Macromolecular Research, Vol. 20, No. 1, pp 106-111 (2012)
DOI 10.1007/s13233-012-0074-6
www.springer.com/13233

Chondrocyte 3D-Culture in RGD-Modified Crosslinked Hydrogel with Temperature-Controllable Modulus

Hyesun Lee[1,2], Bo Gyu Choi[2], Hyo Jung Moon[2], Jiyeon Choi[1], Kwideok Park[1], Byeongmoon Jeong*[,2], and Dong Keun Han*[,1]

[1]*Center for Biomaterials, Korea Institute of Science and Technology, Seoul 130-650, Korea*
[2]*Department of Bioinspired Science (WCU), Department of Chemistry and Nano Science, Ewha Womans University, Seoul 120-750, Korea*

Received September 13, 2011; Revised October 4, 2011; Accepted October 6, 2011

Abstract: Based on the thermosensitivity of Pluronic® F127 and the cytointeractability of Arg-Gly-Asp (RGD) sequences, RGD-modified F127 dimethacrylate (FM-RGD) hydrogel was investigated as a 3-dimensional culture matrix for chondrocytes. Chondrocytes were encapsulated in the hydrogel by radical copolymerization of FM-RGD and FM in the presence of cells. The FM-RGD hydrogel modulus could be modulated by temperature variation from 15 ºC (1,300 Pa) to the culture temperature of 37 ºC (8,900 Pa), at which the hydrogel provided a condrocyte-compatible microenvironment. Compared with the hydrogel prepared from F127 dimethacrylate (FM) without RGD, the RGD-modified hydrogel produced significant (p<0.01) improvements in cell proliferation, DNA production, and viability while allowing the chondrocytes to maintain their original spherical phenotypes.

Keywords: stimuli-sensitive polymers, biomimetic, modulus, hydrogel, biomaterial.

Introduction

Tissue engineering has been considered as an inevitable approach for treatment of damaged tissues or organs due to the shortage of donors and demands for advanced medical care.[1,2] A knowledge of cells, signaling molecules, and extracellular matrix (ECM) is a prerequisite for this approach, where the design of the biomimetic extracellular matrix plays a critical role.[3,4] Hydrogels have been considered as one of the most promising materials as a synthetic ECM due to their high water content and controllable physicochemical properties.[5] Initially, bioinert hydrogels based on poly (ethylene glycol), poly(vinyl alcohol), hyaluronic acid, alginate, and collagen were used as a 3-dimensional (3D) culture matrix for cells.[6-8] By adding cyto-interactive moieties such as RGD to the synthetic ECM, the design principles changed from using bioinert ECM to using bioactive ECM.[9] RGD is an active sequences of adhesive proteins in the extracellular matrix, and acts as a ligand for cell adhesion receptors, thereby increasing cell attachment and spreading on surfaces in a dose-dependent manner.[10,11]

Recently, details of the physicochemical control of hydrogels for cell culture have been reported. For example, stem cells can be induced to differentiate into adipocytes or osteocytes by changing the nature of the hydrogel surface to *t*-butyl group or phosphate groups, respectively.[12] In addition, depending on the hydrogel modulus, stem cells undergo neurogenesis (low modulus), myogenesis (intermediate modulus), or osteogenesis (high modulus).[13,14] The modulus of the hydrogel can be controlled by varying the cross-linking density of the hydrogel, adding inorganic silica into the 3D matrix, or varying the initial concentration of the hydrogel.[13-16] A controllable modulus during the cell culture also was realized through photodegradation of the hydrogel during the cell culture.[17]

Here, we report that the modulus of a hydrogel can be controlled by varying the temperature by using temperature-sensitive Pluronic® F127. F127 itself could not be used as a 3D cell culture matrix due to its short gel duration of <1 day, poor tissue adhesion, and its cellular toxicity at high concentrations.[18,19] In this study, the hydrogel was chemically crosslinked by radical polymerization of the temperature-sensitive polymer,[20] and RGD was introduced to give bioactive properties to the hydrogel. First, we confirmed the temperature-sensitive modulus control of the hydrogel, and optimized the modulus of hydrogel for chondrocyte culture to 1,000-30,000 Pa at the cell-culture temperature of 37 ºC. Second, the proliferation and viability of chondrocytes during the 3-dimensional cell culture were compared by varying the RGD content in the hydrogel to confirm the significance of cell-material interactions.

