

Perspective Article

Effects of basic fibroblast growth factor combined with an injectable in situ crosslinked hyaluronic acid hydrogel for a dermal filler

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ABSTRACT

Hyaluronic acid (HA) has been applied as a biomaterial for injectable dermal filler due to the unique viscoelastic property. Although HA derivatives are promising dermal filler materials, poor durability by degradation is the limitation of HA dermal filler. The chemical crosslinking of HA hydrogel is necessary to restrain the degradation rate. Herein, we present an injectable in situ HA hydrogel containing basic fibroblast growth factor (bFGF) crosslinked via a visible light-induced thiol-ene reaction. The thiol-ene reaction between thiolated HA (SH-HA) and methacrylated HA (MA-HA) is triggered by riboflavin phosphate with blue light (BL) exposure. The mechanical property, matrix degradation, and protein release of HA hydrogels were evaluated regarding SH-HA and MA-HA ratio. The HA hydrogels improve NIH/3T3 fibroblast proliferation and migration, and the bFGFs incorporated in HA hydrogel additionally induce the cell proliferation and scratch closure rate. In addition, the HA hydrogel precursor solution under a porcine skin forms hydrogel by transmitted BL through the skin, so the gelation can occur under the skin after injection. According to the results, the in situ crosslinked HA hydrogel is a promising injectable and long-lasting dermal filler that can deliver therapeutic factors.

1. Introduction

Hyaluronic acid (HA) is a biocompatible and biodegradable material and is widely used for dermal filler and soft tissue regeneration. HA is a type of glycosaminoglycan (GAG) and plays a vital role in wound healing, angiogenesis, and cell proliferation [1,2]. In addition, the viscoelastic property of HA protects tissues and controls tissue hydration and water transportation [3]. In terms of chemical structures, the hydrogen bond and hydrophobic interaction, both intramolecular and intermolecular, form physical crosslinks [4]. The intra- and interchain interactions are weak and temporarily maintained the network structures, so the physically crosslinked HA has short durability with the rapid degradation by enzymes such as hyaluronidase [5,6]. Chemical crosslinking has been introduced to the HA chains with crosslinkers such as divinyl sulfone and diglycidyl ether to avoid rapid degradation; the degree of crosslinking increases a storage modulus (G') [7–10]. The chemical crosslinking promotes the durability of HA, but the gel injection becomes difficult. Generally, the matrix is chemically crosslinked before injection, and a small amount of crosslinker is used to maintain the injectability of HA gels. Thus, in situ crosslinked dermal filler must

resist compressive forces with a longer retention time after injection into the skin.

In situ crosslinking can be achieved by thiol-ene chemistry, which forms an alkyl sulfide bond by a free radical-mediated reaction between a thiol and an alkene group [9–18]. In our previous study, HA hydrogels were synthesized in situ by the visible light-induced thiol-ene reaction; riboflavin phosphate (RFP) was a photoinitiator, and blue light (BL) triggered the gelation of HA via a thiol-ene reaction between thiolated HA (SH-HA) and methacrylated HA (MA-HA) [19]. RFP-mediated thiol-ene crosslinking is safer than other photo-mediated thiol-ene reactions. RFP is a form of riboflavin, a water-soluble essential micronutrient, naturally occurring vitamin B complex. It absorbs the light at wavelengths at 370 and 445 nm and thus absorbs the blue spectrum as well as lights in the UV spectrum [19,20]. Although other photoinitiators, such as eosin Y and rose Bengal, mediate thiol-ene crosslinking, RFP is considered safer since it was approved in 2016 by the FDA for crosslinking collagen with UV light [21–23]. The physical properties of the hydrogel were modulated by adjusting the concentration of HA, RFP, and BL exposure time. The HA hydrogel precursor solution had viscoelastic behavior ($G' < G''$) before BL exposure, and the HA hydrogel after

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BL exposure was elastic ($G' > G''$) [19]. The light-mediated in situ crosslinked elastic hydrogel, which has a resistance to skin tension forces, is more suitable for aesthetic corrections [24,25].

The chemically crosslinked HA hydrogel is useful as not only a tissue structure reconstruction but also a carrier for hydrogel-based drug delivery systems. HA hydrogels are biocompatible and capable of encapsulating biomolecules, are beneficial for clinical uses such as drug dosage adjustments or repeated administrations [26,27]. The hydrogel matrices can protect protein or growth factors against physical and chemical denaturation, such as aggregation, enzymatic degradation, so crosslinked HA hydrogels have been extensively studied as drug delivery matrices [10–14]. The spatiotemporal control of therapeutic delivery is an additional advantage of hydrogels, and it can be applied not only to topical applications but also to injection systems [28,29].

The drugs or proteins loaded in hydrated HA hydrogel are easily leaked from the hydrogel matrix during storage, so preserving drugs inside HA hydrogel is difficult. In situ crosslinking is a way to solve the problem. The therapeutic factors can be incorporated immediately before hydrogel formation without storage in the hydrogel matrix. We included the basic fibroblast growth factor (bFGF) as a model growth factor, which induces fibroblast proliferation and migration [30,31]. bFGF plays a significant role in the wound healing process, but it is labile by proteolysis and loses bioactivity under normal physiological conditions [32]. Immobilizing proteins in the HA hydrogel matrix can improve the drug delivery efficiency of bFGF [33–35]. Still, there was no biocompatible injectable in situ HA hydrogel that was completed gelation with visible light irradiation in a minute.

In this study, the BL exposure time and SH-HA and MA-HA compositions were adjusted during the hydrogel fabrication process to investigate the therapeutic effect and feasibility of the HA hydrogel as a dermal filler. We fabricated HA hydrogels and evaluated the mechanical properties, degradation characteristics, and protein release. Also, bFGF was incorporated into the crosslinked HA hydrogel by thiol-ene reaction, and the therapeutic effects of bFGF and HA hydrogels were assessed through fibroblast proliferation and scratch assays. HA binds the CD44 receptor, which activates various signaling pathways related to cell proliferation, adhesion, and migration by facilitating the binding of adaptor proteins to intracellular domains [36,37]. To eliminate these effects of HA, we blocked the CD44 receptors in fibroblasts and evaluated the therapeutic effect of only bFGF. Lastly, in situ gelations were performed under the porcine skin, assuming an injection situation to demonstrate the feasibility of BL-assisted HA hydrogel as a dermal filler.

2. Materials and methods

2.1. Materials

Unless otherwise noted, all chemicals and solvents were used as provided by the manufacturers. Thiolated HA (SH-HA) and methacrylated HA (MA-HA) were purchased from Blafar (Dublin, Ireland). Riboflavin phosphate (RFP), phosphate-buffered saline (PBS), fluorescein isothiocyanate conjugated bovine serum albumin (FITC-BSA), dimethyl sulfoxide (DMSO), and pH test strips (pH 4.5–10) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's phosphate-buffered saline (DPBS), trypsin-EDTA, and penicillin/streptomycin were purchased from Gibco (Waltham, MA, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) with a high concentration of glucose were purchased from HyClone Laboratories Inc. (South Logan, UT, USA). Basic fibroblast growth factor (bFGF) was purchased from Peprotech (London, UK). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and CD44 monoclonal antibody (Hermes-1) were purchased from Invitrogen (Carlsbad, CA, USA). Cell inserts were purchased from IBIDI (IBIDI GmbH).

