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Synthesis of *In situ* Chondroitin Sulfate Hydrogel through Phosphine-Mediated Michael Type Addition Reaction

Seongyeon Jo, Sumi Kim, and Insup Noh*

Department of Chemical Engineering, College of Engineering, Seoul National University of Science and Technology, Seoul 139-743, Korea

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Abstract: Novel *in situ* chondroitin sulfate (CS) hydrogel was synthesized *via* phosphine-mediated Michael type addition reaction by mixing precursor solutions of both CS-acrylate and CS-tri(2-carboxyethyl)phosphine (TCEP) as an electrophile and a nucleophile, respectively. CS-acrylate and CS-TCEP were synthesized in advance by chemical grafting of acrylic acid and TCEP to adipic dihydrazide (ADH)-grafted CS. After verification of chemical grafting of TCEP to CS-ADH by phosphorous peaks in the proton nuclear magnetic resonance spectra (^{1}H NMR) and Fourier transform infrared spectroscopy (FTIR), gel formation was evaluated with a tilting method under different pHs, temperatures and concentrations of the precursor solutions. Precursor solutions spontaneously turned into a gel within a minute to several hours depending on the solution conditions, where a basic pH and higher concentrations and reaction temperatures of the precursor solutions induced quicker gel formation. The fabricated CS hydrogel was thermally stable at low temperatures as observed by both differential scanning calorimeter (DSC) and thermogravimetric analysis (TGA), and also swelled to equilibrium state, leading to 1 to 2 times increase in mass, where a basic medium induced more gel swelling than an acid one. The morphologies of the equilibrated swollen gel showed expansion of its polymer network having pores with a diameter of 13 to 16 μm in dehydrated state. The hydrogel released rhodamine B, a model drug, over approximately 10 h and had 1.08 MPa of compressive modulus for the 10% hydrogel. On the other hand, degradation of the CS hydrogel was controlled by the addition of chondroitinase ABC, along with the reduction of compression strength. The hydrogel had excellent *in vitro* biocompatibility both inside and on the surface of the CS hydrogel when tested with the live and dead assay of fibroblasts. From these experimental results, we concluded that when TCEP acted as a nucleophilic cross-linking agent of Michael type addition reaction in the synthesis of CS hydrogel, the CS hydrogel demonstrated adequate physicochemical and biological properties.

Keywords: chondroitin sulfate, tris(2-carboxyethyl)phosphine, *in situ* hydrogel, synthesis.

Introduction

Chondroitin sulfate (CS), an anionic linear polysaccharide with a molecular weight of 50-100 kDa and disaccharide repeating units of 1-3 linkage of *D*-glucuronic acid and *N*-acetylgalactosamine with sulfate groups, is mainly attached to proteoglycans in connective tissues as a component of structural matrix, or on cell surface and basement membranes as a receptor.[1] In structural, CS has played a major role in creating high osmotic swelling pressure in articular cartilage among many important structural properties, leading to expansion of the matrix and subsequent placement of collagen network under tension.[2] However, loss of CS component from the cartilage matrix degrades its structure, leading to a major cause of osteoarthritis. In biological, it possesses various characteristic properties including binding and modulation of certain growth factors such as insulin-like growth factor-1, heparin-binding growth factor, fibroblast growth factor-1.[3-5] CS also binds with core proteins to produce highly absorbent aggrecan, which is a major structure inside cartilage. It can produce syndecan, a cell receptor, which can interact with adhesion proteins, cells and extracellular matrix. Furthermore, CS readily interacts with proteins in the extracellular matrix through its negative charges of the sulfate side chains. These electrostatic interactions are also important for regulating diverse cellular activities such as anti-inflammatory activity, articular functions, swelling/pains reductions as well as prevention of narrowing of the joint space of the knee and fingers.[6]

Despite these interesting structural and biological properties, the readily water-soluble properties of CS limit its applications both in a delivery system of biomolecules and cells,

*Corresponding Author. E-mail: insup@seoultech.ac.kr

and in tissue engineering, requiring transformation of CS into insoluble matrix. Diverse synthesis methods and mechanisms have been developed to get insoluble CS biomaterials by combining with other biocompatible polymers such as chitosan,[7] gelatin,[8] collagen,[9,10] hyaluronic acid,[11] poly (ethylene oxide) (PEO),[12-17] polylactide,[18] poly(vinyl alcohol)[19,20] and poly(*N*-isopropylacrylamide).[21] By combining with one of those polymers, CS was transformed into various forms of biomaterials such as hydrogels, scaffolds, films and nanostructures, according to their application purposes. These CS-based hybrid materials have been applied as biomaterials such as a carrier in delivery of biomolecules, and a scaffold in wound healing and tissue engineering of skin and cartilage.[11,16,22-25] For therapeutic applications, CS-hyaluronic acid-gelatin was synthesized into a hydrogel by modifying both CS and hyaluronic acid with 11-azido-3,6,9-trioxaundcant-1-amine and gelatin with propionic acid.[8] CS-PEO hydrogels were fabricated as either a pH-sensitive hydrogel through amide linkage or a self-cross-linking hydrogel through mechanism of Michael type addition reaction.[15,17] As natural biomaterials, CS-collagen was also fabricated into a temperature-sensitive hydrogel by combining CS with type I collagen by utilizing polyacrylamide and acrylic acid.[26] As injectable biomaterials, CS-based hydrogel was fabricated by modifying CS with N-acryloxysuccinimide and poly(trimethylene carbonate)-hydroxyethyl methacrylate for delivery of growth factors and cells.[26] For tissue engineering applications, a porous CS-based scaffold was fabricated by combining with either polylactide or type II collagen for regeneration of cartilage tissue.[18] CS-collagen-hyaluronic acid was utilized for regeneration of nucleus pulposus and dermis tissue.[27] Fabrication of CS-chitosan was also reported as a sponge for bone regeneration by prolonged delivery of platelet-derived growth factors,[28] and as a self-assembly hydrogel for regeneration of intervertebral disc injury.[29]

