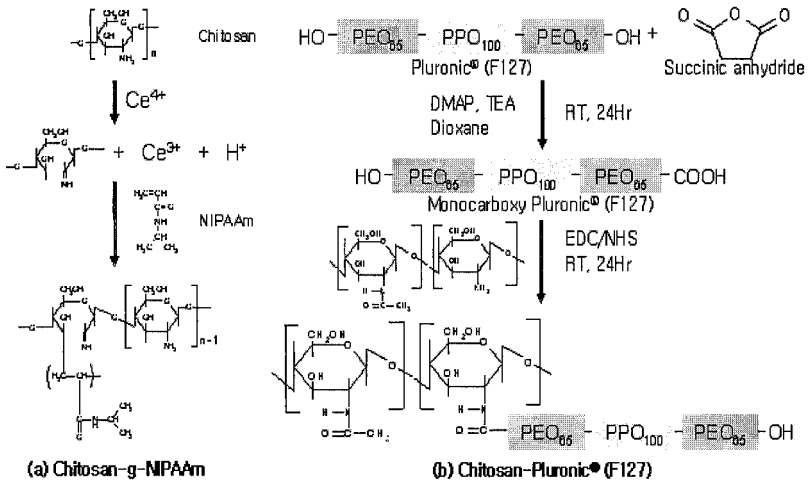

Figure 1. Schematic procedure in preparation of (a) chitosan-g-NIPAAm, (b) chitosan-Pluronic® (F127).

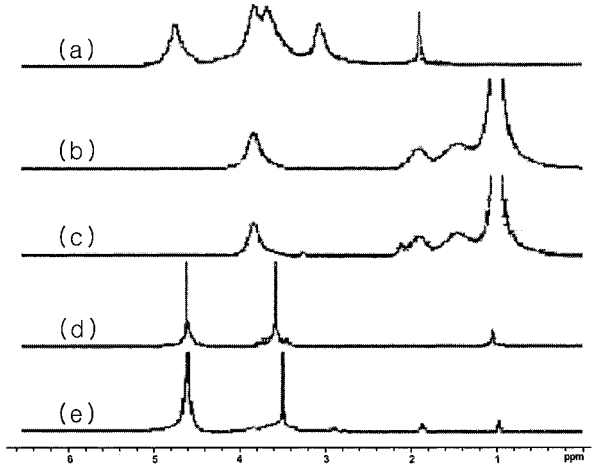

Figure 2. ^{1}H-NMR spectra of (a) chitosan, (b) PNIPAAm, (c) chitosan-g-NIPAAm, and (d) Pluronic®, (e) chitosan-Pluronic®.

and a strong peak of the methyl group at 1.11 ppm. Also, the peaks of vinyl protons (around 5.60-6.20 ppm) were made to disappear by the polymerization of NIPAAm. The spectrum of the chitosan-g-NIPAAm copolymer in Figure 2 (c) is similar to that of PNIPAAm, excepting a weak peak at 2.12 ppm and a proton peak at 3.11 ppm from the carbon bearing amine (partially acetamido) groups of chitosan. The spectrum of Pluronic® (Figure 2, d) exhibits a peak at 3.65 ppm (PEOs methylene protons) and a weak peak at 1.12 ppm due to the protons of PPOs methyl pendant groups. In the same manner as chitosan-g-NIPAAm, the spectrum of chitosan-Pluronic® in Figure 2 (e) is similar to that of Pluronic® excepting a weak peak at 2.12 ppm and a proton peak at 3.11 ppm from the newly formed amide bond by the EDC/NHS chemistry. These results indicated that chitosan copolymers had been well synthesized.

Solubility test. Chitosan, PNIPAAm, chitosan-g-NIPAAm, and Pluronic®, chitosan-Pluronic® were used for the solubility test in several solvents including water. Chitosan is insoluble in water and other common solvents, because of its strong hydrogen bond. However, it is soluble in acidic solutions such as acetic acid or formic acid due to the effect of the amine group. [19] As shown in Table 1, the chitosan-g-NIPAAm is soluble in water in spite of its chitosan moiety, but insoluble in several organic solvents. Chitosan-Pluronic® is soluble in both water and DMSO, therefore it can be applied for the injectable and coating system.

