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The use of acetylation to improve the performance of hyaluronic acid-based dermal filler

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Abstract−Injectable dermal fillers, which are used for various plastic surgery purposes, are experiencing explosive market growth due to increasing interest in appearance management. Hyaluronic acid (HA) hydrogels have been considered an ideal material for fillers due to their high-water retention, biodegradability, and biocompatibility. However, their application is limited by shortcomings in durability and persistence caused by rapid enzymatic degradation. Therefore, in this study, we introduce acetylated hyaluronic acid-divinyl sulfone (AcHA-DVS) hydrogels for a novel approach for improving the physical properties and gel retention time of HA. The AcHA-DVS hydrogels showed significant advantages in terms of longevity and performance as dermal fillers compared to HA-DVS hydrogels. These results suggest that our new AcHA-DVS hydrogel is a promising biomaterial as an injectable filler or scaffold for tissue engineering.

Keywords: Filler, Hyaluronic Acid, Acetylation, Mechanical Property, Enzyme Resistance

INTRODUCTION

Hyaluronic acid (HA) is one of the most popular materials used in injectable dermal fillers to reduce skin aging and laxity [1]. As a natural polymer with a broad molecular weight spectrum, HA can form hydrogels that act as an ideal filler material due to their injectability, biocompatibility, and biodegradability [2,3]. Moreover, the mechanical properties of HA hydrogels can be adjusted according to molecular weight, crosslinking method, and degree of crosslinking, making them highly advantageous for forming hydrogels with a natural texture even when they are injected into various areas [1,4-6]. However, despite these advantages, HA hydrogels often last only for several months *in vivo* due to rapid enzymatic degradation [7,8]. The short residence time of HA hydrogels due to rapid enzymatic degradation is one of the major challenges in the development of long-lasting dermal fillers. To address this issue, various modification strategies have been explored to increase the residence time of HA hydrogels at injection sites and prolong their aesthetic effect while maintaining their appropriate physical properties [1,9-11]. Chemical crosslinking is a common strategy for modifying the properties of HA hydrogels. By incorporating functional reagents during the crosslinking reaction, it is possible to obtain hydrogels with improved resistance to enzymatic degradation and control over physical properties such as stiffness and degradation rate. Increasing the resistance of HA hydrogels to enzymatic degradation through chemical crosslinking can often result in an increase in the hardness of the gel, which can lead to a feeling of a foreign body and potentially cause inflammation or other adverse reactions [12]. Therefore, it is important to balance the desired level of resistance to degradation with the required mechanical properties for the intended application, as well as the biocompatibility and safety of the material.

In this study, acetylation of HA is proposed as a potential solution to the issue of rapid enzymatic degradation and hardness of the gel. Acetyl groups were induced onto the HA backbone for acetylation, which can increase the hydrophobicity of the polymer and prolong the degradation time under aqueous conditions. This modification can also affect the physical and mechanical properties of the hydrogel, such as its stiffness and swelling behavior. To evaluate the suitability of AcHA hydrogel as dermal fillers, factors such as the degradation rate mechanical properties, and biocompatibility of AcHA hydrogel were compared to those of conventional HA hydrogel to determine *in vitro* whether they offer significant advantages in terms of longevity and performance as dermal fillers.

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EXPERIMENTAL

1. Materials

Hyaluronic acid with a molecular weight of 800 kDa was obtained from Hyundai Bioland Co. (Cheongju, Korea). Chemicals and reagents, such as formamide, pyridine, acetic anhydride, sodium hydroxide, divinyl sulfone (DVS), hyaluronidase from bovine testes, sodium tetraborate decahydrate, sulfuric acid, carbazole, ethanol, and D-(+)-Glucuronic acid γ-lactone, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dialysis membranes with a molecular weight cut-off (MWCO) between 10 and 12 kDa were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin (P/S) were obtained from Gibco-Invitrogen (Grand Island, NY, USA).

2. Synthesis of Acetylated Hyaluronic Acid (AcHA)

AcHA was synthesized by acetylating HA using a previously reported method [13]. First, HA (0.5 g) was dissolved in 100 mL of formamide at 50 °C. Pyridine (1.25 ml) was added to the HA solution and the mixture was stirred for 1 h at room temperature. Next, acetic anhydride (2.5 ml) was added to the HA and pyridine solution, and the mixture was allowed to react for 24 h at room temperature with stirring. The feed molar ratio between acetic anhydride and the hydroxyl group of HA was 5 : 1. After stirring for 24 h at room temperature, the reaction solution was dialyzed using a dialysis membrane with a molecular weight cut-off of 10-12 kDa, and then lyophilized to obtain a white solid powder of AcHA.

