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Modulation of biomechanical properties of hyaluronic acid hydrogels by crosslinking agents

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Abstract: Modulation of both mechanical properties and biocompatibilities of hyaluronic acid (HA) hydrogels is very importance for their applications in biomaterials. Pure HA solution was converted into a hydrogel by using butanediol diglycidyl ether (BDDE) as a crosslinking agent. Mechanical properties of the HA hydrogels have been evaluated by adding up different amount of BDDEs. While the mechanical properties of the obtained HA hydrogels were evaluated by measuring their crosslinking degrees, elastic modulus and viscosity, their *in vitro* biocompatibilities were done by measuring the degrees of anti-inflammatory reactions, cell viabilities and cytotoxicity. The degrees of anti-inflammatory reactions were determined by measuring the amount of nitric oxides (NOs) released from lipopolysaccharide(LPS)(+)-induced macrophages; cell viability was evaluated by observing differences in the behaviors of fibroblasts covered with the HA hydrogels, compared with those covered with the films of Teflon and Latex. Cytotoxicity of the HA hydrogels was also evaluated by measuring the degrees of viability of the cells exposed on the extracts of the HA hydrogels over those of Teflon, Latex and pure HA solutions by the assays of thiazoly blue tetrazolium bromide (MTT), neutral reds, and bromodeoxyuridine (BrdU). The results showed that employment of BDDEs beyond critical amounts showed lower biocompatibility of the crosslinked HA hydrogels but higher crosslinking degrees and mechanical properties, indicating the importance of controlling the HA concentrations, BDDE amounts and their reaction times for the synthesis of the crosslinked HA hydrogels for their clinical applications as biomaterials. J Biomed Mater Res Part A: 103A: 3072–3080, 2015.

Key Words: hyaluronic acid, mechanical property, anti-inflammatory, biocompatible, hydrogel

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INTRODUCTION

Hyaluronic acid (HA), a linear anionic non-sulfated, high molecular weight glycosaminoglycan with an alternating repeating unit of D-glucuronic acid and *N*-acetyl-D-glucosamine, has been recognized as an important biomedical polymer. HA, distributed as a component of the extracellular matrix in connective, epithelial and neuronal tissues in body, is physiologically functional due to its physiochemical and biomechanical properties such as viscoelasticity, lubricity with high water retention, biodegradation by both hyaluronases and reactive oxygen species,[1,2] biocompatibility, morphogenesis, wound repair and suppression of inflammatory reactions through cell surface receptors.[3–5] Recently, Kim et al.[6] demonstrated experimentally that pure HA polymers induced less *in vivo* expression of inflammatory cytokines such as nitric oxide (NO), interleukin-1 (IL-1), cyclooxygenase-2 (COX-2), and prostaglandin E2 (PGE2) than that of the control group of a mouse model. Furthermore, inhibitions of NO-induced apoptosis and differentiation of articular chondrocytes *in vitro* by HA were also

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Author's contributions: The corresponding authors of the manuscript have equal contributions to this study. SC primarily worked on the experiments, Ms. ML, Ms. JJ and Mr. DS did on the tests of rheology and anti-inflammatory reaction, and Ms. SL and HL did the tests of both biocompatibility and cell toxicity. All authors read and approved the final manuscript.
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reported.[7–9] In human chondrocytes, HA affected mitochondrial function and mitochondria-driven apoptosis.[9]

Even though excellent mechanical and biochemical properties of HA polymers have been observed in natural states, chemical crosslinking of HA polymers through either inter- or intra-functional groups of their side chains, such as hydroxyl and carboxylic acid groups, has been tried to meet the requirements of its applications to biomaterials such as increased resistance to both biodegradation and lubrications and also needed to fabricate medical devices in desired forms such as hydrogel, porous degradable scaffolds and delivery vehicles of bioactive molecules and cells.[10] Chemical crosslinking of pure HA polymers showed, as expected, induced increase in their resistance to mechanical stress under applied living tissues and slower biodegradation than those of the unmodified HA polymers, meeting some important properties required in biomaterials. These improved properties came from the formation of both inter- and intra-molecular crosslinking of HA polymers.[11,12] Chemical cross-linkings of HA polymers have been obtained in many research groups by using reactive crosslinking agents such as butanediol diglycidyl ether (BDDE)[13,14] and divinyl sulfone (DVS)[15] among others, and have been widely used as medical devices such as dermal fillers, drug delivery and adhesion barriers. For examples, the commercial dermal fillers such as Hylaform and Restylane were fabricated by using DVS and BDDE as crosslinking agents, respectively.[16,17] Nishi et al. reported that degradation of the HA hydrogel crosslinked with BDDE showed excellent biocompatibility during hydrolyzation of the BDDEs into simple diols.[18]

The crosslinked HA polymers showed improved properties, leading to their applications to medical devices, but the chemically crosslinked HA hydrogels have yet to be studied in detail to observe the effects of the amount of BDDEs and other reaction conditions such as HA concentrations, dynamics and reaction times on their biomechanical properties and biocompatibility. Particularly, the degrees of both anti-inflammatory reactions and cell compatibility should be investigated as important factors to its clinical applications to medical devices. Furthermore, the relationships between biocompatibility and the mechanical properties of the crosslinked HA hydrogels, including the degrees of crosslinking and biodegradation of HA polymers, should be analyzed at the same time to predict their clinical applications to other possible medical devices such as osteoarthritis, drug delivery, and dermal fillers.[19–21]

Further expansion of the ranges of its biomedical applications to clinical biomaterials has been required through modulated modifications of its chemical structures and rigorous evaluations of their mechanical and biocompatibility properties. We here report the effects of the crosslinking agent BDDEs and related reaction conditions on the mechanical and biocompatibility properties of the crosslinked HA hydrogels, by observing both rheological properties and degrees of crosslinking of HA polymers as well as anti-inflammatory properties and other biocompatibility properties of the crosslinked HA hydrogels. This study was to investigate the importance of the control of mechanical properties and biocompatibility of the crosslinked HA hydrogels.

