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Development and Physicochemical Evaluation of Chondroitin Sulfate-Poly(ethylene oxide) Hydrogel

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Abstract: Novel chondroitin sulfate (CS) - poly(ethylene oxide) (PEO) hydrogel was synthesized and evaluated by a mechanism of self cross-linking of CS derivative with PEO with *hexa*-thiols (PEO-SH). A derivative of CS was synthesized by the sequential grafting of adipic acid dihydrazide (ADH) and acrylic acid: chemical grafting of ADH to the carboxylic acid in CS (CS-ADH) followed by grafting of the acrylic acid to the free amine groups in the CS-ADH (CS-ADH-Ac). The synthesis of CS-ADH-Ac molecules was confirmed by observing new acrylate peaks in CS-ADH-Ac by FTIR, ESCA, and NMR. The CS-PEO hydrogel was self cross-linked through a Michael type addition reaction between the acrylate end groups of CS-ADH-Ac and the thiol end groups of the PEO-SH. the gelation behavior of 10% CS-PEO was evaluated by rheological analyses from the changes in the solution properties, such as phase angles and visco-elasticities. Rheological analysis indicated that the gelation process was complete within 2 min after mixing two polymer solutions of CS-ADH-Ac and PEO-SH. The fabricated CS-PEO hydrogel was analyzed by measuring both its swelling under different water pHs and its mechanical strength against compression. The morphological shapes of both its surface and cross sections were also evaluated after the sequential processes of gel swelling to equilibrium followed by dehydration. Both the gelation time and swelling of the fabricated hydrogel were dependent on the pH of the polymer solutions and swelling medium, showing quicker gel formation and better swelling behaviors under basic conditions than under acidic conditions. The equilibrated gel showed different morphologies depending on its location, i.e. its cross sections demonstrated more homogeneous morphologies than the surfaces. While the dehydrated hydrogel demonstrated 8-10 μm pore sizes on its cross sections, the compression strength of the hydrogel ranged from 1.4 to 2.8 Pa depending on its gel concentration. Toluidine blue molecules as a model drug were released from the hydrogel over a period of more than 5 days. These hydrogel properties, such as formation of *in situ* gel, release behaviors of toluidine blue, and porous structures and mechanical properties of the fabricated gel, highlighted the potential of a hydrogel as a carrier for local drug delivery and a scaffold for tissue engineering.

Keywords: chondroitin sulfate, poly(ethylene oxide), self cross-linking, hydrogel.

Introduction

Chondroitin sulfate (CS) is an anionic linear polysaccharide with a molecular weight of 50-100 kDa, consisting of alternating disaccharide repeating units of 1-3 linkage of D-glucuronic acid and *N*-acetylgalactosamine. The galactosamine residues are sulfated either in position 4, 6 or 4 and 6. CS has been known to be a major component of the extracellular matrix in many connective tissues including cartilage, blood vessel, bone, skin, ligament, tendons and etc. The CS polysaccharide has shown interesting biological properties such as anti-inflammatory activity, reduction of pain and improvement of articular functions, reduction of swelling and effusion as well as prevention of narrowing of the joint space of the knee and fingers, leading to classification as a symptomatic slow acting drug in osteoarthritis.[1] It has been reported to down regulate proteolytic enzymes, proinflammatory enzymes and proinflammatory cytokines. As another example of its biological effects, high contents of CS have played a major role in creating a considerable osmotic swelling pressure in articular cartilage, leading to expansion of the matrix and then placement of collagen network under

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tension.[2] Based on these and other biological activities and biological sources, it has been used in human in the treatments of osteoarthritis, psoriasis, atherosclerosis, and central nervous systems.

CS has been applied in numerous forms such as either a bioactive material itself,[2] a natural polymer scaffold for tissue engineering[3] or a carrier polymer network for cell therapy[4] and drug delivery.[5] In recent pharmaceutical study, CS has been employed directly as an anti-cancer or anti-osteoarthritis agent,[1,2] or for efficient drug delivery system.[6,7] As another form of CS fabrications, a porous three-dimensional scaffold has been fabricated for its application in cartilage tissue regeneration by combining CS with either biodegradable polylacitide or type II collagen, which is abundant in the extracellular matrix of cartilage.[8,9] A terpolymer composed of collagen/hyaluronan/chondroitin-6-sulfate has been also synthesized as a scaffold for tissue engineering of nucleus pulposus and dermis.[10] CS-based hydrogel has been fabricated for its applications in either cell therapy or drug delivery and employed as a bioactive molecule to measure its diffusion properties in chitosan hydrogel or its biological properties to induce chondrogenic differentiation of mesenchymal stem cells.[11-13]

Recently many fabrication methods of *in situ* CS hydrogel have been developed for its applications in drug delivery system, cell therapy and tissue engineering.[14,15] From the perspective of its clinical applications, this *in situ* hydrogel has demonstrated many advantages such as injection of a large amount of hydrogel into the defect sites via minimally invasive surgery and at the same time gel formation from a liquid precursor at the implantation site, thus matching conformation of the final hydrogel implant to the irregular and complex defect shapes. Another example of advantages of *in situ* hydrogel was easy and safe encapsulation of bioactive substances such as cells in the matrix by simply mixing them in precursor liquids and then transforming the liquid solution into a cross-linked hydrogel network, thus confining the cells in its cross-linked network and then localizing them into the defect sites.[1] Furthermore, this hydrogel was reported to have many functional advantages such as biological wastes release from the hydrogel and delivery of nutrients and other important bioactive molecules to the cells in the hydrogel through its porous network, which are important properties in its applications in tissue engineering and cell therapy.[16,17]

We here developed and evaluated a self cross-linked CS-PEO hydrogel via self cross-linking reaction for its possible applications in drug or cell delivery and tissue engineering. The mechanism of formation of the self cross-linking hydrogel has been in detail reported in our previous reports,[18,19] even though different polymer has been employed. After synthesis of CS-ADH-Ac and fabrication of hydrogel, we evaluated its physico-chemical properties such as morphologies, swelling behaviors, and mechanical and rheological properties of the CS-PEO hydrogel. We expected that the CS-PEO hydrogel could be employed as a carrier in drug delivery system and as a scaffold for tissue engineering.