3. Preparation of HA-DVS and AcHA-DVS Hydrogels

HA or AcHA was dissolved at a concentration of 4 wt% (w/v) in 0.2 N NaOH (pH=13). Once fully dissolved, DVS was added to the solution in a molar ratio of 1 : 1 between DVS and the hydroxyl group of both HA and AcHA. The mixtures were vortexed and centrifuged to remove air bubbles, and the final precursor solution was incubated at 37 °C overnight to complete the crosslinking reaction. The crosslinked gels were then washed in DI water to remove hydroxyl ions and unreacted DVS.

4. Characterization of the AcHA-DVS Hydrogels

The prepared AcHA hydrogel was analyzed using ^{1}H-NMR (AdvanceIII 600 spectrometer) to determine its chemical structure. Samples were prepared by dissolving them in D_2O. A Nicolet iS5 ATR-FTIR (Thermo Fisher Scientific, Madison, WI, USA) was used to perform ATR-FT-IR spectroscopy analysis on the HA-DVS and AcHA-DVS hydrogels to confirm the chemical structure of the samples produced.

The morphology of the hydrogels was characterized using a field emission scanning electron microscope (FE-SEM, S-4800, Hitachi, Japan). The hydrogels were frozen in liquid nitrogen and lyophilized for 24 h before sectioning. The cross-sections of the hydrogels, sputter-coated with platinum for 60 s, were then observed.

5. Rheological Analysis of Hydrogel

The rheological properties of the hydrogels were measured using a Haake Mars 40 rheometer (Thermo Scientific, Karlsruhe, Germany) with a 20 mm parallel plate geometry at a gap of 1 mm. The hydrogel and crosslinker solution mixture (500 μL) was filled into a 24-well plate and incubated at 37 °C overnight to complete the crosslinking reaction. The hydrogel was then loaded onto the measuring geometry. The dynamic rheological parameters used to evaluate the gel network were the elastic modulus (G′) and viscous modulus (G″). An oscillation frequency sweep test was performed from 0.01 Hz to 1 Hz at a constant strain of 0.1%, and the thermoelectric Peltier device was maintained at 25 °C.

6. Water Uptake Ability of Hydrogel

The hydrogel samples were lyophilized prior to measuring their swelling ratio. The freeze-dried hydrogels were weighed to obtain the dry mass weight (W_d). Then, the samples were incubated in PBS at 37 °C, and the swollen hydrogels were weighed (swelling mass weight (W_s)) at specified time points. The equilibrium swelling ratio (ESR) of the hydrogel was calculated using the following equation: $ESR=(W_s-W_d)/W_d$.

7. Hydrogel Degradation Assay

The *in vitro* enzymatic degradation of the hydrogels was measured by incubating the gels in hyaluronidase and monitoring their weight loss over time. A hydrogel sample (200 μL) was placed into a 96-well plate and incubated at 37 °C overnight to complete the crosslinking reaction. After overnight crosslinking, 100 μL of PBS containing 10 and 25 U/ml of hyaluronidase was added to the hydrogel in the 96-well plate, which was then incubated in a 37 °C shaking water bath (70 rpm) for various intervals. At each time point, the hydrogels were weighed and the supernatant was collected. A fresh enzyme solution was then added and the degradation of the hydrogels was analyzed through a carbazole assay using the collected solution.

