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Hyaluronic acid/chondroitin sulfate-based hydrogel prepared by gamma irradiation technique

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ABSTRACT

Gamma-ray irradiation of novel hydrogels was used to develop a biocompatible hydrogel system for skin tissue engineering. These novel hydrogels are composed of natural polymers including hyaluronic acid (HA) and chondroitin sulfate (CS), and the synthetic polymer, poly(vinyl alcohol) (PVA). The γ-ray irradiation method has advantages, such as relatively simple manipulation without need of any extra reagents for polymerization and cross-linking. We synthesized HA and CS derivatives with polymerizable residues. The HA/CS/PVA hydrogels with various compositions were prepared by using γ-ray irradiation technique and their physicochemical properties were investigated to evaluate the feasibility of their use as artificial skin substitutes. HA/CS/PVA hydrogels showed an 85–88% degree of gelation under 15 kGy radiation. All HA/CS/PVA hydrogels exhibited more than 90% water content and reached an equilibrium swelling state within 24 h. Hydrogels with higher concentrations of hyaluronidase solution and HA/CS content had proportionally higher enzymatic degradation rates. The drug release behaviors from HA/CS/PVA hydrogels were influenced by the composition of the hydrogel and drug properties. Exposure of human keratinocyte (HaCaT) culture to the extracts of HA/CS/PVA hydrogels did not significantly affect the cell viability. All HaCaT cell cultures exposed to the extracts of HA/CS/PVA hydrogels exhibited greater than 92% cell viability. The HaCaT growth in HA/CS/PVA hydrogels gradually increased as a function of culture time. After 7 days, the HaCaT cells in all HA/CA/PVA hydrogels exhibited more than 80% viability compared to the control group HaCaT culture on a culture plate.

1. Introduction

Hydrogel has been considered to be an interesting vehicle for drug delivery and a biomaterial for tissue engineering because of its good tissue compatibility and the ability to manipulate the permeability for drug molecules (Hoare & Kohane, 2008; Hoffman, 2002). Over the past three decades, chemically and physically diverse hydrogels have become standard materials for drug delivery, contact lenses, corneal implants, and scaffolds for the regeneration of new skin, tendons and cartilage, and the encapsulation of cells (Deligkaris, Tadele, Olthuis, & Berg, 2010; Hamidi, Azadi, & Rafiei, 2008; Kraehenbuehl, Ferreira, Zammaretti, Hubbell, & Langer, 2009).

In recent years there has been a growing interest in hydrogel systems prepared by γ-ray irradiation. Gamma-ray irradiation is a valuable technique for the modification of the chemical and physical properties of polymeric materials (Gottlieb, Schmidt, & Arndt, 2005; Jha, Jha, Gupta, & Guha, 2010). The γ-ray irradiation method has advantages, such as relatively simple manipulation without need of any extra agents for polymerization and cross-linking, By contrast, the thermal activation method requires radical initiators and cross-linkers (Gottlieb et al., 2005; Jha et al., 2010; Park et al., 2013). For these reasons, γ-ray irradiation method is useful for preparing hydrogels for medical applications, for which even a small contamination is undesirable, and is often used to sterilize biomedical devices for medical and veterinary applications (Gad, 2008; Juby et al., 2012).

Natural polysaccharides such as hyaluronic acid (HA) have been extensively studied in medical applications since they provide intrinsic biological activity when used as basis for biomaterials (Leach & Schmidt, 2005). HA plays a prominent role in lubrication, cellular processes, wound healing, and is naturally angiogenic when enzymatically degraded to small fragments. Cellular interactions

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Table 1
Composition and water content of HA/CS/PVA hydrogels.

No.	Hydrogel sample[a]	Feed weight ratio (%)		Water content[c]	
		HA/CS[b]	PVA	Water	PBS
1	HA/CS/PVA-73-G15k	70	30	98.63 ± 0.11	96.85 ± 0.13
2	HA/CS/PVA-55-G15k	50	50	97.56 ± 0.12	95.71 ± 0.51
3	HA/CS/PVA-37-G15k	30	70	97.18 ± 0.36	93.93 ± 0.81

[a] Hydrogel samples were prepared under 15 kGy radiation, which exhibited the highest degree of gelation.
[b] Feed weight ratio of HA:CS = 1:1.
[c] Water content (%) = $(W_s - W_d)/W_s \times 100$, where W_s is the weight of the swollen gel and W_d is the weight of the dry gel.

with HA occur through cell surface receptors and influence tissue formation, inflammation, and morphogenesis (Leach & Schmidt, 2005; Oudshoorn, Rissmann, Bouwstra, & Hennink, 2007; Toh, Lim, Kurisawa, & Spector, 2012).

In addition, chondroitin sulfate (CS) is sulfated glycosaminoglycan (GAG) composed of repeating disaccharide units of D-glucuronic acid and *N*-acetyl galactosamine, sulfated at either the 4- or 6-positions (Strehin, Nahas, Arora, Nguyen, & Elisseeff, 2010). CS is an important structural component in connective tissues and cartilage. CS can bind to a core protein to produce highly absorbent aggrecan, which is a major structure inside cartilage and acts as a shock absorber, or it can produce syndecan, which is a cell receptor which can interact with adhesion proteins, cells and the extracellular matrix (ECM) (Hu, Li, Zhou, & Gao, 2011; Piai, Rubira, & Muniz, 2009; Wang, Shen, & Lu, 2003). This evidence suggests that HA and CS could be ideal candidate materials for promoting wound healing and tissue regeneration, provided they can be modified to improve their mechanical properties. Although various natural polymer hydrogel systems which prepared using radical initiators and cross-linkers have been developed (Strehin et al., 2010; Toh et al., 2012; Wang et al., 2003), natural polymer hydrogel system crosslinked by γ-ray irradiation methods has been rarely reported.

