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RGD-Conjugated Chitosan-Pluronic Hydrogels as a Cell Supported Scaffold for Articular Cartilage Regeneration

Kyung Min Park, Yoon Ki Joung, and Ki Dong Park*

Department of Molecular Science and Technology, Ajou University, Suwon 443-749, Korea

Sang Young Lee and Myung Chul Lee

Department of Orthopedic Surgery, Seoul National University, College of Medicine, Seoul 110-744, Korea

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Abstract: A RGD (Arg-Gly-Asp) conjugated chitosan hydrogel was used as a cell-supporting scaffold for articular cartilage regeneration. Thermosensitive chitosan-Pluronic (CP) has potential biomedical applications on account of its biocompatibility and injectability. A RGD-conjugated CP (RGD-CP) copolymer was prepared by coupling the carboxyl group in the peptide with the residual amine group in the CP copolymer. The chemical structure of RGD-CP was characterized by ^{1}H NMR and FT IR. The concentration of conjugated RGD was quantified by amino acid analysis (AAA) and rheology of the RGD-CP hydrogel was investigated. The amount of bound RGD was 0.135 μg per 1 mg of CP copolymer. The viscoelastic parameters of RGD-CP hydrogel showed thermo-sensitivity and suitable mechanical strength at body temperature for cell scaffolds (a > 100 kPa storage modulus). The viability of the bovine chondrocyte and the amount of synthesized glycosaminoglycans (GAGs) on the RGD-CP hydrogels were evaluated together with the alginate hydrogels as a control over a 14 day period. Both results showed that the RGD-CP hydrogel was superior to the alginate hydrogel. These results show that conjugating RGD to CP hydrogels improves cell viability and proliferation, including extra cellular matrix (ECM) expression. Therefore, RGD conjugated CP hydrogels are quite suitable for a chondrocyte culture and have potential applications to the tissue engineering of articular cartilage tissue.

Keywords: chitosan, Pluronic, hydrogel, cartilage regeneration, biomedical applications.

Introduction

Current therapies for articular cartilage disease, including subchondral drilling and autologous chondrocyte implantation (ACI), have many limitations such as incomplete defect healing and secondary site morbidity.[1] Recently, to overcome these restrictions, various studies have been investigated for cartilage regeneration.[2-4] Over the past decade, biomaterials using polymers such as poly(D,L-lactide) (PLA), poly(glycolide) (PGA), poly(D,L-lactide-*co*-glycolide) (PLGA), and polysaccharides have widely used for tissue engineering.[5-8] Particularly, out of these biomaterials, polysaccharides have been in receipt of much attention as materials of biomimetic scaffold because their structures are similar with several extracellular matrix (ECM) molecules found in natural tissue. In various polysaccharides, chitosan, alginate, collagen, hyaluronic acid, and fibrin glue are used representatively.[9] In particular, chitosan-based scaffolds have been mainly used for tissue regeneration due to their biocompatibility, biodegradability and minimal immune response.[9-12]

Chitosan, a partially deacetylated derivative of chitin, is a linear polysaccharide, composed of a relatively simple glucosamine and *N*-acetyl glucosamine unit. Its chemical structure is analogous with diverse glucosaminoglucans (GAGs) found in articular cartilage. Also, cationic property of chitosan provides binding affinity with anionic GAGs, proteoglycans, so chitosan can encapsulate the ECM molecules in articular cartilage. Since GAGs play a vital role of regulating cell function and chondrogenesis, chitosan is attractive biomaterials for cartilage regeneration.[13] At last decades, a number of studies have been investigated to improve cell viability and proliferation of scaffolds for tissue regeneration. Preparing ideal scaffolds for tissue engineering requires mimicking natural environments in the ECM.[14]

The ECM has specific peptide motifs that bind to receptors of cell surface such as integrins and transmembrane proteoglycans.[15-19] In these peptide moiety, RGD sequence stimulate adhesion and spreading of cells such as chondro-

*Corresponding Author. E-mail: kdp@ajou.ac.kr

cyte, fibroblast and endothelial cell. So, peptides containing RGD sequence has been conjugated with various polymers to improve cell adhesion and proliferation.[20,21] Ming-Hua Ho and coworkers reported RGD-conjugated chitosan scaffolds.[22] In their studies, the conjugation of RGD improved adhesion and proliferation of osteoblast, resulting in improved regeneration of bone tissue. J. A. Burdick and coworkers also reported RGD-conjugated PEG hydrogels as a scaffold and the results showed that the conjugation of RGD enhanced attachment, spreading, and proliferation of human fibroblast.[23]

In our previous study, we concluded that thermosensitive chitosan-Pluronic (CP) hydrogel has the potential for various biomedical applications because of their biocompatible and injectable property based on minimal invasive technique.[24] The hypothesis of our study is that introducing RGD sequence to CP hydrogel can improve adhesion and proliferation of chondrocyte through the interaction with integrins. In this manuscript, we introduce a thermosensitive RGD-conjugated CP hydrogel (RGD-CP) prepared as a cell supporting scaffold for articular cartilage regeneration. Preparation of RGD-CP copolymer and properties of RGD-CP hydrogel are introduced and proliferation of bovine chondrocyte study and the expression of GAGs are described and discussed as compared with alginate hydrogel as a control group.