*Corresponding Authors. E-mails: dkh@kist.re.kr or bjeong@ewha.ac.kr

Experimental

Materials. Pluronic® F127 ((ethylene glycol)$_{99}$-(propylene glycol)$_{65}$-(ethylene glycol)$_{99}$; M_w =12,500 Da), stannous octoate, *N*-hydroxysuccinimide (NHS), dicyclohexyl carbodiimide (DCC), 4-(dimethyl amino)-pyridine (DMAP), triethyl amine, ammonium persulfate (APS), *N*,*N*,*N*',*N*'-tetramethyl ethylene diamine (TEMED), anhydrous toluene, anhydrous tetrahydrofuran (THF), dimethylformamide, and ethyl ether were used as received from Sigma-Aldrich. 4-Methacryl oxyethyl trimellitic anhydride (4-META; SMC Co., Korea) was used as received. RGD was purchased from Anigen Inc., Korea. Fetal bovine serum (FBS), Dulbeco's Modified Eagle's Medium (DMEM), penicillin/streptomycin, trypsin, and phosphate buffered saline were purchased from GIBCO, Inc. USA. Papain was used as received from Sigma. The Live/Dead kit and the Picogreen assay kit were used as received from Invitrogen, USA.

Synthesis. The synthetic procedure for the FM-RGD hydrogel is presented in Figure 1.[21,22] Pluronic® F127 (9.0 g, 0.72 mmol) was dissolved in anhydrous toluene (100 mL), and the solvent was distilled-off to a final volume of 30 mL. To the solution were added 4-META (0.52 g, 1.71 mmol), DMAP (0.087 g, 0.71 mmol), and triethylamine (87 μL, 0.72 mmol). After 15 h of stirring at room temperature, the product (FM) was isolated by precipitation in diethyl ether and residual solvent was removed under vacuum. Then, the product was purified by dialysis using membrane (cut-off molecular weight: 8,000 Da), followed by freeze-dried under vacuum. The final yield was ~80%.

Oligopeptide-grafted FM (FM-OligoP) copolymer was synthesized using two steps: (1) NHS-activation of carboxyl groups of FM and (2) addition of oligopeptides. FM (3.0 g, 0.23 mmol) and NHS (0.80 g, 6.96 mmol) were dissolved in anhydrous THF (20 mL). To the solution were added DCC (1.42 g, 6.88 mmol) and DMAP (5.7 mg, 0.046 mmol). The mixture was stirred at room temperature for 15 h. By-products of dicyclohexylurea were removed by filtration and the remaining solution was poured into diethyl ether to precipitate the product. The polymer was redissolved in THF, and then precipitated by slow addition of diethyl ether. The product was filtered, dried under vacuum, and the final yield was ~70%. The NHS-activated FM (1.5 g, 0.11 mmol) and RGD (273 mg, 0.67 mmol) were dissolved in *N*,*N*-dimethylformamide (10 mL) and stirred at room temperature for 24 h. The final product (FM-RGD) was precipitated by adding chilled methanol (100 mL). Then, the product was purified by dialysis using a membrane (cut-off molecular weight: 8,000 Da), and freeze-dried under vacuum. The final yield was ~60%.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra in $CDCl_3$ (500 MHz NMR spectrometer; Varian) were used to determine the composition of the polymer.