8. Biocompatibility Assays of Hydrogels

The cytotoxicity of AcHA hydrogel was evaluated using MTT assay and Live/Dead assay. Dermal fibroblasts (KCLB, Seoul, Korea) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, New York, NY, USA) in a humidified incubator at 37 °C with 5% CO_2. The medium was changed every other day. For cytotoxicity measurement in the hydrogel, cells were seeded in 96-well plates at 4×10^3 cells/well and cultured overnight. The culture medium was then replaced with 100 μL of fresh medium containing varying concentrations of HA or AcHA. After 24 h of media change, cells in the 96-well plate were incubated with 200 μL of fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 2 mg/mL in PBS, Sigma, Milwaukee, WI, USA). After 4 h of incubation at 37 °C, the MTT solution was removed, and 100 μL of dimethylsulfoxide (DMSO) was added to dissolve the formazan crystals formed by the live cells. Absorbance (A) was measured at 570 nm, and cell viability (%) was calculated by comparison with the non-transfected control using the following equation: Cell viability (%)=(A570 (sample)/A570 (control))×100%. Cell viability was also assessed using a Live/Dead assay (Thermo Fisher Scientific, Madison, WI, USA). Fibroblasts in each well were briefly stained using 0.15 mM of Calcein-AM and 2 mM of ethidium homodimer at 37 °C for 30 min. The fluorescence images of the cells in wells were digitally captured using an inverted epifluorescence microscope (Zeiss Axiovert 200, Göttingen, Germany).

9. Statistical Analysis

The data obtained were presented as mean±standard deviation. Statistical analysis was conducted using Student's t-test, and $p<0.05$ was considered statistically significant.

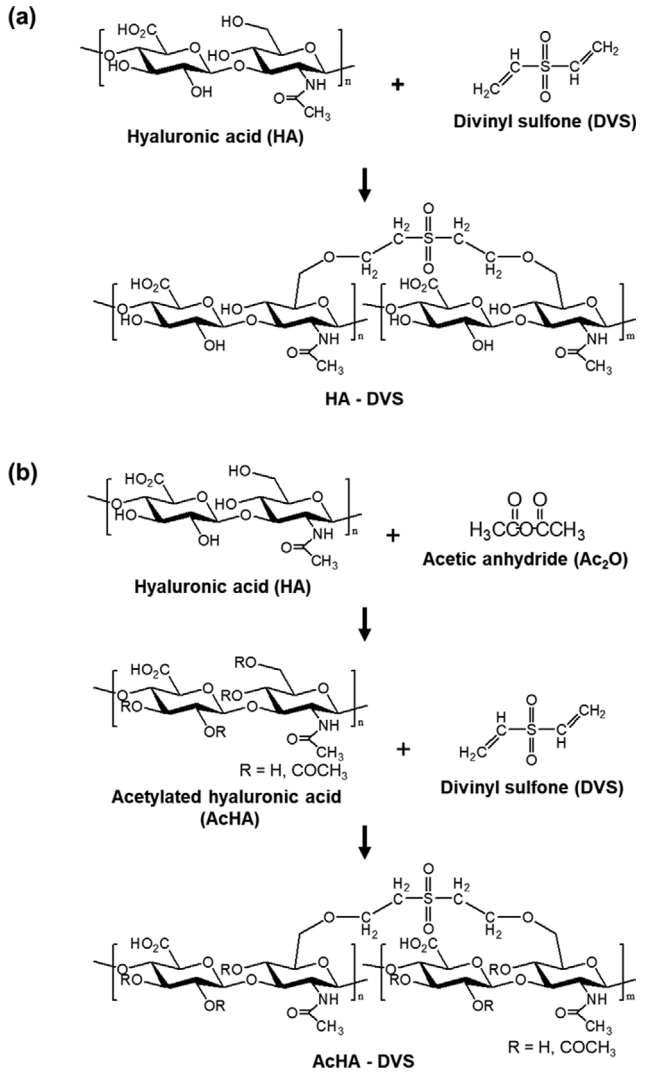

Fig. 1. Schematic representations of (a) hyaluronic acid (HA) hydrogel and (b) acetylated hyaluronic acid (AcHA) hydrogel preparation using a crosslinking reagent of divinyl sulfone (DVS).