Experimental

Materials. Chitosan (high molecular weight, 75-85% deacetylated), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECD), *N*-hydroxysuccinimide (NHS), succinic anhydride, 4-dimethylaminopyridine (DMAP) and 4-morpholineethanesulfonic acid (MES) were purchased from Aldrich (St. Louis, MO, USA). Pluronic F127 was provided from BASF (Seoul, Korea). The peptide GRGDS (Gly-Arg-Gly-Asp-Ser) was obtained from Chem-Impex (USA). Other chemicals and solvents were used without further purification.

For cell culture, low-viscosity alginate, L-ascorbic acid, chondrotin sulfate C, collagenase and dimethylmethylene blue (DMB) was purchased from Sigma (St. Louis, MO, USA). High-glucose Dulbecco's modified Eagle medium (DMEM) and 10 % fetal bovine serume (FBS) was supplied from Life Technology (Carlsbad, CA, USA). Papain buffer was obtained from CalBiochem (Indianapolis, IN, USA). CellTilter 96 A_{queous} assay kit was obtained from Promega (Madixon, WI, USA).

Synthesis of Chitosan-Pluronic (CP) Graft Copolymer. Thermo-responsive CP copolymer was synthesized by grafting Pluronic onto chitosan chain using EDC and NHS as described in our previous report.[24] Briefly, after carboxylation of the hydroxyl terminal group of Pluronic with succinic anhydride, DMAP and TEA as a catalyst in dioxane for 24 h at room temperature, the resulting monocarboxylated Pluronic was grafted onto chitosan chain. The grafting reaction was performed at room temperature for 24 h, using EDC and NHS as a catalyst in 0.1 M MES buffer including 1 N HCl. Finally, the product was dialyzed against distilled water using a cellulose membrane (molecular weight cut-off (MWCO) = 50 kDa) for 3 days and then lyophilized to give powdery product of CP graft copolymer.

Grafting yield (%) for conjugation was calculated by the difference between weights before and after conjugation according to the following equation:

$$\text{Grafting yield (\%)} = (W_f - W_c) / W_c \times 100$$

where W_f and W_c denote the weight of the final CP graft copolymer and initial chitosan, respectively.[24]

Synthesis of RGD Conjugated CP Copolymer. There are various methods to conjugate RGD peptides to polymers using functional groups such as hydroxyl, amine, or carboxyl groups.[25] In our study, RGD-CP was synthesized by coupling GRGDS with CP via forming the amide bond between residual amine groups in CP copolymer and carboxyl groups in peptide using EDC and NHS as coupling agents. After dissolving GRGDS peptide (2 mg, 4.08×10^{-6} mol) in 0.1 M MES buffer (2 mL), EDC (0.94 mg, 4.9×10^{-6} mol) and NHS (0.28 mg, 2.44×10^{-6} mol) were added to the peptide solution (molar ratio of GRGDS:EDC:NHS= 1:1.2:0.6). Then, this solution was incubated for 30 min to activate the terminal carboxyl group of serine. After activating the carboxyl group, this solution was added to CP solution dissolved in 0.1 M MES buffer (400 mL, 0.5 wt%). The coupling reaction was performed for 24 h at room temperature. Finally, the products were dialyzed against distilled water using a cellulose membrane (MWCO = 25 kDa) for 3 days to remove uncoupled peptides and lyophilized to give white powder. The chemical structure of synthesized copolymer was characterized by ^{1}H NMR and IR with a Bruker AMX-500 NMR spectrometer and Nicolet Model Magma IR 550 spectrometer, respectively.

Amino Acid Analysis (AAA) of RGD-CP. Samples (1 mg) of RGD-CP copolymer were mixed with 2 mL of 6 N HCl solution to hydrolyze the conjugated peptides. Chitosan and GRGDS peptide were measured as a control group using same methods. The acidic mixtures were allowed to keep at 110 °C for 24 h in dry oven. The hydrolyzed polymer and peptides mixture was analyzed with amino acids analyzer.[22]

Scanning Electron Microscope Observations (SEM) of RGD-CP Hydrogel. For SEM analysis, 20 wt% RGD-CP hydrogels were prepared by dissolving the polymer in PBS (0.01 M and pH 7.4) at 0-4 °C and the polymer solution was kept in oven at 37 °C to form gel. The specimens of dehydrated RGD-CP hydrogel were prepared by freeze drying of the hydrogels and cross sections; vertical and horizontal direction of the specimens. Then, a gold layer was coated on the samples. Surface morphology of prepared specimens was observed by SEM, JEOL (JSM-6380, Japan).