2.2. Fabrication of HA hydrogels by thiol-ene reaction

SH-HA and MA-HA were dissolved in PBS at 10 mg/mL, and the pH of the solutions was adjusted to 7 using 1 M NaOH. SH-HA and MA-HA solutions were mixed in volume ratios of 1:4, 1:1, and 4:1, and the resultant mixtures were named SH1MA4, SH1MA1, and SH4MA1, respectively. RFP was dissolved in PBS at 1% w/v and added to the mixed HA solution at the final concentration of 0.01% w/v. The HA hydrogel precursor solution was exposed to blue light (BL) for 10 or 20 s.

The HA hydrogel rheology was evaluated using an MCR 302 rheometer (Anton Paar, Germany). The HA hydrogels were incubated at 37 °C overnight and mounted onto the rheometer. The storage and loss moduli were measured using frequency sweeps from 0.1 to 10 Hz with a fixed 1% strain were performed.

The HA hydrogel matrix structure was observed using a scanning electron microscope (SEM) (S-4700, Hitachi, Japan) at the Smart Materials Research Center for IoT at Gachon University. The cross-sections of the HA hydrogels were prepared by freezing hydrogels in liquid nitrogen, and the frozen hydrogel samples were broken into some segments and lyophilized. The analytes were coated with platinum under vacuum before the SEM measurements.

2.3. HA hydrogel degradation and protein release

HA hydrogel degradation was evaluated through weight losses. HA hydrogels were fabricated, and the weights of the hydrogels were measured immediately after gelation (day 0). The hydrogels were incubated in 5 mL PBS at 37 °C. PBS was refreshed on certain days, and the weights of hydrogels were measured.

Protein release profiles were evaluated using FITC-BSA as a model protein. FITC-BSA was added at 0.01% w/v concentration to the HA hydrogel precursor solution, and HA hydrogels were fabricated with a volume of 100 μ L. The fabricated HA hydrogels were rinsed with PBS and incubated at 37 °C for 15 min, and the solution was collected (day 0). Fresh PBS was replaced to each well and incubated at 37 °C in a dark environment. The solutions containing FITC-BSA were collected on days 1, 2, 4, 7, 14, and 21.

The amount of FITC-BSA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The absorbance of the collected solutions was measured at 479 nm, and the concentration was calculated according to the standard curve.

2.4. Cell culture and proliferation & migration assay

NIH/3T3 fibroblasts were purchased from ATCC (Manassas, VA, USA) and cultured in high-DMEM supplemented with 10% v/v FBS and 1% v/v penicillin/streptomycin and incubated at 37 °C in 5% CO₂ and 95% air. The cells (0.5×10^4 cells/cm²) were seeded on one side of each well of the 24-well plates. Cell proliferation was evaluated by MTT assay, and it was performed on days 2, 4, and 6. The control group cells were cultured in the culture medium (500 μ L), and 1 μ g/mL of bFGF was added to culture media for the soluble bFGF group. For the HA hydrogel without bFGF group, HA hydrogel (SH1MA1, 50 μ L) was transferred onto the cell insert of 24-well plates, and 500 μ L of culture medium was added to the 24-well plates. For the HA hydrogel with bFGF groups, SH1MA4, SH1MA1, and SH4MA1 hydrogels containing 10 μ g/mL of bFGF were transferred to the well plates at a volume of 50 μ L, and 500 μ L of the culture medium was added to the 24-well plates. The culture media was refreshed every two days. The culture media and HA hydrogels were removed, and culture media with 10% v/v MTT solution (6 mg/mL) was added on days 2, 4, and 6. The cells were incubated for 1 h at 37 °C, and the formazan crystals transformed from MTT by mitochondrial reductase were dissolved in DMSO. The absorbance was measured at 540 nm using a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA).

A scratch wound healing assay was performed to evaluate the wound

healing effects of bFGF and HA hydrogel. NIH/3T3 fibroblasts (2×10^4 cells/cm²) were seeded on 24-well plates with scratch inserts and incubated until they formed an optically confluent monolayer for 24 h. The scratch inserts were then removed to create the gap. The experimental groups were the same as the cell proliferation assay. The gap closure was observed using an optical microscope (IX 51, Olympus, Tokyo, Japan), and the images were taken at 0, 24, and 48 h for each group. The remained scratch areas were quantified via the wound healing measurement tool of ImageJ. The scratch closure was assessed by dividing the remaining opened area by the initial scratch area.

The proliferation and migration assays were repeated after CD44 receptor blocking of NIH/3T3 fibroblasts to eliminate the HA effect. The cells were seeded in the same manner as the cell proliferation and wound healing scratch assays and incubated at 37 °C in serum-free media with anti-CD44-neutralizing antibody (Anti-CD44) for 20 min. The culture medium was then refreshed and incubated at 37 °C for 18 h. MTT and scratch wound healing assays were performed in the same ways.

2.5. HA hydrogel formation under porcine skin

The porcine skins were purchased from Apures (Gyeonggi, Republic of Korea), and the dimension was 15 mm × 15 mm × 400 μm. The HA hydrogel precursor solution of 1:1 ratio of SH-HA and MA-HA was prepared, including 0.01% w/v RFP. The precursor solution was applied to a petri dish and covered by a piece of porcine skin. BL was exposed for 10 or 60 s toward porcine skin at a 5 mm distance. The resultant HA hydrogels were mounted onto the rheometer, and storage and loss moduli were measured.

2.6. Statistical analysis

All data are expressed as mean ± standard deviation (SD). Each experiment was repeated at least three times unless otherwise indicated. Statistical evaluation was performed using one-way ANOVA. A value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed using the statistical software GraphPad Prism 7.

3. Results and discussion

3.1. HA hydrogel formation by BL-induced thiol-ene reaction

A hyaluronic acid (HA) hydrogel was created by a thiol-ene reaction with riboflavin phosphate (RFP) following a previous study [19]. An alkyl sulfide bond between thiolated HA (SH-HA) and methacrylated HA (MA-HA) was formed with initiation by RFP under blue light (BL) exposure. RFP was used as the photoinitiator and activated by BL. HA hydrogel was fabricated in a well-controlled environment to characterize properties. A mixture solution of MA-HA, SH-HA, and RFP was dropped on a glass substrate, and BL was irradiated to the HA hydrogel precursor solution (Fig. 1). The SH-HA and MA-HA composition of the HA hydrogel was varied to reveal the effect on physical properties and growth factor release profiles (Table 1). The volume ratios between the thiol and methacrylate groups were 1:4, 1:1, and 4:1, and the resultant hydrogels were named SH1MA4, SH1MA1, and SH4MA1, respectively. The RFP concentration was 0.01% w/v, which was optimized in a previous study [19].

The molecular weights of both MA-HA and SH-HA were 450 kDa, and the degrees of substitution of MA-HA and SH-HA were 49.0% and 69.4%, respectively. The volume ratios of the SH and MA groups (1:4, 1:1, and 4:1) are converted to molar ratios of 1:5.6, 1:1.4, and 2.9:1, respectively. The SH and MA groups form a bond at a 1:1 M ratio theoretically; therefore, it is expected that the 1:1 M ratio would create the maximum number of crosslinking sites. However, it was revealed in a previous study that not all the SH and MA groups participated in the thiol-ene reaction [19]. Thus, we crosslinked the HA hydrogels by using the thiol-ene reaction with different ratios of the SH and MA groups and determined the physical properties of the resultant hydrogels.