FTIR Spectroscopy. The FTIR measurements were performed using a JASCO615 FTIR spectrometer in the frequency range 400-4000 cm^{-1} with a resolution of 4 cm^{-1}.

Dynamic Light Scattering. The apparent sizes of F127, FM, and FM-RGD in water (0.05 wt%) were studied by a dynamic light scattering instrument (Malvern Nano ZS) as a function of temperature. A He-Ne laser operated at 633 nm was used as a light source. The results of dynamic light scattering were analyzed by the Automeasure Software. The instrument was calibrated by an aqueous dispersion of polystyrene particles with a size of 60 nm.

Dynamic Mechanical Analysis. Changes in modulus of the F127 aqueous solution (7.5 wt%), and hydrogels of FM and FM-RGD prepared from their aqueous solution (7.5 wt%) were investigated by dynamic rheometry (Thermo Haake, Rheometer RS 1). The sample was placed between parallel plates of 25 mm diameter and a gap of 0.5 mm. The data were collected under a controlled stress (4.0 dyne/cm^2) and a frequency of 1.0 rad/s. The heating rate was 0.5 °C/min.

Human Chondrocyte 3D Culture. The human chondrocytes were cultured as monolayers in DMEM containing 10.0% FBS and 1.0% penicillin/streptomycin under 5.0% CO_2 atmosphere at 37 °C, and then harvested by trypsinization. The harvested cells (5×10^5 cells) were suspended in polymer aqueous solutions of FM/FM-RGD mixtures (0.50 mL DMEM; 7.5 wt%) with 100/0, 90/10, and 50/50 by mole and crosslinked by the redox system using APS/TEMED (4.0 mM of each) in 24-well culture plates at 37 °C for 10 min. DMEM (0.5 mL) containing 10.0% fetal bovine serum and 1.0% penicillin/streptomycin was added to the cell-encapsulated hydrogel under 5.0% CO_2 atmosphere at 37 °C and then the medium was replaced every 3 days.

Cell Proliferation and Viability. The proliferation of human chondrocytes in hydrogels was assessed by biochemical assay. After samples were homogenized in a papain solution, each sample was digested for 15 h at 60 °C; then, DNA contents were measured using a Picogreen assay in 96 well plates. The Picogreen solution (1×, 0.1 mL) was added to each well and incubated for 2 to 5 min at room temperature while protecting the sample from the light. The fluorescence of the samples was measured using a fluorescence microplate reader using the wavelengths of 480 nm (excitation) and 520 nm (emission).

Viability of chondrocytes in hydrogels was evaluated by the Live/Dead kit after 1 day, 7 days, and 14 days of incubation. Chondrocyte encapsulating hydrogels were incubated at room temperature for 30 min in a solution of 4.0 μM ethidium homodimer-1 (EthD-1) and 2.0 μM calcein AM in phosphate buffered saline. Viable cells were stained with calcein AM (green), while dead cells were stained with EthD-1 (red). All images were captured using a confocal laser scanning microscope (FV1000/IX81, Olympus, Japan).

Results and Discussion

A synthetic scheme for the polymer is presented in Figure 1. F127 with hydroxyl end groups was reacted with the anhydride functional groups of 4-META to give FM which has polymerizable methacrylate and carboxylic acid functional groups. Carboxylic acid groups of FM were activated with NHS, followed by reaction with the amino group of GRGD to give FM-RGD. FM-RGD copolymerized with FM to control the RGD content of the final hydrogel. Methyl protons of F127 (1.0-1.3 ppm) and vinyl protons of 4-META (5.5-6.5 ppm) in the ^{1}H NMR spectra suggest the progress of the reaction (Figure 2(a)).[21] In addition, carbonyl bands of ester (1700-1760 cm^{-1}) and amide (1600-1700 cm^{-1}) functional groups indicate the progress of the reaction from F127 to FM and to FM-RGD (Figure 2(b)). Elemental analysis of the FM-RGD was conducted to obtain quantitative structural information. The ratios of observed/theoretical composition (by wt%) of the FM-RGD were C (55.8/56.1), H (9.2/9.1), N (1.3/1.4), and O (33.7/33.5), indicating 92.4% conversion to FM-RGD.