Viscoelastic Analysis of RGD-CP Hydrogel. The rheological measurements of RGD-CP hydrogel were carried out with 20 wt% hydrogel in PBS (0.01 M and pH 7.4) using dynalyser 2000 (Reological, Sweden). The elastic (G') and viscous (G'') shear modulus were measured at a frequency of 0.1 Hz. For the oscillatory shear rheology measurement, parallel plate geometry was used (plate diameter = 25 mm, gap = 0.45 μm and stress = 10 Pa). The hydrogel samples were placed into the plate of rheometer using temperature changes of 2 °C intervals over the range 5-60 °C.[26,27]

Isolation and Expansion of Chondrocyte. Bovine chondrocytes were isolated from articular cartilage derived from the knee of a 4-week-old calf by enzyme digestion. Cartilage was washed in calcium- and magnesium-free phosphate buffered saline (DPBS) and finely minced. Bovine chondrocytes were released from articular cartilage after being digested for 1 h with 0.2 % pronase, followed by digestion for 3 h with 0.2 % collagenase at 37 °C in DMEM containing antibiotic-antimycotic solution (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 g/mL amphotericin B). After exclusion of the undigested cartilage using a 70 μm nylon sieve, the chondrocytes were collected by centrifugation, washed twice, resuspended in DMEM supplemented with 10 % fetal bovine serum (FBS), 25 μg/mL L-ascorbic acid and antibiotics, and finally expanded to get adequate cell numbers for seeding at 37 °C in a humidified atmosphere of 5 % CO_2.

***In vitro* Cell Culture of RGD-CP Hydrogel.** The cell viability test of RGD-CP hydrogel was investigated using bovine articular chondrocyte. For this assay, aqueous polymer solutions (20 wt%) of the RGD-CP copolymer was mixed with the cells (6×10^6 cells/mL). The cell suspended polymer solutions were placed in a 96-well plate (100 μL of RGD-CP suspension per well) and incubated at 37 °C for 5 min to form gel. Then cell suspended hydrogels were cultured in DMEM, which was changed everyday. At 1, 3, 7 and 14 days, samples were collected for assays (n = 4). Chondrocyte proliferation assay was performed using the 3-(4, 5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) method using a commercially available kit (CellTilter 96 A_{queous} Assay) according to the manufacture's instruction. At determined times, MTS solution was added into each well containing the samples in DMEM and incubate the plate for 90 min at 37 °C in a humidified atmosphere of 5% CO_2. A hundred μL of incubated medium was transferred to a 96-well culture plate and the optical density was recorded in a microplate reader. For the measurement of synthesized GAG contents, the media