MATERIALS AND METHODS

Materials

Hyaluronic acid sodium salt (HA) with a molecular weight of ~1000 kDa was prepared in Hanmi Pharmaceutical (Pyeongtaek, Korea) and employed in this study. While 1.4-butanediol diglycidyl ether (BDDE), lipopolysaccharide (LPS) endotoxins, hyaluronidases (Type IV), and sulfuric acid solution were purchased from Sigma–Aldrich (MO, USA), the nitrate/nitrite colorimetric assay kit was purchased from Cayman Chemical (MI, USA). All the chemicals were employed as received.

Synthesis of crosslinked HA hydrogel

After dissolving 26.5 mg mL^{-1} HA polymers in 0.1 M NaOH solution at room temperature, different amounts of BDDE were added and then mixed for crosslinking of HA polymer. The mixture solution was homogenized with a propeller impeller at 500 rpm for 60 min by top-driving stirrer (Heidolph, Germany). After crosslinking HA polymers for certain reaction times, the crosslinked HA solution was dialyzed in phosphate buffered solution (PBS). Crosslinking HA solutions were controlled by employing different conditions of BDDE amount and reaction times, that is, 0.5–10.0 ppm BDDEs and 6~36h reaction.

Characterizations of HA polymers

The ferment, employed HA polymers were characterized by using glucuronic acid solution as a standard according to the standard protocols supplied by the vendor (Sigma–Aldrich; MO). Detailed information of purifications of the fermented HA polymers has been reported in our previous work.[18] After addition of HA polymers (0.17 g) in 20 mL sulfuric acid solution, the solution was heated in boiling water bath for 15 min. The prepared HA solutions and glucuronic acid as standard solutions were diluted with sulfuric acid solution to final concentrations of ~6.5–65 μg g^{-1} D-glucuronic acid in separate vessels (0.5 mL). Both HA and standard solutions were reacted with borax-sulfuric acid (2.5 mL) in boiling water bath for 15 min. After addition of 0.1 mL carbazole ethanol, the solutions were heated for 15 min. After cooling down, the absorbance of both the obtained HA and standard solutions were measured by UV spectroscopy (Ultrospec 2100 Pro, GE Healthcare; USA) at the wavelength of 530 nm. The HA concentration of each sample was calculated by comparison of the absorbance values of the samples with that of the D-glucuronic acid.

Measurement of rheological properties of HA solutions

Rheology and viscosity properties of the HA solutions were measured by employing the 26.8 mg mL^{-1} HA solutions according to the method suggested by Kinexus (Malvern Instruments; Malvern, England). The solutions were crosslinked in advance by additions of 0.5–10 ppm BDDEs as a crosslinker. Oscillatory rotation of parallel plates was

performed with a 1 mm gap. Oscillation measurements of the HA solutions were taken at a frequency range of 0.1–10.0 Hz. All measurements were carried out at 25 °C.

Measurement of the degree of crosslinking of HA polymers

The degrees of crosslinking of HA polymer were measured as described below according to the method suggested by Kablik *et al*.[22] HA polymers were degraded by bovine hyaluronidases (Type IV) in acetate buffer (100 unit/mL) at pH 5.0 and 37 °C for 72 hr. High performance liquid chromatography (Agilent 1200 HPLC system, Agilent Technologies; CA), consisting of a quaternary pump, an auto-sampler, a thermo-stated column compartment, a solvent cabinet with degasser and an ultraviolet detector, was employed for measurement of molecular weight of the degraded HA polymers. Column anionic exchanger (4×250 mm^2, CarboPac PA100, Dionex; CA) was used with different ratios of the mobile phases consisting of both water and 0.4*M* sodium phosphate (pH 5.8) over time. Specifically, the ratios of the mixture solutions were 90:10, 20:80, and 90:10 at the time points of 0, 55, and 57 min, respectively. After injection of 50 μL sample solutions with a 1 mL glass syringe and control of flow rate at 0.8 mL min^{-1}, UV detection of the processed samples was performed at the wavelength of 232 nm.

In vitro cell culture

Human foreskin fibroblasts (CRL-2429) and 264.7 macrophage (mouse macrophage cell line) were employed for the tests of *in vitro* biocompatibility of HA samples. The fibroblast and 264.7 macrophages obtained from American Type Culture Collection (ATCC; MD) were used for the tests of both anti-inflammation and biocompatibility of HA samples. *In vitro* cell culture was performed at 37 °C in a humidified atmosphere (5% CO_2/95% air) in Dubellco's modified Eagle's medium (DMEM, Gibco BRL; Life Technologies; FL) containing 10% fetal bovine serum (FBS) and 1% penicillin (10,000 U mL^{-1})-streptomycin (10,000 U mL^{-1}). While fibroblast growth medium-2 (FGM-2) and penicillin-streptomycin were purchased from Lonza Inc. (Basel, Switzerland), the assay kit of live & dead viability/cytotoxicity for mammalian cells was bought from Life Technologies (FL). The assay kit of *in vitro* toxicology by bromodeoxyuridine (BrdU) was purchased from Roche Biomedical (Mannheim, Germany), the methylthiazolyl tetrazolium assay (MTT) and neutral red were purchased from Sigma–Aldrich (MO). All chemicals were used as received.

Measurements of both anti-inflammatory activity and cell viability assay

Both raw 264.7 cells (1×10^5 macrophages/mL) and 0.11 mmol HA hydrogels in fetal bovine serum (FBS)-free DMEM with lipopolysaccharide (LPS; 1 μg mL^{-1}; Sigma, MO) were prepared in a 96 well plate and then incubated in an *in vitro* incubator (5% CO_2 at 37 °C) overnight. 100 μL supernatant from each culture media was transferred onto a new 96-well plate, and the equivalent amount of Griess reagent mixture was added as below, according to the method suggested by the vendor (Cell Biolabs; CA). The amount of NOs produced by the HA samples ($n = 4$) was measured as the following. Either the uncrosslinked pure HA hydrogel or crosslinked HA hydrogels in PBS was located in an *in vitro* incubator for 24 h. A top layer was obtained by centrifuging the sample solutions at 1000 rpm for 10 min (Hanil Science Industrial; Seoul, Korea). The top layer extracts were added in the polystyrene tissue culture flask containing macrophages, which were activated in advance by LPS(+) for 24 h. The degrees of crosslinking of the pure HA solutions were controlled by the amounts of BDDEs, frequencies and their reaction times as described in the session of HA solution preparations. The culture plates were incubated at room temperature for 10 min, and the degrees of anti-inflammatory reactions of the samples were measured by using an enzyme-linked immuno-absorbent assay (ELISA) reader (Tecan; Mannedorf, Germany) at the wavelength of 540 nm. By considering the values obtained from the LPS-induced macrophages in PBS as 100%, the reduced percentage obtained by addition of HA samples into the LPS-induced samples were used as indications of the degrees of anti-inflammations. Calibration curves of LPS standards were prepared in advance by using sodium nitrite as the standard agents. Nearly 540 nm was employed as a reference wavelength.