Even though numerous CS-based biomaterials have been applied as biomaterials for fabrication of either hydrogels or scaffolds for tissue regeneration, development of homogeneous CS hydrogel is required to fully utilize its unique biological and physicochemical properties through new methods and mechanisms. CS polymer could supply unique and pivotal biological properties to the homogeneous CS hydrogel compared to those of above hybrid biomaterials. This homogeneous CS hydrogel is expected to expand the scopes of injectable hydrogel applications to medical biomaterials, drug delivery vehicles and cell therapy. In this study, we synthesized a new homogeneous *in situ* CS hydrogel *via* phosphine-mediated Michael type addition reaction of the CS-acrylate and CS-phosphine, which is a new method in biomaterials areas. This novel CS hydrogel is expected to have its own biological properties compared to those of our previously reported CS-PEO hydrogel.[17] While PEO is a synthetic polymer with chemical and biological inertness, CS is a natural polymer with diverse and unique biological properties.[17] While PEO in the CS-PEO hydrogel is not biodegradable to enzymes, the CS hydrogel is expected to show biodegradability by chondroitinases. Evaluations of CS hydrogel demonstrated successful formation of *in situ* CS hydrogel *via* self cross-linking and excellent physico-chemical and biological properties.

Experimental

Materials. Chondroitin sulfate (CS: MW 50 kDa) from shark cartilage, adipic dihydrazide (ADH), acrylic acid, *N*-(3-diethylpropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), tris(2-carboxyethyl)phosphine (TCEP) and rhodamine B were purchased from Sigma-Aldrich (ST. Louis, MO, USA). While fibroblast growth medium-2 (FGM-2) and penicilin-streptomycin were purchased from Lonza Korea (Switzerland), cell counting kit-8 (CCK-8) and live & dead viability/cytotoxicity kit for mammalian cells were bought from Dojindo Laboratories (Japan) and Invitrogen (USA), respectively. Fetal bovine serum (FBS) was gotten from Gibco (Australia), and minimum essential medium-α (MEM-α), *in vitro* toxicology assay kit and neutral red were purchased from Sigma-Aldrich (ST. Louis, MO, USA). All chemicals were employed as received.

Synthesis of CS Derivatives. Both CS-acrylate and CS-TCEP were synthesized by grafting of acrylic acid and TCEP to CS-ADH through EDC chemistry. Chemical grafting of ADH to CS was previously reported by us in detail.[17] Briefly, CS-ADH was synthesized by making the carboxylic acid of CS react with the free primary amines of ADH. Subsequent grafting of either acrylic acid or TCEP molecules to the CS-ADH was performed with the same mechanism through EDC chemistry, thus obtaining either CS-acrylate or CS-TCEP, respectively. The resulting CS-derivatives of both CS-acrylate and CS-TCEP were dialyzed in a dialysis tubing with 6-8 kDa of molecular weight cut off against 100 mM NaCl solution (pH 6.8), followed by sequential dialysis against distilled water (pH 6.8) and then 20% ethanol solution. Powders of both CS derivatives were obtained by lyophilizing them for 2 days.

ATR-FTIR Spectroscopy. Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra of CS and CS-TCEP samples were recorded and compared with, 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 and penetration depth of about 2 μm.

^{1}H NMR and ^{31}P NMR. Spectra of ^{1}H NMR of both CS and CS-TCEP samples were obtained in solutions (10 mg/1 mL). The CS-TCEP samples were further evaluated by ^{31}P NMR to verify existence of phosphine molecules in the CS-TCEP chemical structure. Both CS and CS-TCEP samples in 1 mL deuterium oxide (D_2O) were analyzed with UI 500 MHz FT-NMR Spectrometer (Varian, Japan). Chemical

shift (δ) was measured in ppm by employing sodium-2,2-dimethyl-2-silapentane-5-sulfonate and H_3PO_4 as internal standards for ^{1}H NMR and ^{31}P NMR, respectively.

Fabrication of *In Situ* CS Hydrogel. The synthesized CS derivative powders were separately dissolved in either 5 or 10% PBS concentrations. *In situ* CS hydrogel was spontaneously synthesized via Michael type addition reaction by mixing two precursor solutions of CS-acrylate and CS-TCEP at a molar ratio of 1:1.

Gelation Behaviors of CS Precursor Solutions. Behaviors of 10% CS hydrogel formation were analyzed by using both precursor solutions and a vial tilting method. Gelation time was measured by tilting the mixed precursor solution in a 1 mL conical vial over time. Gelation time was regarded a point, when there was no further flow for more than 1 min after inverting 100 μL mixture solution in a 1 mL conical vial.[17]

Thermal Analysis. Both CS and 5% CS hydrogel was thermally analyzed with differential scanning calorimeter (DSC) (TA-50 series, Shimadzu; Japan). DSC was operated under the conditions of heating rate of 10 °C/min, argon flow rate of 60 mL/min, and temperatures ranging from 22 to 800 °C. The thermal analyses were made in a tightly closed container to minimize humidity loss. Another thermogravimetric analysis of both CS and 5% CS hydrogel was also performed with a thermogravimetric analyzer (TGA: TA-50 series, Shimadzu; Japan) at the conditions of a heat flow rate of 10 °C/min under argon atmosphere and broad temperatures, ranging from 22 to 800 °C.