The main objective of this study is to develop a biocompatible hydrogel system prepared using γ-ray irradiation without any radical initiators and cross-linkers as scaffold materials for skin tissue engineering applications. We designed hydrogels composed of biocompatible natural polymers, HA and CS, and a synthetic polymer with relatively good mechanical properties, poly(vinyl alcohol) (PVA) (Jiang, Liu, & Feng, 2011; Lohakan, Jamnongkan, Pintavirooj, Kaewpirom, & Boonsang, 2010; Stammen, Williams, Ku, & Guldberg, 2001; Varshney, 2007). HA and CS derivatives with polymerizable residues were synthesized. The HA/CS/PVA hydrogels were prepared using γ-ray irradiation and their physicochemical properties were investigated to evaluate the feasibility of using the hydrogels in artificial skin substitutes. The water content, swelling kinetics, enzymatic degradation kinetics, and *in vitro* drug release behaviors of HA/CS/PVA hydrogels were investigated. *In vitro* cytotoxicity and the growth of human keratinocytes in HA/CS/PVA hydrogels were also evaluated.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA, Mw 1.6×10^6 Da, from Streptococcus equi.), chondroitin sulfate (CS, from bovine trachea), poly(vinyl alcohol) (PVA; Mw 89,000–98,000), triethylamine, glycidyl methacrylate and methacrylic anhydride were purchased from Sigma (St. Louis, MO, USA). Hyaluronidase, cefazolin, theophylline and sodium hydroxide were obtained from Aldrich (Milwaukee, WI, USA). Dulbecco's phosphate-buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM, high glucose, with L-glutamine, with pyridoxine hydrochloride, without sodium pyruvate), and heat-inactivated fetal bovine serum (FBS) were purchased from GIBCO BRL (Grand Island, NY, USA). Bovine serum albumin (BSA) was purchased from Sigma. Distilled and deionized water was prepared using a Milli-Q Plus System (Millipore, Bedford, MA, USA). All other chemicals used were reagent grade and were used as purchased without further purification.

2.2. Methods

2.2.1. Synthesis of HA and CS derivatives

One gram HA was dissolved in 100 ml distilled water. 2.2 ml triethylamine and 2.2 ml glycidyl methacrylate were added separately and thoroughly mixed for 1 h at 60 °C and then stirred overnight at room temperature. After reaction, the solution was precipitated in a 20-fold volumetric excess of acetone and dissolved in distilled water twice to remove excess reactants. The methacrylated hyaluronic acid (HA-MA) solution was lyophilized and stored desiccated at 4 °C.

0.5 g CS was dissolved in the 25 ml distilled water. After complete dissolution, methacrylic anhydride (MAA) (8 ml) was added drop-wise into the CS solution for 30 min. Then, a 5 N NaOH solution was carefully added to keep the reaction mixture at ~pH 8.0 (mol ratio of MAA/NaOH is 1/1.12). The reaction solution was stirred at room temperature for 2 h, at 4 °C for 8 h, and then moved to a refrigerator for another 14 h. The reaction mixture was precipitated in cold methanol and the precipitate was centrifuged and washed with a large amount of cold methanol several times until no methacrylic anhydride residue was traced by NMR. The resulting methacrylated chondroitin sulfate (CS-MA) was dried in a freeze-dryer. The chemical structures of HA and CS derivatives were characterized by 400 MHz ^{1}H NMR measurement (JNM-AL400, JEOL, Tokyo, Japan).

2.2.2. Preparation of HA/CS/PVA hydrogels

An aqueous solution of HA-MA, CS-MA and PVA at a concentration of 5% (w/v) were mixed in various ratios, as listed in Table 1. The mixed solutions were poured into a 24-well plate and then irradiated with various of doses γ-rays from a ^{60}Co source (MDS Nordion, CA, IR221n wet storage type C-188, Advanced Radiation Technology Institute, Korea) with 5–25 kGy under a nitrogen atmosphere at room temperature. Following cross-linking, the HA/CS/PVA hydrogel was washed three times in excess ultrapure water to remove compounds that had not reacted. The chemical structures of HA/CS/PVA hydrogels prepared by γ-ray irradiation were confirmed by Fourier transform infrared (FT-IR) spectroscopy. FT-IR spectra were recorded on a FT-IR-460 PLUS spectrometer (JASCO, Tokyo, Japan) ranging between 4000 and 650 cm^{-1}, with a resolution of 2 cm^{-1} and 64 scans. To determine the degree of gelation of hydrogels of various radiation doses, dried samples were cut from each hydrogel (5 mm × 5 mm) and weighed. The HA/CS/PVA hydrogel samples were then immersed in distilled water for 48 hr to remove uncross-linked fractions. The swollen hydrogel samples were dried in a vacuum oven at 37 °C for 48 h and then weighed.

The degree of gelation (%) is defined as follows (Park et al., 2013; Park, Nho, & Kim, 2004):

$$\text{Degree of gelation}\ (\%) = \left(\frac{W_{d2}}{W_{d1}}\right) \times 100 \quad (1)$$

where W_{d2} is the dry weight of the gel after 48 h immersed in distilled water and W_{d1} is the dry weight of the gel, before immersion.

2.2.3. Water content and swelling kinetics of HA/CS/PVA hydrogels

The HA/CS/PVA hydrogel samples were freeze-dried overnight. The freeze-dried hydrogel samples were weighed upon removal from the freeze-dryer and immersed in excess distilled water and PBS for 24 h at 37 °C. The water content was calculated on the basis of the weight difference of the hydrogel samples before and after swelling:

$$\text{Water content}\ (\%) = \frac{W_s - W_d}{W_s} \times 100 \quad (2)$$

where W_s is the weight of the swollen gel and W_d is the weight of the dry gel, respectively. The swelling kinetics of HA/CS/PVA hydrogels was measured in distilled water and PBS at 37 °C. The weight change of the hydrogels was recorded at each given time.