To confirm the temperature-sensitivities of the F127, FM, and FM-RGD, dynamic light scattering of the polymer aqueous solutions was investigated as a function of temperature. Based on hydrophobic dye (1,6-diphenyl-1,3,5-hexatriene) solubilization method, the critical micelle concentration of FM-RGD is about 0.1 wt% (Supporting Information, Figure S1). Therefore, the size and size distribution of the unimers and micelles were studied by dynamic light scattering at 0.05 wt%, where the unimer-to-micelle transition could be observed. The peak-average size (d_p) of F127 unimers slightly decreased from 7.5 to 6.5 nm as the temperature increased from 5 to 30 °C. On the other hand, as the temperature increased from 30 to 35 °C, significant changes in d_p of F127 from 6.5 to 24.4 nm and a significant broadening of the size distribution were observed, which can be assigned to a unimers-to-micelle transition of the F127.[23] Due to the difference in the end groups and thus the hydrophilicity of the polymers, F127, FM, and FM-RGD underwent unimer-to-micelle transitions at 30→35 °C, 35→40 °C, and 30→35 °C, respectively (Figure 3). As the hydroxyl end groups of F127 were modified into methacrylate end groups with ionizable carboxylates of FM, the polymer became more hydrophilic and the transition temperature increased from 30 to 35 °C. By conjugating the zwitterionic RGD to FM, the aggregation tendency of FM-RGD increased and lowered the transition temperature from 35 °C (FM) to 30 °C (FM-RGD).[22]

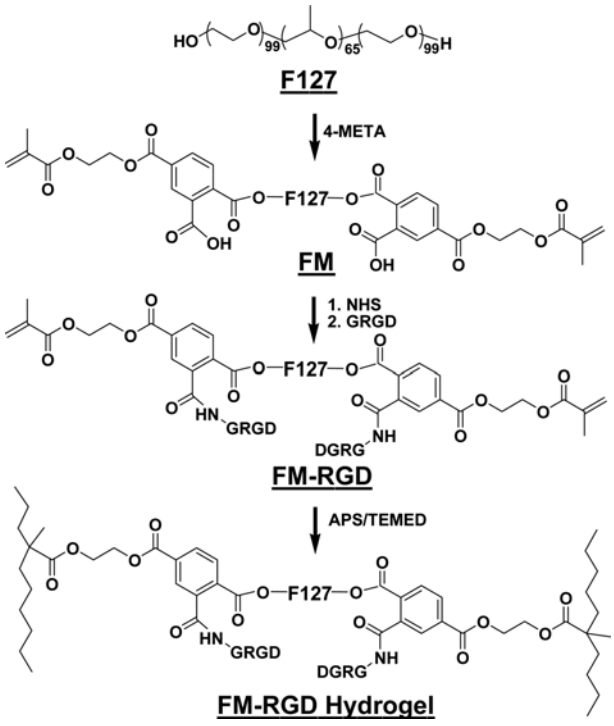

Figure 1. Synthetic scheme of the FM-RGD hydrogel.