Swelling Behaviors of CS Hydrogel. Swelling of 5% CS hydrogel (200 μL) was measured in 3 mL phosphate buffered saline (PBS) solution or distilled water (DW) at the pHs of 4.0, 7.4, and 10.0 at 37 °C for 4 weeks. After weighing the fabricated hydrogel with a microbalance, its swelling was measured by comparing the weights of the hydrogels obtained before and after its immersion in water. Adherent water was removed by blotting the wet CS hydrogels before weighing them on an electronic balance. The percentage of gel swelling was calculated by employing a formula (1).

$$\text{Swelling percent (\%)} = (W_t/W_i)\times 100 \quad (1)$$

(W_t=weight of the swollen hydrogel at time t, W_i=weight of the hydrogel at initial gelation point)

Morphologies of the Dehydrated CS Hydrogel. Morphologies of both 5% and 10% CS hydrogel were observed by scanning electron microscopy (SEM) (Jeol Ltd., Japan) after their dehydration as follows. The hydrogel was dehydrated by sequential freezing of the swollen hydrogel in liquid nitrogen and then at -55 °C overnight. The dry specimen was mounted on an aluminum stub with a double-sided tape, and then gold-coated with a sputter coater for 1 min. The morphologies of the surface and cross-sections of the dehydrated CS hydrogel were analyzed with SEM in different magnifications.

Release Experiment. Rhodamine B was used as a model drug for evaluation of its release from the CS hydrogel. After mixing 2% rhodamine B solution (10 μL) with 10% precursor solution, 200 μL CS hydrogel with rhodamine B was fabricated. Release of rhodamine B from either 5 or 10 % CS hydrogel was measured at room temperature during shaking in 100 rpm for 24 h. The amount of rhodamine B was estimated by measuring the peak area of rhodamine B around 550 nm of the 100 μL sink solution by UV-vis (JP/V-560, Jasco, USA). The amount of the released rhodamine B was calculated by employing a formula (2).

Percentage of the amount of rhodamine B released at time t (R_s)

$$R_x\ (\%) = R_t/R_0\times 100$$

$$\text{Cumulative amount of rhodamine released (\%)} = \sum_{n=1}^{x} R_x \quad (2)$$

Where, R_x=amount of rhodamine B released at time t,
R_0=amount of rhodamine B initially loaded.

Compressive Modulus of CS Hydrogel. Compression strengths of both 5% and 10% CS hydrogel were evaluated by employing microforce test system (Tytron 250, MTS system; MN, USA). The hydrogel specimen were fabricated by addition of precursor solutions into a cylindrical specimen fixture of aluminum (ϕ=6 mm, h=12 mm) without either any compression or distortion. The specimen was mounted on the micro-force test system by inserting it into a confined compression apparatus. After compression of the hydrogel specimen at room temperature (n=3) by imparting the loading at a rate of 1 mm/min to 60% strain (n=3), their compressive modulus were calculated from the slope of the initial linear portion (<5% strain) of the stress-strain curve.

Degradation of CS Hydrogel by Enzyme. *In vitro* degradation of CS hydrogel by enzyme was evaluated by addition of 0.1 unit chondroitinase ABC at a regular interval. Buffer solution was made in advance in an aqueous solution with 0.01% bovine serum albumin, containing 50 mM Tris buffer at pH 8.0, 60 mL sodium acetate and 0.02% bovine serum albumin. After swelling of 200 μL CS hydrogel to equilibrium in 2 mL buffer solution for 30 h, it was weighed (W_s) after blotting with kimwipe paper. *In vitro* biodegradation of CS hydrogel was evaluated by measuring its weight after adding 0.1 unit chondrotinase ABC in 10 μL buffer solution over the hydrogel every other day. The same amount of buffer solution without chondroitinase ABC was compensated into the bath of the hydrogels (n=3) after each aliquot. The weight percentage of the remaining hydrogel was calculated by employing a formula (3).

$$\text{Remaining weight (\%)} = (W_t/W_s)\times 100 \quad (3)$$

W_t-weight of CS hydrogel treated at time t.
W_s-weight of the maximally swollen CS hydrogel before degradation process.

***In vitro* Cell Culture.** Fibroblasts at a density of approximately 1×10^5 cells/hydrogel were cultured either on the surface of or inside 200 μL CS hydrogel in an incubator with 5% CO_2 at 37 ºC for 1 week by feeding fibroblast growth medium-2 supplemented with 10% fetal bovine serum and 1% antibiotics of penicillin and streptomycin. Cell adhesion and proliferation on the surface of 200 μL hydrogels in a 24 well plate were quantitatively and qualitatively measured with an assay of CCK-8 through the microplate reader (Tecan: Australia) after insertion of 100 μL CCK-8 solution into 900 μL FGM-2 cell culture medium. Optical density of the aliquoted 100 μL medium was measured by the microplate reader at a wave length of 450 nm.

