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히알루론산 피부용 필러의 분자량에 따른 팽윤도와 탄성계수 영향

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Influence of Molecular Weight on Swelling and Elastic Modulus of Hyaluronic Acid Dermal Fillers

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초록: 분자량이 다른 세종류 히알루론산(HA)에 1,4-butanediol diglycidyl ether(BDDE)가 가교된 HA를 첨가하여 하이드로겔을 제조하고 HA 분자량에 따른 팽윤도와 탄성계수의 효과를 조사하였다. 최종 투석 후 잔류 BDDE 양은 0.5 ppm 이하이었다. 비가교된 분자량이 큰 HA에서 최대 팽윤도가 관찰되었다. 팽윤도는 비가교 HA 양과 가교도에 각각 비례와 반비례하였다. 1.0 w/v% BDDE 가교 HA와 HA(1368 kDa)를 혼합한 monophasic 하이드로젤의 탄성계수는 152~325 Pa이었다. 15% 가교 HA(687 kDa)과 85% HA(1368 kDa)로 구성된 biphasic 하이드로젤의 탄성계수는 178 Pa이었다.

Abstract: 1,4-Butanediol diglycidyl ether (BDDE) crosslinked hyaluronic acid (HA) suspended in three different HAs (697, 1058, 1368 kDa) were prepared to investigate the effect of HA molecular weight on swelling property and elastic modulus of the hydrogels. The amount of the residual BDDE after final dialysis was less than 0.5 ppm. The highest swelling ratio was observed for the uncrosslinked HA having the largest molecular weight. The expansion capacity of HA rose with increasing the amount of pure HA and was inversely proportional to the crosslinking degree due to an increased number of coiled HA chain interactions. Elastic modulus (G') of monophasic fillers having different ratios of 1.0 w/v% BDDE crosslinked HAs to pure HA (1368 kDa) were within 152 and 325 Pa. 178 Pa was observed for the biphasic fillers consisting of 15% crosslinked HA (697 kDa) nanoparticles suspended in 85% of uncrosslinked HA (1368 kDa).

Keywords: hyaluronic acid hydrogel, dermal filler, 1,4-butanediol diglycidyl ether, crosslink, elastic modulus.

Introduction

Hyaluronic acid (HA) is an unbranched glycosaminoglycan with a disaccharidic repeating unit composed of glucuronic acid and *N*-acetylglucosamine linked through alternating β-1-4 and β-1-3 glycosidic bonds.[1-14] The human dermis has a high percentage of HA, which hydrates the skin because of high water-binding properties. In addition, it also serves as a lubricant to provide a protective environment to the cells by facilitating the movement. It is the predominant part of the viscoelastic intracellular matrix, which provides and maintains proper tissue volume and acts as a cushion to help protect the skin.[7-9]

A 70 kg person has just 15 g of dry-weight HA.[11] Naturally occurring HAs are constantly being turned over the skin (5g/day). One-third of the body's HA is known to be degraded and replenished every day. Approximately 50% of the total quantity of HA in the human body is concentrated in the skin and

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it has a half-life of 24-48 h.[11] To improve the longevity of HA, it must be made more robust and stable by crosslinking.[1-16] The non-animal derived and stabilized HA (NASHA) products and the crosslinking agent, 1,4-butanediol diglycidyl ether (BDDE), are used in the majority of the market-leading HA hydrogels (HAHs) due to its stability, biodegradability, safety, biocompatibility, hydrophilicity, good tissue integration, and tunable duration of action spanning the entire range of the temporary filler category (6-24 months).[7,9-14]

The epoxide groups in BDDE preferentially react with the most accessible primary alcohol in the HA backbone, forming an ether bond connection.[7,9-14] It is known that the BDDE has a significantly lower toxicity than other ether-bond crosslinking chemistry based agents. Hydrogels are 3-D high-molecular weight networks composed of a polymer backbone, a water and a crosslinking agent. Since the hydrogels have structural similarities to body tissue, they can be used as soft tissue fillers for defected tissue regeneration by replacing water with biological body fluid, cell, and/or medicine.[7]

Although the hydrogel was not strong enough to support the mechanical loading of tissues (cartilage), this soft hydrogel could be used for the regeneration of the non-loading areas of bones and the skin. HA dermal fillers, the most widely used filler substance currently on the market, are employed to lift and fill wrinkles in the skin. In the present study, the HAHs are prepared by varying the molecular weight of HAs (697, 1058, 1368 kDa) and the degree of BDDE crosslinking (0, 0.1, 0.3, 0.5, 1.0 w/v%) to investigate physical properties of the HAHs.