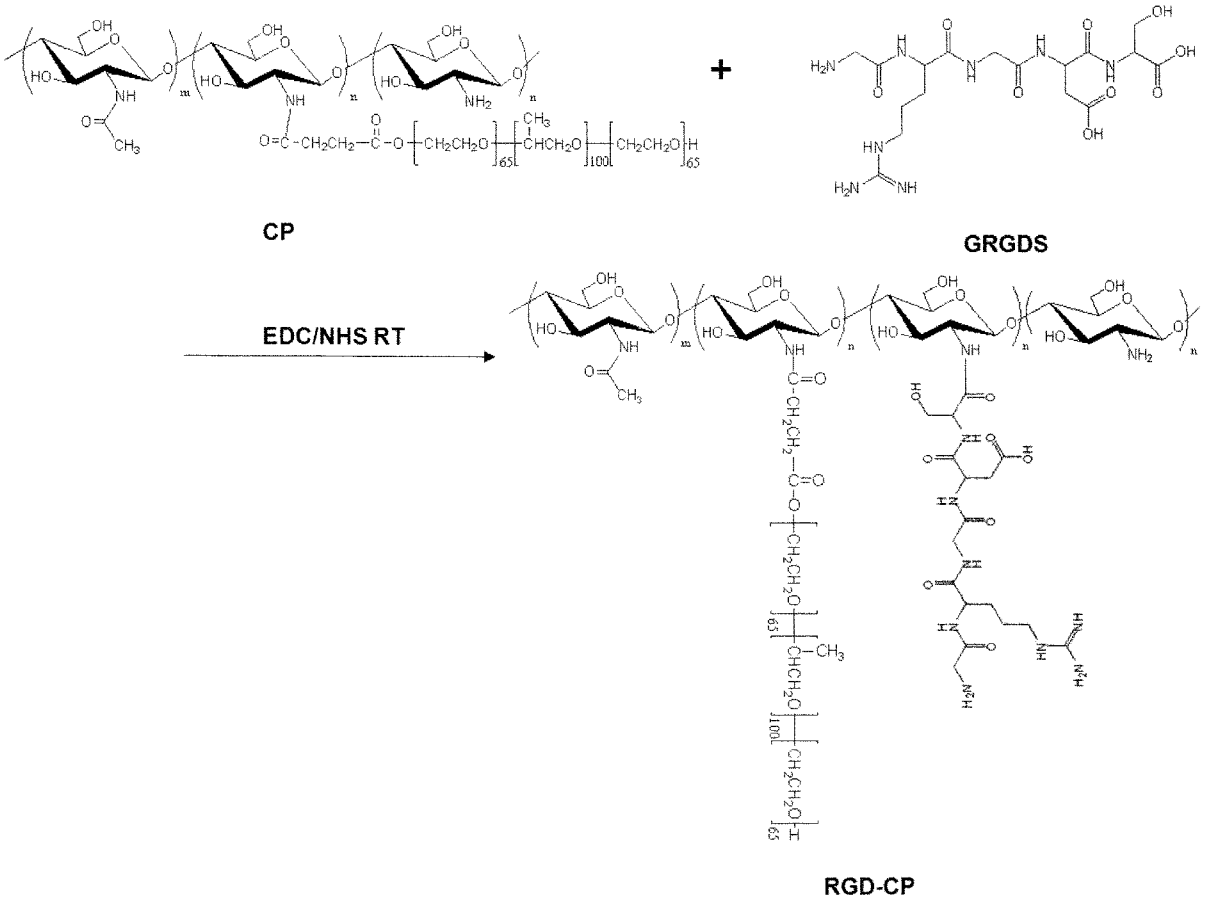

Figure 1. Synthetic scheme of RGD-CP copolymer.

were harvested everyday and pooled for each 14 day period. At each predetermined time, the RGD-CP hydrogels were dissolved, obtaining fractions: supernatant containing macromolecules from the further removed compartment and a pellet containing cells with cell-associated matrix. The media, supernatant and pellet were digested for 12 h at 55 °C in papain buffer (200 μg/mL papain in 50 mM EDTA, 5 mM L-cystein). The metachromatic reaction of GAG with DMB was monitored using spectrophotometer and the ratio of A530 and A590 was used to determine GAG contents with chondrotin sulfate C as a standard.

Results and Discussion

Synthesis and Characterization of RGD-CP Copolymer. The synthesis of RGD-CP copolymer is illustrated in Figure 1. Carboxyl groups of GRGDS were activated with EDC and NHS. Figure 2(a) presents the ^{1}H NMR spectrum of CP. Proton assignments are as follow: $\delta_{2.12}$ = CH_3 (peak a, the methyl proton of partially acetylated group in chitosan), $\delta_{1.12}$ = CH_3 (peak b, the methyl proton of PPOs), $\delta_{3.65}$ = CH_2 (peak c, the methylene proton of PEOs). All other signals were attributed to the protons on the anomeric carbon.[24,28] As shown in Figure 2(b) for RGD-CP, new peaks at $\delta_{3.1\text{-}3.3}$ (peaks e and f) and $\delta_{3.89}$ (peak d) were clearly detected due to the conjugation of GRGDS groups. However, other proton peaks of peptide were not clear by typical peaks of the CP copolymer.

The FT IR spectra shown in Figure 3 demonstrate that GRGDS was successfully conjugated onto CP copolymer. In the spectra of the CP (b), a peak appeared at 1530 cm^{-1}, corresponding to the primary amine bending region (1500-