After the completion of gelation, the dynamic moduli of the HA hydrogels were measured to characterize their physical properties

Table 1

Compositions of HA hydrogel precursor solutions.

	SH-HA (wt%)	MA-HA (wt%)	RFP (wt%)
SH1MA4	0.2	0.8	0.01
SH1MA1	0.5	0.5	0.01
SH4MA1	0.8	0.2	0.01

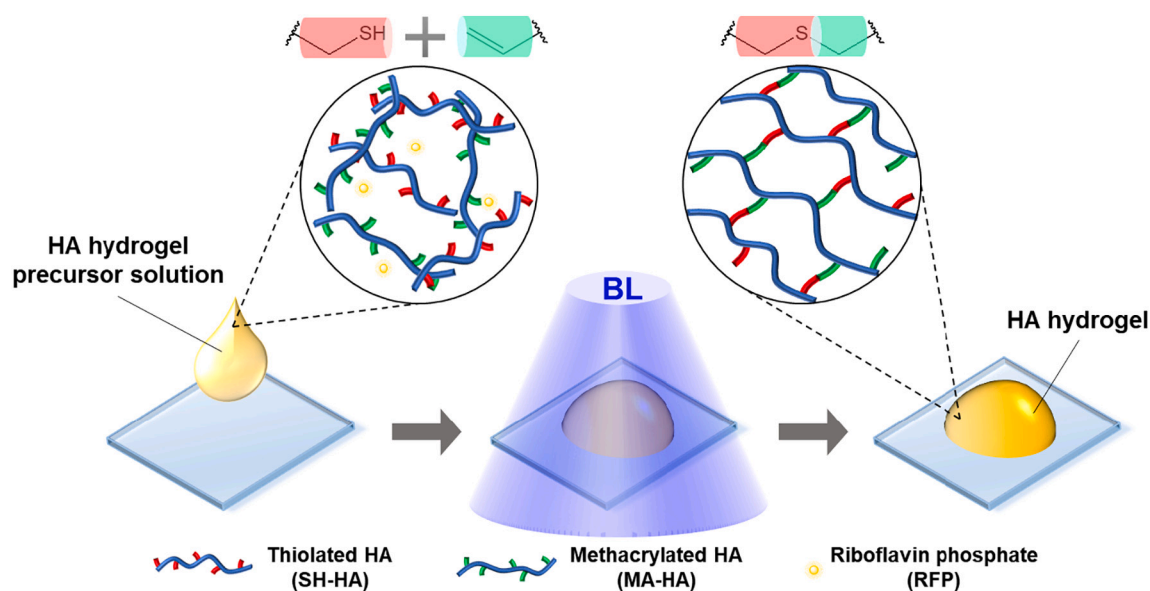


Fig. 1. Schematic images of hyaluronic acid (HA) hydrogel formation by the blue light (BL)-induced thiol-ene reaction of thiolated HA (SH-HA) and methacrylated HA (MA-HA) with riboflavin phosphate (RFP). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

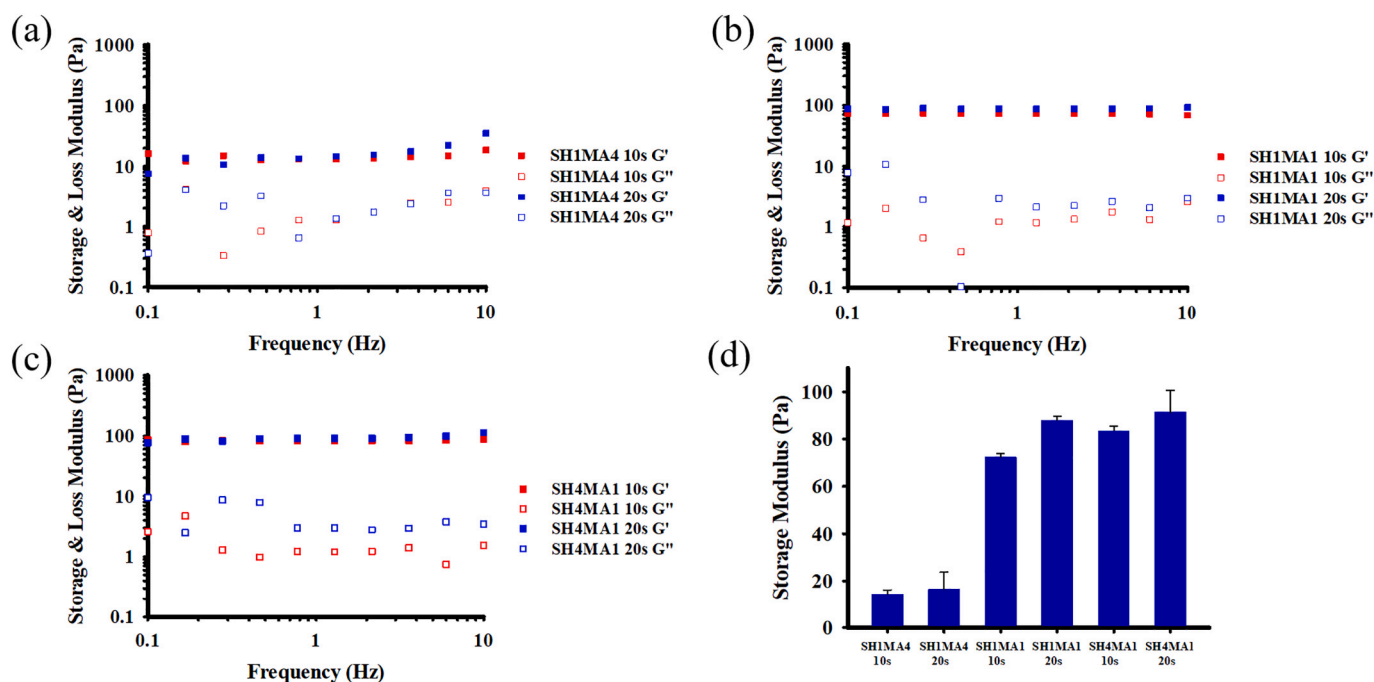


Fig. 2. Dynamic moduli (G' : storage modulus and G'' : loss modulus) plotted as a function of the frequency of (a) SH1MA4, (b) SH1MA1, and (c) SH4MA1 from 0.1 and 10 Hz of frequency. (d) Average storage moduli of HA hydrogel.

(Fig. 2). For all the dynamic moduli results, the storage moduli were higher than the loss moduli. The hydrogels maintained their viscoelastic behavior from 0.1 and 10 Hz of frequency, which indicates that SH-HA and MA-HA showed elastic behavior due to the hydrogel formation with crosslinking. In addition, a longer exposure time increased the storage and loss moduli for SH1MA1 and SH4MA1, so BL exposure for 20 s induced more thiol-ene crosslinking sites than 10 s. Although the hydrogels exposed for 20 s showed higher modulus than when exposed for 10 s, the increase in the storage modulus was not significant considering that the time was doubled. Thus, we fixed the BL exposure time to 10 s to fabricate HA hydrogels for the subsequent experiments because a shorter irradiation time could be advantageous in biomedical

and therapeutic applications.