Evaluation of cytotoxicity of the crosslinked HA hydrogels

The crosslinked HA hydrogels were synthesized by adding up different amounts of BDDEs ranging from 0.5, 1.0, 5.0, to 10.0 ppm. The cytotoxicity of the extracts from the HA samples ($n = 4$) were measured in 37 °C by the assays of methylthiazolyl tetrazolium (MTT), neutral red, bromodeoxyuridine (BrdU) after locating 26.5 mg pure and crosslinked HA hydrogels and the films of Teflon and Latex in 1 mL FGM-2 cell culture medium for 72 h. The biological assays described below were performed by using the protocols as thoroughly reported in our previous works.[23,24]

MTT assay. Cell viability of the HA hydrogels were *in vitro* evaluated by using human foreskin fibroblasts (passage 10) by the MTT assay.[25] The HA hydrogels were evaluated according to the protocol suggested by the vender (Sigma; MO). In brief, the fibroblasts were *in vitro* cultured in a 96 well polystyrene plate at a density of 1×10^4 cells for 24 h. Cell culture lasted for another 24 h after addition of the extracts of the HA hydrogels (200 μL). We employed the extracts of Teflon and Latex films as positive and negative controls, respectively. After adding up 20 μL MTT/PBS solution in 100 μL culture medium, *in vitro* cell culture was performed for 4 h. Sequentially, the removal of the culture medium and then addition of 100 μL dimethylsulfoxide (Sigma; MO) followed. The optical density of the final solution was measured by the microplate reader at the wavelength of 570 nm.

Neutral red assay. About 100 μL extracts of the HA hydrogel and the films were dispensed into the well plates with fibroblasts at a density of 1×10^4 cells/well. Cell culture lasted in 5% CO_2 incubator at 37 °C for 24 h. Subsequent to

removal of the medium from the well, cell culture lasted for another 24 h after addition of the prepared extracts. The culture medium and 0.33% neutral red solution [10:1 (v/v)] (Gibco BRL, Australia) were added in the *in vitro* incubator, and cell culture lasted for another 2 h. After washing with the fixation solution of the assay and proceeding reaction for 10 min, 100 μL solubilization solution was added per well. Optical densities of the samples were measured at an absorbance wavelength of 550 nm by the microplate reader by referencing the wavelength at 690 nm.

BrdU assay. Fibroblasts cells at a density of 1×10^4 cells were *in vitro* cultured in the 96-well culture plate for 24 h after adding up 100 μL of 1 mL extract of the crosslinked HA hydrogels and films on the cell culture medium. A 10 μL BrdU labeling solution was added into the culture medium and then cell culture lasted for another 2 h. After removing the labeling medium, a solution of both 200 μL Fixdent and 100 μL anti-BrdU peroxide-labeled anti-BrdU antibody per well was loaded according to the manufacturer's protocol (Roche Biomedical, Germany). A 25 μL H_2SO_4 solution (1*M*) was added into each well after washing the wells, and then optical density of the samples was measured at the absorbance wavelength of 450 nm by the microplate reader by referring to that of 690 nm as a standard peak.

Covering of the HA hydrogels on the cells and live and dead assay. Effects of direct contact of the 15% crosslinked HA hydrogels (150 mg mL^{-1}) on the fibroblasts on the *in vitro* culture plate have been evaluated by covering the cells with the crosslinked HA hydrogels. For the assay, 100 ppm BDDEs were employed for crosslinking of 150 mg mL^{-1} HA solutions for 24 h. After seeding fibroblasts (passage 10) at a density of 2×10^4 cells cm^{-2} on the six-well plate, the cells were covered with either the HA hydrogels (200 μL) or the films of Teflon and Latex (diameter = 10 mm).

To observe *in vitro* cell viability, a live and dead assay was performed over the HA hydrogels. After incubation of the cells for another 24 h, 200 μL reagent of the live and dead assay was added into the cell culture plate. Both viability and morphological changes of the cells were observed by a fluorescein microscope (Leica DMLB, Leica; Wetzlar, Germany).

Statistical analysis

Data were expressed as mean ± standard deviations. Statistical significance was assessed with one-way and multi-way ANOVA by employing the SPSS 12.0 program (ver. 18.0, SPSS, IL). The comparisons between two groups were carried out using a *t* test. The samples were considered as significantly different when $p < 0.05$.

RESULTS

Characterization of HA polymer and measurement of its rheological properties

HA, a polysaccharide polymer with a repeating unit of D-glucuronic acid and *N*-acetyl-D-glucosamine, was verified by measuring the chemical structures of glucuronic acid. Various rheological and biological behaviors were measured in aqueous solutions by changing the experimental conditions such as concentrations of pure HA polymers, amounts of BDDE crosslinking agent and the crosslinking reaction times. There has been a report that the mechanical properties of HA samples were highly affected by the molecular weights of HA polymer itself as well as the degrees of crosslinking and the concentrations of its crosslinked hydrogel.[13,26,27] In this study, we evaluated the rheological properties and biocompatibilities of the crosslinking and crosslinked HA hydrogels by changing the concentrations of BDDEs and reaction times. While both elastic modulus and viscosity of the crosslinking HA hydrogel and the degrees of crosslinking were measured for observations of rheological behaviors, both anti-inflammatory behaviors and cell viabilities were measured to observe biocompatibility of the crosslinked HA hydrogels. The crosslinking mechanism of pure HA solutions via the reaction of hydroxyl groups of HA polymer side chains with the epoxide groups of the crosslinking agent BDDE is shown in Figure 1. While crosslinking of HA polymer were processed by employing different amount of BDDE ranging from 0.5 to 5.0 ppm and reaction times from 6 to 24 h, the frequencies of rheological tests were modulated from 0.1 to 1.0 Hz for the HA solutions, which was treated with 1.0 ppm BDDEs for 6–24 h.