2.2.4. Enzymatic degradation of HA/CS/PVA hydrogels

The enzymatic degradation of HA/CS/PVA hydrogels was investigated by monitoring the mass loss of the hydrogel samples as a function of exposure time to an enzyme solution (Kim & Healy, 2003). The degradation behavior of the HA/CS/PVA hydrogels depending on the concentration of hyaluronidase solution and the composition of HA/CS/PVA was investigated for 14 days. Each HA/CS/PVA hydrogel sample (dimensions = 2.0 mm × 5.0 mm × 5.0 mm) was placed in PBS (pH 7.4) in a shaking water bath at 37 °C. When the hydrogel samples had attained a swollen state after 24 h immersion in PBS at 37 °C, the initial weight of the swollen samples was measured. The scaffolds were then placed in PBS containing hyaluronidase, 0.2 mg/ml sodium azide and 1 mM $CaCl_2$, and shaken gently at 37 °C. The mass loss of the samples was tracked over time based on the initial swollen weight. Three specimens were tested for each sample. The remaining weight (%) of HA/CS/PVA hydrogels was calculated using the following equation:

$$\text{Remaining weight}\ (\%) = \left(\frac{W_t}{W_0}\right) \times 100 \quad (3)$$

where W_0 is the initial weight of the hydrogel and W_t is the swollen weight of the hydrogel at each time point.

2.2.5. In vitro drug release behavior from HA/CS/PVA hydrogels

In this experiment, cefazoline as an ionic drug and theophylline as a nonionic drug were respectively adopted. These two model drugs were loaded into each HA/CS/PVA hydrogel sample by the swelling-loading technique. That is, dried HA/CS/PVA samples were soaked into each aqueous drug solution for 2 days at 25 °C, and allowed to swell to an equilibrium state to achieve a high loading content in the hydrogel. The fully swollen hydrogels removed from the drug solution were blotted with filter paper to eliminate the surface water and dried. The amount of drug loaded into HA/CS/PVA hydrogel was determined from the weight difference of hydrogel between the fully swollen state in drug aqueous solution and completely dried state.

Dried, drug-loaded HA/CS/PVA hydrogels were placed in 25 ml sealed vials containing PBS (0.1 M, pH 7.4) solution. All release studies were conducted in a shaker agitating at 50 rpm at 37 ± 0.5 °C. At predetermined time intervals, 3 ml aliquots of aqueous solution were withdrawn from the release medium and replaced with the same volume of fresh buffer solution. The medium was then analyzed using UV–visible spectrophotometry (Shimadzu Model UV 2101PC, Kyoto, Japan). The UV absorbances of cefazoline and theophylline were measured at 274 and 270 nm, respectively. The cumulative amount of released drug was determined by using the standard calibration curve. All release experiments were carried out in triplicate.

2.2.6. Cytotoxicity study of HA/CS/PVA hydrogels

The *in vitro* cytotoxicity of the HA/CS/PVA hydrogels was evaluated using an indirect extraction method (Song, Kim, Chun, Byun, & Lee, 2006). Extracts were obtained by immersing fragments of each of the hydrogels (0.2 g/mL) in a culture medium at 37 °C. After incubation for 2 days, the extracts of the hydrogel samples were collected and diluted to 25%, 50%, 75% and 100% strength with culture media. Human keratinocyte (HaCaT) cells, maintaining the full epidermal differentiation capacity *in vitro* but without tumorigenicity, were incubated using a culture medium composed of DMEM, 10% FBS, and 100 U/mL of penicillin-streptomycin. The cells were harvested using 0.05% trypsin-EDTA after washing with PBS. HaCaT cells were seeded in 96-well plates at a density of 1.0×10^4 cells/well and incubated at 37 °C in a wet 5% CO_2 atmosphere for 48 h. After the cells had attached to the wells, the initial culture medium was removed and replaced with the hydrogel-extract medium and incubated for 48 h at 37 °C. At the end of the incubation period, the extract medium was discarded and the cell viability was determined using the CCK-8 assay ($n = 4$). The untreated cells served as a positive control and were taken as being 100% viable.

2.2.7. Cell growth in HA/CS/PVA hydrogels

The ability of the HA/CS/PVA hydrogels to allow cell growth was investigated in an *in vitro* study. The dried HA/CS/PVA hydrogel samples were sterilized under UV for 12 h. The samples were cut into a disk shape (about 14 mm diameter and 1 mm thickness) and transferred a 24-well plate. Each well contained 2 ml culture media (DMEM, 10% FBS, and 100 U/mL of penicillin-streptomycin) and the plate was kept in an incubator (37 °C, 5% CO_2) for 24 h to allow the hydrogel disks to swell. The cultured HaCaT cells were seeded (1.0×10^4 cells/sample) onto each sterilized HA/CSA/PVA hydrogel sample. Fresh medium was added to each culture dish prior to incubation at 37 °C in a humidified 5% CO_2 atmosphere. The medium was changed every 2 days for 7 days. Cell viability in the hydrogels was investigated by MTT assay. 100 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (12 mM) was added to each well. After 4 h of incubation at 37 °C, the MTT solution was removed, and the insoluble formazan crystals that formed were dissolved in 100 μl of dimethylsulfoxide (DMSO). The absorbance of the formazan product was measured at 570 nm using a microplate reader (MQX200, Bio-Tek Instruments Inc., VT, USA).