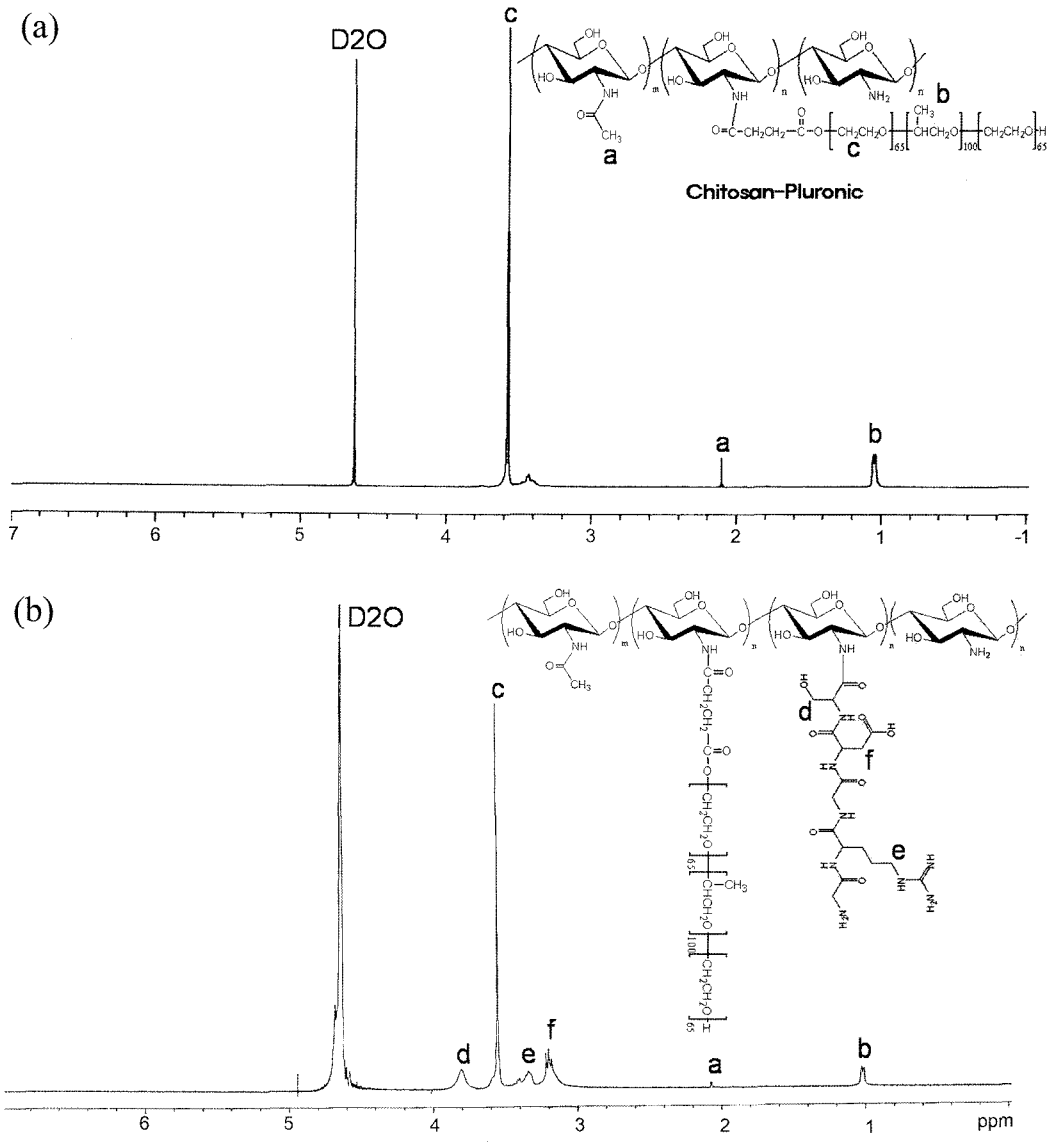

Figure 2. ^{1}H NMR spectra of (a) CP and (b) RGD-CP in D_2O.

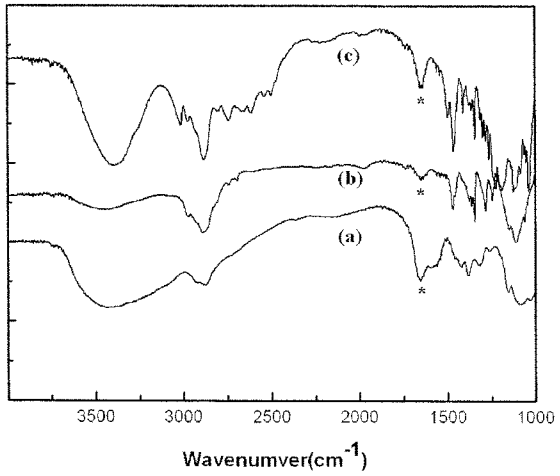

Figure 3. FT IR spectra of (a) chitosan, (b) CP, and (c) RGD-CP.

1550 cm^{-1}).[29] In the spectrum of the RGD-CP copolymer (c), an increase in intensity of the characteristic peaks (1530 cm^{-1}) of amine group was observed as compared with CP copolymer, indicating that the amine group was increased by grafting the peptide which has a lot of primary amine group onto CP copolymer. This result suggests that the GRGDS peptide was conjugated successfully onto CP copolymer. Amount of conjugated GRGDS was 0.135 μg per 1 mg of CP copolymer and the conjugation yield was 13.5%, quantitatively determined by AAA method.

Rheological Analysis and Surface Morphology of RGD-CP Hydrogel. The shear modulus of injectable RGD-CP hydrogel with thermo-sensitivity was measured by oscillatory rheological experiments. The kinetics of hydrogel formation was observed by monitoring the storage modulus (G') and loss modulus (G'') versus temperature. Figure 4 shows the storage modulus, loss modulus, and viscosity of 20 wt% RGD-CP hydrogel and 20 wt% Pluronic as a control in PBS (0.01 M and pH 7.4) solution versus temperature at a frequency 0.1 Hz. Pluronic has been well known as a thermosensitive copolymer composed of polyethylene oxide-polypropylene oxide-polyethylene oxide (PEO-PPO-PEO). The storage modulus was increased gradually by increasing temperature, over around the body temperature. The G' of RGD-CP hydrogel was recorded as 100 kPa over body temperature. The storage moduli of common hydrogels were ~10 kPa according to some literatures[27,30] and its value was higher than Pluronic hydrogels. So, we could confirm that RGD-CP hydrogel has a enough mechanical strength as a cell supported scaffold.