The storage modulus was compared concerning the ratios of the SH and MA groups. The storage moduli of SH1MA1 and SH4MA1 were 72.40 ± 1.44 and 83.52 ± 2.04 Pa, respectively. They were remarkably higher than the storage modulus of SH1MA4 (14.35 ± 1.80 Pa), suggesting that the SH groups provide more crosslinking sites. Since disulfide bonds rapidly form by the Michael addition in mild conditions, more linkages exist; however, the linkage between the SH groups is temporary because the disulfide bond is reversible and weak [12,38].

The structures of the hydrogel matrices were observed by collecting their scanning electron microscopy (SEM) images. The HA hydrogels showed a highly porous structure, which is characteristic of typical

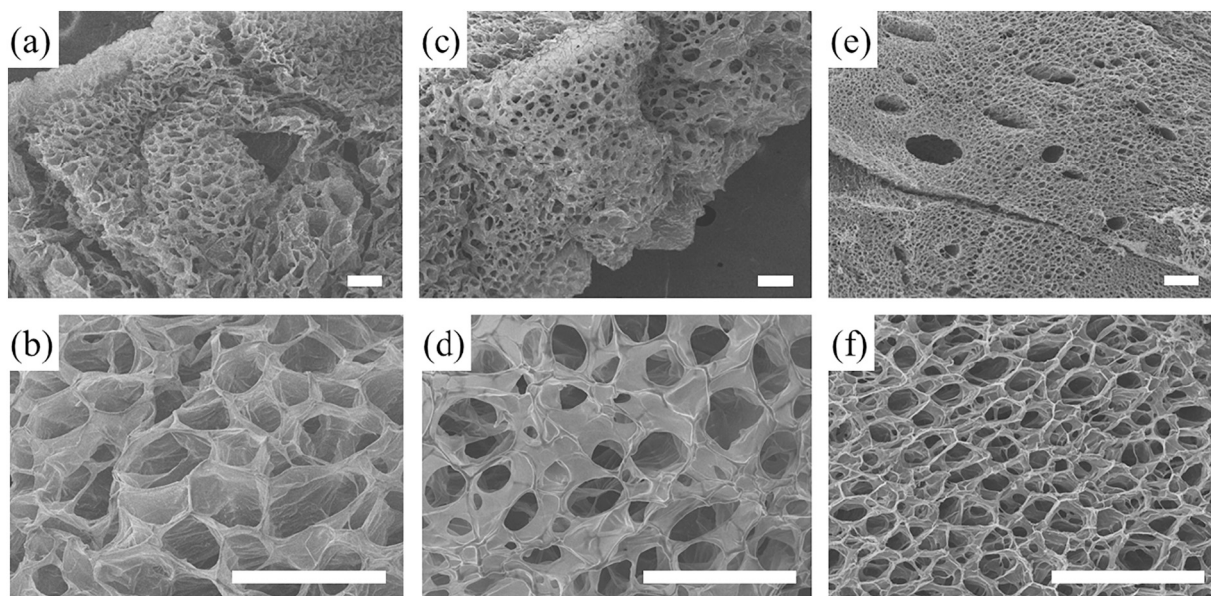


Fig. 3. Scanning electron microscopy (SEM) images of the HA hydrogels. SH1MA4 hydrogels (a, b), SH1MA1 hydrogels (c, d), and SH4MA1 hydrogels (e, f). Scale bars represent 100 μm.

hydrogel matrices (Fig. 3). The HA hydrogels contained interconnected pores, and the pore sizes varied depending on SH-HA and MA-HA ratios. The geometry of the SH1MA4 hydrogel resembled a honeycomb structure, and the pores converged with shared planes. The geometry gradually changed to mesh structures with an absence of planes when the thiol compositions were increased. SH1MA1 showed an intermediate microstructure between SH1MA4 and SH4MA1. The HA hydrogel matrices showed decreasing pore sizes with increasing compositions of SH-HA, although the variations were not statistically significant (Table 2). The microstructure of crosslinked HA hydrogels is related to its mechanical property, and a dense pore structure is connected to a firm mechanical property [10]. We confirmed the relation between pore size and mechanical property for the SH4MA1 HA hydrogel, which had the highest storage modulus and the smallest pore size with a dense structure.

3.2. HA hydrogel degradation and protein release

The degradation of the HA hydrogels was observed by photo images (Fig. 4a). After BL exposure, dome-shaped HA hydrogels were formed on the glass substrates, and all the HA hydrogels were tinged with yellow due to the residual RFP. The next day, RFP diffused out of the hydrogel matrix, and the HA hydrogels became transparent. From day 4, the dome shape of SH1MA4 and SH4MA1 became noticeably rippled, while SH1MA1 maintained its original structure without degradation. The degradation process of SH1MA4 and SH4MA1 lasted for two weeks, and there were no visible changes in the construction of SH1MA1. The results indicate that the composition ratio determines the degradation rate, and the 1:1 ratio of SH to MA forms a hydrogel with a slow degradation rate. When the hydrogels moduli were measured, SH1MA4 had a markedly lower storage modulus than SH1MA1 and SH4MA1 (Fig. 2), so it was expected that the crosslinking density would be lower than the other two compositions. In addition, the microstructure of SH1MA4 was less dense than that of SH1MA1 and SH4MA1 (Fig. 3). Thus, it was evident that the degradation of SH1MA4 was faster than that of the other two HA hydrogels. SH4MA1 showed slightly higher moduli and smaller pore sizes than SH1MA1 (Figs. 2 and 3), so it was expected that the crosslinking density of SH4MA1 was higher than that of SH1MA1, and the degradation rate of SH4MA1 would be slower than that of SH1MA1. However, the degradation of SH4MA1 was faster than that of SH1MA1 (Fig. 4a).

Generally, the disulfide bond results from side reactions and is formed when a higher SH than MA ratio is involved in the thiol-ene reaction [26]. Although there are no previous studies on the formation of photo-crosslinked HA hydrogels from SH-HA, the disulfide bond has been utilized to fabricate HA hydrogels by the oxidation of thiols [12,13]. The result means that the disulfide bond is strong enough to crosslink a hydrogel. SH4MA1 had more disulfide bonds due to the high composition of SH, and it exhibited the highest increase in storage modulus immediately after gelation. However, the disulfide bond is easily decomposed to free thiol groups by reduction [13]. In contrast, the resultant bond of the thiol-ene reaction is strong enough to bear hydrolytic degradation compared to the ester bond [27]. Thus, SH1MA1, with the most similar proportion of SH and HA content, promoted the most alkyl sulfide bond content, and the resulting hydrogel maintained its original shape with the slowest degradation rate over two weeks. The results indicate that the proportions of SH and MA can control the degradation rate of the HA hydrogel along with the initial

mechanical properties.