Experimental

Materials. HA solution was prepared by dissolving 3 sodium hyaluronates (HA(1.3)=697 kDa, HA(1.8)=1058 kDa, and HA(2.2)=1368 kDa, Shiseido Co., Tokyo, Japan) in 0.05 mol/L NaOH at room temperature, as shown in Figure 1. The HA solution was crosslinked with BDDE (0.1~1 w/vol%) by using a homogenizer for 10~20 min at 50 °C. Then, pH of the crosslinked solution was adjusted to ~7.4 by adding 1 N HCl. Dialysis is performed for 3 days against PBS for 2 days and distilled water for 1 day to eliminate the residual crosslinkers. The product was then lyophilized for 2 days to obtain solid HA. For gel preparation, HAs dissolved in a PBS solution were incubated for gelation. The HAHs (20 mg/mL) were then prepared.

Properties of HA Hydrogels. The elastic and viscous response of hydrogel depend on the concentration and molecular weight of the HA and on the frequency used during the measurements.[7-12] Rheological behaviors of HAHs were analyzed with a Thermo Haake RS1 Rheometer (Newington, USA), using a plate and plate geometry with a 1.2 mm gap. All measurements were performed using a 20 mm titanium sensor at 25 °C. Oscillation measurements were taken at 5 Pa tau over a frequency range of 0.01 to 100 Hz. The mechanical properties of hydrogels was evaluated by measuring the storage modulus(G'), loss modulus(G''), and viscosity. The G' measured at frequency of 5 Hz for these gels was compared in the present study.[12]

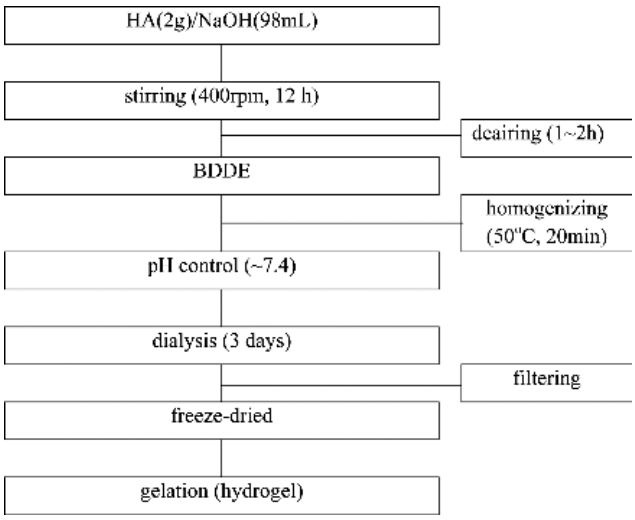

Figure 1. A flowchart for the fabrication of crosslinked HA hydrogel.

Swelling Studies. In order to measure the swelling properties of HAHs, it was placed in distilled water for 24 h at room temperature.[9] The swelling ratio was measured by comparing the change of hydrogel weight before and after incubation. The percentage of water absorbed was calculated by the following eq. (1):

$$\text{Swelling rate}(\%) = \{(W_w - W_i)/W_i\} \times 100 \quad (1)$$

Where W_w and W_i are the wet weight and initial weight of hydrogel, respectively.

Gas Chromatography. BDDE preferentially reacts with the primary alcohol groups in the HA backbone. Dialysis is performed against PBS for 2 days and distilled water for 1 day to eliminate the residual crosslinkers. The presence of BDDE after cleaning may cause adverse, allergic reactions and potential noxiousness of the dermal fillers because they are used within the dermis for several months. The presence of the unreacted residual crosslinker in HAHs is evaluated by using gas chromatography (YL6100 GC, Younglin Co., Ltd., Yongin, Korea).[1]

Results and Discussion

Three different HAs are crosslinked with BDDE (0, 0.3, 0.5, 1.0 w/v%) to extend the duration because uncrosslinked HA is completely metabolized a few day after injection.[12] The epoxide groups in BDDE preferentially react with the alcohol in the HA backbone, forming an ether bond connections, as depicted in Figure 2. The presence of residual BDDE after dialysis was evaluated by using a GC, as shown in Figure 3. Since the BDDE, detected at 4.8 min in GC graph (Figure 3), is likely to be the mutagenic potential, the amount of unreacted residual BDDE is maintained at trace amounts of <2 ppm, which has been determined to be safe after a Food and Drug Administration (FDA).[7] GC results of 0.3 w/v% BDDE crosslinked HA (1.8) revealed that the amount of the unreacted residual BDDE after final dialysis was less than 0.5 ppm, suggesting that the crosslinker was successfully removed.