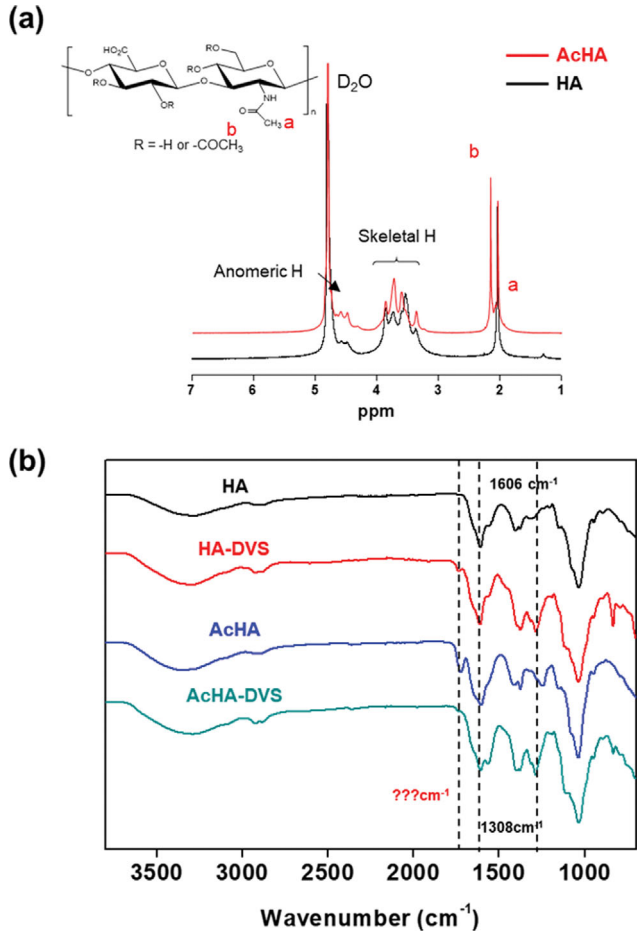

Fig. 2. (a) ^{1}H-NMR spectra of HA-DVS and AcHA-DVS hydrogels. (b) FT-IR spectra of HA-DVS and AcHA-DVS hydrogels.

RESULTS

1. Synthesis of AcHA-DVS Hydrogels

HA-DVS and AcHA-DVS hydrogels were prepared as shown in Fig. 1. AcHA was synthesized by acetylating the hydroxyl group in HA with acetic anhydride in the presence of pyridine. The degree of acetylation was controlled by adjusting the molar ratio between HA and acetic anhydride. The hydroxyl groups of HA and AcHA were then crosslinked via DVS under alkaline conditions.

The chemical modification of HA into AcHA was evaluated using ^{1}H-NMR (Fig. 2(a)). The D_2O peak at δ4.8 ppm was used as a reference to analyze the spectra of AcHA. In the disaccharide unit of HA and AcHA, the proton signal in the acetamido moiety was evident at δ=2.0 ppm. A new characteristic peak appeared at δ=2.1 ppm corresponding to the acetyl group of AcHA. FT-IR spectral analysis was conducted to compare the functional groups of the HA, AcHA, HA-DVS, and AcHA-DVS hydrogels (Fig. 2(b)). The peaks at 3,200-3,600 cm^{-1} were attributed to O-H bond stretching, and the peaks at 1,606 cm^{-1} corresponded to the carbonyl stretching band of carboxylates. The peak at 1,735-1,750 cm^{-1} of AcHA appeared after acetylation, indicating the carbonyl stretching of the ester. The peaks at 1,308 cm^{-1} that appeared in both HA-DVS and AcHA-DVS were attributed to S=O asymmetric stretching due to divinyl. The results of ^{1}H-NMR and FT-IR demonstrate the successful acetylation of HA and crosslinking of the polymers mediated by DVS.

The cross-sectional morphology of the lyophilized HA-DVS and AcHA-DVS hydrogels was observed using FE-SEM to evaluate their interior structure and porosity (Fig. 3). SEM images of both hydrogels exhibited similar structures with heterogeneously formed pores. The pore network of the hydrogels is expected to facilitate the delivery of oxygen and nutrients to cells.

2. Changes of Physical Properties in AcHA Hydrogels

The effect of acetylation of HA on the swelling ratio of the hydrogels was investigated (Fig. 4(a)). Both types of hydrogels reached a maximum water absorption of over three equilibrium swelling ratios (300% of their initial dried weight) after 12 h. However, the AcHA-DVS hydrogels showed a lower swelling ratio than the HA-DVS hydrogels, indicating that the acetyl group on the AcHA backbone decreased hydrophilicity and water absorptivity.