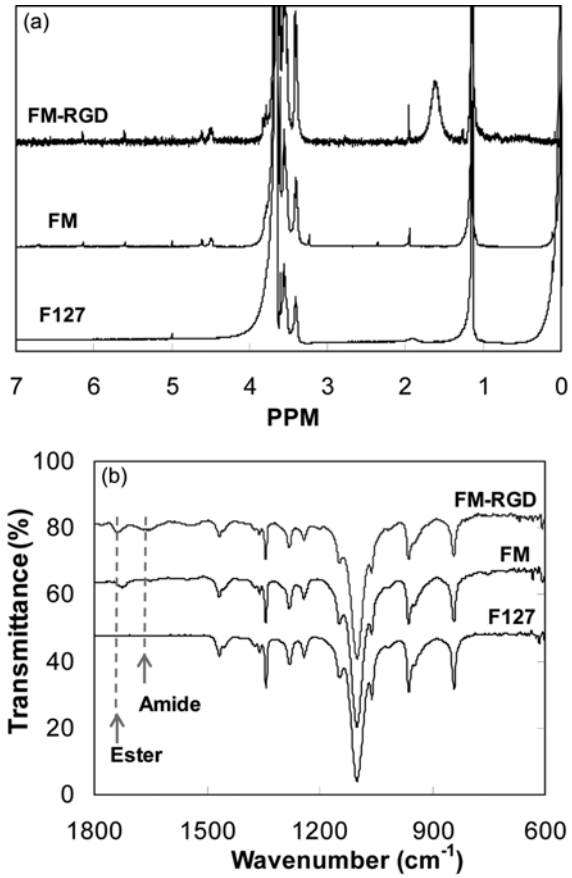

Figure 2. ^{1}H NMR ($CDCl_3$) (a) and FTIR (b) spectra of F127, FM, and FM-RGD.

Polymerization of the FM-RGD was carried at a 4.0 mM concentration of APS and TEMED to lower the cell damage during polymerization. A preliminary study showed that the cells were healthy at this concentration of the initiator and

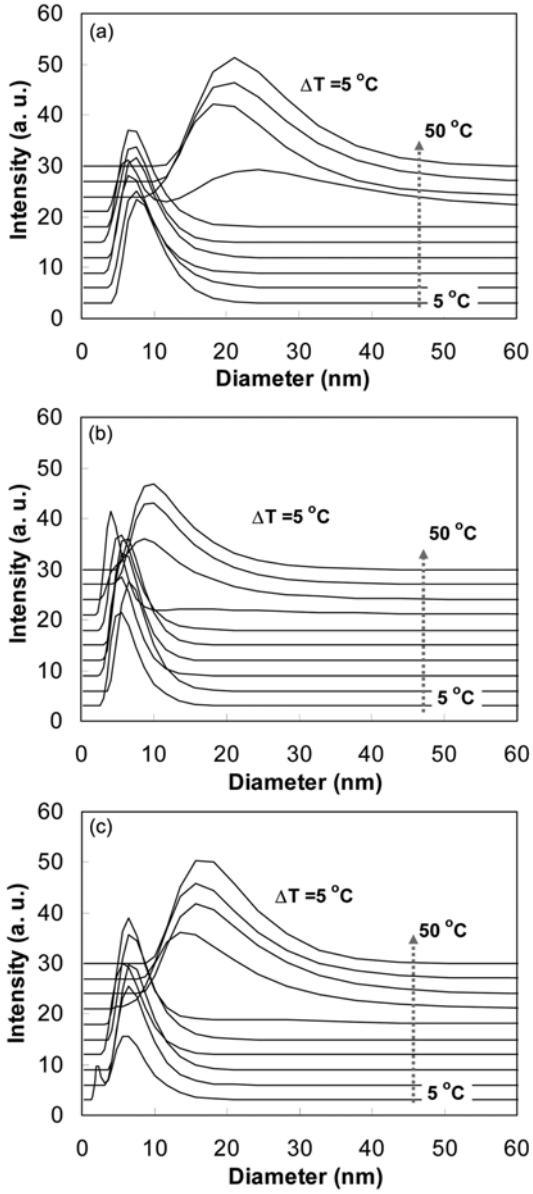

Figure 3. Dynamic light scattering study for size and size distribution of F127 (a), FM (b), and FM-RGD (c) aqueous solutions (0.05 wt%) as a function of temperature.