Figure 5 shows the SEM images horizontally and vertically cleaved surfaces of lyophilized RGD-CP hydrogel. Surface images showed irregular pores with an average pore size of 100 nm. Observed inner pores could be constructed by swelling due to water penetration and interfaces are consisted of hydrophilic chains of RGD-CP copolymers.[31] But,

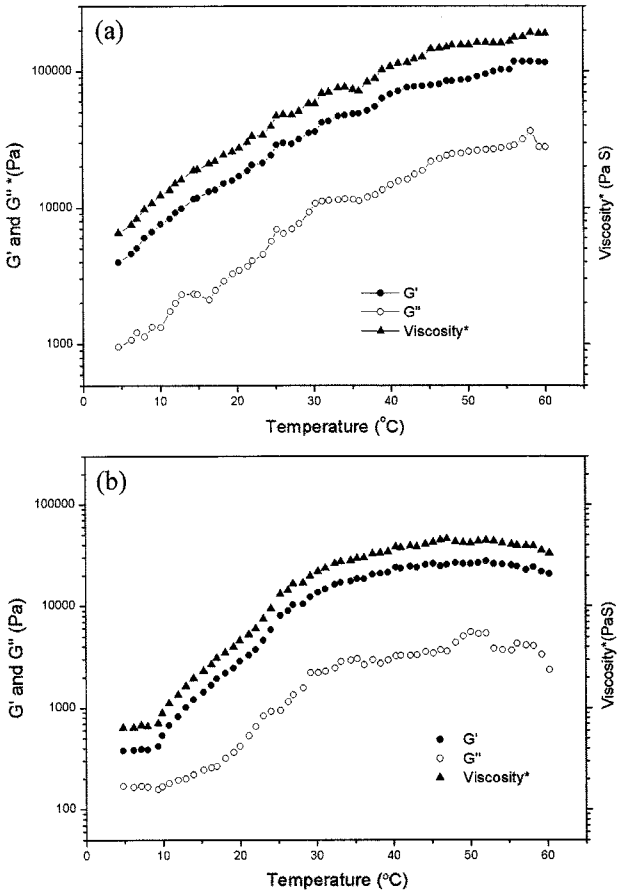

Figure 4. Storage modulus (G'), loss modulus (G''), and complex viscosity of (a) 20 wt% RGD-CP hydrogel and (b) 20 wt% Pluronic in PBS (0.01 M and pH 7.4).

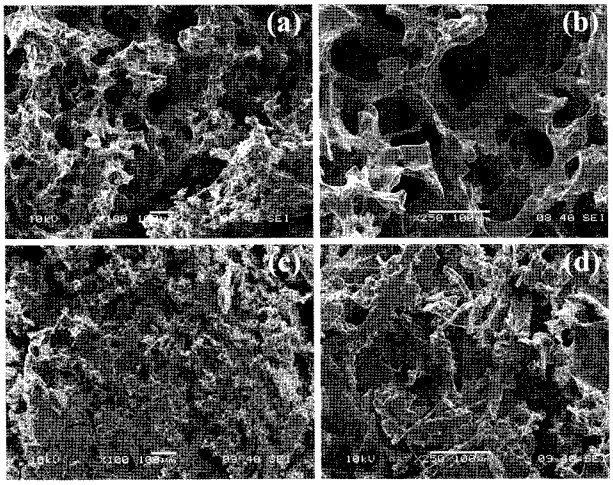

Figure 5. SEM images of horizontally cleaved surface at magnitude of vertically cleaved surface of lyophilized 20 wt% RGD-CP hydrogel at magnifications of ×100 (a and c) and ×250 (b and d).

the size of pores observed in images may be not enough small for cell growth if the size is not different with that of swelled RGD-CP hydrogel. The hydrophobic interaction

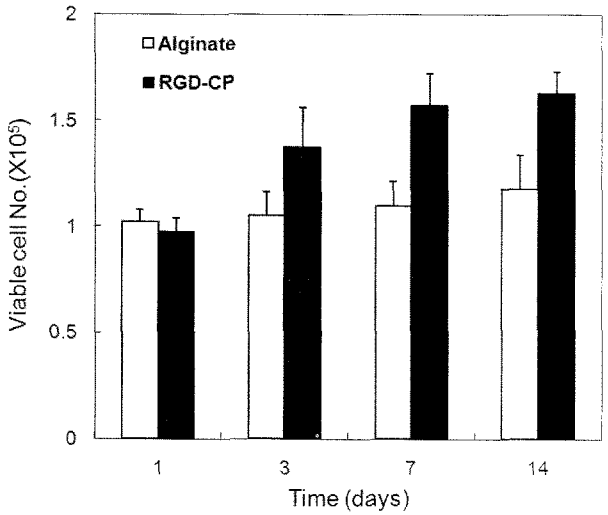

Figure 6. The viability of bovine chondrocyte on RGD-CP hydrogel and alginate hydrogel for 14 days culture, viable cell numbers were detected by MTS assay.