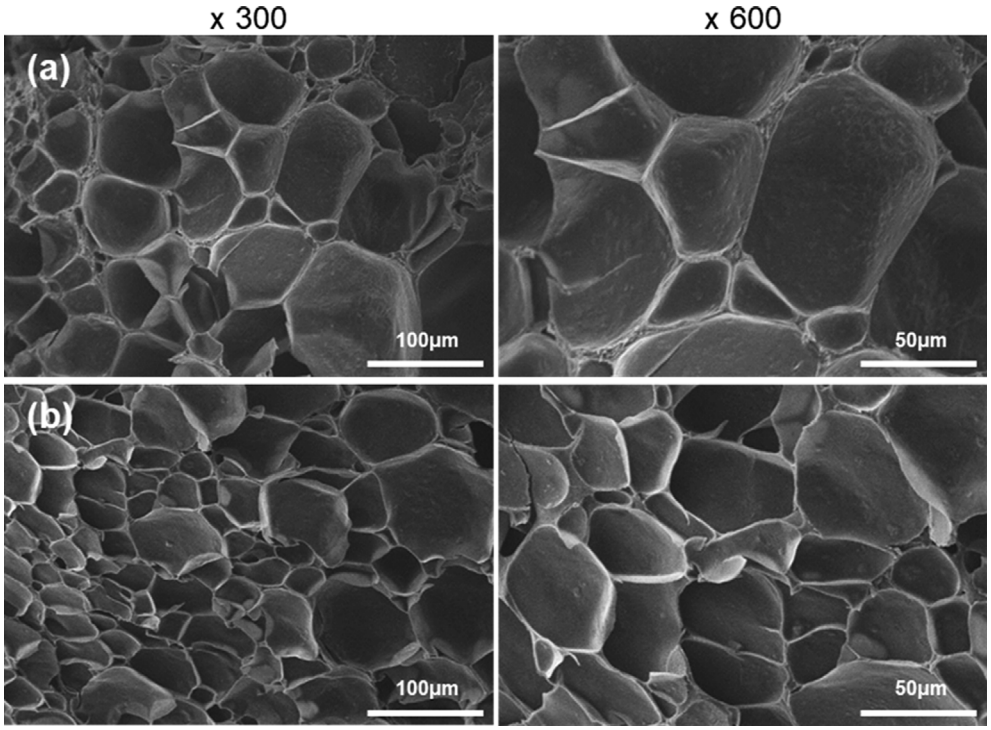

Fig. 3. The FE-SEM images of lyophilized (a) HA-DVS hydrogel and (b) AcHA-DVS hydrogel.

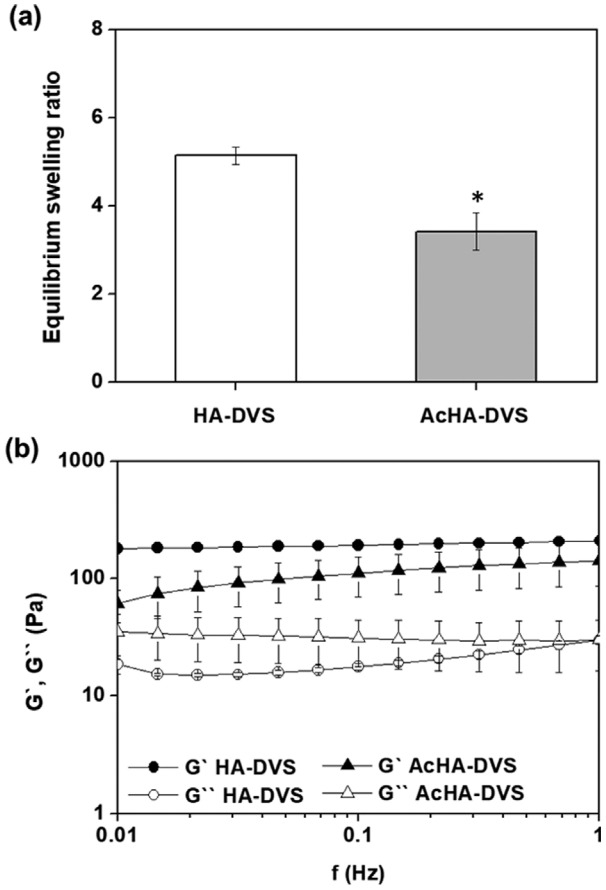

Fig. 4. (a) Equilibrium swelling ratio (ESR) and (b) rheological analysis of HA-DVS and AcHA-DVS hydrogels.