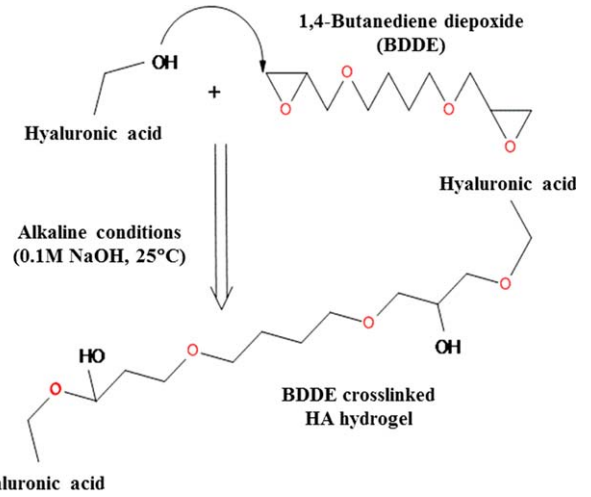

FIGURE 1. Mechanism of synthesis of HA hydrogel by the crosslinking reaction between the hydroxyl group of HA and epoxide of butadiene diepoxide (BDDE). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The experimental results indicated that as we increased the amount of BDDEs, its elastic modulus and viscosity were increased as we expected. Precisely, when we increased BDDE amounts from 0.5, 1.0, 5.0 to 10.0 ppm at a fixed frequency of 1.0 Hz, its solutions showed corresponding elastic modulus of 1904, 2324, 2699 Pa and ND, which is beyond the limit of elastic moduli of the samples by the employed rheometer [Fig. 2(A)]. In addition, at a fixed 0.5 ppm BDDE, its elastic modulus increased from 1668, 1668, 1904, and 2319 Pa as we increased the frequency from 0, 0.1, 1.0, and 10.0 Hz at the fixed reaction time of 24 h. The

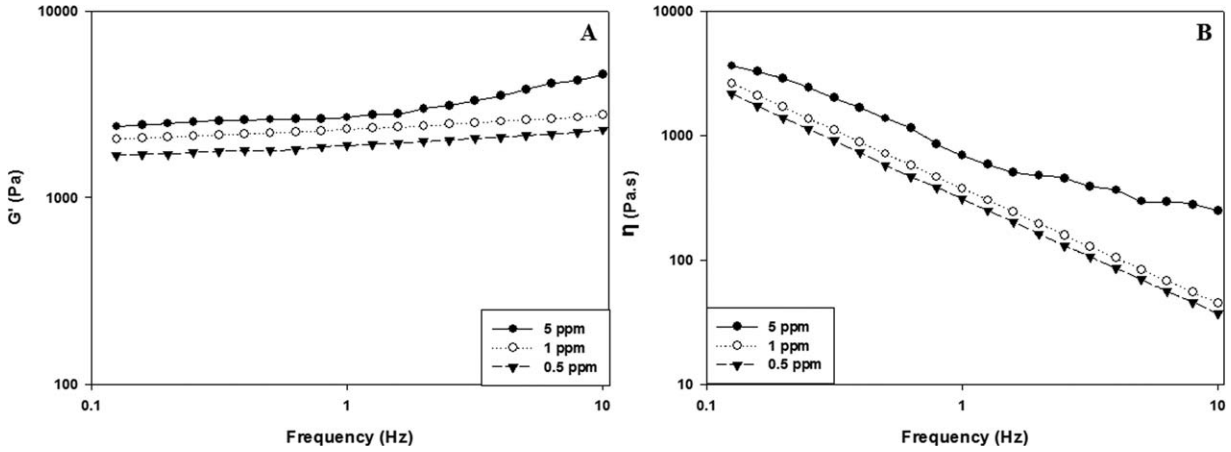

FIGURE 2. Elastic modulus (a) and viscosity (b) of crosslinking HA hydrogels at different BDDE amounts at the fixed reaction time, 24 h.

results showed that while the viscosities of the crosslinking HA solutions increased at the fixed frequency when we increased the amount of BDDEs, increases in frequency induced decrease in their elastic modulus and viscosities at a fixed BDDE and reaction time [Fig. 2(b)]. When we increased BDDE amounts from 0.5, 1.0, 5.0, to 10.0 ppm at a fixed frequency of 1 Hz, the crosslinking HA solutions showed corresponding increases in their viscosities from 308, 374, 690 Pa and ND, which is beyond the limit of detection by the employed rheometer. Also, at a fixed condition of 0.5 ppm BDDE and 24-h reaction time, their elastic viscosities decreased from 2696, 2167, 308 to 37 Pa as we increased the frequency from 0, 0.1, 1.0, and 10.0 Hz.

Next, we measured both elastic modulus [Fig. 3(a)] and viscosity [Fig. 3(b)] of the crosslinking HA solutions by changing the reaction times while fixing the amount of BDDE at 1 ppm. In specific, when we increased the reaction times from 6, 9, 18, to 24 hr at a frequency of 0.1 Hz, its solutions showed increase in elastic modulus from 513, 903, 1662 to 2070 Pa, correspondingly [Fig. 3(a)]. At a fixed 1.0 ppm BDDE, its elastic modulus increased from 2027, 2070, 2324 to 2776 Pa as we increased the frequency from 0, 0.1, 1.0 to 10.0 Hz at the fixed reaction time for 24 h. Viscosities of the crosslinking HA hydrogels showed increase in both elastic modulus and viscosity at the fixed BDDE and frequency as we increased the reaction times [Fig. 3(b)]. When we increased reaction times from 6, 9, 12, 18 to 24 hr at the fixed frequency of 1.0 Hz, the crosslinking HA solutions showed increase in the viscosity from 118, 168, 218, 310 to 374 Pa s^{-1}, correspondingly. At a fixed reaction time of 24 h, its viscosity decreased from 3255, 2644, 374, to 45 Pa s^{-1} as we increased the frequency from 0, 0.1, 1.0 to 10.0 Hz at the fixed 1.0 ppm BDDE.