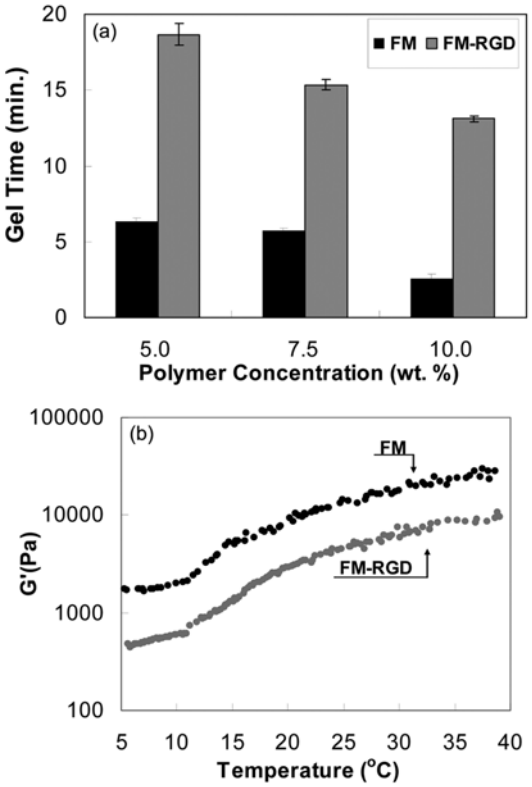

Figure 4. (a) Gelation kinetics of FM and FM-RGD aqueous solutions as a function of polymer concentration. *n*=3. The concentrations of both APS and TEMED were fixed at 4.0 mM. The reaction temperature was fixed at 15 °C under air. Gel time was determined by the test-tube tilting method, where sol (flow)-gel (no flow) was defined. (b) Storage modulus (*G*') of chemically crosslinked thermosensitive hydrogels (FM and FM-RGD) prepared from aqueous polymer solutions (7.5 wt%) as a function of temperature. The modulus of F127 aqueous solution (7.5 wt%) was less than 0.01 Pa (Supporting Information, Figure S2).

was verified in the Live/Dead assay. FM was copolymerized with FM-RGD to control the density of RGD in the hydrogel. The gel times of FM and FM-RGD, measured by flow (sol)-no flow (gel) criterion when the vial was tilted, were 5-6 and 14-18 min, respectively. The gel times for both polymers decreased as the initial concentration of the polymer increased from 5.0 to 10.0 wt% (Figure 4(a)). The modulus of the hydrogel prepared from the initial polymer concentration of 7.5 wt% was monitored as a function of temperature. As the temperature increased from 15 °C to the cell culture temperature of 37 °C, the modulus of the hydrogel increased from 5,400 to 28,400 Pa for FM and from 1,300 to 8,900 Pa for FM-RGD, respectively (Figure 4(b)). Because the polymer concentration of 7.5 wt% was lower than the critical gel concentration of F127, a significant change in modulus (< 0.01 Pa) was not observed for the F127 aqueous solution over the current experimental temperature range (Supporting Information, Figure S2).[24]

DNA content in the encapsulated cells was compared as a function of RGD content. As RGD content increased from 0% (FM) to 10% and to 50% by mole, the DNA content per well significantly (p<0.01) increased from 480 to 790 ng, and to1100 ng after 14 days of incubation (Figure 5(a)). The proliferation of the chondrocytes in the hydrogel assayed by the Live (green)/Dead (red) kit pronouncedly increased as the RGD content increased, indicating the significance of RGD in the 3D matrix of the hydrogel (Figure 5(b)). Also, the chondrocytes maintained their original spherical phenotypes in the 3D culture system, which was in contrast with the change of the chondrocytes morphology into a fibro-