The total weights of the HA hydrogels were measured to estimate the water uptake and HA matrix degradation (Fig. 4b). The initial weights of all HA hydrogels increased by more than three times until day 4, indicating that hydrogel matrices absorbed water. SH1MA4, which had the weakest mechanical properties and a low crosslinking density, possessed more water than SH1MA1 and SH4MA1. After day 4, the degradation rate increased, and the total weights of the HA hydrogels decreased for all groups. SH1MA4 showed a rapid degradation rate at day 7, and measurements could not be made until complete degradation at day 14. The degradation of both SH1MA1 and SH4MA1 was not completed until day 21. The slopes of the weight changes were similar from day 7 to day 21 for both SH1MA1 and SH4MA1, indicating that the matrix degradation rates were similar. The weights of SH1MA1 and SH4MA1 were 0.50 ± 0.02 g and 0.25 ± 0.02 g at day 21, respectively. The higher storage modulus and smaller pore structure can be expected that SH4MA1 had a higher crosslinking density than SH1MA1. The water uptake capability of SH4MA1 was lower than that of SH1MA1, which is the reason for the weight difference.

The protein release profiles were evaluated using fluorescein isothiocyanate (FITC)-conjugated bovine serum albumin (BSA) as a model protein (Fig. 4c). The excitation and emission wavelengths of FITC were 490 and 525 nm, respectively. The bands overlapped with residual RFP excitation and emission wavelengths at 445 and 518 nm, respectively [39]. The amount of RFP released from the HA hydrogels was measured, and we subtracted the value from the overall amount to calculate the amount of released BSA. The HA hydrogels were rinsed after crosslinking by BL exposure. The amounts of FITC-BSA in the rinsed solutions were measured, and we calculated the protein encapsulation efficiency by subtracting this amount from the initially added amount of protein.

The protein release profiles showed a massive initial burst of release followed by sustained-release, and FITC-BSA was wholly released within 96 h for all the HA hydrogels (Fig. 4c). While HA hydrogel degradation was not completed within 96 h, all the proteins were discharged within 96 h, so the effect of HA hydrogel degradation on the release was low. Leach et al. showed that the BSA release from a photo-crosslinked glycidyl methacrylate-HA (GMHA) hydrogel was rapid, and 60% of the protein was released within 6 h [8]. The mesh size of photo-crosslinked GMHA hydrogels was estimated to be several hundred nanometers [14]. It is estimated that our HA hydrogel mesh size crosslinked by a thiol-ene reaction without a crosslinker is similar to that of the GMHA hydrogel. Thus, the network structure dimension was much larger than the size of BSA, which is approximately 4 nm in diameter and 14 nm long [40]. The driving force of BSA release was diffusion rather than HA structure degradation.

Protein release was completed within 96 h for all the HA hydrogels, although slight differences in the initial release profiles depended on the composition of the thiol and methacrylate groups. The HA hydrogel containing fewer thiol groups showed a rapid protein release profile (Fig. 4c). The percentages of the protein released within 0.25 h were approximately 67%, 54%, and 46% from SH1MA4, SH1MA1, and SH4MA1. The composition differences greatly affected the initial burst in the release of the protein. As shown in Fig. 3, the pore sizes were more prominent when the composition of the thiol group was lower. Therefore, protein diffusion was rapid from SH1MA4, a loose matrix with a low crosslinking density and large pores.

General growth factors have smaller sizes compared to the BSA that we utilized as a model protein. Thus, the incorporated growth factors in our HA hydrogel would be released faster than the BSA since the diffusion is faster when the molecular size is smaller. The sustained release of growth factor could be achieved by tethering functional groups to the growth factors via NHS ester coupling reaction [41]. However, the initial burst of growth factor can be effective in some specific situations, such as prompt recruiting and proliferation of fibroblasts after dermal filler formation. Thus, we incorporated bFGFs into the in situ HA hydrogel and evaluated the cell migration and

Table 2
Pore size of the HA hydrogels ($n = 10$).

	Pore size (μm)
SH1MA4	27.92 ± 5.18
SH1MA1	24.99 ± 4.48
SH4MA1	21.25 ± 5.98

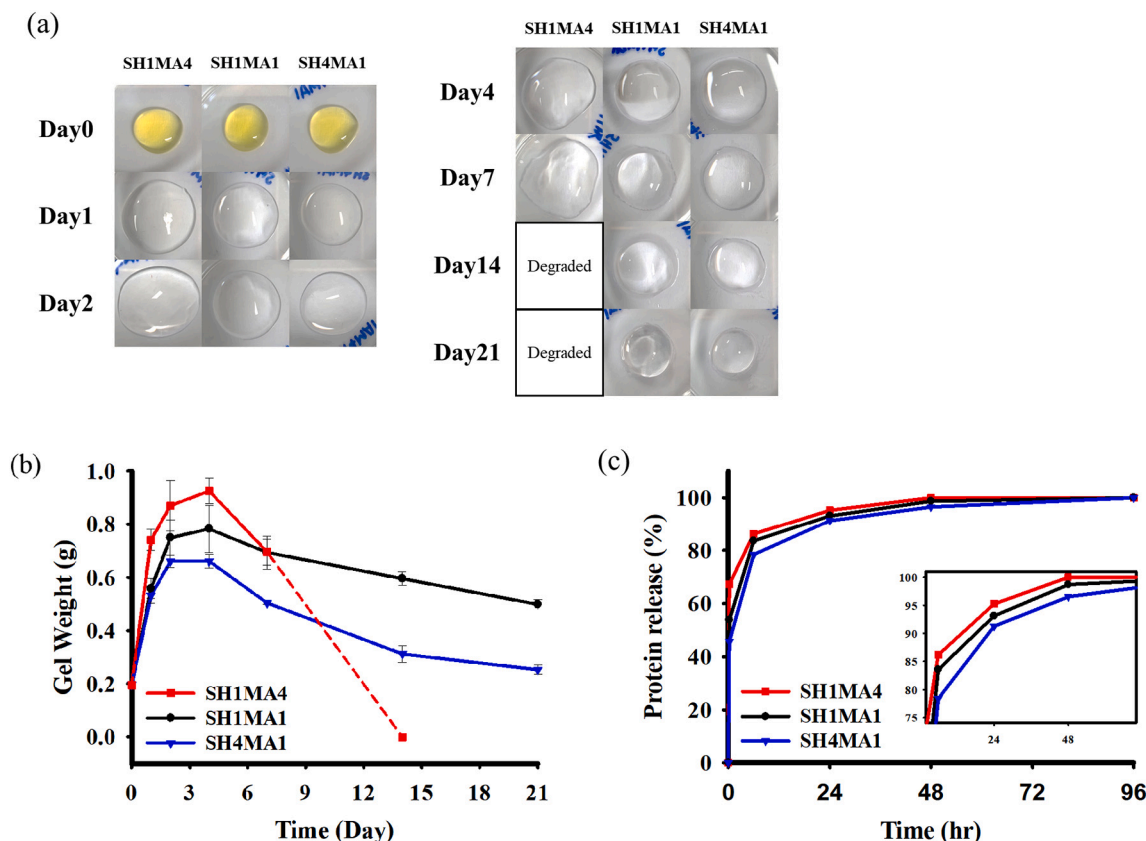


Fig. 4. (a) Images of HA hydrogels showing degradation behaviors. (b) Weights of HA hydrogels with time. (c) FITC-BSA release profile from HA hydrogels depending on different ratios of functional groups.

proliferation for a short period.