On the basis of these results of the rheological properties of the crosslinking HA hydrogels, we went on to measure the degrees of crosslinking of the pure 26.9 mg mL^{-1} HA solutions by changing BDDE amounts (Fig. 4) and reaction times (Fig. 5) at the fixed conditions of 0.7 Hz frequency and 1.0 ppm BDDE. When we increased the degrees of crosslinking from 5.0, 5.6, 5.8 to 6.3 by using 0.5, 1.0, 5.0 to 10.0 ppm BDDEs, the elasticity of the crosslinking HA hydrogels saw increases from 1876, 2286, 2645 Pa to ND. Correspondingly, their viscosities increased from 382, 463, 855 Pa s^{-1} to ND. The changes in elasticity of the crosslinking HA hydrogels were due to increases in degrees of their crosslinking, which were measured as 5.0, 5.6, 5.8 and 6.3%, accordingly (Fig. 4). These experimental data were also supported by the experimental results, where the degrees of the crosslinking of the HA hydrogels increased according to the reaction times increased from 6, 9, 12, 18 to 24 h at the fixed conditions of 0.7 Hz frequency and 1.0 ppm BDDE for 26.0 mg mL^{-1} HA solution (Fig. 5). While elastic modules of the crosslinking HA hydrogels were measured as 683, 1010, 1304, 1883, and 2286 Pa, their viscosities were done as 143, 208, 267, 383, and 464 Pa s^{-1}, respectively. Correspondingly, the degrees of crosslinking of the HA solutions were measured as 2.8, 2.8, 4.5, 5.0, and 5.6%.

Measurement of anti-inflammatory activity of crosslinking HA hydrogels

After exposing the crosslinked HA hydrogels on the LPS(+)-induced macrophages, the amount of NO produced was measured to observe the degrees of anti-inflammations by the crosslinked HA hydrogels. Before tests of the crosslinked HA hydrogels, the degrees of suppression of NO productions by the uncrosslinked pure HA solutions were measured by considering the amount of NO produced by the LPS(+)-induced macrophages in the cell culture medium only as 100% [Fig. 6(A)]. When we added different concentrations of pure HA solutions ranging from 0.1, 0.3 to 0.5% (w/v) into the LPS(+)-induced macrophages in the medium, the degrees of NO production decreased to 89, 82 to 80%. The results indicated that while the pure HA solutions with 0.1 ppm BDDE showed no difference in statistics in NO production, those crosslinked with either 0.3 or 0.5% BDDE showed in statistic significantly reduced amount of NO production compared to that of HA only [Fig. 6(A)] ($p < 0.05$).

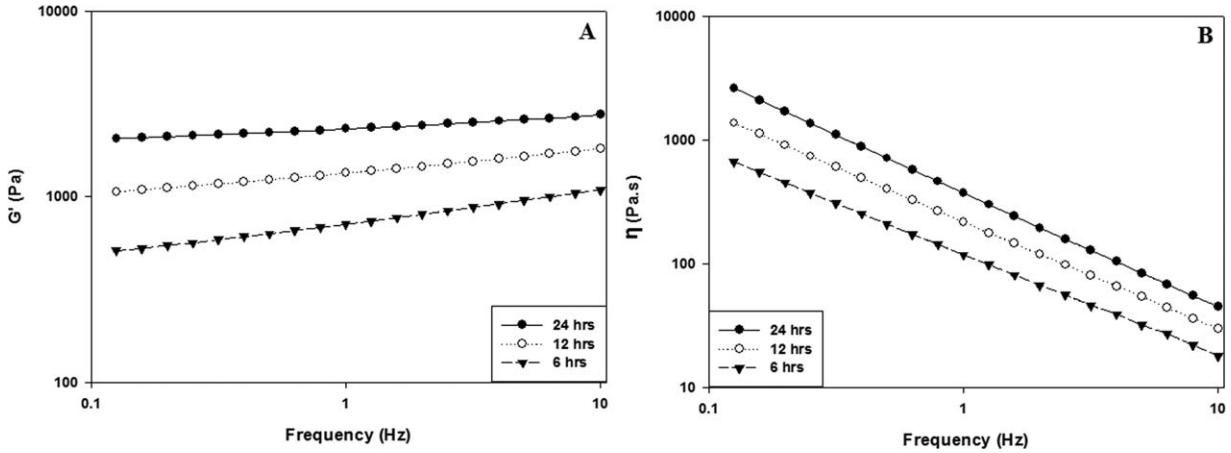

FIGURE 3. Elastic modulus (A) and viscosity (B) of the HA hydrogels depending on the reaction time at 1 ppm BDDE.

These results indicated different degrees of anti-inflammatory effects of pure HA polymer. Next, the effects of BDDE cross-linkers on anti-inflammations of the crosslinked HA hydrogels were measured by adding different amount of BDDEs at different concentrations of HA hydrogels [Fig. 6(B)]. When we added different of BDDE from 0.5, 1.0, 5.0 to 10.0 ppm into the medium, there were no differences in the amount of NO productions between the samples, and we considered this value as nearly 100% inflammation. When we added the HA hydrogels crosslinked with 0.1% BDDE into the medium, there were no differences in statistics in the degrees of anti-inflammations, showing nearly 100% NO productions. However, when we employed 0.3% HA solutions, the amount of NO productions by the crosslinked HA hydrogels decreased from 100% to 64, 68, 97 and 99% by additions of 0.5, 1.0, 5.0 and 10.0 ppm BDDEs, respectively. These results indicated that while the HA hydrogels crosslinked with 0.5 and 1.0 ppm BDDEs suppressed inflammatory reactions, those with 5.0 and 10.0 ppm BDDEs had not induce significant amount of inflammation suppression in statistics. The 0.5% HA solutions showed similar trends in the degrees of NO productions, showing significant reduction of NO productions to 58, 65, 95, and 97%, respectively, in statistic ($p < 0.5$). Further reduction of NO productions followed as we increased the concentrations of the crosslinked HA hydrogels. The NO productions of the HA hydrogels crosslinked by 0.5 ppm BDDEs decreased to 50% for the 1, 3, 5% crosslinked HA hydrogels, but when we increased the amount of BDDEs to 5.0 and 10.0 ppm, there were no significant amount of reductions in NO productions, showing approximately 98% NO productions for the conditions employed. On the basis of these results, we concluded that the HA hydrogels crosslinked with higher amount of BDDEs induced higher inflammatory reactions. However, when we employed 1.0 ppm BDDEs, the amount of NO productions decreased to 54, 51, 51, and 43% for the 1.0, 3.0, 5.0, and 10.0% crosslinked HA hydrogels. These results indicated that the degrees of NO productions were affected by the amount of both BDDEs depending on the concentrations of both the pure HA solution and crosslinked HA hydrogels.