In addition, HaCaT viability in HA/CS/PVA hydrogels was assessed with a live/dead staining assay kit (Invitrogen, Eugene, OR, USA) composed of fluorescein diacetate (FDA) and ethidium bromide (EB). This assay is based on the simultaneous determination of live and dead cells with the detection of intracellular esterase activity by FDA and of plasma membrane integrity by EB, respectively. In this assay, FDA is cleaved by cellular esterases present within viable cells to form a fluorescent green product that is membrane impermeable, whereas EB is a fluorescent red marker that only passes through the compromised membrane of nonviable cells and binds to nucleic acids, enhancing its fluorescence (Jeon, Bouhadir, Mansour, & Alsberg, 2009). The cell-cultured samples were washed and stained with FDA and EB mixture in PBS (FDA (3 μM): EB (1 μM)) for 10 min. After staining, the samples were washed in PBS twice, and the stained hydrogel samples

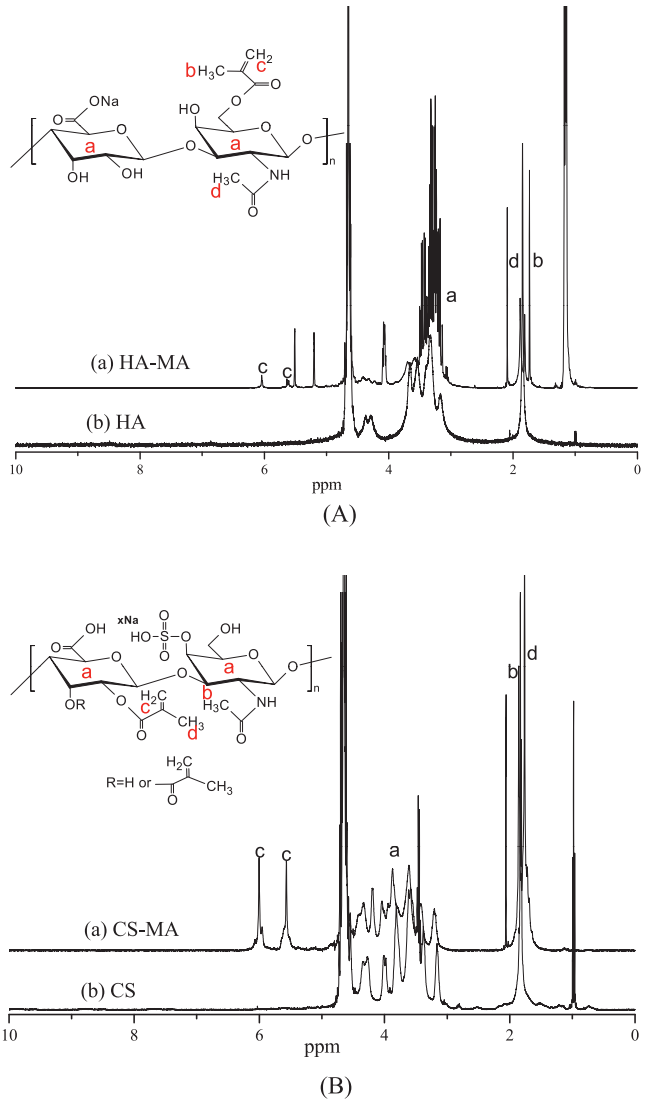


Fig. 1. ^{1}H NMR spectra of (A) HA and methacrylated HA (HA-MA), and (B) CS and methacrylated CS (CS-MA).

were observed using confocal laser scanning microscopy (Leica DMI4000B, Wetzlar, Germany) (50× magnification).

3. Results and discussion

3.1. Characterization of HA and CS derivatives

To prepare the HA and CS derivatives with polymerizable residues, HA-MA and CS-MA precursors were synthesized. The chemical structures of HA and CS derivatives were characterized by ^{1}H NMR measurement as shown in Fig. 1. Fig. 1(A) shows the ^{1}H NMR spectrum of HA and the HA-MA precursor. Evidence of the substitution of methacrylate groups on HA backbone was observed in the ^{1}H NMR spectrum (Fig. 1(A); (a)). It were observed not only peaks at about 1.8 ppm (peak d) and 3.0 to 4.4 ppm (peak a) corresponding to the methyl protons and the protons on the ring of original HA, but also two distinctive peaks at 5.6 and 6.0 ppm (peak c) attributed to the two protons of the double bond of the methacrylate group. The peak at about 1.7 ppm (peak b) was attributed to the methyl group adjacent to the double bond, which was not present in the original HA. The ^{1}H NMR spectrum of CS and the CS-MA precursor are shown in Fig. 1(B). The CS-MA spectrum shows peaks at

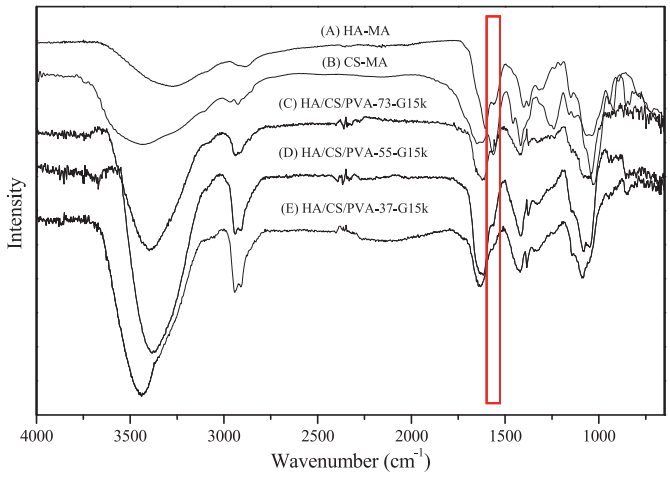


Fig. 2. FT-IR spectra of (A) HA-MA, (B) CS-MA, (C) HA/CS/PVA-73-G15k hydrogel, (D) HA/CS/PVA-55-G15k hydrogel, and (E) HA/CS/PVA-37-G15k hydrogel.

about 1.8 ppm (peak b) and 3.0 to 4.4 ppm (peak a) corresponding to the methyl protons and protons on the ring of the original CS molecule as well as two distinctive peaks at 5.6 and 6.0 ppm (peak c) attributed to the two protons of the double bond of the methacrylate group. The peak at about 1.7 ppm (peak d) is attributed to the methyl group adjacent to the double bond, which was not present in the original CS.