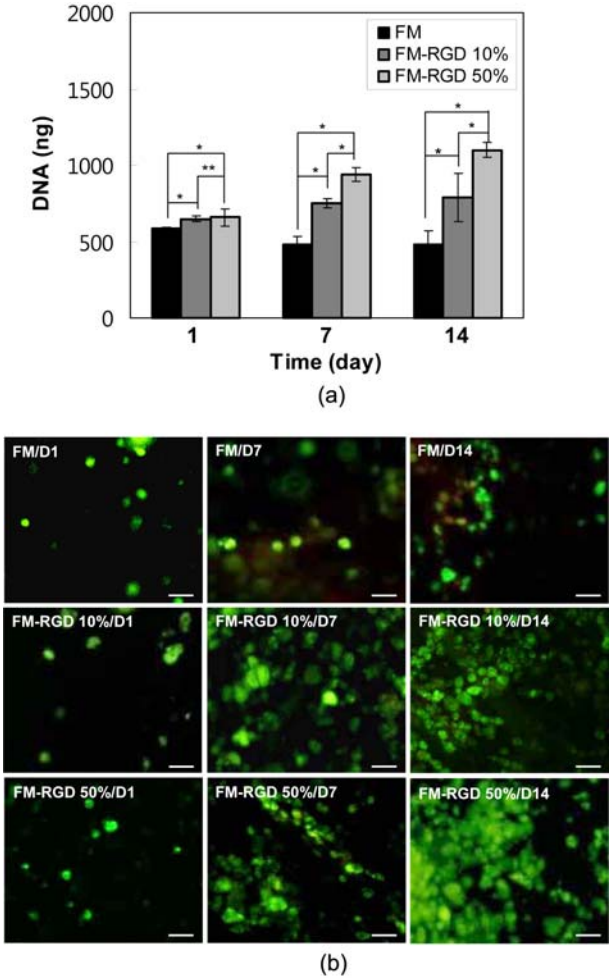

Figure 5. (a) Comparison of DNA contents in the encapsulated cells as a function of RGD content of the hydrogel. n=3. The fraction (%) in the legend is the mole percent of FM-RGD used in copolymerization of FM and FM-RGD to prepare the hydrogel. The amount of the DNA per well was calculated by the Picogreen assay. The * and ** indicate $p< 0.01$ and $p>0.01$ by the Student t-test. (b) Confocal microscopy images of hydrogel encapsulating chondrocytes analyzed using the Live/Dead kit. D1, D7, and D14 indicate the images of cells at 1, 7, and 14 days after the incubation of the cells in the hydrogel, respectively. Green and red images indicate live and dead cells, respectively. The scale bar is 20 μm.

blastic shape in 2-dimensional culture system or in the Matrigel™.[25,26]

Conclusions

The control of gel modulus in the cell culture matrix has been an important issue. By using the temperature-sensitive property of the F127, the hydrogel was modulated from a flexible soft gel (15 ºC, G~1,300 Pa) to a rigid matrix (37 ºC, G~8,900 Pa) which provides a cyto-compatible microenvironment for chondrocytes.[16,27] In addition, the incorporation of RGD provides the hydrogel favorable interactions with chondrocytes. Therefore, the cells maintained their original spherical phenotypes in the current hydrogel and showed significant DNA production as the RGD content increased.

This paper emphasizes that physicochemical control of hydrogel is very important in designing an artificial ECM, and suggests that a biomimetic ECM with a variable modulus can be a promising platform for the 3D cell culture.

Acknowledgements. This study was supported by the Pioneer Research Center Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2011-0001696), a grant from the Fundamental R&D Program for Core Technology of Materials funded by the Ministry of Knowledge Economy, Republic of Korea (K0006028), KIST grant (2E22360), and National Research Foundation of Korea (Grant number: 2011-0000376 & 2011-0001340).

Supporting Information: Information is available regarding the UV-vis spectra of FM-RGD aqueous solution containing 1,6-diphenyl-1,3,5-hexatriene and storage modulus (G') of chemically crosslinked thermosensitive hydrogels (FM and FM-RGD). The materials are available *via* the Internet at http://springer.com/13233.