3.3. Effects of HA hydrogel with bFGF on fibroblasts

NIH/3T3 fibroblasts were cultured to evaluate the proliferative and migrative effects of bFGF within the HA hydrogel vehicle (Fig. 5). The cells were cultured in a culture medium, including serum for a control group, and a soluble bFGF group was additionally tested with 1 $\mu\text{g/mL}$ of bFGF. The plain HA hydrogel group is a crosslinked HA hydrogel fabricated with a 1:1 ratio of SH-HA and MA-HA, without bFGF incorporation. For the SH1MA4, SH1MA1, SH4MA1 + bFGF groups, bFGF was added to the HA hydrogels before BL exposure, and the resultant hydrogels were transferred to each well plate. On day 2, the soluble bFGF group showed statistically higher proliferation compared to the control group. On day 4, compared to the control group, SH1MA1 + bFGF and SH4MA1 + bFGF showed increased absorbance, while there were no differences in the soluble bFGF, plain HA hydrogel, and SH1MA4 + bFGF groups. The absorbance of SH4MA1 + bFGF was slightly higher than that of SH1MA1 + bFGF, but the difference was not statistically significant. bFGF showed a proliferative effect on day 4 when the SH1MA1 and SH4MA1 hydrogels loaded the growth factor. On day 6, all HA hydrogel groups enhanced the proliferation of fibroblasts. The bFGF-loaded HA hydrogel showed a similar effect, regardless of the ratio of SH-HA and MA-HA.

The soluble bFGF affects fibroblast proliferation, although the promotion was not statistically different. The combination of HA hydrogel and bFGF additionally elevated cell proliferation compared to the summation of each effect of bFGF and HA hydrogel. The soluble bFGF showed a slightly higher proliferation level than the control group, but there were no statistical differences except day 2. In previous *in vitro* studies, the application of soluble growth factors did not significantly affect cell proliferation compared to the standard conditions without

growth factor [41,42]. The culture media were refreshed, and bFGF was also provided for the soluble bFGF group on days 0, 2, and 4. The total amount of bFGF released from the HA hydrogels was matched with the quantity of soluble bFGF. Thus, the release profiles of the soluble bFGF and bFGF from the HA hydrogel were different, but the total amounts of bFGF treated to the cells were the same. The plain HA hydrogel increased cell proliferation without bFGF on day 6, and there was a rapid increase between days 4 and 6. It is known that HA induces the rate of cell division or DNA synthesis [43], and the proliferative effect of HA occurs after day 4 if the cell density is sparse [44]. The initial cell density was 0.5×10^4 cells per well, a mild condition, so a few days were required for the cells to divide actively.

The combination of bFGF and HA hydrogels significantly increased fibroblast proliferation. Since the plain HA hydrogel was similar to the control on day 4, it is suggested that the differences on day 4 were derived from the bFGF. The culture media was refreshed every two days, and the remaining amounts of bFGF would affect cell proliferation. The total amount of bFGF released from HA hydrogel would be higher for SH4MA1 than SH1MA1 and SH1MA1 between day 2 and day 4, so the absorbances were proportional to the amounts of released bFGF. However, the result on day 6 was not related to bFGF since the hydrogel released the proteins entirely within 4 days (Fig. 4C). Although the exact mechanism is unknown, cell proliferation was promoted by HA hydrogels with bFGF exposure at the initial stage. A hypothesis is that the degraded HA molecules stimulate cells and enhance cell proliferation. The degradation of the SH4MA1 hydrogel matrix was remarkably rapid than SH1MA1 and SH1MA4 after day 4, and the degraded HA molecules could increase the absorbance level without bFGFs.

The scratch-based wound healing assay was performed to evaluate the effects of HA hydrogels and bFGF incorporation on the migration of NIH/3T3 fibroblasts (Fig. 5b and c). As with the proliferation test, the soluble bFGF, plain HA hydrogel, or bFGF-loaded HA hydrogels were