3.2. Characterization of HA/CS/PVA hydrogels

The HA/CS/PVA hydrogels with various compositions were prepared using the γ-ray irradiation technique as shown in Table 1. FT-IR spectroscopy measurements were carried out to confirm the synthesis of the HA/CS/PVA hydrogels. Fig. 2 illustrates FT-IR spectra of (A) HA-MA, (B) CS-MA, (C) HA/CS/PVA-73-G15k, (D) HA/CS/PVA-55-G15k, and (E) HA/CS/PVA-37-G15k hydrogels. The CS-MA and HA-MA show a characteristic peak at 1562 cm^{-1} which can be attributed to the C=C stretching vibration of methacrylate groups. After γ-ray radiation, these peaks in the FT-IR spectra of HA/CS/PVA hydrogels disappeared (Fig. 2(C)–(E)). As shown in Fig. 2, a sharp peak was observed in the region of 3401 cm^{-1}, it is due to the hydrogen bonded O—H stretching and N—H stretching vibration of *N*-acetyl side chain. The peaks at 1633 and 1419 cm^{-1} assigned to amide I group of C=O carboxyl and aromatic primary amine C=N stretching, respectively. The peak at 2938 cm^{-1} was due to the methyl C—H stretch responsible for glucuronic acid. The peak at 1079 cm^{-1} was observed for the primary alcohol C—O stretch.

The degree of gelation was estimated by measuring the insoluble parts after washing the HA/CS/PVA hydrogels. Fig. 3 shows the degree of gelation of the HA/CA/PVA hydrogels as a function of the irradiation dose. Irradiation doses greater than 15 kGy reduced the degree of gelation of the HA/CS/PVA hydrogels as shown in Fig. 3. This result indicates that a high dose of γ-rays could cause the destruction of the natural polymers, HA and CS, and reduce cross-linking structure in hydrogel. Therefore, HA/CS/PVA hydrogel samples prepared with a 15 kGy irradiation dose were used for this study.

3.3. Water content and swelling kinetics of HA/CS/PVA hydrogels

Table 1 shows the water content of the HA/CS/PVA hydrogels in deionized water and PBS, respectively. All of the HA/CS/PVA hydrogel samples exhibited relatively high water contents exceeding 90%. The water content somewhat increased as the HA/CS content in the hydrogel increased; this is due to the ionizable groups

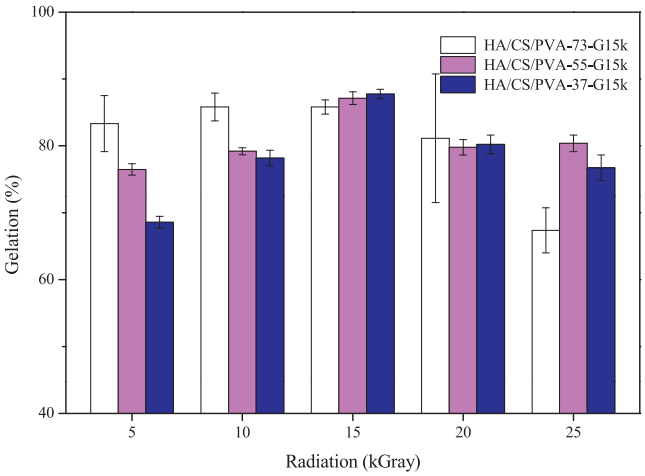
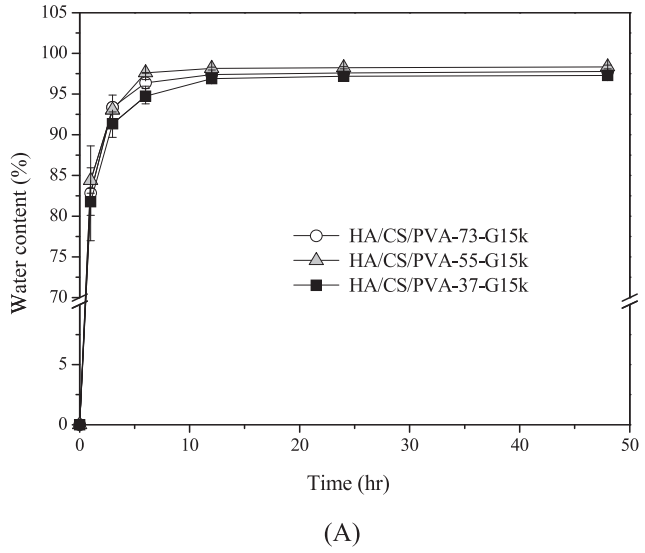


Fig. 3. Degree of gelation (%) of HA/CS/PVA hydrogels depending on irradiation dose [Degree of gelation (%) = $(W_{d2}/W_{d1}) \times 100$, where W_{d2} is the dry weight of the gel after 48 h immersed in distilled water and W_{d1} is the dry weight of the gel, before immersion].



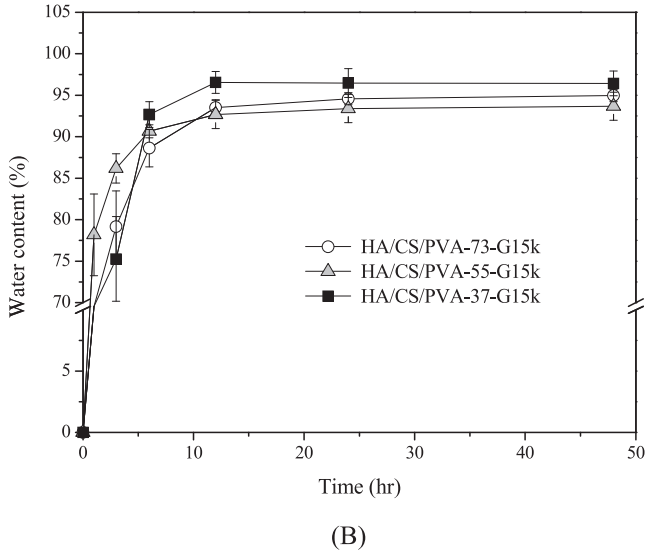
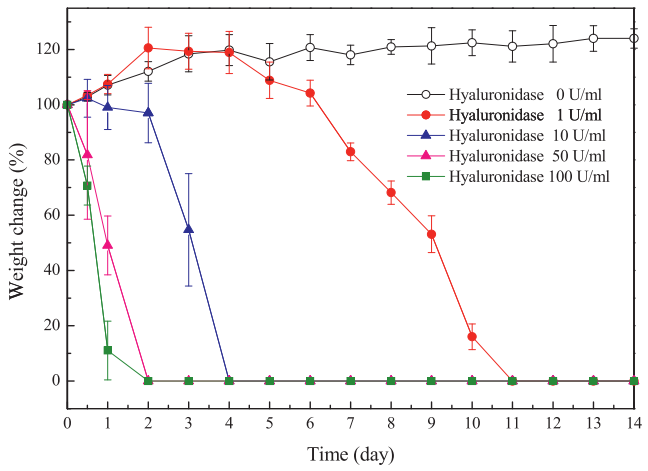