Experimental

Materials. Chondroitin sulfate (CS: MW=50 kDa) from shark cartilage, adipic dihydrazide (ADH), acrylic acid, hydrochloric acid and triethanolamine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Poly(ethylene oxide) (PEO) with *hexa*-thiols (MW=10 kDa) (PEO-SH) was purchased from Sunbio Inc. (Seoul, Korea). *N*-(3-diethylpropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) was obtained from Fluka Chemie GmbH (Buchs, Switzerland). All other chemicals were used as received.

Synthesis of CS-ADH-Ac. Sequential grafting of ADH and acrylic acid to CS was performed as follows. After dissolving 0.2 g CS in 40 mL distilled water, ADH (0.15 g) and EDC (0.16 mL) were added to the CS solution at defined ratios. The EDC-mediated coupling reaction between the carboxyl group of CS and the primary amines of ADH was proceed by stirring the mixture solution with a magnetic bar at room temperature for 4 h, thus obtaining CS-ADH solution. After addition of acrylic acid (0.12 mL) and EDC (0.32 mL) to the CS-ADH solution, the reaction continued with stirring for another 3 h. Solid, acrylated CS powders (CS-ADH-Ac) was obtained after lyophilizing for 2 days.

Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) Spectroscopy. To confirm sequential grafting of ADH and acrylic acid to CS, ATR-FTIR spectra of CS, CS-ADH and CS-ADH-Ac samples were recorded ranging from 650 to 4000 cm^{-1} with a spectrometer (Travel IR; Smiths, USA). A diamond crystal refractive index of 2.4 was employed at 45 degrees of incidence angle. The ATR depth of penetration was about 2 μm.

^{1}H Nuclear Magnetic Resonance (NMR) and Two-Dimensional (2-D) Homo-Nuclear Correlation Resonance. Spectra of both ^{1}H NMR and 2-D NMR correlation spectroscopy (COSY) were obtained by employing the 0.01 g CS-ADH-Ac samples. The CS-ADH-Ac sample in 1 mL deuterium oxide (D_2O) with UI 500 MHz FT-NMR Spectrometer (Varian, Japan) was employed to observe an extent of acrylation of the CS polymer. Chemical shift (δ) was measured in ppm by employing sodium-2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard for ^{1}H NMR spectra. COSY NMR was used for detailed detection of acrylates grafted to the CS derivatives. At the first step, the acrylate structure of the CS-ADH-Ac was identified by sequential assignment of the COSY spectra. Since the protons in acrylate group of the expected CS-ADH-Ac sample contains two protons, ready identification was performed in the spectra.

Electron Spectroscopy for Chemical Analysis (ESCA). Changes in the chemical composition of the CS derivatives were observed with an ESCA spectrometer (PHI 5800 ESCA

SYSTEM; Perkin-Elmer, USA) after turning the powders into a disk film. Disk samples (diameter = 1 cm, thickness = 40 μm) were prepared by pressurizing the obtained powder into a cylindrical sample holder upto 70 MPa. ESCA spectra of the survey scans and high resolutions of C1s and N1s were analyzed after irradiating the 1,400 eV ion beams from an Al K-α (350 W, 15 kV) source on their surfaces under 10^{-9} torr.

Fabrication of CS-PEO Hydrogel. The synthesized CS-ADH-Ac (0.01 g) powders were dissolved in triethanol amine (0.3 M, pH 8), leading to formation of 10% (w/v) CS-ADH-Ac solution. After mixing the PEO-*hexa* thiols solution in TEA-buffered solution with the above CS-ADH-Ac solution (1:1), CS-PEO hydrogel was spontaneously synthesized via Michael type addition reaction without any further treatment. Their hydrogelation times were determined by employing the methods of both vial tilting[18,19] and rheological measurement as below.

Evaluation of Formation of CS-PEO Hydrogel. Behaviors of the formations of CS-PEO hydrogel were analyzed by using the methods of both vial tilting and rheological measurement. Measurement of gelation times by the vial tilting method was processed by tilting the mixture solution in a vial over time. Gelation time was regarded at the point, when there was no flow for more than 1 min after inverting 100 μL mixture in the 1 mL conical vial. CS-PEO gel formation was further analyzed in detail by measuring rheological changes of the mixture solution with the Rotational Rheometer Gemini (Bohlin Instruments Ltd., Germany) after mixing the solutions of both CS-ADH-Ac and PEO-SH, 150 mL each. Its rheological behaviors such as phase angles and modulus of both viscosity and elasticity were observed on a sandblast parallel plate (d=15 mm). Its rheological behaviors were measured under the conditions of both frequency sweep at 0.1~20 rad/s and percentage strain of 0.1% at 25 °C. While frequency sweep was analyzed by changing frequency rates ranging from 0.01 to 100 rad/sec for approximately 30 min, percentage strains were measured by changing hydrogel strain ranging from 0.01 to 40% for 4 min at a frequency rate of 1 rad/sec.