The highest swelling ratio (4080%) is observed for the uncrosslinked HA (2.2) having the largest molecular weight (1368 kDa), as displayed in Figure 4. The swelling ratio decreased with increasing the BDDE concentration and decreasing the molecular weight due to an increased number of coiled HA chain interactions. SEM results revealed that pore size and porosity decreased and increased with increasing the BDDE concentration, respectively, as illustrated in Figure 5.

A 3-D network of HAHs is formed when crosslinks between

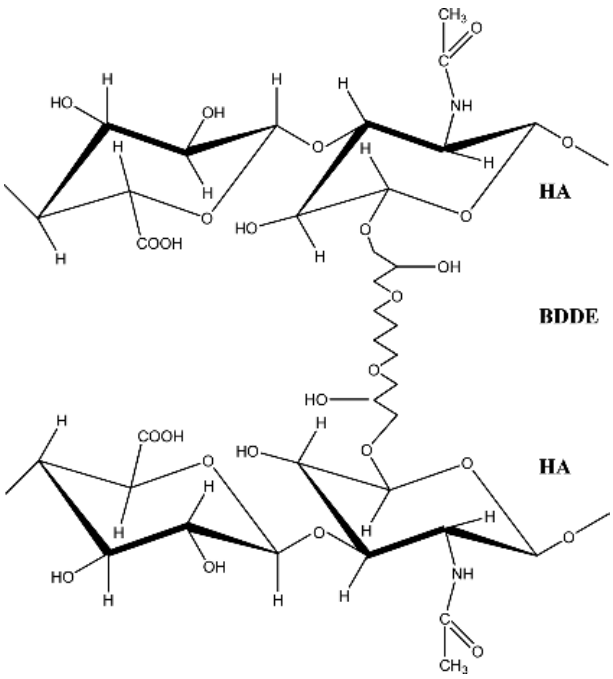

Figure 2. Chemical structure of crosslinked HA with BDDE.

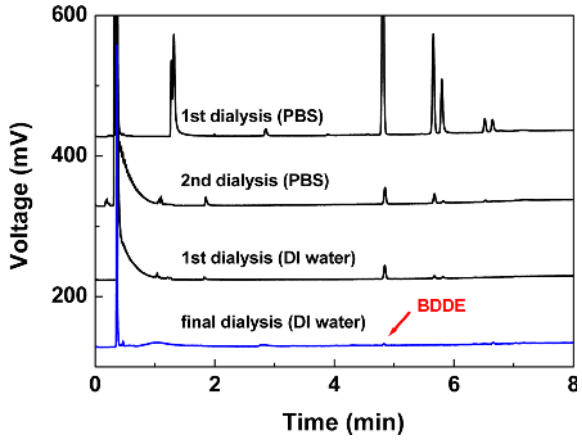

Figure 3. GC graphs of 0.3 w/v% BDDE crosslinked HA(1.8).

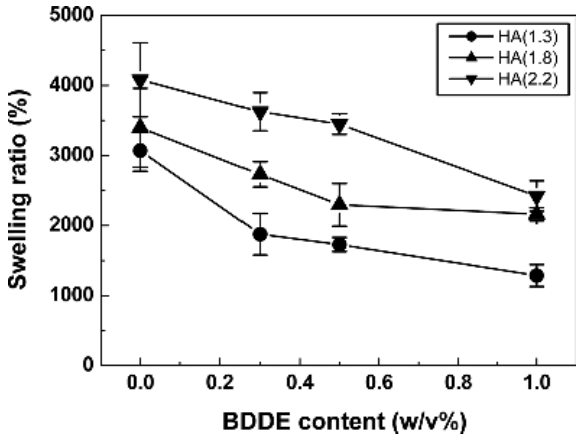

Figure 4. Swelling ratio of various HA membranes as a function of BDDE content.