Live & Dead Assay. *In vitro* cell viability of CS hydrogel was evaluated by evaluating the fibroblasts both on the surface of and encapsulated in the 200 μL 10% hydrogel. Live & dead viability/cytotoxicity kit for mammalian cells (Invitrogen, USA) was prepared by adding solutions of both 1.2 μL ethidium homodimer-1 (EthD-1) (2 mM) and 4 mM calcein AM (0.3 μL) into 600 μL PBS. After performing the reaction with prepared kit for 30 min in the *in vitro* incubator, cell viability was observed by a fluorescence microscope.

Results and Discussion

Synthesis and Chemical Analysis of CS-TCEP. CS-acrylate and CS-TCEP have been synthesized by sequential grafting of ADH and then either acrylic acid or TCEP to CS through EDC-mediated coupling reaction (Figure 1). Since chemical synthesis and analysis of CS-acrylate were previously reported,[17] chemical analysis of CS-TCEP only was in detail performed by ATR-FTIR, ^{1}H NMR, and ^{31}P NMR. In ATR-FTIR spectrum, the CS-TCEP spectrum demonstrated new peaks compared to those of the control CS spectrum, which are evidences of chemical grafting of TCEP to CS as a cross-linking agent (Figure 2). In specific, the phosphorus atoms connected to the carbon atom (P-C) were clearly

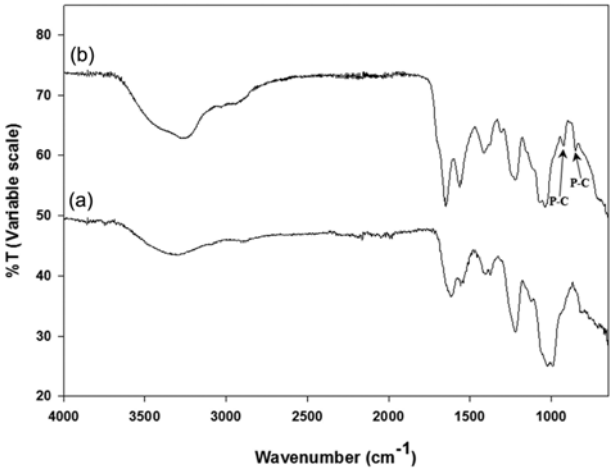

Figure 2. ATR-FTIR spectra of CS (a) and CS-TCEP (b).

observed at the wavelengths of both 910 and 890 cm^{-1} in the spectrum of ATR-FTIR (Figure 2(b)). Another chemical analyses of both CS and CS-TCEP polymers were performed with ^{1}H NMR (Figure 3(a)). Compared with the results of ^{1}H NMR spectrum of control CS, the CS-TCEP polymer demonstrated new peaks from the ADH and TCEP cross-linking agents, which were sequentially grafted to CS at the positions of 2.65-2.40 (the peak positions 1 and 4 in Figure 3(a); O=C-C$\underline{H_2}$-CH_2), 2.30-2.18 (the peak position of 3 in Figure 3(a); P-C$\underline{H_2}$-CH_2) and 1.25 ppm (the peak position of 2 in Figure 3(a); O=C-CH_2-C$\underline{H_2}$) from their proton signals. While the proton peaks from the ADH cross-linking agent were clearly observed at the peak position of 1.25 ppm, those from the TCEP molecules were observed at the positions of 2.30 to 2.18 ppm. The protons attached directly to carbonyl group in either ADH or TCEP molecules made complex peaks at around 2.65-2.40 ppm due to their chemical shifts. The CS-TCEP compound was also analyzed for detection of phosphorous atoms with ^{31}P NMR (Figure 3(b)). Its phosphorous

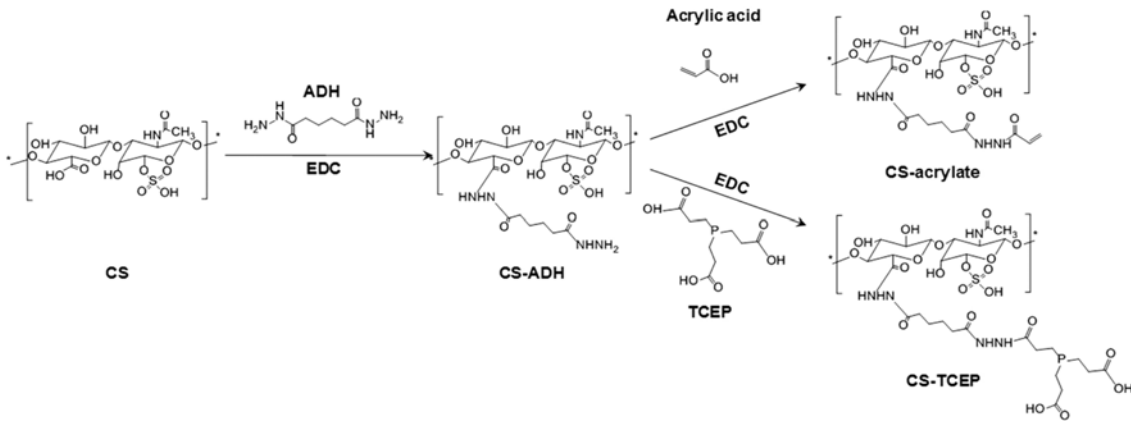

Figure 1. Schematic representation of syntheses of both CS-acrylate and CS-TCEP, which are precursor polymers for Michael type addition reaction.