Swelling Behaviors of CS-PEO Hydrogel. Swelling of either 5 or 10% CS-PEO hydrogel was measured in water at the conditions of different pHs, 2.0, 4.0, 6.0, 7.4 or 9.0, at 37 °C for upto 24 h. After measuring the weight of the prefabricated hydrogel with a microbalance, swelling of CS-PEO hydrogel was determined by comparing the weights of the hydrogel sample before and after immersing it in water. Adherent water was removed by blotting the wet CS-PEO hydrogels with a piece of kimwipe paper before weighing them on an electronic balance. The percentage of swelling was calculated by employing a formula (1).

$$\text{Swelling percent (\%)} = [(W_s - W_i) / W_i] \times 100(\%) \quad (1)$$

W_s=wet weight of the CS-PEO hydrogel at time t, W_i=weight of the CS-PEO hydrogel at initial hydrogelation point.

Morphologies of the Dehydrated CS-PEO Hydrogel by Scanning Electron Microscopy (SEM). Observation of the morphologies of CS-PEO hydrogel was performed by SEM (JEOL Ltd, Japan) after processes of dehydration and coating its surface with gold as follows. Dehydration of the hydrogel was processed by freezing the swollen hydrogel in liquid nitrogen and then freeze-drying at -55 °C overnight. The dry sample was mounted on an aluminum stub with a double-sided tape, and then gold-coated with a sputter coater for 1 min. The morphology of the dehydrated CS-PEO hydrogel was analyzed with SEM.

Release Experiment. Toluidine blue (Sigma-Aldrich Chemical Co.) was used as a model drug for observation of the behaviors of drug release from the CS-PEO hydrogel. After loading 50 μL (0.1% w/v) toluidine blue in 200 μL CS-PEO hydrogel, its release from either 5 or 10% CS-PEO hydrogel was measured at either 37 °C or room temperature for 10 days by collecting 100 μL from the reservoirs. The amount of toluidine blue was measured with the peak area of the toluidine blue around 610 nm by utilizing the ELISA microplate reader (TECAN, Switzerland). The amount of the toluidine blue released was calculated by employing follow eq. (2).

$$\text{Release percent (\%)} = W_t / W_o \times 100(\%) \quad (2)$$

W_t = the peak area of the toluidine blue at the wavelength of 610 nm released from the hydrogel at time t, W_o=total weight of the initially loaded toluidine blue in the CS-PEO hydrogel

Compression Strength of CS-PEO Hydrogel. Texture analyses were performed by employing MT-LQ material testers (Stable Micro Systems, UK) equipped with a 5 kg load cell for measurement of compression strength of the CS-PEO hydrogel. Three times per condition were performed for the tests of both 5 and 10% hydrogel at room temperature. The apparatus was equipped with a circular probe of 12.77 mm in diameter, which descended on the sur-

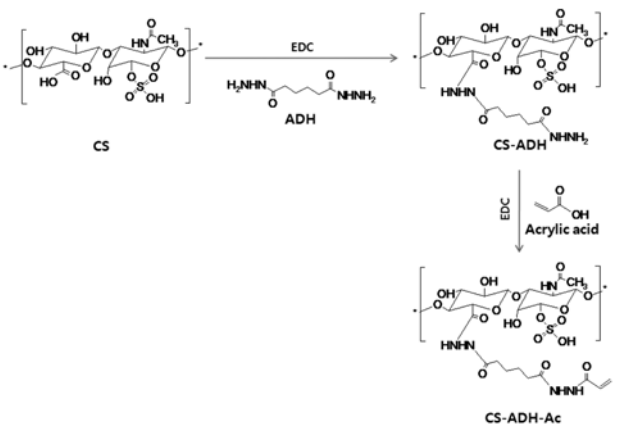

Figure 1. Schematics of CS-ADH-Ac synthesis by sequential grafting of ADH and then acrylic acid to CS.

face of the hydrogel sample moving at a rate of 0.2 mm/sec. After loading the hydrogel sample into a mold (d=1 cm, h=3 cm) to half of the mold height, a probe cell (d=0.8 cm) was compressed onto the sample until it was completely crushed.

Results and Discussion

Synthesis of CS. Synthesis of CS-ADH-Ac has been performed by sequential grafting of ADH and acrylic acid to CS via EDC chemistry. We chemically analyzed CS derivatives such as CS-ADH and CS-ADH-Ac to verify the existence of ADH and acrylate in the CS-ADH-Ac chemical network by FTIR, NMR, and ESCA (Figures 2-4). Grafting of both ADH and acrylate molecules to CS was observed by the appearances of new FTIR peaks compared to those of CS (Figure 2). In specific, the control CS sample demonstrated its characteristic IR peaks at the positions of 3320 cm^{-1} for both -OH stretch vibration and -NH symmetrical vibration; 2920 cm^{-1} and 2878 cm^{-1} for -CH stretch vibration; 1613 cm^{-1} for amide II carbonyl (C=O) stretch vibration; 1658 cm^{-1} for amide II (-NH) deformation and -CN stretch vibration; 1413 cm^{-1} for -OH and -CH deformation (ring); 1378 cm^{-1} for $-CH_3$ deformation (bend) vibration; 1210 cm^{-1} for sulfonic group (S=O) stretch vibration; 1122 cm^{-1} primary or secondary alcohol (saccharide); 1062 cm^{-1} for -C-O stretch vibration; 862 cm^{-1} for aliphatic aldehyde (saccharide) (Figure 2(A)).[20,21] The CS-ADH samples, an intermediate molecule in this study, demonstrated clearly a new secondary amine (-NHNH-) peak at the position of 1700 cm^{-1} (Figure 2(B)). The IR spectrum of the CS-ADH-Ac sample demonstrated two new peaks as the evidences of sequential grafting of both ADH and acrylic acid to CS in addition to its characteristic peaks observed in the above CS ones. In specific, the protons of the acrylate bond (H_2C=CH-) connected to the amide bond of the CS-ADH-Ac was observed at the positions of both 3200 and 814 cm^{-1} (Figure 2(C)).