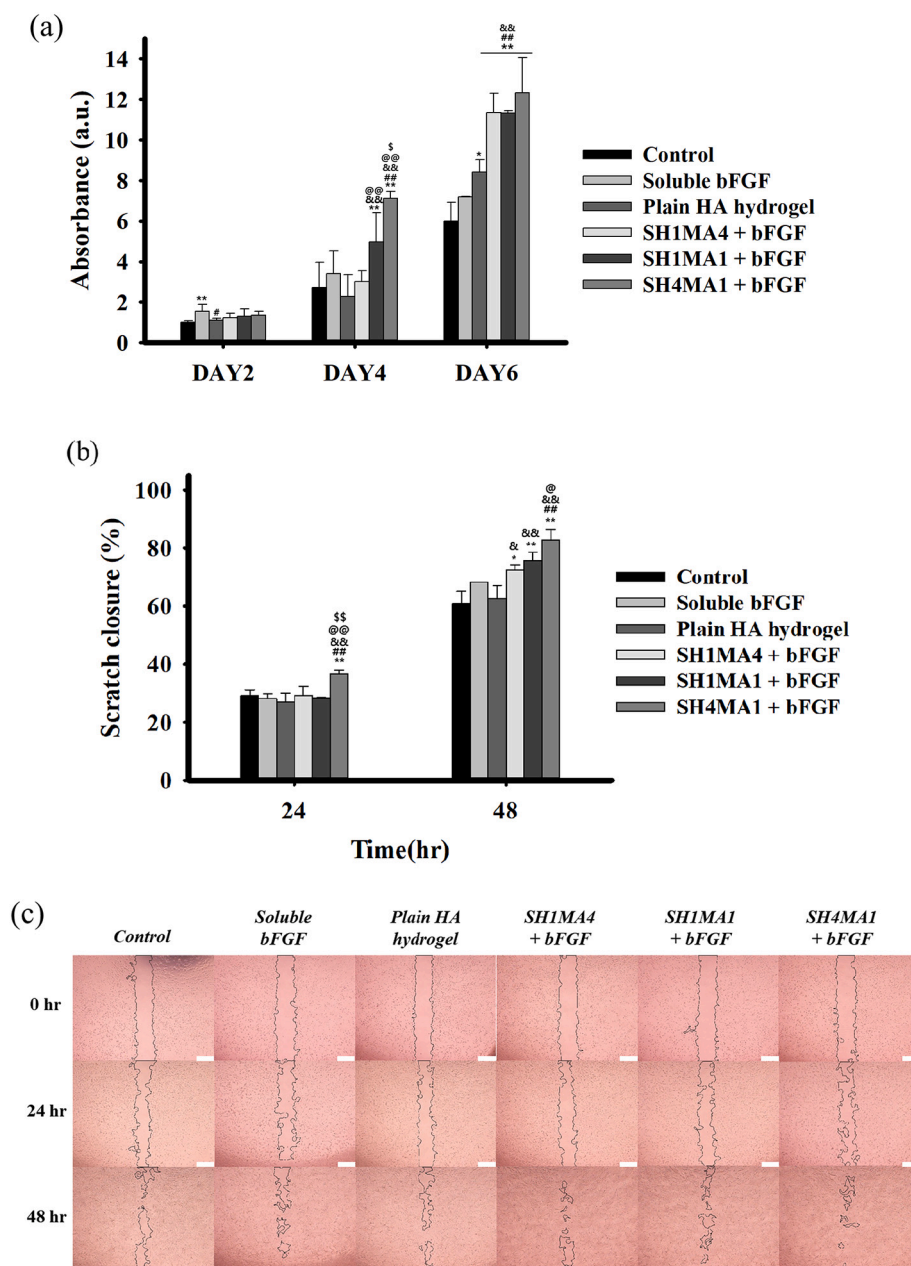


Fig. 5. Effects of bFGF loaded HA hydrogels on NIH/3T3 fibroblast proliferation and wound healing scratch assay. Cell proliferation was evaluated using the MTT assay. (a) Statistical analysis was performed between the groups for each time (Day 2, Day 4, or Day 6). (b) Quantification of scratch closure area using the wound healing measurement tool of ImageJ. The scratch closure was evaluated by dividing the open area by the initial scratch area. Symbols represent significant differences: control (*), soluble bFGF (#), plain HA hydrogel (&), SH1MA4 + bFGF (@), and SH1MA1 + bFGF (\$). The levels of significant differences are also represented by symbols: $0.01 < p < 0.05$ (*, #, &, @, and \$) and $p < 0.01$ (**, ##, &&, @@, and \$\$). (c) Representative images at 0, 24, and 48 h. The black lines generated by the wound healing measurement tool of ImageJ represent the wound borders. Scale bars represent 400 μ m.

added to the well plate on which a scratch was generated. The lateral movement of the scratch border was assessed using the wound healing measurement tool of ImageJ, and the internal areas were measured. At 24 h after the scratch formation, only the SH4MA1 + bFGF group showed a statistical increase in scratch closure compared to the control group, and the scratch closures were 36.70 ± 1.14 and 29.14 ± 2.07 for the SH4MA1 + bFGF and control groups, respectively. At 48 h after the scratch formation, except for the plain HA hydrogel group, all the bFGF-applied groups significantly progressed scratch closure. In particular, the scratch closure was faster when bFGF was incorporated into the HA hydrogels (SH1MA4, SH1MA1, and SH4MA1 + bFGF) compared to the soluble bFGF group, and the SH4MA1 + bFGF group showed the most effective results on cellular migration.

Soluble bFGF exhibited an effect in the wound healing scratch assay. The chemokinetic effect of 0.1–100 ng/mL bFGF on fibroblast migration was measured, and the migrated cell number was over three times that of the cells without bFGF treatment [45]. Moreover, incorporating bFGF in the HA hydrogel enhanced the scratch closure compared to the

soluble bFGF treatment. The combination of bFGF and HA hydrogel should increase the scratch closure rate by the sustained release of bFGF from the HA hydrogel matrix and CD44 interaction with HA because it is known that simultaneous bFGF stimulation and CD44 interactions mediate cell migration [46,47]. The SH4MA1 + bFGF group provided more bFGF between 24 and 48 h since it showed minimum initial bursts in the release of bFGF. The more amounts of bFGF of SH4MA1 affected the scratch closure rate than SH1MA4 and SH1MA1, and degraded HA of SH4MA1 was also able to stimulate the CD44 receptor compared to the soluble bFGF group. Therefore, the SH4MA1 + bFGF group was most effective in scratch closure.

3.4. Effect of bFGF without CD44 receptor stimulation by HA

The proliferation and scratch closure results indicated that both the release profile of bFGF and the CD44 interactions with degraded HA are crucial factors in stimulating cellular proliferation and migration (Fig. 5). The sole influence of bFGF without CD44 receptor stimulation

by HA was evaluated using the CD44 receptor block. Anti-CD44-neutralizing antibody (Anti-CD44) has been used to block the CD44 receptors [37]. The NIH/3T3 fibroblasts, except for the control group, were treated with Anti-CD44 before applying soluble bFGFs or HA hydrogels, and then the proliferation and scratch assays were performed.

Although all groups showed no statistical differences with or without bFGF and HA hydrogel, the groups including bFGF (soluble bFGF and HA hydrogel + bFGF) showed a slight increase in cell proliferation than the control group (Fig. 6a). The effect of the plain HA hydrogel group on proliferation after the Anti-CD44 treatment was almost the same or slightly lower than the control group (Fig. 6a), while SH1MA1 enhanced cell proliferation regardless of bFGF incorporation when the cells were not treated with Anti-CD44 (Fig. 5a). We confirmed that the proliferative effect of the HA hydrogel was derived from the CD44 receptor stimulation. Moreover, there was no additional influence from the incorporated bFGF in the HA hydrogel after the Anti-CD44 treatment. In the scratch assay, similar to the proliferation result, there were no significant differences among the groups after the Anti-CD44 treatment (Fig. 6b).

The stimulation of the CD44 receptor by HA was a significant factor in enhancing cell proliferation and migration, demonstrated in previous studies [46,47]. Thus, we expected blocking CD44 receptors decreased the proliferation and migration. Nevertheless, we anticipated that the bFGF incorporated in HA hydrogel showed better influence than soluble bFGF due to the protective effect of the HA hydrogel matrix despite the absence of CD44 receptor stimulation of HA. However, there was no significant difference between soluble and incorporated bFGFs (Fig. 6). It seems that the release rate of bFGF was too fast to observe the protective effect of the hydrogel matrix, as we evaluated that most of the proteins released out from the matrix within 24 h.

Nevertheless, we demonstrated that the use of HA hydrogel as a degradable delivery matrix could improve the efficiency of therapeutic

factors by stimulating the CD44 receptor of cells and acting as a drug delivery carrier. bFGF was used as a model therapeutic factor to confirm its effectiveness in this study, but other biomolecules may also benefit from the stimulation of the CD44 receptor.

3.5. In situ gelation under porcine skins

The realistic environment for the dermal filler application was created, and in situ gelation was performed under porcine skins. The skin consisted of the epidermis and dermis, and the thickness was approximately 400 μm . The HA hydrogel precursor solution was put under the porcine skin, and then the BL was transmitted through the skin. We tested 10 s exposure first under the same time as when there was no porcine skin. The HA hydrogel was successfully crosslinked under the skin (Fig. 7a). The light intensity was measured according to the presence of porcine skin, and the skin membrane decreased intensity from about 160 mW/m^2 to 30 mW/m^2 . Therefore, the 60 s was tested as a condition with the same total light energy. The HA hydrogels had elastic property ($G' > G''$) for both 10 and 60 s exposure, which represents that the resultant HA hydrogel is appropriate to be used as in situ dermal filler (Fig. 7b and c).