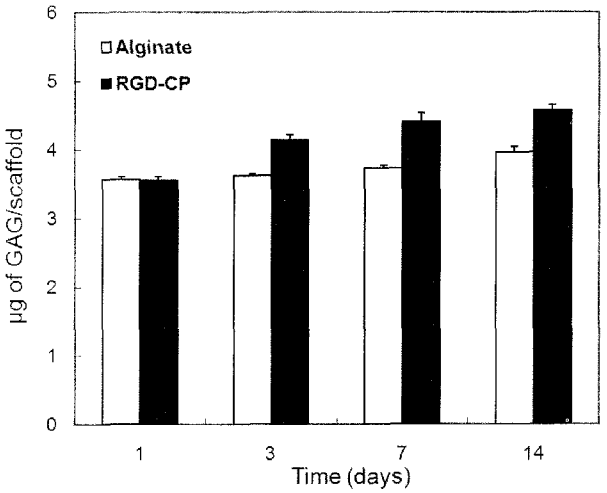

Figure 7. The synthesized GAG amounts in RGD-CP hydrogel and alginate hydrogel for 14 days culture. GAGs amounts were detected by DMB assay.

between polypropylene oxide (PPO) segments in Pluronic created the crystalline phase, meaning the physical cross-link supporting three dimensional network structures. As a result, hydrated spaces (observed pores) and crystalline phase are correlated in terms of cell growth and mechanical strength, can be modulated by the ratio between hydrophilic and hydrophobic chains.

Chondrocyte Proliferation and Synthesized GAG Amount on RGD-CP Hydrogel. The cell compatibility of RGD-CP hydrogel was investigated with bovine chondrocyte for cartilage regeneration. Figure 6 shows the cell viability of the bovine chondrocyte in the RGD-CP hydrogel and alginate hydrogel. Alginate hydrogel was used as the control group because it has shown encouraging results for chondrocyte proliferation.[9] The number of chondrocyte was recorded four times during 14 days. In the result by MTS assay, there is not significant difference in cell numbers between both hydrogels after 1 day. But, the cell number at third day showed considerable difference between both hydrogels. Up to second week, viable cell numbers cultured on RGD-CP hydrogel were more than those on alginate hydrogel. This indicates that RGD-CP hydrogel has more compatible environment for chondrocyte growth due to the integrin-mediated signal transduction. Integrins have been known to bind various cells and control the cell function such as proliferation, differentiation and expression of ECM components. Especially, RGD is specific peptide motifs that bind to the integrins. RGD sequence in the RGD-CP hydrogels was allowed to bind to the integrins, so the signal transduction was controlled between chondrocyte and the hydrogels. Therefore, we suggest that this improved proliferation of chondrocyte on RGD-CP hydrogel was derived from RGD conjugation as compared with alginate hydrogels because RGD effects on cell viability were already demonstrated by many studies.

To evaluate maintenance or the chondrocyte phenotype, GAG construction was investigated because GAG is an important component of articular cartilage ECM.[1] Figure 7 shows the amount of synthesized GAG content on hydrogels by DMB assay. The result showed similar tendency with the result of cell viability. At first day of culture, there is no difference in GAG content of both hydrogels. But, GAG amount on RGD-CP hydrogel were slightly higher than those on alginate hydrogel after third day of culture. Obtained results of cell viability and synthesized GAG amounts can be inferred from suggestions as follows: 1) chitosan chain of RGD-CP hydrogel was more compatible for GAG synthesis of chondrocyte than alginate chain, 2) conjugated RGD sequence stimulated GAG synthesis of chondrocyte and 3) conjugated RGD sequence enhanced adhesion or proliferation of chondrocyte on RGD-CP hydrogel, resulting in more viable cell and GAG synthesis. Suggestion 1 and 2 is not clear only with our results of GAG content and cell viability. The effect of chitosan on chondrocyte viability and GAG synthesis, directly compared with alginate, also were nearly reported. But, suggestion 3 can be supported by other results from many references. Although different cells were cultured on RGD-modified surfaces, many effects of RGD-modified surfaces in terms of interaction with cells were reported. Examples of the effect are mainly about suppressed attachment and spreading of cells, which involved with affinity or interaction of RGD with surface receptors of cells.

Conclusions

In the present study, the RGD-CP hydrogel was devel-

oped as a cell supported scaffold for articular cartilage regeneration. The amount of RGD conjugated to CP was 0.135 μg per 1 mg of CP copolymer and RGD-CP hydrogel contained hydrated spaces for cell growth. RGD-CP hydrogel could be readily injected by using syringe to the defect site and solidified at body temperature. RGD-CP hydrogel showed higher cell numbers and GAGs amounts as compared with alginate hydrogel. Therefore, these results demonstrate that RGD-CP hydrogel has a potential as an injectable cell carrier for cartilage regeneration and also can be useful for other biomedical applications.