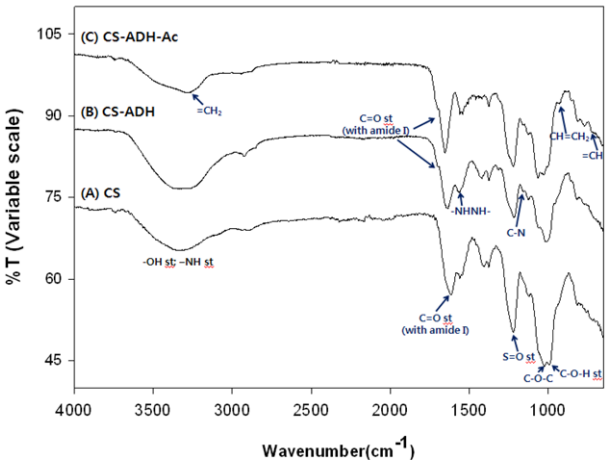

Figure 2. Analysis of CS derivatives by ATR-FTIR; CS (A), CS-ADH (B), and CS-ADH-Ac (C).

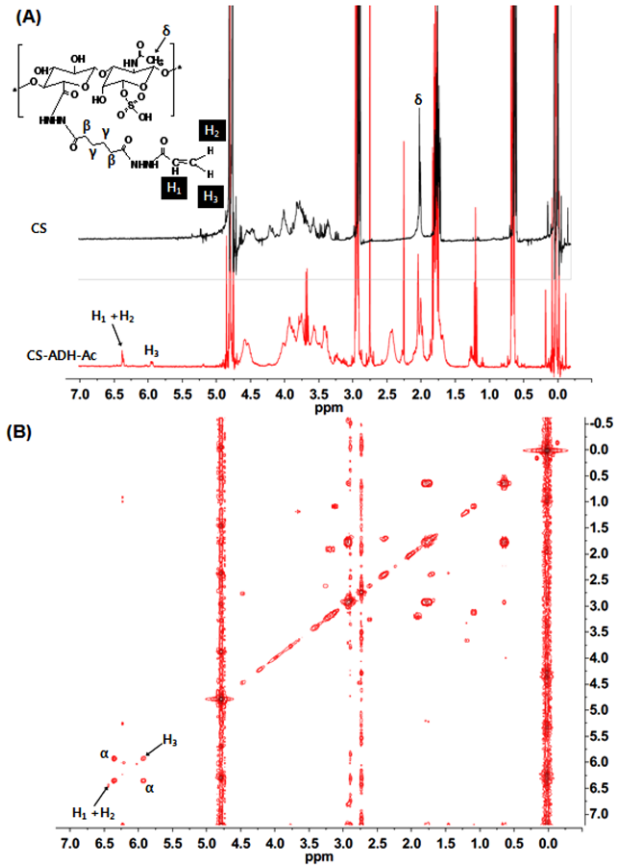

Figure 3. Analysis of CS derivates by ^{1}H NMR (A) and COSY spectrum (B).

Increase in the intensities of both the carbonyl absorption peak at 1650 cm^{-1} and the N-H stretching one at 1550 cm^{-1} of CS-ADH-Ac was observed compared to those of either CS or CS-ADH. Other chemical bonds appeared with little shifts from the peak positions of those shown in the control CS spectrum.

CS-ADH-Ac molecules were further analyzed with both ^{1}H NMR and COSY spectroscopy by comparing those of the native CS (Figure 3(A)). Sequential grafting of both ADH and acrylic acid to CS was clearly verified as judged by the new peaks at 6.4 (H_1 + H_2), 5.9 (H_3), 2.4 (β), and 1.2 (γ) ppm on the spectrum of the CS-ADH-Ac samples. The peaks at the positions of both 2.4 and 1.2 ppm were considered as the hydrogen atoms of ADH molecule attached to CS. While the peaks at the positions of 6.4 and 5.9 ppm were considered as the hydrogen atoms attached to the acrylate end group (H_2C=CH-). Further analysis of the chemical identification of the CS-ADH-Ac samples was performed with its COSY spectrum (Figure 3(B)). Chemical analysis of CS-ADH-Ac was made with the COSY spectrum to determine grafting and verification of acrylates. Starting from the hydrogen atom H_3, two lines parallel to its x and y axes were drawn. Another two lines from the sum of H_2, *cis*

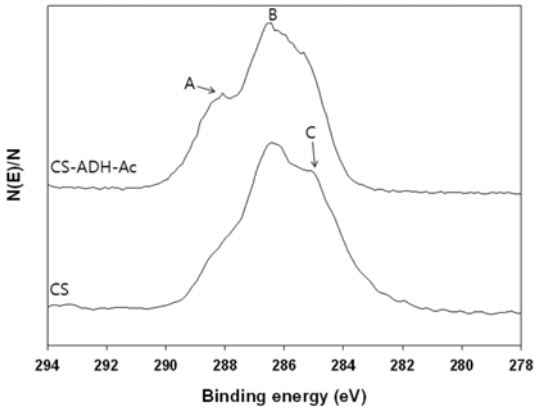

Figure 4. Chemical analyses of CS and CS-ADH-Ac by ESCA C1s high resolution, where (A) 288.1 eV; (B) 286.5 eV; (C) 285.0 eV.