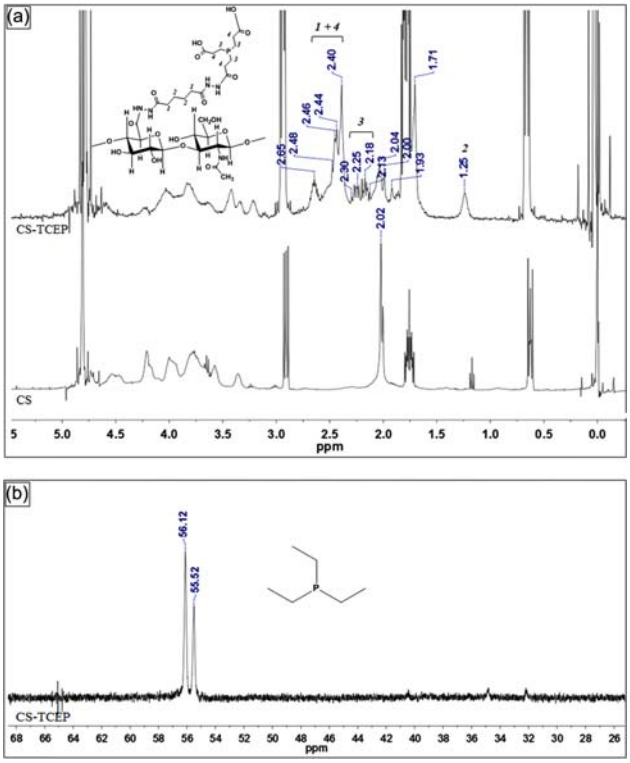

Figure 3. NMR spectra of CS-TCEP; ^{1}H NMR (a) and ^{31}P NMR (b).

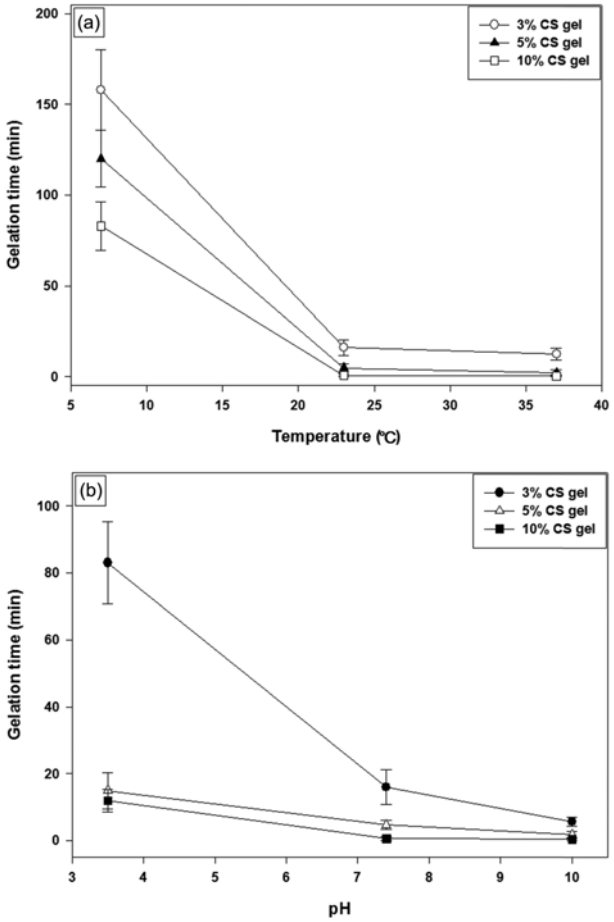

Figure 4. Gel formation of CS precursor solutions under different temperatures (a) and pHs (b).

signal appeared clearly at the position of 55 ppm of the ^{31}P NMR spectrum, confirming grafting of TCEP to the CS-ADH polymer network.

Gelation of CS Precursor Solutions. CS hydrogel was fabricated through the phosphine-mediated mechanism of Michael type addition reaction between CS-TECP and CS-acrylate, by mixing the precursor solutions in PBS. Gel formation behaviors measured by the vial tilting method depended on the experimental conditions of pHs, temperatures and concentrations of precursor solutions (Figure 4). Overall, higher concentrations, reaction temperatures and pHs of the precursor solutions induced quicker gel formation (Figure 4(a)). In specific, while 10% precursor solutions turned into a gel approximately in 83 min at 7 ºC, and they did in less than 1 min at the temperatures of both 23 and 37 ºC. Gel formation was significantly delayed when lower concentrations of precursor solutions were tried at the same solution temperature. While the 5% precursor solution turned into a gel in 120 min, the 3% precursor solution formed a gel in 158 min at 7 ºC. When we increased the solution temperatures from 7 ºC to 23 and 37 ºC, the precursor solutions quickly became a gel in 4.8 and 2.2 min for the 5% precursor solutions but at 16 and 12.5 min for the 3% precursor solutions, respectively. Next, when we changed the pHs of the precursor solutions from basic to acid condition, significant delay of gel formation time was observed (Figure 4(b)). In specific, the 10% precursor solution turned into a gel in 1 min at both pH 10 and 7.4, but it took approximately 12 min at pH 3.5. As expected, the concentrations of the precursor solutions also affected its gel formation time. When we decreased its concentrations from 5% to 3%, gelation time delayed from 15 to 83 min, respectively. Overall it took several minutes for the precursor solutions at neutral pH and room temperature to be a gel. Quicker gel formation was attributed possibly due to the effects of hydroxyl groups from the basic mediums on the inflation of hydrogel.