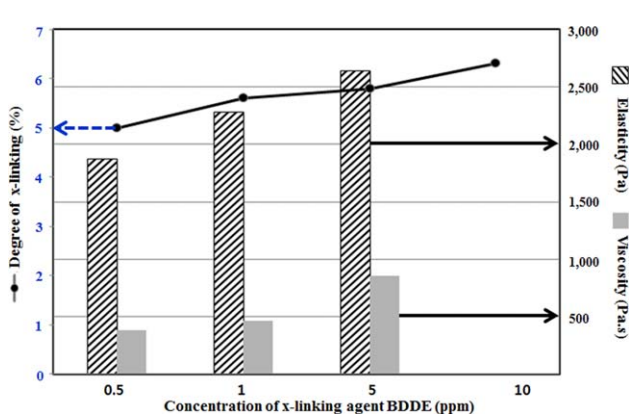

FIGURE 4. Degrees of crosslinking of pure HA polymers and rheological properties of the crosslinking HA hydrogels as a function of BDDE amount at 0.7 Hz frequency and 24-h reaction ($n = 2$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

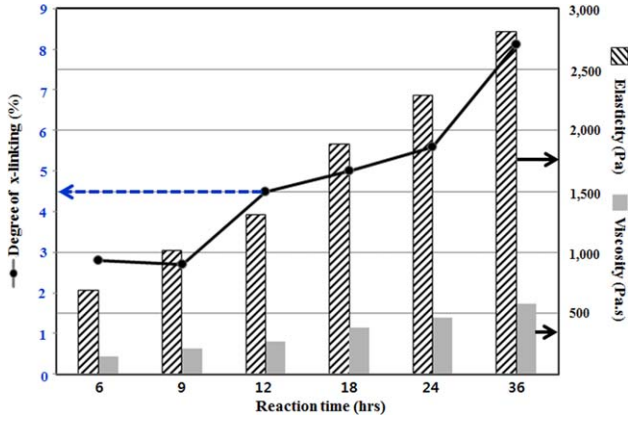

FIGURE 5. Degrees of crosslinking and rheological properties of HA hydrogels as a function of reaction time at 0.7 Hz frequency and 1 ppm BDDE ($n = 2$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