Fig. 4. Swelling profiles of HA/CS/PVA hydrogels; (A) in distilled water and (B) in PBS [Water content (%) = $(W_s - W_d)/W_s \times 100$, where W_s is the weight of the swollen gel and W_d is the weight of the dry gel].



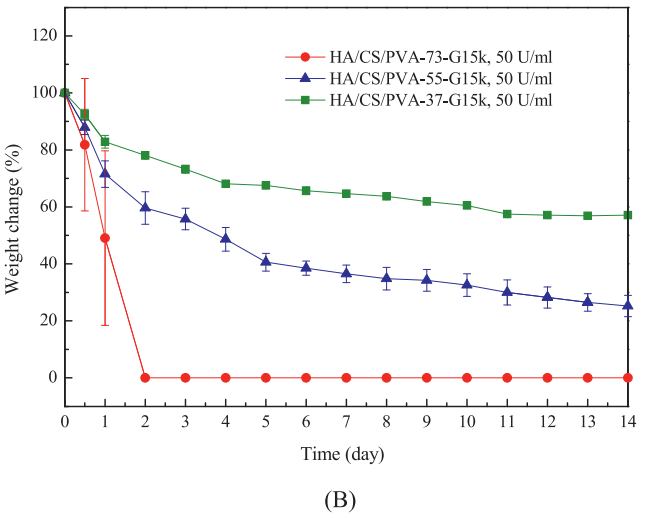


Fig. 5. Enzymatic degradation of HA/CS/PVA hydrogels; (A) depending on the concentration of hyaluronidase solution, and (B) depending on the composition of hydrogels in 50 U/ml hyaluronidase solution. Each point represents the mean ± S.D. of three samples.

(—COO^- and —OSO_3^-) on HA and CS. Ionized —COO^- or —OSO_3^- groups within the HA/CS/PVA hydrogel repel each other, resulting in increased free volume in the polymeric matrix, and thus, an increase in the swelling of the hydrogel. In addition, the water contents of the hydrogels were lower in PBS than in deionized water. The effect of the swelling media on the swelling behavior can be attributed to the shielding of —COO^- or —OSO_3^- repulsion, which prevents collapse of the gel, by the interactions between —COO^- or —OSO_3^- groups in HA/CS and the ions present in the PBS.

The swelling kinetics of HA/CS/PVA hydrogels in distilled water and PBS are plotted in Fig. 4. All hydrogels swelled so rapidly, reaching equilibrium within 24 h. The swelling kinetics of HA/CS/PVA hydrogels in PBS (Fig. 4(b)) are somewhat slower that of those in distilled water (Fig. 4(a)).

3.4. Enzymatic degradation behavior of HA/CS/PVA hydrogels

The enzymatic degradation of HA/CS/PVA hydrogels was investigated by monitoring the mass loss of the hydrogel samples as a function of exposure time to a hyaluronidase solution. Fig. 5 shows the *in vitro* degradation kinetics of the HA/CS/PVA

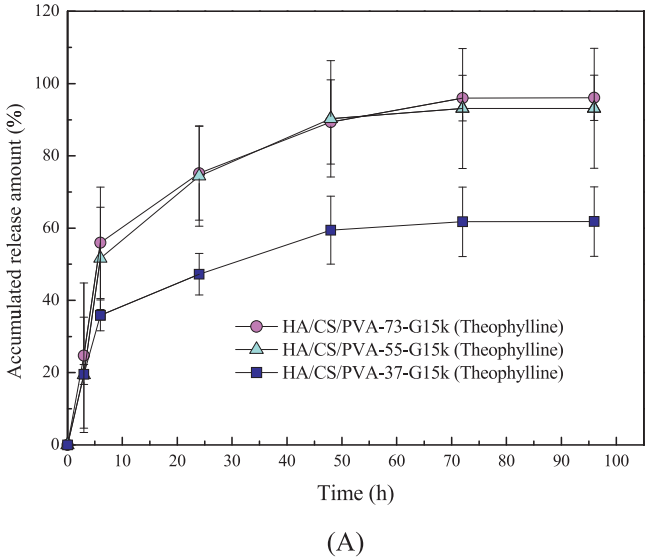


Fig. 6. *In vitro* drug release kinetics from HA/CS/PVA hydrogels at 37 °C; (A) release behavior of theophylline from HA/CS/PVA hydrogels, and (B) release behavior of cefazoline from HA/CS/PVA hydrogels. Each point represents the mean ± S.D. of three samples.

hydrogels. Degradation kinetics depended on both the concentration of hyaluronidase solution (0–100 U/ml) and the composition of the HA/CS/PVA hydrogel. The HA/CS/PVA-73-G15k hydrogels in hyaluronidase solution showed loss of mass proportional to the concentration of enzyme (Fig. 5(A)). The HA/CS/PVA-73-G15k hydrogels placed in blank solution without enzyme did not show any decrease in their mass as a function of time. While HA/CS/PVA hydrogels placed in 100 U/ml hyaluronidase solution were completely degraded within 2 days, hydrogels in 1 U/ml hyaluronidase solution showed complete degradation after 11 days of exposure time. In addition, the degradation behavior of hydrogels depended on composition of the HA/CS/PVA hydrogel. Fig. 5(B) exhibits the degradation profiles of HA/CS/PVA hydrogels prepared using different feed molar ratio of HA/CS to PVA (as shown in Table 1) in 50 U/ml hyaluronidase solution. As the composition of the natural polymer HA/CS increased in the HA/CS/PVA hydrogel, the degradation rate of the hydrogel gradually increased.