Elastic modulus (storage moduli G′) and viscous modulus (loss moduli G″) were analyzed for the HA-DVS and AcHA-DVS hydrogels using a rheometer (Fig. 4(b)). Both hydrogels exhibited higher G′ values than G″ at all frequencies, indicating that the hydrogels crosslinked by DVS have solid-like properties. Interestingly, the AcHA-DVS hydrogels showed a higher modulus than the HA-DVS hydrogels, as we hypothesized. This may be due to the property of the AcHA polymer to form additional self-organized networks in aqueous conditions through increased hydrophobicity.

3. Increased Resistance to Enzymatic Degradation of AcHA Hydrogels

We hypothesized that the increased hydrophobicity of AcHA would slow enzymatic degradation, making it a promising material for dermal fillers with longer-lasting effects [14-16]. To evaluate hydrogel degradation, we measured the weight of hydrogels cultured in PBS containing 10 or 25 U/ml of hyaluronidase at various time points (Fig. 5). HA-DVS hydrogels in 10 U/ml hyaluronidase were completely degraded within 48 h. However, AcHA-DVS hydrogels showed a much slower degradation profile than HA-DVS hydrogels. AcHA-DVS hydrogels in 10 U/ml hyaluronidase underwent approximately 50% degradation in 36 h, and even after 84 h of incubation, about 10% of the hydrogel remained undegraded. AcHA-DVS hydrogels cultured in 25 U/ml hyaluronidase showed relatively faster degradation than those in the 10 U/ml condition. Nevertheless, the AcHA-DVS hydrogels exhibited longer resistance to enzymatic degradation than the HA-DVS hydrogels in 10 U/ml hyaluronidase. The increased resistance to enzymatic degradation of the AcHA-DVS hydrogels may be due to the increased hydrophobicity, which hinders interaction with the enzyme in aqueous conditions.

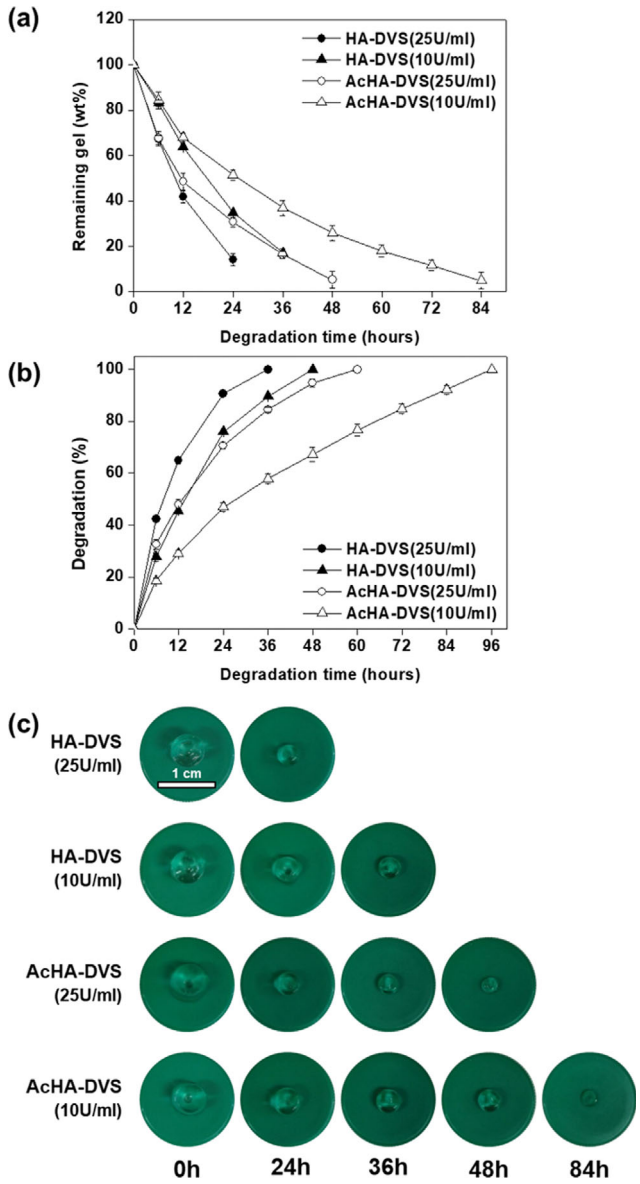

Fig. 5. *In vitro* degradation of HA-DVS hydrogels and AcHA-DVS hydrogels in PBS containing 10 or 25 U/mL of hyaluronidase. (a) weight changes, (b) carbazole assay and (c) representative images of the degraded of HA-DVS and AcHA-DVS hydrogels during 96 h of indication.