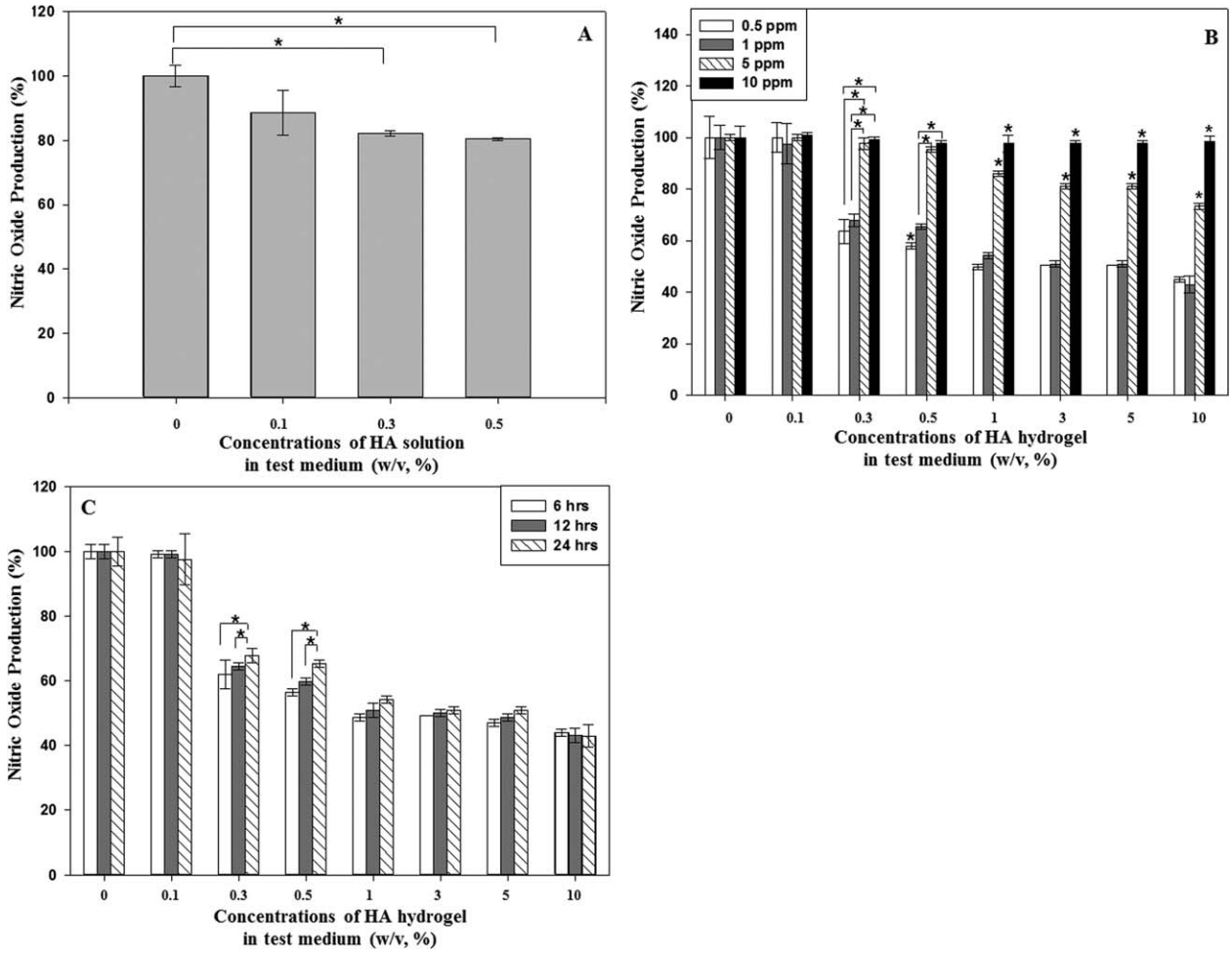

FIGURE 6. Effects of the concentrations of pure HA solutions (A), and HA hydrogels on the productions of nitric oxide (NO) by LPS (+)-induced macrophages as measured by the Griess assay. The hydrogels were fabricated by different amounts of BDDE (B) for different reaction times (C). While the graft of 0 in A is the test medium without any HA solutions, those in B and C are the pure HA solutions without BDDE. * indicates difference in statistics ($n = 4$).

Next, we changed the reaction times of the HA solutions crosslinked with fixed 0.1 ppm BDDEs from 6 to 24 h, which showed reduction of NO production in previous anti-inflammatory tests [Fig. 6(C)]. When we employed the crosslinked 0.1% HA hydrogels, there was no reduction in the amount of NO, showing 98–100%. However, when we increased the concentrations of the crosslinked HA hydrogels, the degrees of NO productions decreased to 62, 56, 49, 49, 47, and 44%, dependent upon the concentrations of the crosslinked HA hydrogels, that is, 0.3, 0.5, 1.0, 3.0, 5.0, and 10.0%, respectively. When we increased the reaction times of crosslinking from 6 to 24 h, there were no significant differences in the degrees of NO production for the HA hydrogels with from 1.0 to 10.0%, indicating that the reaction time for 6 h might be enough to obtain the crosslinked HA hydrogels. However, the hydrogels with $<0.5\%$ showed dependence of their NO productions in statistic ($p < 0.5$).

In vitro cytotoxicity of the crosslinked HA hydrogels

MTT/Brd-U/neutral red assay. The extracts of the crosslinked 26.5% HA hydrogels by reacting for 24 hr demonstrated toxicity depending on the amount of BDDEs when measured by the assays of MTT, neutral red and BrdU [Fig. 7(A–C), respectively]. The test medium showed 105, 98, and 118% cell viabilities by the assays of MTT, neutral red and BrdU, respectively. While the extracts of positive control of Teflon films showed no cell toxicity, those of the negative control of Latex showed 28, 22, and 30%, respectively, when tested with the three kinds of assays above. The HA hydrogels crosslinked with 0.5, 1.0, and 5.0 ppm BDDEs showed excellent cell viabilities such as 132, 118 and 106% by MTT assay. While the cell viabilities by neutral assay were measured as 130, 123, and 109%, those by BrdU were done as 114, 107 and 104%, respectively. However, the HA solutions crosslinked with 10.0 ppm BDDEs showed 97, 95, and 87% cell viabilities by the assays of MTT, neutral red and BrdU, indicating a little bit of cell toxicity. From these results, we concluded that the HA hydrogel crosslinked with more than 0.5% BDDE induced significant cell toxicity in statistics.

Cell viability assay by direct covering with crosslinked HA hydrogel. Biocompatibilities of the crosslinked HA hydrogels were supported by observing their cell viabilities by both