Thermal Analysis of CS Hydrogel by TGA and DSC. 5% CS hydrogel was thermally analyzed with both TGA and DSC by comparing with that of native CS. Weight loss of the specimens over the temperatures ranging from 27 to 639 ºC was recorded during processing of 4 TGA stages of DSC (Figure 5(a)). In specific, 1.32 mg native CS showed 16.5% weight loss during the first stage of 27~230 ºC. It showed more weight losses of 25.8%, 20.7%, and 24.1% during the 2nd, 3rd, and 4th stages of 230~260, 250~490, and 490~639 ºC, respectively. However, the hydrogel, a cross-linked CS, showed delayed weight loss during thermal anal-

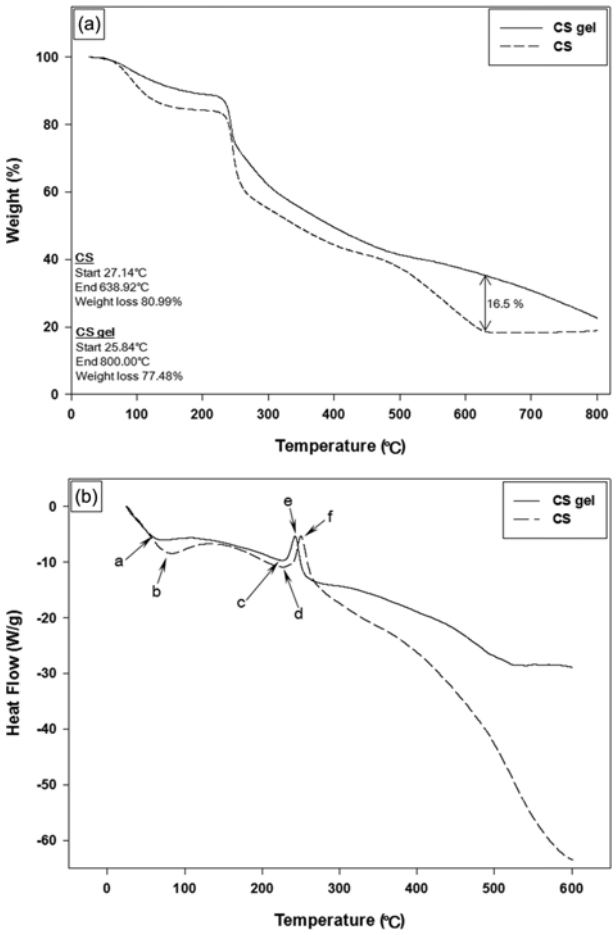

Figure 5. Thermal analyses of CS and 5% CS hydrogel; TGA (a) and DSC (b).

ysis. Hydrogel's weight losses were 9.9%, 12.9%, 26.3% and 22.2% during the 1st, 2nd, 3rd, and 4th stages of 26~225, 225~253, 253~475, and 475~800 ºC, respectively.

Another thermal analysis of both native CS and CS hydrogel

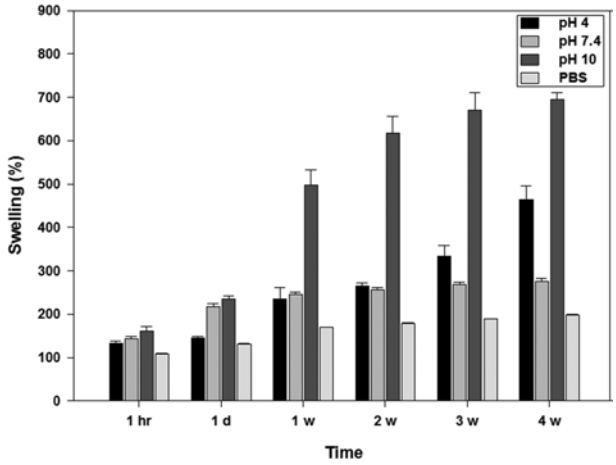

Figure 6. Swelling behaviors of 5% CS hydrogel in water under different conditions.

was also measured with DSC, where endothermic peaks were observed at 70 and 80 ºC for native CS and CS hydrogel, respectively (Figure 5(b)). While the exothermic peaks of CS started at 228 ºC and culminated at 242 ºC, those of CS hydrogel started at 239 ºC and then culminated in 251 ºC.

Swelling Behaviors. Next, we measured swelling behaviors of the 5% hydrogels by changing the mediums such as PBS and water in acidic, neutral and basic. Degrees of hydrogel swelling were dependent on the conditions of hydrogel-soaking mediums (Figure 6). Overall while PBS induced least amount of gel swelling, basic water induced highest amount of gel swelling among the employed mediums regardless gel soaking time. Hydrogel in acidic water showed more swelling than that in neutral water did, but less than that in basic water. While PBS and acidic water at pH 4 induced hydrogel swelling to approximately 131% and 145%, respectively, basic water at pH 10 did approximately 235% gel swelling. When we measured swelling of the hydrogels during their immersion in water at different pHs for upto 4