hydrogen, and H_3, *trans* hydrogen, were drawn parallel to the x and y axes. Two new lines were intersected with previous parallel lines at two cross peaks and they were designated as 'α' in common (Figure 3(B)). If the cross peak 'α' is located at the position of some hydrogen peaks, they indicated that the peaks of H_1, designated as *geminal*, and H_2 diagonal to H_3 had coupled to the H_3 hydrogen atom.[22-24] Next we measured the ratio of peak areas of *geminal* hydrogen and sum of the peaks of *cis* and *trans* hydrogens to verify the existence of the acrylate end group. The symmetry of acrylate end group was destroyed by the attachment of a carbonyl group (C=O) to the acrylate double bond, and three different hydrogen atoms were observed such as H_1, H_2 and H_3. These three hydrogen atoms had their own substituent values (σ). In specific, while the H_1 hydrogen atom has the *geminal* substituent constant value of the carbonyl group, the H_2 and H_3 hydrogen atoms have those of *cis* and *trans*, respectively. Since the electron-withdrawing ability of carbonyl group decreased the electron density around the proton, less shielding electron density of the *geminal* hydrogen occurred. Though the substituent constant value of *geminal* was normally larger than that of either *cis* or *trans,* that of *geminal* was similar to that of *cis* due to the effect of carbonyl group. By overlapping the H_2 peak on that of H_1 in the CS-ADH-Ac spectrum, we obtained a value of 1.7:1 by comparing the sum of the peak areas of $H_{geminal}$ and H_{cis} to that of H_{trans}, and it was considered as 2:1. These chemical shifts led to resonance of both H_1 and H_2 protons at the lower field of 6.3 ppm, and that of *trans*-H_3 did at 5.9 ppm. Degree of acrylate grafting to CS-ADH was estimated as 28% by calculating the ratio of the peak area of the acrylate at both 6.3 and 5.9 ppm to that at 2.0 ppm. The peak at 2.0 ppm (δ) was designated as the hydrogen atoms of the methyl groups connected to the carbonyl group of the *N*-acetylgalactosmine sugar molecule.

Chemical analysis of further grafting of acrylic acid to CS-ADH was performed by employing both the spectra of their survey scan and high resolutions of ESCA. From the survey scans, significant increase in nitrogen atoms of the CS-ADH-Ac has been noticed from 5.88 to 11.19%. The control CS sample demonstrated atomic compositions of 57.43% carbon, 5.88% nitrogen, 35.08% oxygen, and 1.60% sulfur atoms, leading to 9.8% ratio of carbon to nitrogen atom percent (Table I). The CS-ADH-Ac samples, however, showed atomic compositions of 55.46% carbon, 11.19% nitrogen, 30.58% oxygen, and 1.73% sulfur, leading to reduction of atomic percentage ratio of carbon to nitrogen to 4.9%. This chemical composition change was also observed in the spectra of their corresponding C1s high resolution spectrum (Figure 4). While the CS sample demonstrated clear hydrocarbon peak at 285 eV, the CS-ADH-Ac did a relatively smooth peak at 285 eV and a new sharp peak at around 288.45 eV, indicating carbonyl peak from the ADH and Ac grafted to CS.

Table I. Chemical Composition of Both CS and CS-ADH-Ac Polymers Measured by ESCA

Elements	Atomic Composition (%)		Atomic Percent Ratio (carbon/nitrogen)	
	CS	CS-ADH-Ac	CS	CS-ADH-Ac
Carbon	57.43	55.46	9.78	4.95
Nitrogen	5.88	11.19		
Oxygen	35.08	30.58		
Sulfur	1.60	1.73		

Fabrication of CS-PEO Hydrogel. Analysis of the CS-PEO hydrogel formation was performed by utilizing a rheometer for real time measurement of changes in both viscosity and elasticity of the polymer solutions. CS-PEO hydrogel formation was indicated by instantaneous changes of both viscosity and elasticity modulus as well as phase angles of the CS-ADH-Ac and PEO-SH solutions over time. The results indicated that hydrogelation began right after loading the mixture solutions in the rheometer (Figure 5(A)), which was observed by sharp decrease in viscosity modulus and increase in elastic modulus of the solutions, showing cross-linking of CS-ADH-Ac and PEO-SH within 3 sec (Figure 5(B)). Hydrogelation was also completed approximately within 2 min judged by the values of phase angles [tan(θ)], which was calculated by the value of viscous modulus (G') / elastic modulus (G'')]. The point where its elastic modulus reached plateau was considered as the time of gelation completion. Completion of hydrogelation was also supported by the data of the elastic modulus values over the values of frequency and percentage strain (Figure 5(C)), where the elastic modulus was measured as nearly constant over both frequency rates and percentage strains employed. Furthermore, these gelation behaviors were supported by the results of the tilting method, i.e. no fluid flow was observed in a couple of minutes after the precursor polymer solutions in a vial were inverted.