Table 1. Solubility of chitosan, PNIPAAm, chitosan-g-NIPAAm, Pluronic®, and chitosan-Pluronic®

Sample [a]	Acetic Acid	Water	MeOH	DMSO	DMF	THF	Hexane
Chitosan	O [b]	× [b]	×	×	×	×	×
PNIPAAm	O	O	O	O	O	O	O
ChitoNIPAAm	O	O	×	×	×	×	×
Pluronic®	O	O	**O**	O	O	O	×
ChitoPluronic®	O	O	×	O	×	×	×

[a] Sample (10mg) was dissolved in 10 ml of each solvent with shaking for 2 days.
[b] O : soluble, × : insoluble.

Thermosensitive behaviors. The LCST of thermoreversible chitosan copolymers was determined by cloud point measurement and the vial-tilting method. The chitosan-g-NIPAAm exhibit thermosensitive behavior and become opaque, whereas chitosan-Pluronic® gel is transparent, and precipitates or forms a gel as the temperature increases. Figure 3 represents the transmittance of polymer solutions upon temperature for chitosan copolymers and PNIPAAm with different ratios of chitosan. As was shown, the transparency of the chitosan-g-NIPAAm solution was decreased dramatically. This means that phase transition takes place upon heating, at that polymer transformation from coil to compact agglomerate conformation. This process occurs due to stabilization of the agglomerate conformation with inter- and intra- chain hydrophobic interactions.[20] The more PNIPAAm with hydrophobic isopropyl groups in the copolymer structure, the lower the temperature that is required for copolymers to accept dense conformation. In the case of chitosan-Pluronic®, there was no cloud point due to the different mechanism from the gelling system of chitosan-g-NIPAAm.

The phase transition diagram of chitosan-g-NIPAAm aqueous solution is shown in Figure 4. It was found that there was a transition temperature dependency on the concentration of the copolymer solution (from 3 to 7 wt%). The kinetics of thermo-induced phase

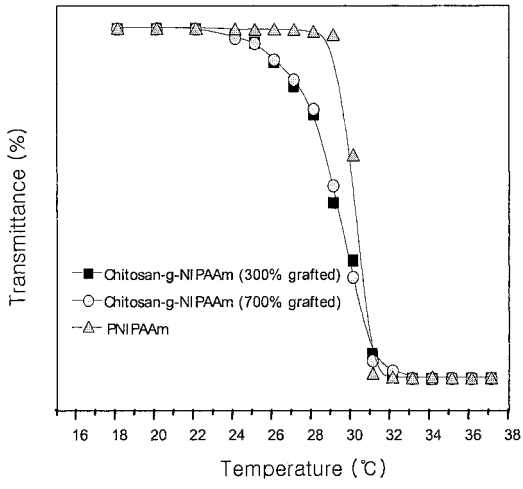

Figure 3. Transmittance of copolymer aqueous solutions with the temperature: (a) chitosan-g-NIPAAm (the percent of grafting=300%), (b) chitosan-g-NIPAAm (the percent of grafting=700%), and (c) PNIPAAm.

transition were clearly also heat transfer-dependent, as larger volumes of precursor solution took a longer time to gel under similar conditions. These results are obviously related to thermal induction time. With further heating, thermal-induced gelation occurred, resulting in lost fluidity of the solutions. Copolymers in a globule conformation probably aggregate with each other and lose their mobility at some certain temperature.

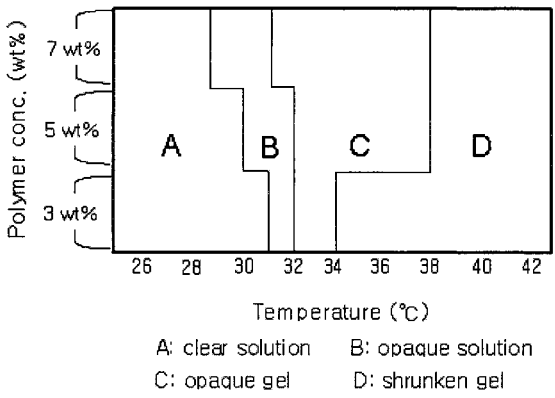

Figure 4. Phase transition diagram of chitosan-g-NIPAAm copolymer aqueous solution with the temperature.

The phase diagram of chitosan-Pluronic® is shown in Figure 5. The change of gelation temperature was dependent on the content of chitosan and the concentration of Pluronic®. The more content of chitosan, the higher was the gelation temperature. This result suggested that hydrophilic properties of chitosan interfered with the hydrophobic interaction of Pluronic®. Also, the sol-gel transition of the graft copolymer did not occur if chitosan was over 17 wt% in the copolymer owing to the increased hydrophilic property of chitosan.