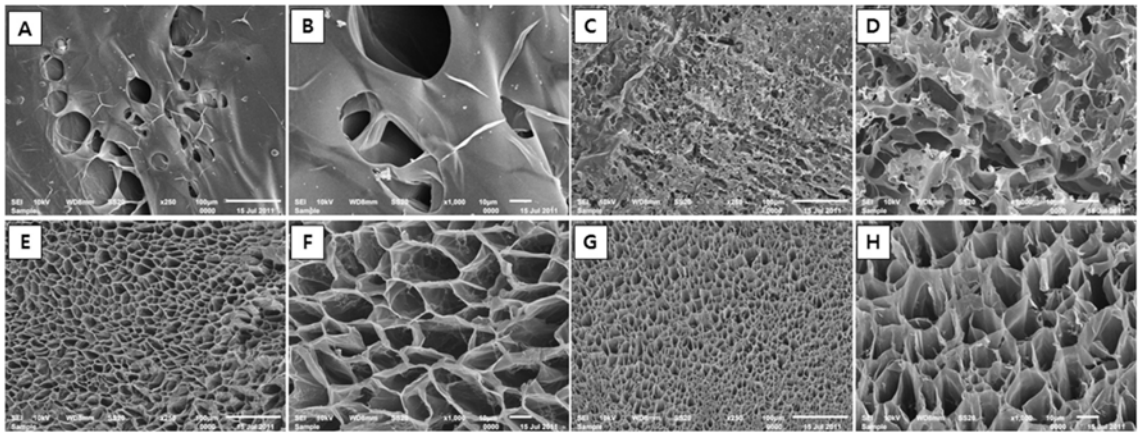

Figure 7. Morphologies of the surfaces (A, B, E, F) and cross-sections (C, D, G, H) of CS hydrogel; 5% (A~D) and 10% hydrogel (E~H) at the magnifications of both ×250 (A, C, E, G) and ×1000 (B, D, F, H).

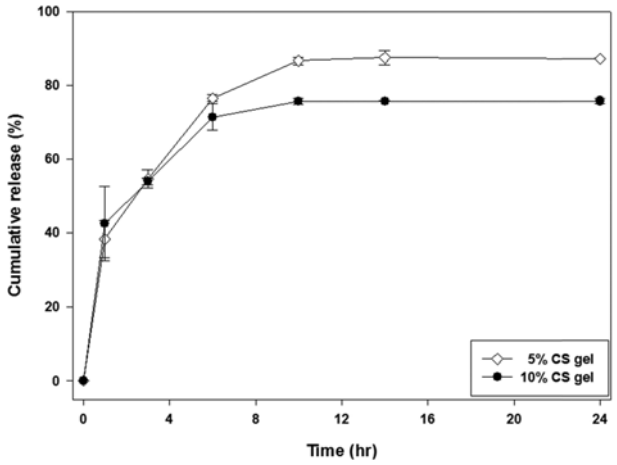

Figure 8. Release of rhodamine B from the CS hydrogels.

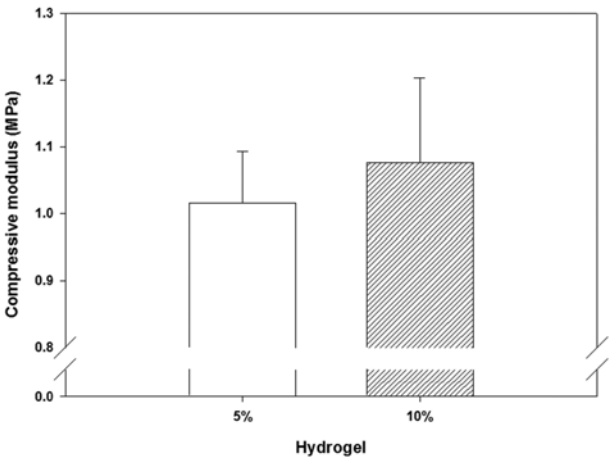

Figure 9. Compressive modulus of CS hydrogels.

weeks, effects of the medium's pHs on hydrogel swelling became remarkably distinctive. The hydrogles immersed in PBS and neutral water for 4 weeks swelled to 98% and 275%, respectively, but those in water pH at 4 and 10 swelled to 464% and 695%.

Morphologies of Dehydrated CS Hydrogel. Both surface and cross-section morphologies of 5% (Figure 7(A), (B), (E), and (F)) and 10% (Figure 7(C), (D), (G), and (H)) hydrogels were evaluated with SEM after dehydration of the swollen gel. While their surface morphologies showed relatively smooth surface with irregular pore shapes and sizes, their corresponding cross-sectional morphologies had more homogeneous and organized pore shapes. The average pores sizes of the cross-sectional morphologies were measured as approximately 16 and 13 μm for 5% and 10% hydrogel, respectively.

Release of Rhodamine B from CS Hydrogel. Release of rhodamine B from CS hydrogel was evaluated by measuring cumulative amount of rhodamine B molecules released over time under different precursor solutions (Figure 8). The rhodamine B encapsulated in both 5% and 10% CS gel was sustain-released overtime and reached to a plateau status approximately at 10 h. When the rhodamine B molecules reached to a plateau state, approximately 87% and 76% of the initially loaded amount was released from 5% and 10% hydrogels, respectively.

Compressive Modulus of CS Hydrogel. Mechanical property of CS hydrogels was evaluated by measuring compressive strengths of both 5% and 10% hydrogels (Figure 9). While the 5% hydrogel had 1.02 MPa of compression strength, the 10% one had 1.08 MPa. The hydrogel with higher concentrations demonstrated higher compression strengths, possibly due to higher degrees of crosslinking of the CS network.