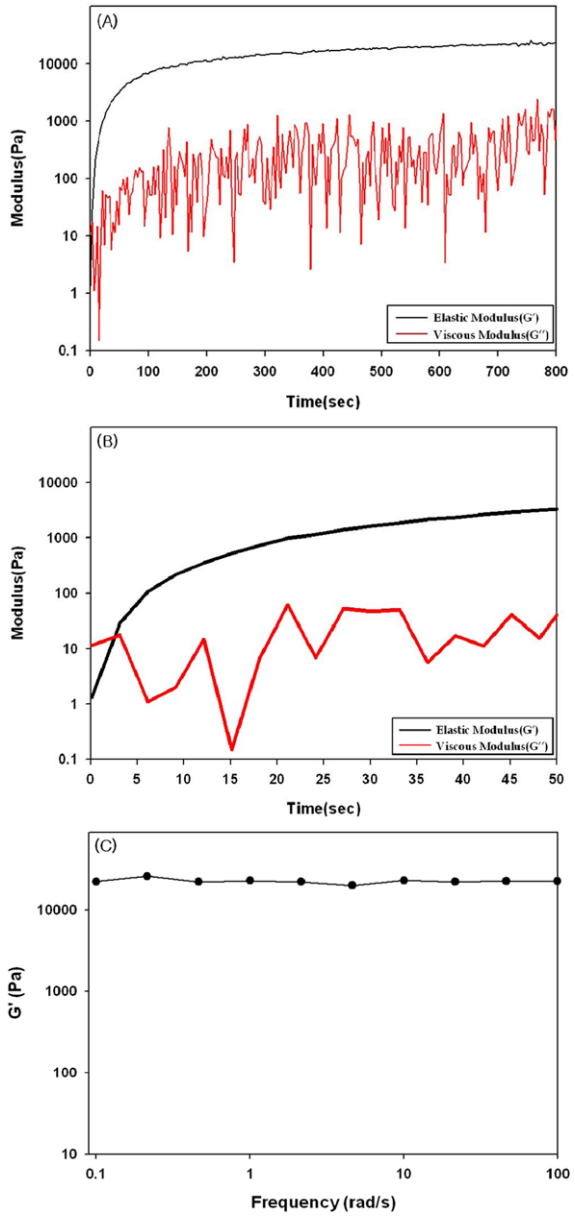

Figure 5. Rheological analysis of CS-PEO gel formation; viscoelasticity (A) and its initial behaviors (B), and frequency sweep (C).

Effects of pHs on CS-PEO Hydrogel Formation. Dependence of hydrogel formation on the pHs of the mixture solutions of CS-ADH-Ac and PEO-SH was also analyzed by the tilting method (Figure 6). CS-PEO hydrogel was formed nearly instantaneously in mild basic conditions, i.e. in pH 8.06 and 7.47 in this experiment. However, as the pH of the mixture solutions changed from neutral status to acidic ones, hydrogel formation was significantly delayed regardless their concentrations, i.e. either 5 or 10%. In specific, while hydrogels were formed in 1.5 and 2.8 min for both 10 and 5% solutions at pHs 9.3; 2.6 and 3.9 min at pHs 8.8; 7.3 and 32 min at pH 7.5; and 105 and 128 min at pH 6.7,

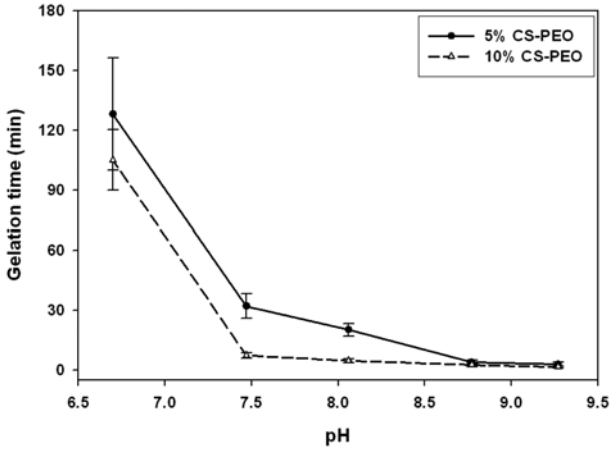

Figure 6. CS-PEO gelation behaviors dependent upon the polymer solutions with different pHs.

respectively. These results indicated that higher concentrations in basic conditions of the CS-ADH-Ac and PEO-SH solutions induced quicker hydrogel formation than the solutions with lower concentrations and acidic conditions. The effects of pHs of the polymer solutions on the gelation time were higher than those of the concentrations of the precursor polymer solutions in inducing quicker gel formation time. In specific, while the gap of the gelation times between the solution concentrations between 10 and 5% at pH 6.7 was 23 min, that at pH 9.3 was 1.3 min. This result indicated that while the acidity of the precursor polymer solutions retarded the hydrogel formation, the basic polymer solutions induced quicker gel formation. In the other aspect, while there was a big gap in hydrogel formation time between the different concentrations of the polymer solutions under acidic conditions, there was no difference in hydrogel formation time under basic conditions.

Swelling Behaviors of CS-PEO Hydrogel. Physical properties of the CS-PEO hydrogel were evaluated by measuring swelling behaviors of the formed hydrogels under different pHs and over time. While higher pHs of the swelling medium induced more gel swelling, higher concentrations of the gel did less swellings (Figure 7(A)). In acidic condition, i.e. pH 2, the CS-PEO hydrogels demonstrated approximately nearly the same swelling behaviors regardless their concentrations. In specific, 170% and 166% swellings for 5% and 10% hydrogels, respectively, were observed over their initial weights. However, remarkable differences in the swelling behaviors of these hydrogels were observed in basic mediums. In specific, while the 5% CS-PEO hydrogel swelled to 170% in water at pH 2, 224% in pH 4, 324% in pH 7.4, and 525% in pH 9, the 10% hydrogel did to 166% in pH 2, 218% in pH 4, 319% in pH 7.4, and 456% in pH 9. Even though there were no significant differences in the swelling of the hydrogels at the acidic conditions, their swelling in water at pH 9 demonstrated significant difference in swelling behaviors. Specifi-

cally, while the 5% hydrogel demonstrated 525% swelling, the 10% hydrogel did 456%, indicating that hydrogel fabricated with lower concentration imbibed higher water content than that with higher concentrated did. These results indicated that the differences in the degree of swellings between the 5% and 10% hydrogel increased from 2.4% to 13.1% when we changed the water environment from pH 2 to 9. This higher percentage of swelling under basic condition was attributed to the repulsive force of hydroxyl ions between the negatively charged carboxylic acid groups of the chondroitin sulfate network.[16]