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References

(1) H. S. Park, J. S. Temenoff, T. A. Holland, Y. Tabata, and A. G. Mikos, *Biomaterials*, **26**, 7095 (2005).
(2) M. Brittberg, A. Lindahl, A. Nilsson, C. Ohlsson, O. Isaksson, and L. Peterson, *New. Engl. J. Med.*, **331**, 889 (1994).
(3) E. Bel, B. Ivarsson, and C. Merrill, *Proc. Natl. Acad. Sci. USA*, **76**, 1274 (1979).
(4) J. F. Hansbrough, D. Christine, and W. B. Hansbrough, *J. Burn. Care. Rehabil.*, **13**, 519 (1992).
(5) G. Khang, J. M. Rhee, P. Shin, I. Y. Kim, B. Lee, S. J. Lee, Y. M. Lee, H. B. Lee, and I. Lee, *Macromol. Res.*, **10**, 158 (2002).
(6) J. S. Park, J. M. Kim, S. J. Lee, S. G. Lee, Y. K. Jeong, S. E. Kim, and S. C. Lee, *Macromol. Res.*, **15**, 424 (2007).
(7) H. S. Kim, J. T. Kim, Y. J. Jung, S. C. Ryu, H. J. Son, and Y. G. Kim, *Macromol. Res.*, **15**, 65 (2007).
(8) S. H. Lim, Y. Son, C. H. Kim, H. Shin, and J. I. Kim, *Macromol. Res.*, **15**, 370 (2007).
(9) B. L. Seal, T. C. Otero, and A. Panitch, *Mater. Sci. Eng.*, **R34**, 147 (2001).
(10) Q. Hu, B. Li, M. Wang, and J. Shen, *Biomaterials*, **25**, 779 (2004).
(11) M. V. Risbud and R. R. Bhonde, *Drug. Deliv.*, **7**, 69 (2000).
(12) K. S. Chow and E. Khor, *Biomacromolecules*, **1**, 61 (2000).
(13) D. M. Albert, S. Michael, and V. R. Makarand, *Biomaterials*, **26**, 5983 (2005).
(14) M. Tatsuya, I. Norimasa, Y. Shintaro, F. Tadanao, M. Tokifumi, M. Akio, O. Noriko, O. Takashi, and N. Shin-Ichiro, *Biomaterials*, **26**, 5339 (2005).
(15) M. D. Pierschbacher and E. Ruoslahti, *Nature*, **309**, 30 (1984).
(16) E. Ruoslahti and M. D. Pierschbacher, *Science*, **238**, 491 (1987).
(17) S. P. Massia and J. A. Hubbell, *Anal. Biochem.*, **187**, 292 (1990).
(18) H. B. Lin, W. Sun, D. F. Mosher, C. Garcia-Echeverria, K. Schaufelberger, P. I. Lelkes, and S. L. Cooper, *J. Biomed. Mater. Res.*, **28**, 329 (1994).
(19) J. Graf, Y. Iwamoto, M. Sasaki, G. R. Martin, R. K. Kleinman, F. A. Robey, and Y. Yamada, *Cell*, **48**, 989 (1987).
(20) S. Jo, P. S. Engel, and A. G. Mikos, *Polymer*, **41**, 7595 (2000).
(21) K. J. Jung, K. D. Ahn, D. K. Han, and D. J. Ahn, *Macromol. Res.*, **13**, 446 (2005).
(22) H. Ming-Hua, W. Da-Ming, H. Hsyue-Jen, L. Hwa-Chang, H. Tzu-Yang, L. Juin-Yoh, and H. Lein-Tuan, *Biomaterials*, **26**, 3197 (2005).
(23) B. A. Jason and A. S. Kristi, *Biomaterials*, **23**, 4315 (2002).
(24) H. J. Chung, D. H. Go, J. W. Bae, I. K. Jung, J. W. Lee, and K. D. Park, *Curr. Appl. Phys.*, **5**, 485 (2005).
(25) H. Ulrich, D. Claudia, and K. Horst, *Biomaterials*, **24**, 4385 (2003).
(26) A. Martinez-Ruvalcaba, E. Chornet, and D. Rodrigue, *Carbohyd. Polym.*, **67**, 586 (2007).
(27) D. R. Nisbet, K. E. Crompton, S. D. Hamilton, S. Shirakawa, R. J. Prankerd, D. I. Finkelstein, M. K. Horne, and J. S. Forsythe, *Biophys. Chem.*, **121**, 14 (2006).
(28) C. Qing, W. Yuqing, B. Jianzhong, and W. Shenguo, *Biomaterials*, **24**, 3555 (2003).
(29) D. L. Pavia, G. M. Lampman, and G. S. Kriz, *Introduction to Spectroscopy*, Second edition, Saunders College Publishing, 1979, pp. 81-84.
(30) R. Jin, C. Hiemstra, Z. Zhong, and J. Feijen, *Biomaterials*, **28**, 2791 (2007).
(31) X. Jia, J. A. Burdick, J. Kobler, R. J. Clifton, J. J. Rosowski, S. M. Zeitels, and R. Langer, *Macromolecules*, **37**, 3239 (2004).