Cell viability and chondrogenic differentiation *in vitro*. Figure 6 shows cell viability of hMSCs on the chitosan-g-NIPAAm and alginate gel. The cell cultures in the chitosan-g-NIPAAm and alginate beads were observed from the first time of seeding until Day 10. The cell viabilities under both conditions were maintained at over 90%.

The phenotypic differences between the chitosan-g-NIPAAm-cell suspension and alginate bead chondrogenic differentiation were then compared using RT-PCR. Aggrecan mRNA was rapidly regulated upwards within 3 days in the alginate bead culture. However, it

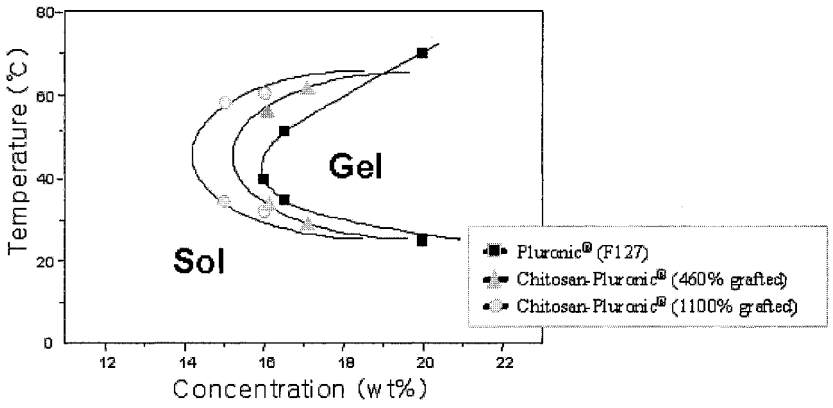

Figure 5. Phase diagram of chitosan-Pluronic® aqueous solution according to the grafting percentage of Pluronic®.

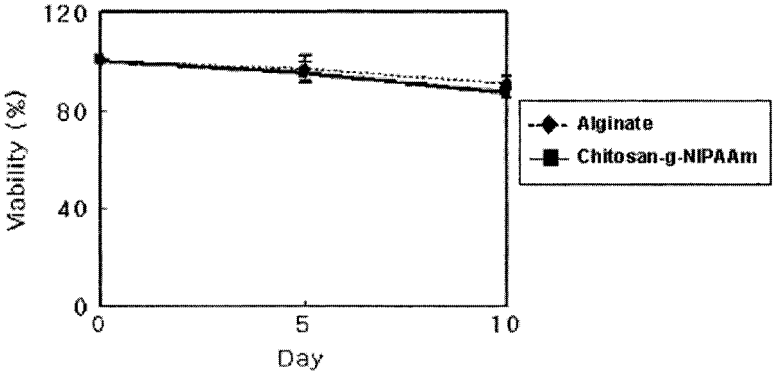

Figure 6. Viability of human MSCs in the chitosan-g-NIPAAm copolymer and alginate gel. This percentage cell viability ranged from 90% to 100% in both groups.

appeared somewhat more slowly in the chitosan-g-NIPAAm encapsulation culture, exhibiting slowly increasing expression levels within 7-14 days. The type I collagen gene

was detected uniformly in the undifferentiated cells and decreased throughout chondrogenesis in both systems. However, the type II collagen gene showed a gradual increase, being readily detected after 7 days in the chitosan-g-NIPAAm encapsulation culture. On the other hand, in an alginate bead culture, type II collagen mRNA was rapidly increased from 5 to 14 days. The type X collagen gene was not detected in the undifferentiated MSCs, and it was rapidly up-regulated at 14 days in the alginate bead culture. However, it was detected after 3 days and gradually increased to a uniform level in the chitosan-g-PNIPAAm encapsulation culture. The RT-PCR results at 3, 5, 7, and 14 days showed that collagen type I, II, X, and aggrecan mRNAs were expressed in the cells. The level of aggrecan expression in the chitosan-g-NIPAAm copolymer gel showed a more gradual increase than that in the alginate bead culture. In addition, the expressions of type II and X collagen in the thermosensitive gel culture were higher, but the type I collagen expression level was either lower or maintained at a uniform level similar to those in the alginate gel culture (Figure 7).