Next, swelling behaviors of the CS-PEO hydrogels at different pHs of water were tested over time (Figure 7(B)). Swelling equilibrium of both 5 and 10% CS-PEO hydrogel was reached at around 1 to 5 h after loading the prefabricated ones in water, dependent upon the conditions of water. In specific, the 5 and 10% hydrogels reached to full swelling 6 h after soaking them in water at pH 2, demonstrating 119 and 111% swellings, respectively. In water at pH 7.4, equilibrium of the hydrogel was reached at 4 h after soaking and they swelled to approximately 310 and 305% for the 5 and 10% hydrogels, respectively, from their initial dry states. More significant changes in their equilibrium time and swelling occurred in water at pH 9 in this experiment, demonstrating that while equilibrium of the 10% hydrogel was reached at approximately at 6 h to the 545% swelling, the 5% hydrogel swelled to 443%, reaching to equilibrium at approximately 3 h after soaking in the medium.

Release Kinetics of Toluidine Blue from CS-PEO Hydrogel. Toluidine blue release from the CS-PEO hydrogels was evaluated by measuring the amount of toluidine blue molecules released as a model drug over time at different temperatures. Toluidine blue was sustain-released from the 10% hydrogel over time and reached a plateau status approximately at 125 and 175 h at 37 and 25 °C, where the hydrogel released 55% and 45% of the amount of the toluidine blue molecules initially loaded at 37 and 25 °C, respectively (Figure 8(A)). However, the 5% hydrogel demonstrated complete release at approximately earlier than 100 and 125 h at 37 and 25 °C, respectively (Figure 8(B)). Toluidine blue molecules seemed to be reached to a plateau state at the point, where 60% and 50% of the initially loaded amount of toluidine blue was released. The toluidine blues remained in the gel was expected to be released during degradation of the hydrogel probably due to its strong interaction with the hydrogel. These results indicated that lower concentrated hydrogel under higher temperature induced quicker release of toluidine blue.

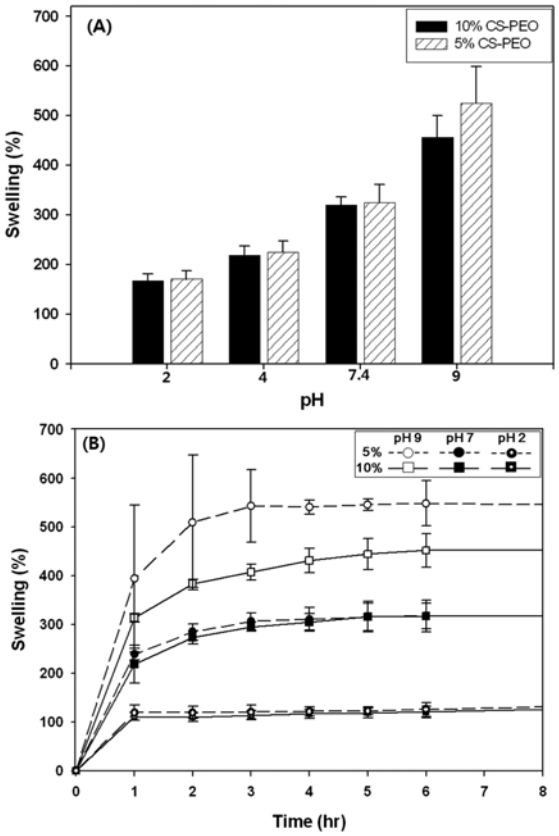

Figure 7. Swelling behaviors of CS-PEO hydrogel in water with different pHs (A) over time (B).

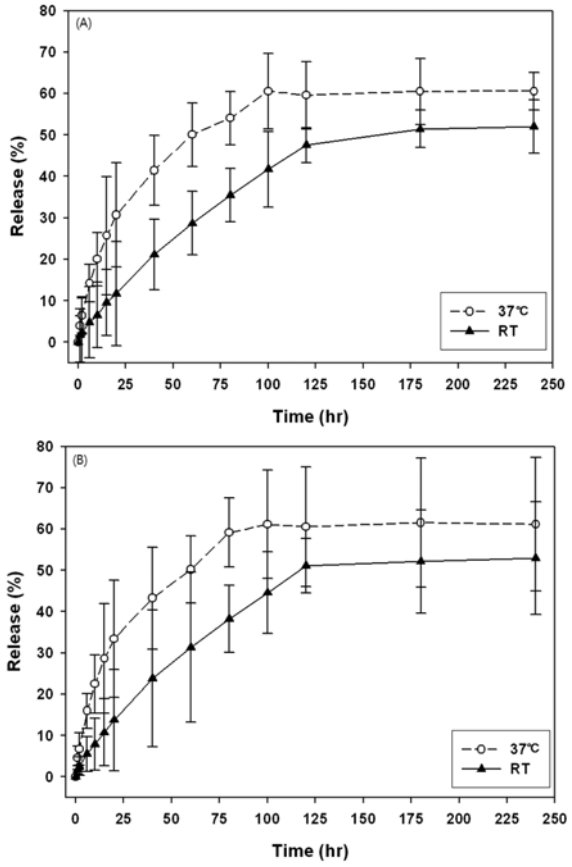

Figure 8. Release kinetics of toluidine blue from CS-PEO hydrogels at pH=7.4; 10% (A) and 5% gel (B).