4. Biocompatibility of AcHA-DVS Hydrogels

We evaluated the biocompatibility of the HA-DVS and AcHA-DVS hydrogels by treating them at two different concentrations (0.25 wt% and 0.5 wt%) with fibroblasts. At day 1, there was no difference in cell viability between the hydrogel-treated groups and the control group (Fig. 6(a)). At day 3, the cell viability of the HA-DVS hydrogel-treated groups increased at both concentrations, while the cell viability of the AcHA-DVS hydrogel-treated groups slightly decreased. However, the decrease in cell viability of the AcHA-DVS hydrogel-treated groups was not statistically significant compared to the control group. The Live/Dead assay also demonstrated the biocompatibility of the AcHA-DVS hydrogels (Fig. 6(b)).

DISCUSSION

HA fillers are commonly used in aesthetic dermatology due to their biocompatibility and ability for minimal invasive application. Moreover, their properties can be modified by varying the concentration and formulation conditions to meet the desired outcomes [9,17,18]. However, to maintain the desired results, repeated procedures may be necessary since the HA molecules are typically degraded in the body within six months to one year [19].

In this study, a new AcHA-DVS hydrogel has been developed to improve the physical properties and prolong the gel retention as filler. This new hydrogel offers advantages over traditional HA hydrogel-based fillers. The AcHA-DVS was created by acetylating HA to slow degradation and increase flexibility. The mechanical properties of the AcHA-DVS hydrogel showed that it is more elastic than the HA-DVS hydrogel, as evidenced by its lower G′ values and higher G″ values across all frequencies (Fig. 4). The AcHA-DVS hydrogel also demonstrated a lower ESR and longer enzymatic degradation time than the HA-DVS hydrogel. The longer degradation time of AcHA-DVS in a hyaluronidase solution suggests that acetylation improves the enzymatic resistance of the HA hydrogel (Fig. 5).

Acetylation is chemical modification that involves the addition of an acetyl group (-$COCH_3$) to a polymer [20]. This modification can increase the hydrophobicity of polymer, as the acetyl group is nonpolar and repels water [21]. This can have important implications for the physical and chemical properties of the polymer, including its solubility, mechanical properties, and interactions with other molecules. In this study, the acetyl group increased the hydrophobicity of the HA main chain and lowered its swelling properties (Fig. 4(a)). The swelling property of hydrogels is an important parameter for the diffusivity of bioactive molecules across the hydrogel network [22,23]. The high ESR values of the hydrogels suggest that they would not impede the delivery of oxygen and nutrients to the cells cultured inside and provide a suitable environment for metabolism [24].

The proliferation of AcHA-DVS treated fibroblasts increased during three days of culture, and their viability was not significantly different from that of the HA-DVS hydrogel-treated cells (Fig. 6). It is known from previous studies that HA has a positive effect on cell proliferation [2], and it is speculated that the 0.5 wt% HA group showed the highest cell viability compared to the other groups on day three due to the effect of HA. However, both of 0.25 and 0.5 wt% AcHA-DVS groups also demonstrated safety of the material by not adversely affecting cell viability. These results indicate that the AcHA-DVS hydrogel is a safe material for dermal filler that does not interfere with the activity of cells at the injected region [25].

In addition, it would be possible to extend the residence time of AcHA hydrogels even longer by modifying the HA molecular weight. Low molecular weight HA can be easily degraded by enzymes, whereas high molecular weight HA can form a denser and more stable network, resulting in a slower degradation rate and longer residence time [26]. Therefore, by combining the acetylation of HA with the use of high molecular weight HA, it may be possible to further increase the longevity of the AcHA hydrogel and enhance