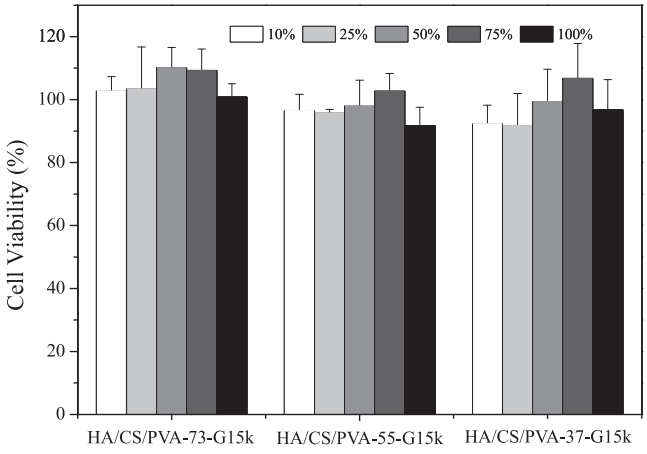


Fig. 7. Viability of HaCaT cells cultured with the extracts of HA/CS/PVA hydrogels with different concentrations. Values represent mean and standard deviation ($N = 4$).

3.5. Drug release study of HA/CS/PVA hydrogels

This study examined the *in vitro* drug release kinetics of the two model drugs, theophylline and cefazoline, from HA/CS/PVA hydrogels in PBS (0.1 M, pH 7.4) solution. The HA/CS/PVA hydrogels with a cross-linked network structure were still a coherent mass and could be physically manipulated during drug release study. The release profiles of theophylline, which is a nonionic drug, from HA/CS/PVA hydrogels, shown in Fig. 6(A), exhibited sustained release behavior and were influenced by the composition of hydrogels. The release of theophylline gradually accelerated [HA/CA/PVA-37-G15k < HA/CA/PVA-55-G15k < HA/CA/PVA-73-G15k] as the water content in PBS increased [HA/CA/PVA-37-G15k = 93.93 ± 0.81 (%); HA/CA/PVA-55-G15k = 95.71 ± 0.51 (%); HA/CA/PVA-73-G15k = 96.85 ± 0.13 (%) water content]. This result was attributed to increasing driving forces which swelled the hydrogels, allowing for drug diffusion. Fig. 6(B) exhibits the release behaviors of cefazoline, which is an ionic drug, from HA/CS/PVA hydrogels. Like the case of theophylline, the release kinetics of cefazoline showed similar tendencies depending on the composition. But, the release of ionic cefazoline slowed compared to that of the nonionic drug theophylline. These results indicated that the drug release behaviors from the HA/CS/PVA hydrogel are significantly influenced by the interaction

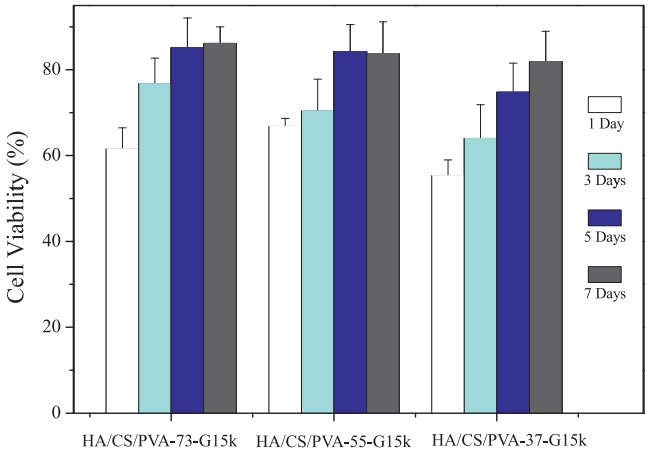


Fig. 8. Viability of HaCaT cells cultured after seeding on the HA/CS/PVA hydrogels. Values represent the mean and standard deviation ($N = 4$).