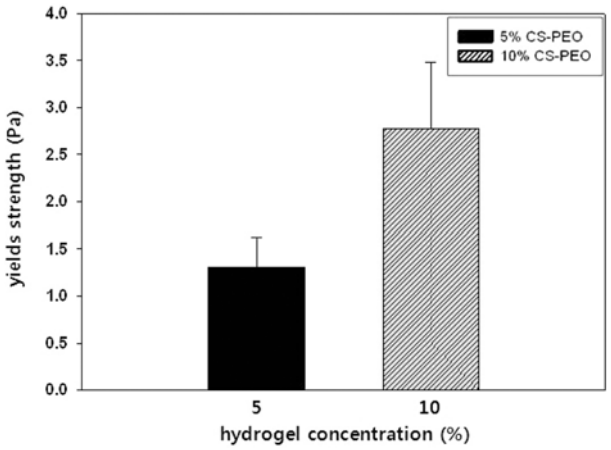

Figure 9. Compression strength of CS-PEO hydrogel.

Mechanical and Morphological Properties of CS-PEO Hydrogel. Mechanical and morphological properties of the CS-PEO hydrogels were also evaluated by measuring both a compression yield strength and pore sizes, respectively. While the 5% hydrogel demonstrated 1.30 Pa, the 10% hydrogel did 2.78 Pa at their yield strengths, when the fully hydrated hydrogel was crushed by compression with no air bubbles or physical entrapment (Figure 9). The 10% CS-PEO hydrogel demonstrated higher value of yield strength than that of 5% gel did because of higher concentration and more cross-linking. Next, the morphologies of the 10% CS-PEO hydrogels were evaluated with SEM after dehydrating the water-expanded gel. Their surface and cross-sections demonstrated surface morphologies with pores ranging approximately from 5 to 15 μm in diameter (Figure 10(A) and (B)). However, their cross-sections demonstrated relatively more homogeneous morphologies with smaller pores, 8~10 μm diameter (Figure 10(C) and (D)), which are smaller than those of their surfaces. This morphological difference of their surface and cross-section might be from the effect of air exposure on the hydrogel surfaces during processing. The surface air-exposed had less number of pores with irregular shapes.

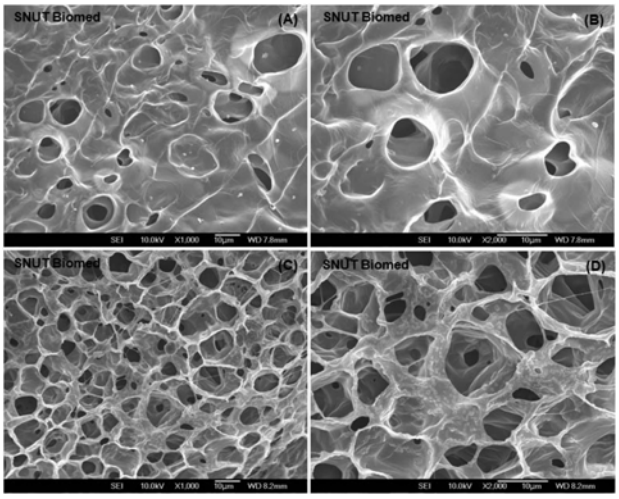

Figure 10. Morphologies of 10% CS-PEO hydrogel; surfaces (A, B) and cross-sections (C, D) magnified to x 1,000 (A, C) and x 2,000 (B, D).

Conclusions

Development of a new method of self cross-linking hydrogel has been recognized as a very interesting tool in biomedical society due to its easy handling and conversion of liquids into a gel *in situ* in defect sites of patient tissues. *In situ* gel formation had many advantages such as encapsulation of cells or drugs and their efficient delivery into specific diseased or damaged tissues with minimal loss of their bioactivities. Furthermore, spatial and temporal control of gelation could be achieved by this controlled self cross-linking of the polymer solutions and homogeneous distribution of bioactive molecules and cells, which are important factors in its applications in drug delivery system and tissue engineering such as bone, skin, and dental regenerations.

A novel CS-PEO hydrogel spontaneously was fabricated in minutes by utilizing the mechanism of a Michael type addition reaction between the synthesized CS derivative and PEO-SH in different conditions of polymer solutions, as suggested in our previous report.[18] After confirming synthesis of the CS derivative by the chemical analyses of FTIR, NMR and ESCA, CS-PEO hydrogelation was completed within several minutes as judged by the changes in its visco-elastic behaviors over time. Hydrogelation behaviors were dependent on the pHs of polymer solutions, demonstrating quicker gel formation and more swelling under basic polymer solutions, probably due to the effects of anionic repulsion of basic hydroxyl ions on the hydrogel networks and thus quicker diffusion of the reacting agents. Swelling equilibrium of the CS-PEO hydrogel was reached in several hours after their loading in water. The cross-sectional morphologies of the equilibrated and then dehydrated hydrogel demonstrated relatively homogeneous pores with approximately 8~10 μm diameters, but its surface showed less homogeneous pores and less number of pores. This hydrogel further demonstrated a potential biomaterial as a carrier for drug release and as a scaffold for tissue engineering. Toluidine blue as a model drug was sustain-released over time for upto 125 h without burst release, and its release was dependent on the environment such as temperature and medium pHs. Both higher temperature of the polymer solution and lower concentrations of hydrogel demonstrated induction of quicker release of toluidine blue molecules from the CS-PEO hydrogel. The SEM morphologies and mechanical properties of the CS-PEO hydrogel demonstrated a potential of its applications in tissue engineering as a porous scaffold. Normally porous structures have been

known to have advantages of their applications to the scaffolds for tissue engineering in cell migration and tissue formation. These hydrogels had different mechanical strengths depending on its concentrations and the existence of PEO as a hydrogel constituent. Possibility of control of different mechanical properties of the hydrogel was also expected to have advantageous potential to be applied in different locations of the damaged tissues or organs for tissue engineering applications, which require different compression stresses.

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