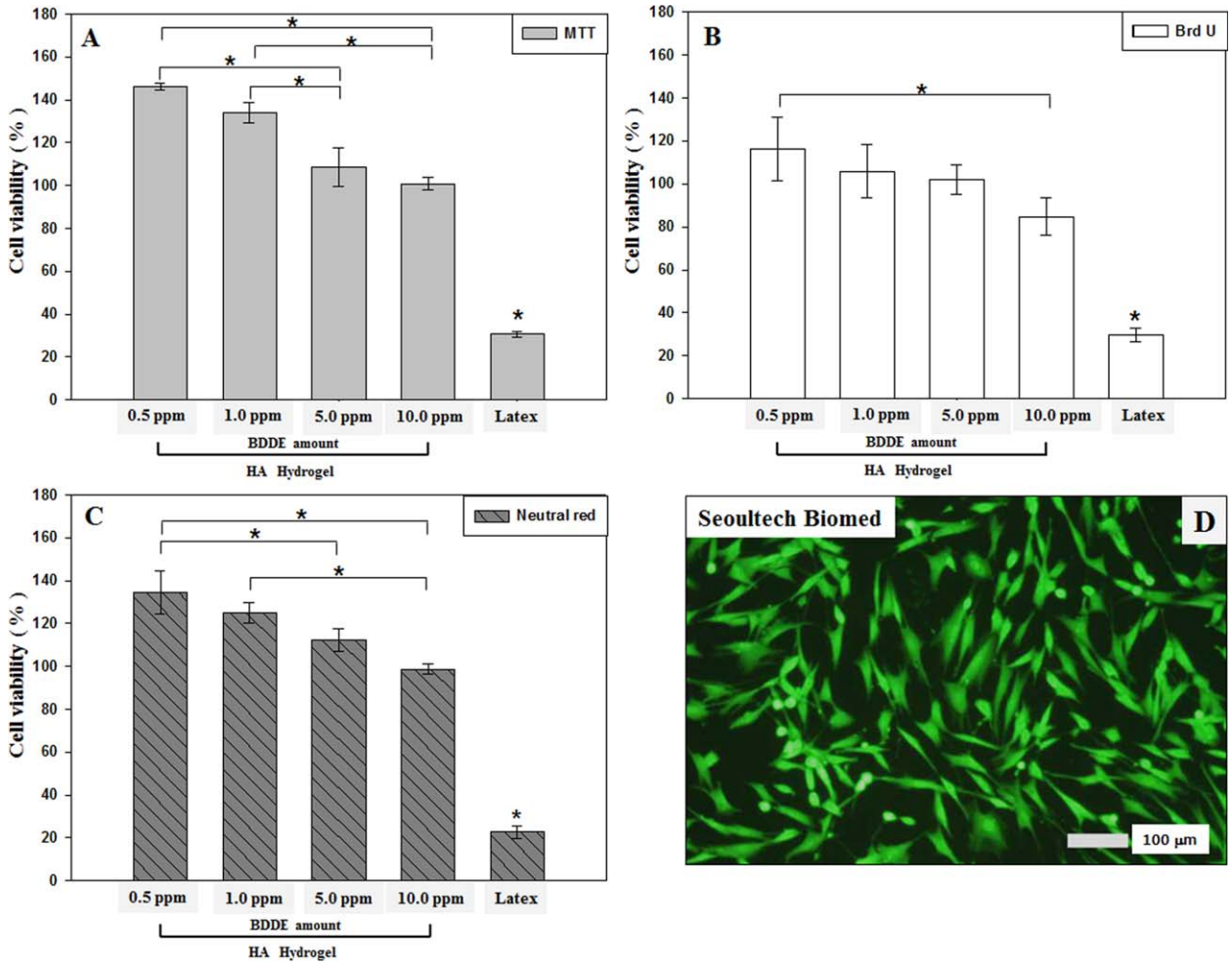

FIGURE 7. Evaluation of cytotoxicity of the extracts of HA hydrogel (26.5%) by the assays of MTT (A), Brdu (B), neutral red (C) and live and dead (D), crosslinked with different amount of BDDE (0.5, 1.0, 5.0, and 10.0 ppm) for 24 hr. The percentage of viable cells after covering with Teflon film was considered as 100%. Live and dead assay was performed by using 15% HA hydrogel crosslinked with 100 ppm BDDE. * indicates difference in statistics ($n = 4$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

light and fluorescence microscopes, after directly covering *in vitro* culturing fibroblasts with the 15% HA samples, that is, the hydrogel crosslinked with 100 ppm BDDEs. After seeding fibroblasts at a density of 2×10^4 cells/cm^2 on the six-well plates, 200 μL crosslinked HA hydrogels were covered on the 50% confluent cells. After *in vitro* cell culture for 24 h, cell spreading was observed by the light microscope, the fluorescence microscope showed that all the cells were viable as indicated by green colors without red colors [Fig. 7(D)]. The red colors indicate cell death.

DISCUSSION

HA has been employed as a biomaterial for medical devices such as dermal fillers, adhesion barriers, drug delivery carriers and scaffolds for tissue engineering such as cartilage, blood vessel, bone, bladders, and so forth. The hydrogels for dermal fillers and adhesion barriers have been currently employed in clinical applications, by using BDDEs as a crosslinking agent. In this study, we have evaluated the effects of BDDEs on the rheological behaviors and biocompatibility of the HA hydrogels by changing the amounts of BDDEs and the reaction times of HA solutions. As we increased the amounts of BDDEs and the frequency of rheometer, the HA solutions showed higher degrees of elastic modulus and viscosity at the fixed conditions of 24 h reaction time, 0.7 Hz frequency and 0.1 ppm BDDEs, indicating that the HA solutions became a hydrogel with higher rheological properties. When the amount of BDDE reached to 10.0 ppm, we could not detect the elastic modulus of the crosslinking HA hydrogels in this study. Furthermore, longer reaction time induced higher elastic modulus and viscosity, which were verified by corresponding increases in the degrees of x-lining of HA hydrogels. Their values were measured as 5.0 and 6.3% when the amount of BDDEs were controlled from 0.5 to 10.0 ppm for the 26.8 mg mL^{-1} HA solution. The reaction times also affected the degrees of crosslinking of HA solutions, leading to 5.6% when the reaction was performed for 24 h at the fixed conditions of 1.0 ppm BDDE, 0.7 Hz frequency and 26.0 mg mL^{-1} HA solutions.

These mechanical properties were related to the anti-inflammatory results of the crosslinked HA hydrogels, when macrophages activated in advanced by LPS(+) were exposed. LPS, also known as lipoglycans and endotoxin, consists of a lipid and a polysaccharide composed of O-antigen, outer core and inner core joined by a covalent bond. The LPS has been known as the prototypical endotoxin because it binds the receptor complex in macrophages, which promotes the secretion of pro-inflammatory cytokines, nitric oxide and eicosanoids.[28] Basically, the HA solution without any crosslinking showed anti-inflammatory behaviors as observed that higher concentrations of pure HA solutions induced less NO productions, indicating that HA polymer itself suppressed inflammation reactions. When pure HA solutions were crosslinked with BDDEs, the crosslinked HA hydrogels reduced the degrees of NO productions by macrophages, where NO is a cellular signaling molecule of inflammation in physiological and pathological process. When we employed higher amounts of BDDEs such as 5.0 or 10.0 ppm there was no significant reduction of amount of NO productions, indicating no effects on anti-inflammatory effects of the crosslinked HA hydrogels. However, the crosslinked HA hydrogels with 0.1 and 1.0 BDDEs showed significant amounts of reduction of NO productions for the employed HA solutions with 0.3–10.0% concentrations. Furthermore, the reaction times ranging from 6 to 24 h affected the degrees of reduction of NO productions of the crosslinked 0.3–10.0% HA hydrogels (w/v). These results, supported by cell viability tests of all the assays of MTT, neutral red, BrdU and live and dead, showed that the HA hydrogels crosslinked with up to 5.0 ppm BDDEs had higher cell viabilities. On the basis of these experimental results, we concluded that higher amounts of both pure and crosslinked HA hydrogels induced reduction of inflammatory reaction generated by LPS-exposed macrophages, but addition of higher amount of crosslinker BDDEs induced higher cell toxicities, indicating the need to modulate the concentrations of crosslinking agents and HA solutions as well as their reaction times for crosslinking if the hydrogels were considered as possible clinical applications to biomaterials.

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