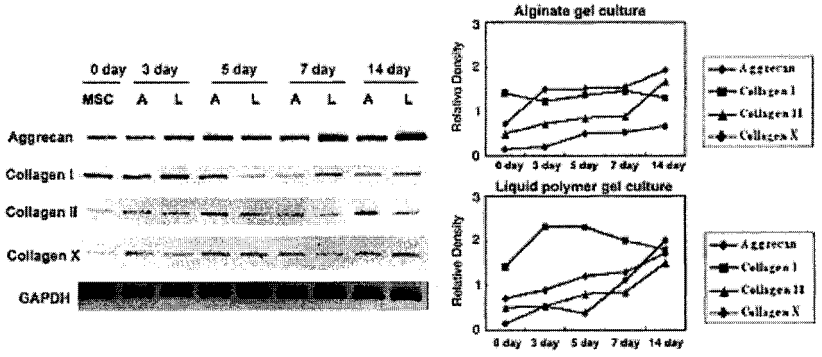

Figure 7. RT-PCR showed the chondrogenic differentiation. Increasing aggrecan and collagen type II expression. There are also collagen type I and X expressions.

Conclusions

Thermosensitive chitosan copolymers, chitosan-g-NIPAAm and the chitosan-Pluronic® were developed. The aqueous solutions of these copolymers exhibited thermoreversible behaviors at body temperature without any treatment. Chondrogenic differentiation in the

thermosensitive chitosan-g-NIPAAm gel was induced from the hMSCs *in vitro*. Analysis of these results revealed that the chitosan copolymer shows viability similar to alginate, as well as differentiation into chondrocyte *in vitro*. This novel thermosensitive chitosan gel might be useful for biomedical and pharmaceutical applications as a novel injectable material for cell or drug delivery.

Acknowledgement

This work was supported by a grant from the Stem Cell Research Center (code: M102KL01001-02K1201-02020) of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea.

[1] A. S. Hoffman, in: *"Controlled Drug Delivery, Challenge and strategies"*, K. Park Ed., ACS, Washington DC, 1997, p. 485.
[2] C. K. Han, Y. H. Bae, *Polymer* **1998**, *39*, 2809.
[3] C. Matschke, U. Isele, P. Hoogevest, A. Fahr, *J. Control. Release* **2002**, *85*, 1.
[4] G. Haipeng, Z. Yinghui, L. Jianchun, G. Yandao, Z. Nanming, Z. Xiufang, *J. Biomed. Mater. Res.* **2000**, *52*, 285.
[5] T. Nonaka, K. Hashimoto, S. Kurihara, *J. Appl. Polym. Sci.* **1997**, *66*, 209.
[6] K. Y. Cho, T. W. Chung, B. C. Kim, M. K. Kim, *Int. J. Pharm.* **2003**, *260*, 83.
[7] J. H. Cho, S. H. Kim, K. D. Park, M. C. Jung, W. I. Yang, S. W. Han, J. Y. Noh, J. W. Lee, *Biomaterials* **2004**, *25*, 5743
[8] Z. Ding, R. B. Fong, C. J. Long, P. S. Stayton, A. S.Hoffman, *Nature* **2001**, *411*, 59.
[9] R. A. Stile, W. R. Burghardt, K. E.Healy, *Macromolecules* **1999**, *32*, 7370.
[10] L. E. Bromberg, E. S. Ron, *Adv. Drug Deliv. Rev.* **1998**, *31*, 197.
[11] B. Vernon, S. W. Kim, Y. H. Bae, *J. Biomater. Sci. Polym. Ed.* **1999**, *10*, 183.
[12] T. Shimizu, M. Yamato, A. Kikuchi, T. Okano, *Tissue Eng.* **2001**, *7*, 141.
[13] H. G. Schild, *Prog. Polym. Sci.* **1992**, *17*, 163
[14] C. Chatelet, O. Damour, A. Domard, *Biomaterials* **2001**, *22*, 261.
[15] S. Zalipsky, C. Gilon, A. Zilkha, *Eur. Polym. J.* **1983**, *19*, 1177.
[16] H. G. Schild, *Prog. Polym. Sci.* **1992**, *17*, 163.
[17] J. O. Hollinger, G. C. Battison, *Clin. Orthop.* **1986**, *207*, 290.
[18] Y. Usami, Y. Okamoto, S. Minami, A. Matsuhashi, Y. Shigemasa, *Carbolydr. Polymr.* **1997**, *32*, 115.
[19] A. V. Ilyina, V. E. Tikhonov, A. I. Albulov, V. P. Varlamov, *Process Biochem.* **2000**, *35*, 563.
[20] C. Boutris, E. G. Chatzi and C. Kiparissides, *Polymer* **1997**, 38, 2567.