The 10 s exposure of BL through the skin induced 44.60 ± 8.09 Pa of storage modulus. In comparison, while the storage modulus of HA hydrogel was 72.40 ± 1.44 Pa when the BL was directly irradiated to the HA precursor solution. The storage modulus was decreased along with the crosslinking since the BL was not wholly transmitted by the presence of the skin. The extension of exposure time to 60 s increased the storage modulus, 93.57 ± 7.18 Pa. Although the storage modulus is not a direct indicator of dermal filler performance, higher storage modulus generally represents a longer retention time of filler material with corrective facial implantation. The storage moduli of commercial dermal fillers are about 0.5–250 Pa, and most of the dermal fillers had lower than 100 Pa due to the usability of injection [48]. Our proposed in situ HA hydrogel dermal

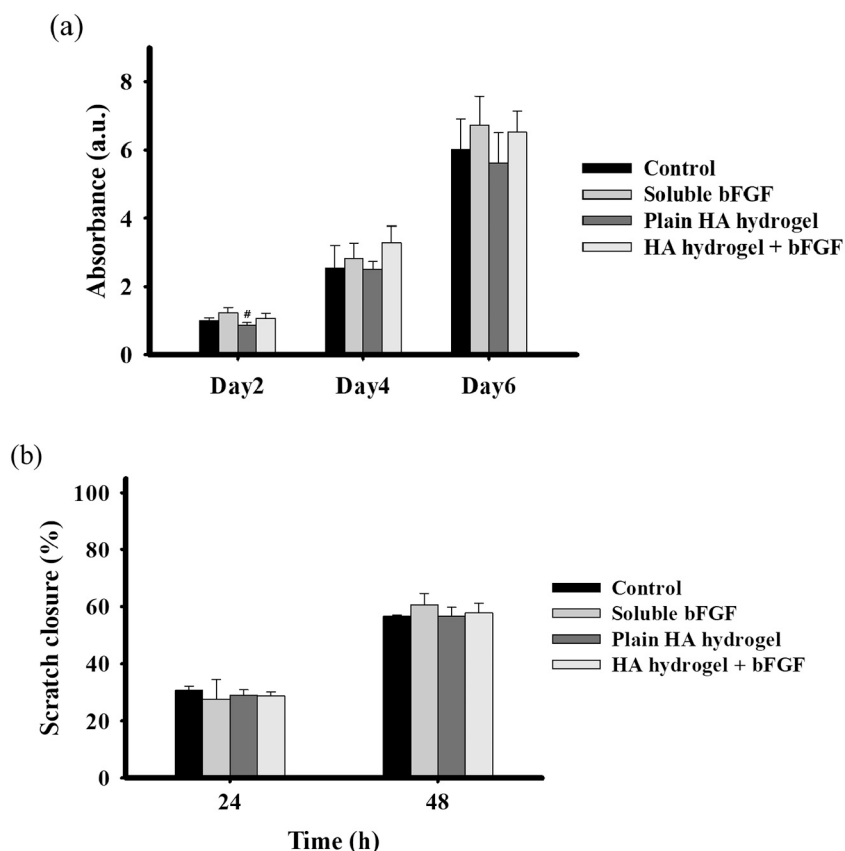


Fig. 6. Effects of bFGF-loaded HA hydrogels on NIH/3T3 fibroblast proliferation after blocking the CD44 receptor. (a) Cell proliferation was evaluated using the MTT assay. (b) Quantification of scratch closure area using the wound healing measurement tool of ImageJ. The scratch closure was evaluated by dividing the open area by the initial scratch area. Statistical analysis was performed between the groups for each time (Day 2, Day 4, or Day 6). The symbol # represent a significant difference to soluble bFGF at the significance level of $0.01 < p < 0.05$ (#). There were no statistical differences for the other comparisons.

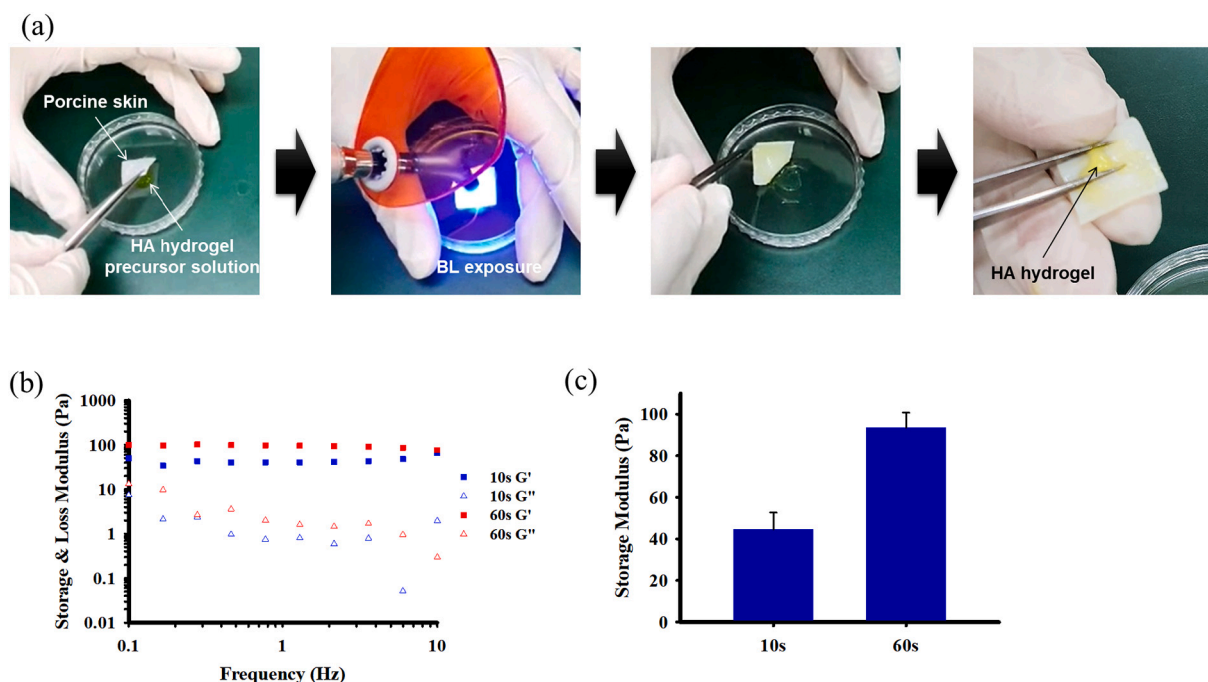


Fig. 7. (a) Fabrication process of in situ HA hydrogel under porcine skins. (b) Dynamic moduli (G' : storage modulus and G'' : loss modulus) plotted as a function of the frequency from 0.1 and 10 Hz of frequency and (c) average storage moduli of in situ HA hydrogels.

filler material is injectable regardless of the physical property of the final HA hydrogel because the HA hydrogel precursor solution is injected before the gelation. Moreover, the physical properties of the HA hydrogel can still be adjusted by the total concentration of HA.

4. Conclusions

HA-based hydrogels are versatile in therapeutic applications, and in situ visible light-induced thiol-ene reactions can extend the applications of HA hydrogels to injectable systems and on-site crosslinking. In this study, we evaluated the therapeutic effects of HA hydrogels fabricated by a visible light-induced thiol-ene reaction with bFGF, which induces fibroblast proliferation and migration. The compositions of SH-HA and MA-HA were adjusted to assess the effects of structure on the physical properties of HA hydrogels. The HA hydrogel with a higher ratio of thiol groups showed relatively higher storage moduli with a dense matrix structure. The release profiles of BSAs from HA hydrogels were not significantly different, but the degradation rate of HA hydrogel matrices could be controlled by the compositions of SH-HA and MA-HA. Cellular proliferation and the scratch closure rate were enhanced by bFGF slightly, and the effect of HA hydrogel was dominant than bFGF. The HA hydrogel fabricated by a visible light-induced thiol-ene reaction served to carry the labile therapeutic factor. The gelation was completed in a practical model situation, and the resultant HA hydrogel had appropriate physical property as a dermal filler. Our proposed in situ HA hydrogel material is injectable, and it could have prolonged retention time as a corrective facial implant due to the enhanced storage modulus. Thus, it can be an ideal in situ injectable therapeutic hydrogel material.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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