***In vitro* Degradation of CS Hydrogel by Chondroitinase ABC.** *In vitro* degradation of 5% CS hydrogel was analyzed by measuring their weight changes by addition of 0.1 U

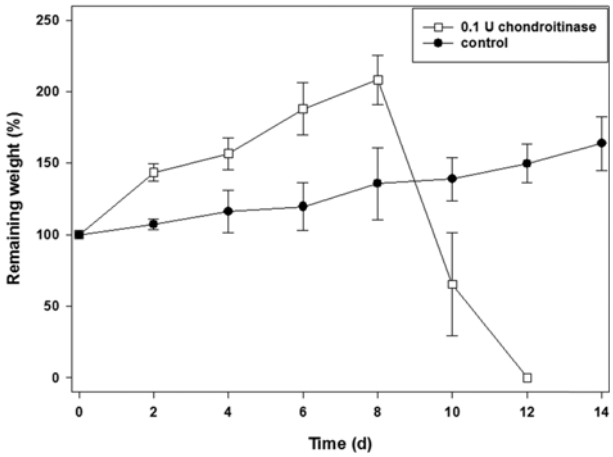

Figure 10. *In vitro* degradation of 10% CS hydrogel by addition of 0.1 unit chondroitinase ABC at an interval of every other day.

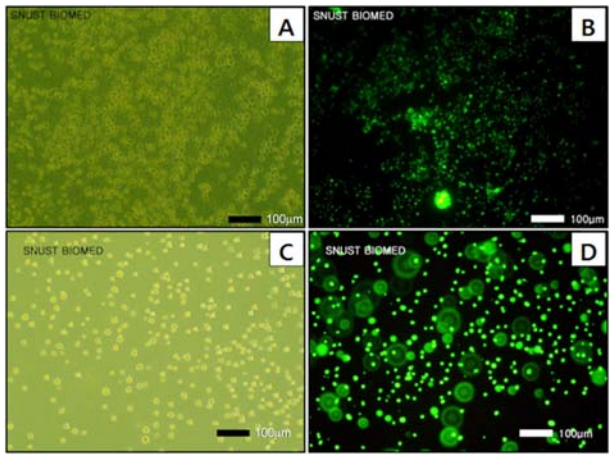

Figure 11. Fibroblast compatibility both on the surface of (A, B) and inside (C, D) the CS hydrogel (5%) at day 3 (A, C) and 7 (B, D) by light (A, C) and fluorescence microscopy (B, D) (×200).

chondroitinase ABC for 2 weeks (Figure 10). Its weight changes were compared with those of the control CS hydrogel in PBS buffer with no enzyme added. Even though the control 5% hydrogel showed 163% increase in its weight during 2 weeks, corresponding 5% hydrogel showed increase in its weight to 208% during 8 days. However, dramatic decrease in its weight was observed for the 5% hydrogel by addition of 0.1 unit chondroitinase ABC onto its surface every other day. Its weight decreased to 65% at day 10 and then completely degraded at day 12 by addition of chondroitinase ABC.

***In Vitro* Cellular Behaviors.** Cell adhesion and viability of 5% hydrogel were observed by both CCK-8 and live & dead assay after seeding 100,000 fibroblast cells both on the surface of and encapsulated in CS hydrogel. All the cells were viable for both 3 (Figure 11(A) and (B)) and 7 days (Figure 11(C) and (D)) as indicated in green color during live & dead assay. When we measured the number of attached cells with the ELISA reader, high number of attached cells was measured indicating cell adhesion. While all the cells inside hydrogel were isolated, the cells on the surface of the hydrogel showed more cells with higher density without spreading.

Conclusions

Novel *in situ* CS hydrogel was developed through the phosphine-mediated mechanism of Michael type addition reaction by mixing the precursor solutions of CS-acrylate as an electrophile and CS-TCEP as a nucleophile after chemical grafting of TCEP to CS-ADH after in advance verification of phosphorous signals from its H^1 and P^{31}-NMR and FTIR spectra. Gel formation of the CS precursor solutions was dependent upon their concentrations, pHs and temperatures, taking a minute to several hours. The *in situ* CS hydrogel showed thermal stability and also swelled to in equilibrium state to 1 to 2 times in weights, depending on the medium conditions. The equilibrated and dehydrated gel showed full expansion of its polymer network having pores with a diameter of 13 to 16 μm. The hydrogel also showed both release of rhodamine B from the CS gel and encapsulation of live cells in the precursor solutions and subsequent self cross-linkings of the precursor polymers. The release kinetics of rhodamine B from the 5% and 10% hydrogels were very similar, possibly due to small molecular sizes of rhodamine B and also no significant effects of both cross linking molecular weights of CS networks and molecular interactions between rhodamine B and CS. Simple mixings of the live cells in the precursor solutions under physiological circumstances did not show damages on them. The 5% and 10% CS hydrogels showed similar compression strengths, and their degradations was also controlled by addition of chondroitinase ABC. The CS hydrogel swelled more by addition of chondroitinase ABC than the gel without enzyme addition did, probably due to gradual breakdown of the polymer network by enzymes and subsequently more water absorption to certain extents. Cell cultures demonstrated excellent *in vitro* cell viability both inside and on the surface of the CS hydrogel, even though the cells encapsulated in the gel did not show spreading or proliferation. Based on all the experimental results, we concluded that when TCEP chemically grafted to the CS network acted as a nucleophilic cross-linking agent during Michael type addition reaction, the spontaneously formed CS hydrogel seemed to have physicochemical and biological properties required for possibly its application as a biomaterial.

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