References

(1) B. V. Slaughter, S. S. Khurshid, O. Z. Fisher, A. Khademhosseini, and N. A. Peppas, *Adv. Mater.*, **21**, 3307 (2009).
(2) A. Atala, *Curr. Opin. Biotechnol.*, **20**, 575 (2009).
(3) T. Dvir, B. P. Timko, D. S. Kohane, and R. Langer, *Nat. Nanotechnol.*, **6**, 13 (2011).
(4) R. C. Dutta and A. K. Dutta, *Biotechnol. Adv.,* **27**, 334 (2009).
(5) M. W. Tibbitt and K. S. Anseth, *Biotechnol. Bioeng.*, **103**, 655 (2009).
(6) J. Zhu, *Biomaterials*, **31**, 4639 (2010).
(7) R. H. Schmedlen, K. S. Masters, and J. L. West, *Biomaterials*, **23**, 4325 (2002).
(8) K. Y. Lee and D. J. Mooney, *Chem. Rev.,* **101**, 1869 (2001).
(9) U. Hersel, C. Dahmen, and H. Kessler, *Biomaterials*, **24**, 4385 (2003).
(10) (a) D. L. Hern and J. A. Hubbell, *J. Biomed. Mater. Res.,* **39**, 266 (1998). (b) S. P. Massia and J. A. Hubbell, *J. Cell Biol.*, **114**, 1089 (1991).
(11) H. Shin, S. Jo, and A. G. Mikos, *Biomaterials*, **24**, 4353 (2003).
(12) D. S. W. Benoit, M. P. Schwartz, A. R. Durney, and K. S. Anseth, *Nat. Mater.,* **10**, 816 (2008).
(13) A. J. Engler, S. Sen, H. L. Sweeney, and D. E. Discher, *Cell*, **126**, 677 (2006).
(14) M. M. Stevens and J. H. George, *Science*, **310**, 1135 (2005).
(15) Y. S. Pek, A. C. A. Wan, and J. Y. Yang, *Biomaterials*, **31**, 385 (2009).

(16) B. G. Choi, M. H. Park, S. H. Cho, M. K. Joo, H. J. Oh, E. H. Kim, K. Park, D. K. Han, and B. Jeong, *Soft Matter*, **7**, 456 (2011).
(17) A. M. Kloxin, A. M. Kasko, C. N. Salinas, and K. S. Anseth, *Science*, **324**, 59 (2009).
(18) H. Lee and T. G. Park, *J. Biomed. Mater. Res. A,* **88**, 797 (2009).
(19) M. K. Joo, M. H. Park, B. G. Choi, and B. Jeong, *J. Mater. Chem.*, **19**, 5891 (2009).
(20) S. A. Robb, B. H. Lee, R. McLemore, and B. L.Vernon, *Biomacromolecules*, **8**, 2294 (2007).
(21) B. G. Choi, S. H. Cho, H. Lee, M. H. Cha, K. Park, B. Jeong, and D. K. Han, *Macromolecules*, **44**, 2269 (2011).
(22) M. H. Cha, J. Choi, B. G. Choi, K. Park, I. H. Kim, B. Jeong, and D. K. Han, *J. Colloid Interface Sci.*, **360**, 78 (2011).
(23) K. Mortensen and S. Perdersen, *Macromolecules*, **26**, 805 (1993).
(24) M. Malmsten and B. Lindman, *Macromolecules*, **25**, 5446 (1992).
(25) B. G. Choi, M. H. Park, S. H. Cho, M. K. Joo, H. J. Oh, E. H. Kim, K. Park, D. K. Han, and B. Jeong, *Biomaterials*, **31**, 9266 (2010).
(26) K. Mark, V. Gauss, H. Mark, and P. Muller, *Nature*, **267**, 531 (1977).
(27) C. Chung, I. E. Erickson, R. L. Mauck, and J. A. Burdick, *Tissue Eng. Part A*, **14**, 1121 (2008).