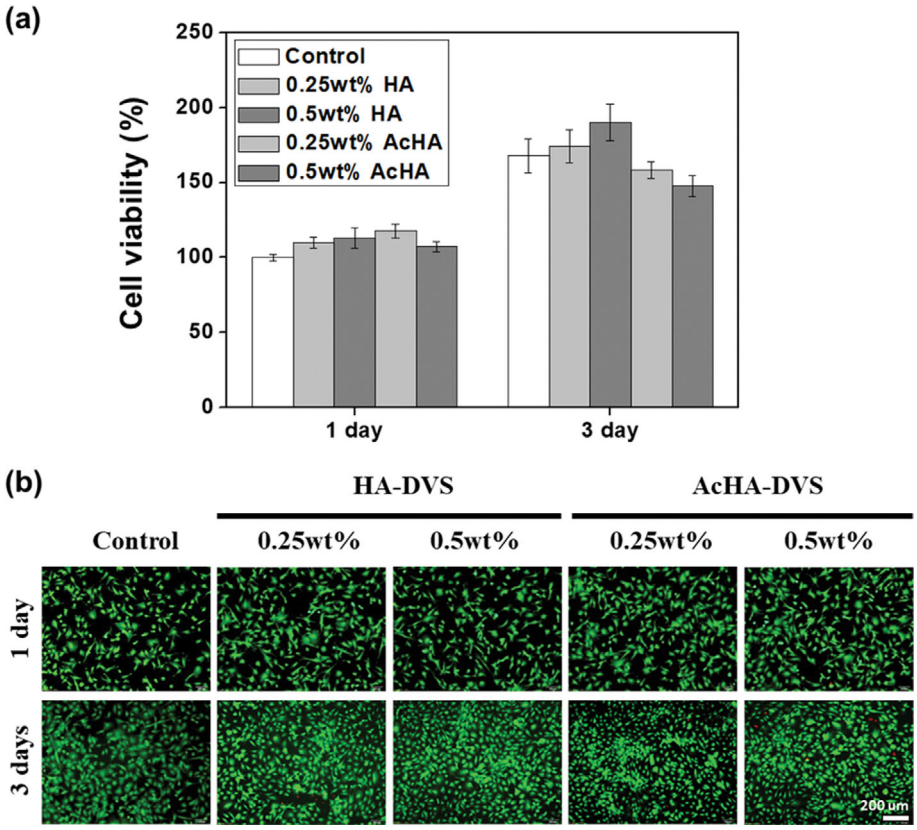

Fig. 6. (a) Proliferation of fibroblasts during 1 and 3 d of incubation treated with HA-DVS and AcHA-DVS hydrogels. (b) Live/Dead assay images of fibroblasts cultured with the hydrogels for 3 d of culture

their performance as dermal fillers.

Addition of other polymers to the AcHA network can improve the mechanical and biological properties for a particular application, such as tissue engineering scaffold, drug delivery, or wound healing. Indeed, collagen is a natural component of the extracellular matrix and can promote cell adhesion, migration and proliferation [27]. Chitosan, a biodegradable and biocompatible polysaccharide, can improve the mechanical strength and stability of AcHA hydrogel, as well as enhance its bioactivity and drug delivery properties [13].

CONCLUSION

The use of acetylation in the development of the AcHA-DVS hydrogel is a promising approach. It slowed the degradation rate and increased its flexibility. However, further studies are needed to fully evaluate its clinical potential and safety as a dermal filler material.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AcHA-DVS : acetylated hyaluronic acid-divinyl sulfone
HA : hyaluronic acid
DVS : divinyl sulfone
MTT : 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide
W_d : dry mass weight
W_s : swelling mass weight
ESR : equilibrium swelling ratio
DMSO : dimethylsulfoxide
DMEM : Dulbecco's modified Eagle's medium
FBS : fetal bovine serum
P/S : penicillin-streptomycin
Calcein-AM : calcein acetoxymethyl ester
FE-SEM : field emission scanning electron microscope

AVAILABILITY OF DATA AND MATERIALS

For data requests, please contact the authors.

AUTHOR CONTRIBUTIONS

E. J. Lee analyzed the characteristics of HA hydrogels. K. H. Park performed cell culture for the cytotoxicity test of the hydrogels. S.-J. Gwak and Y. B. Lee are the major contributors to writing the man-

uscript. K. M. Huh and S.-W. Kang supervised the study. All authors have read and approved the final manuscript.

CONSENT FOR PUBLICATION

All authors have consented to the submission of this manuscript for publication.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

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