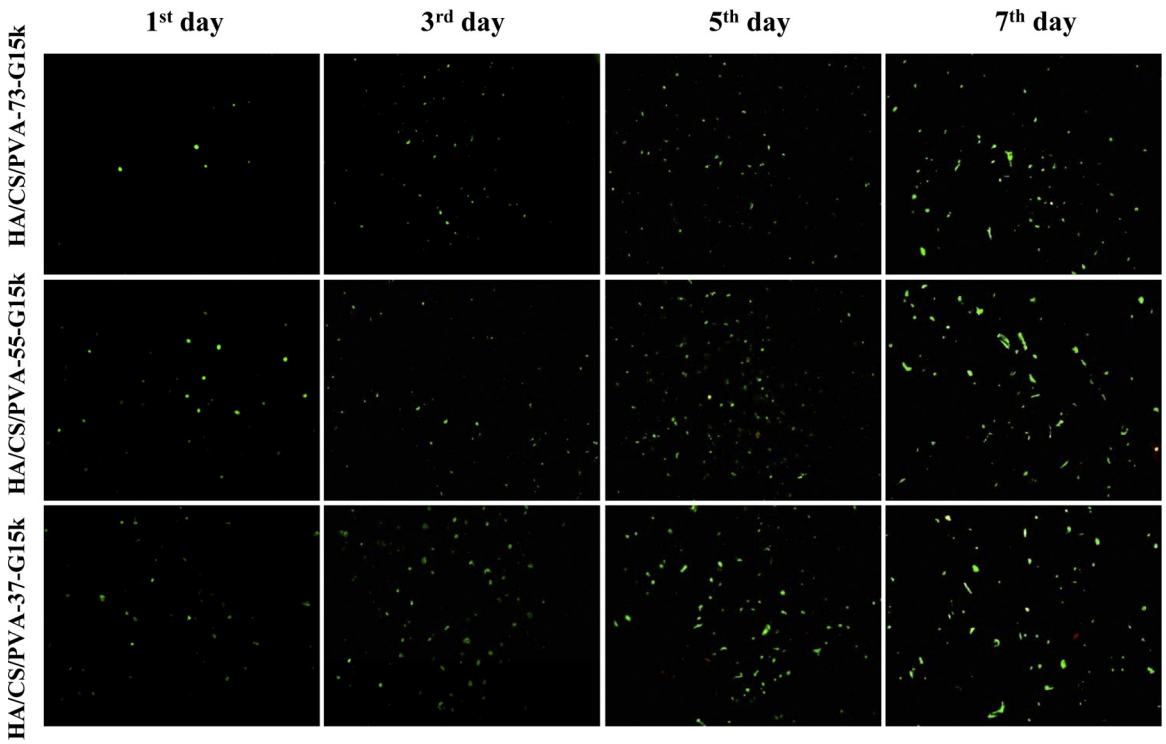


Fig. 9. Live/dead staining fluorescence microscopic images of HaCaT cells cultured on HA/CS/PVA hydrogels. Green and red colors indicate viable and dead cells, respectively. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

between ionic groups in the hydrogel and ionic drug molecules, as well as the swelling of hydrogel.

3.6. Cytotoxicity of HA/CS/PVA hydrogels

The *in vitro* cytotoxicity of HA/CS/PVA hydrogels was investigated using HaCaT cells by indirect extraction method. Fig. 7 shows the viability of HaCaT cells cultured with extracts of the HA/CS/PVA hydrogels. All HA/CS/PVA hydrogel samples tested showed relatively high cell viability. The viabilities of the HaCaT cells were more than 92% within the 48-hr observation period. In addition, the concentration of hydrogel extracts in culture did not significantly influence cell viability, as shown in Fig. 7. This indicates that the HA/CA/PVA hydrogels did not cause any significant adverse effects on cell viability.

3.7. Cell growth study in HA/CS/PVA hydrogels

The ability of the HA/CS/PVA hydrogels to allow cell growth was investigated using HaCaT cells in an *in vitro* study. Cultured HaCaT cells were seeded onto each sterilized HA/CS/PVA hydrogel sample and then incubated at 37 °C in a humidified 5% CO_2 atmosphere for 7 days. As shown in Fig. 8, all HA/CS/PVA hydrogel samples showed that the HaCaT cell growth in hydrogel gradually increased as a function of time. In particular, the growth rate of HaCaT cells in HA/CS/PVA hydrogel increased with increasing of HA/CS composition, natural polymers part, within hydrogel. The HA/CS/PVA-73-G15k hydrogels, which have a higher HA/CS content, exhibited higher cell growth compared with HA/CS/PVA-55-G15k and HA/CS/PVA-37-G15k samples, which have a lower HA/CS content. After 7 days, the HaCaT viability in all HA/CA/PVA hydrogels was more than 80%, compared with the control group of HaCaT cells on cultured on a cell culture plate.

In addition, HaCaT viability in HA/CS/PVA hydrogels was also assessed with a live/dead staining assay which is based on the detection of intracellular esterase activity by FDA and of plasma membrane integrity by EB. Fig. 9 illustrates the confocal fluorescence microscopic images of HaCaT cells cultured on HA/CS/PVA hydrogels. The living and dead cells were respectively stained and visualized in green and red under fluorescence microscope. It was observed that the green fluorescence intensity from living HaCaT cells was well distributed within hydrogel samples and gradually increased as a function of time. These results established that the HA/CS/PVA hydrogels prepared by the γ-ray irradiation method can be used to create a hydrogel system for controlled drug delivery and tissue engineering applications.

4. Conclusions

The objective of this study was to achieve a novel biocompatible hydrogel system prepared using γ-irradiation, for skin tissue engineering applications without any radical initiators and cross-linkers as scaffold materials, We designed hydrogels composed of biocompatible natural polymers, HA and CS, and a synthetic polymer with relatively good mechanical properties, PVA. HA/CS/PVA hydrogels were prepared by γ-ray irradiation using HA and CS derivatives with polymerizable residues. The physicochemical properties of the hydrogels were investigated. The chemical structures of the HA-MA and CS-MA precursors were confirmed by ^{1}H NMR measurement. The HA/CS/PVA hydrogels with various compositions were prepared under 15 kGy radiation, which exhibited the highest degree of gelation. All HA/CS/PVA hydrogels showed relatively high water contents of greater than 90%. These hydrogels reached an equilibrium swelling state within 24 h. The water content gradually increased as the HA/CS content, which have ionizable groups, in hydrogel increased. The enzymatic degradation kinetics of the HA/CS/PVA hydrogels depended on both the concentration of

hyaluronidase solution and the composition of HA/CS/PVA. Enzymatically degradable properties are a necessary feature for the design of artificial extracellular matrixes that promote regeneration of tissues *in situ* (Liu, Jiang, Shi, & Zhang, 2012). In addition, a successful drug delivery device relies not only on well-defined physicochemical properties but also on reproducible drug-release profiles (Lin & Metters, 2006). In this study, *in vitro* drug release behaviors of HA/CS/PVA hydrogels is significantly dependent on the interaction between ionic groups in the hydrogel and ionic drug molecules as well as the swelling of hydrogel.

From the cytotoxicity results of HaCaT cells cultured with extracts of the HA/CS/PVA hydrogels, all HA/CS/PVA hydrogel samples tested showed a relatively high cell viability of greater than 92%. HaCaT cell growth in HA/CS/PVA hydrogels gradually increased as a function of culture time. After 7 days, HaCaT cell viability in all HA/CA/PVA hydrogels was greater than 80%, compared with control group cultures on the cell culture plate. These results suggest that the HA/CS/PVA hydrogels prepared by γ-ray irradiation have great potential to serve as a drug delivery carrier or scaffold material for biomedical applications.

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