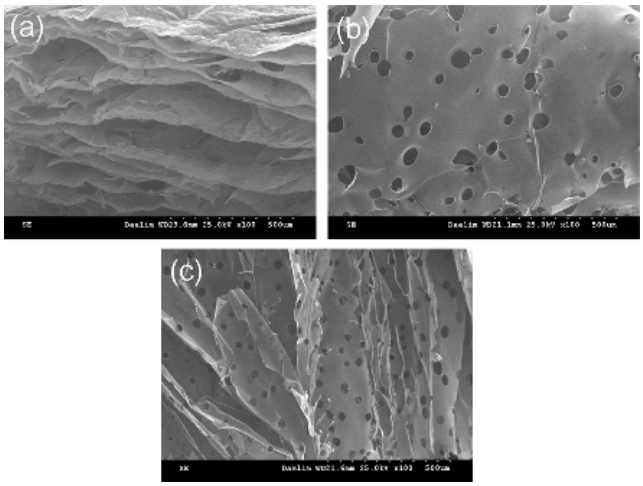

Figure 5. SEM images of 1.0 w/v% BDDE crosslinked: (a) HA (1.3); (b) HA (1.8); (c) HA (2.2) membranes.

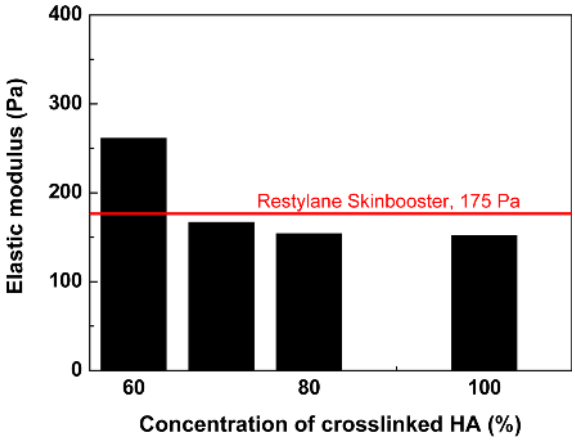

Figure 6. Elastic modulus values of HAHs having different amounts of 1.0 w/v% BDDE crosslinked HAs. Note that molecular weight of HA (2.2) is 1368 kDa.

the HA chains are introduced. A strong gel has a high elasticity, meaning that the response to deformation is mainly elastic. Factors that affect the rheological properties of gels are the HA concentration and the crosslinking density. Uncrosslinked HA is completely metabolized a few day after injection. The ratios of the crosslinked HA concentration to the free HA concentration for commercially available dermal fillers are 98:2 for Hylaform/Prevelle, 75:25 for Restylane/Perlane, 60:40 for Juvederm (30 HV), respectively, which are attributed to their longer duration.[12] Elastic modulus (G') of HAH was evaluated as a function of the ratio of the crosslinked HA concentration to the free HA concentration. Figure 6 revealed that G' decreased with increasing the amount of crosslinked HA.

Approximately 50% of the total quantity of HA in the human body is concentrated in the skin, which is likely to degrade within 24 to 48 h.[7,12] Therefore, HA is crosslinked to increase its longevity, and the crosslinking agent, BDDE, has an important effect on the properties of the dermal fillers. However, the presence of residual BDDE may be harmful to human body due to the reactive nature of the epoxide groups. FDA-approved fillers are manufactured with a design specification of <2 ppm unreacted BDDE in the finished products.[12] GC results revealed that the amount of residual unreacted BDDE in HAHs was maintained at trace amounts of <0.5 ppm, demonstrating that the BDDE-crosslinked HAHs are clinically safe. Also, these remaining trace amounts, which the FDA has determined to be below the level that is safe after a safety risk assessment, are easily converted into CO_2 and water.[7]

Since the filling capacity of a dermal filler is known to be dependent on its water uptake capacity, the swelling ratio was evaluated.[10,13] It is reported that the highest water uptake suggests a lower crosslinking extent with respect to the other gels. On the contrary, the lowest swelling capacity may be due to a higher crosslinking density. The swelling ratio decreased with increasing the BDDE concentration (higher crosslinking) and decreasing the molecular weight due to an increased number of coiled HA chain interactions. Experimental results in Figure 4 are in good agreement with previous results.[13] It is evident that the expansion capacity of HAHs rises with increasing the amount of pure HA and is inversely proportional to the crosslinking degree.

The filling capacity is also affected by the surrounding tissue resistance to gel enlargement and by the rheological behavior of the filler itself during *in situ* implantation. To achieve correction of lines and wrinkles and restore volume, the gel implant should lift the tissue. The strong gel can provide the force required to lift the tissue and resist subsequent deformation, resulting in the desired correction. A high lifting capacity therefore requires high gel strength. The elastic modulus represents the stiffness of the gels and the ease of extrusion of the product. It is known that more deformable gels, having lower G' values ranging from 120 to 430 Pa, can be injected through the finer needles between 29 and 30 gage.[10] In contrast, larger needles (26~27 gage) are required for the extrusion of firmer gels (1400~1800 Pa). G' values of HAHs having different ratios of 1.0 w/v% BDDE crosslinked HAs to pure HA, as shown in Figure 6, are between 152 and 325 Pa. They can be extruded through the 29~30-gage needle, which can be applicable to the treatment of non-severe defects and generally for thinner and softer skin rather than the correction of deep folds and skin deformation.[13,14] The G' value of commercial soft skin filler, Restylane Skinbooster (175 Pa), is indicated as red line in Figure 5. Results of G' values investigated in the present study suggested that the content of 1.0 w/v% BDDE crosslinked HA is likely to be in the range of 60 and 70. The as-prepared HAHs are monophasic monodensified gels produced by mixing the HAs and crosslinking in one step, allowing for ease of injection.[11] The portion of crosslinked HA in HAHs are very similar to Juvederm products (60%) commercially used in the market.[12,14]

However, Restylane is biphasic products consisting of the crosslinked particles (75%) suspended in non-crosslinked HA (25%) used as a carrier.[9] Nanoparticles made from 697 kDa HA (1.3) were blended with 1368 kDa HA (2.2) in different ratios, as depicted in Figure 7. Nanoparticles were synthesized after the consumption of carboxyl groups by two crosslinkers

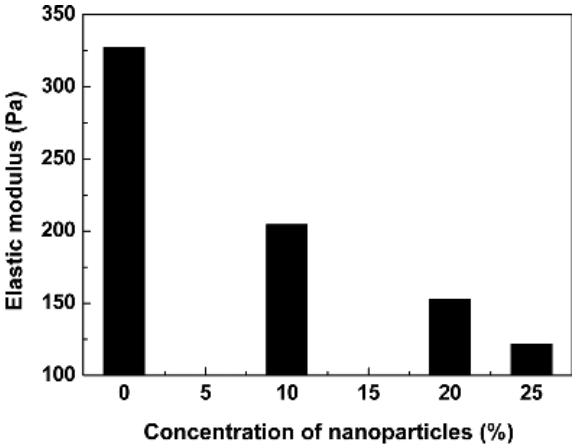

Figure 7. Elastic modulus values of HAHs consisting of the crosslinked HA (1.3) nanoparticles suspended in non-crosslinked HA (2.2).

of 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride and adipic acid dihydrazide. The experimental procedure is described elsewhere.[15,16] The nanoparticle size decreased from 140 to 95 nm with increasing the HA molecular weight from 697 to 1368 kDa. However, the flowability of nanoparticles rose dramatically, which was detrimental to dermal filler. G' value of 178 Pa was obtained for the HAHs composing of 15% crosslinked nanoparticles suspended in 85% of non-crosslinked HA (2.2). The polymer solution is very viscous, however, the nanoparticle suspension could flow easily because of its low viscosity. Polymer chains in an HA solution entangle and can self-associate, resulting in high viscosity. However, HA nanoparticles contain a less-accessible crosslinked polymer structure, thus reducing polymer chain interactions and thereby lowering the viscosity.[15] The viscosity of the nanoparticle suspension was waterlike. Although the G' value is similar to that of Restylane, the portion of the crosslinked HA nanoparticle in HAHs is dramatically decreased from 75% to 15%, which is detrimental to clinical duration as dermal fillers. The 15% portion is also very low as compared to those (60~70%) of monophasic HA fillers.

Conclusions

HAHs containing BDDE crosslinked HAs (0, 0.3, 0.5, 1.0 w/v%) were prepared to investigate the effect of crosslinking degree on elastic modulus, swelling property, and morphology of the hydrogels. GC results of BDDE crosslinked HA revealed that the amount of the unreacted residual BDDE after final dialysis was less than 0.5 ppm. The highest swelling ratio was observed for the uncrosslinked HA (2.2) having the largest molecular weight. The expansion capacity of HA membranes rise with increasing the amount of pure HA and is inversely proportional to the crosslinking degree due to an increased number of coiled HA chain interactions. Pore size and porosity decreased and increased with increasing the BDDE concentration. G' of HAHs having different ratios of 1.0 w/v% BDDE crosslinked HAs to pure HA were between 152 and 325 Pa. They can be extruded through the 30-gage needle, which can be applicable to the treatment of non-severe defects and generally for thinner and softer skin. G' value of 178 Pa was observed for the HAHs composed of 15% crosslinked nanoparticles suspended in 85% of non-crosslinked HA (2.2). However, the amount of crosslinked HA nanoparticle is very low as compared to those (60~70%) of monophasic HAHs, which is detrimental to clinical longevity.

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