가교된 히알루론산 구슬로 제조한 피부용 필러의 점탄성 특성

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Viscoelasticity of Hyaluronic Acid Dermal Fillers Prepared by Crosslinked HA Microspheres

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초록: 디비닐 설폰 가교제로 가교한 히알루론산(HA) 구슬과 비가교된 HA의 부피비가 65/35~95/5로 다른 피부용 HA 필러를 제조하여 가교된 HA 구슬이 필러의 탄성계수와 입자감에 대한 효과를 조사하였다. 구슬 내 2~4±0.5 μm 내부기공을 가진 HA 구슬의 평균 입도는 60~100±4 μm이었다. HA 필러의 가교된 젤 입자 크기는 300±30 μm이었다. 가교된 HA 미세구슬의 부피비가 65에서 95%로 증가함에 따라 필러의 탄성계수는 211에서 700 Pa로 증가하였다. 29~30 게이지 주사바늘을 통과할 수 있는 175~420 Pa 탄성계수를 가진 필러의 가교된 구슬의 부피함량은 65~85%이었다. 실험결과, 모든 필러들은 가교된 HA 구슬의 부피비가 증가할수록 젤 입자 밀도 증가로 인하여 입자감도 증가하였다. 본 연구에서 injectability와 입자감이 우수한 피부용 필러를 성공적으로 제조하였다.

Abstract: Hyaluronic acid (HA) dermal fillers having different ratios (65/35~95/5) of crosslinked HA microspheres (CHMs) to pure HAs (PHs) are synthesized to investigate the effect of CHMs on the variation of elastic modulus (G') and particle texturing feel (PTF). The diameter of CHMs is in the range of 60 to 100±4 μm with a 3-D porous structure channeled with 2 to 4±0.5 μm pores. The fillers consist of gel particles of 300±30 μm size. G' increased from 211 to 700 Pa with raising the volume fraction of CHM from 65% to 95%. The fillers having the ratios of 65% to 85% exhibit the G' values in the range of 175 Pa to 430 Pa, which can be extruded through the 29~30-gage needle. Experimental results reveal that PTF rises with increasing the volume fraction of CHM due to high density of gel particles. Excellent gel injectability and PTF are successfully achieved.

Keywords: dermal filler, hyaluronic acid, divinyl sulfone, crosslink, microsphere, elastic modulus, particle texture feel.

Introduction

Hyaluronic acid (HA) is a linear polysaccharide formed from the disaccharide units of *D*-glucuronic acid and *N*-acetyl-glucosamine linked by β(1,4) and β(1,3) glucosidic bonds.[1-9] The water-soluble polymer having molecular weight in the range of $5×10^4$ to 10^8 Da, is naturally present in vertebrate organisms as well as bacteria.[1] It is especially abundant in the synovial fluid of joints (3~4 mg/mL, wet weight), the extra-cellular matrix of connective tissues, the dermis and epidermis (0.5 mg/g, wet tissue) of the skin and the vitreous humor of the eye (0.1 mg/mL, wet weight). The HA's immunoneutrality and hydrophilic property necessary for hydrogel synthesis make it an excellent building block for biomaterials to be employed for tissue engineering and drug delivery system.[1,2] Despite these advantages of HA, the short half-life of HA limits widespread use.

A variety of HA fillers have been used for the treatment of wrinkles, scars and facial contouring defects.[1-11] HA dermal fillers are classified into two types depending on variations in crosslinking, monophasic and biphasic.[3-8] Monophasic HA fillers (Surgiderm 24XP, England, Juvederm Ultra™, USA) are known as solely stabilized and non-particulate HA gels.[3,4] The monophasic fillers are prepared by varying the amount of high-molecular-weight HA and low-molecular-weight HA. They have weak strength of gel and can easily transform by external force. No appreciable particle texture can be felt when touching gels with hands. In contrast, biphasic fillers (Restylane®, Q-Med, Sweden) consist of gel particles (250 μm in size) of stabilized HA suspended in a non-crosslinked HA.[3,4] The crosslinked HA gel particles are suspended in non-crosslinked HA, which acts as a lubricant, allowing the suspension to be pushed through a fine needle.[4] They are reported to have a rapid initial degradation of non-crosslinked HA and a slower degradation of the stabilized gel particles, whereas monophasic gels are degraded more uniformly.[3-5]

Restylane® products (biphasic gel) are the most widely used fillers in the market. The ratio of crosslinked HA particle to free HA is 75:25, which is attributed to its longer duration.[7] Crosslinked gel nanoparticles are previously prepared to feel the particle texture. Nanoparticles are synthesized after the consumption of carboxyl groups by crosslinker of adipic acid dihydrazide. The experimental procedure is described elsewhere.[7-11] The nanoparticle size decreases from 140 to 95 nm with increasing the HA molecular weight from 697 to 1368 kDa.[12] The decrease in nanoparticle size with increasing HA molecular weight may be due to intramolecular crosslinking rather than intermolecular crosslinking.[9] However, the flowability of nanoparticles rises dramatically due to the loss of viscous modulus, which is detrimental to dermal fillers. The elastic modulus (G') is determined to be 178 Pa for the fillers composed of 15% nanoparticles suspended in 85% of free HA.[12] Although the elastic modulus is similar to that of Restylane® (175 Pa), the amount of the crosslinked HA particles decrease dramatically from 85% to 15%, which is detrimental to clinical duration within the dermis as dermal fillers. The 15% portion is also very low as compared to those (60~70%) of monophasic fillers.[12] In addition, the particle texturing feel (PTF) is not achieved probably due to the size and the elastic properties of nanoparticles.

In the present study, biphasic HA fillers are studied not only to extend the duration of HA in the body but to increase the PTF. PTF is achieved by preparing microspheres instead of

nanoparticles with an aid of a modified spray method.[13-16] The duration of HA in the body by improving its resistance to enzymatic degradation within the dermis is extended by raising the portion of crosslinked particles dramatically from 15% to more than 75%. Covalent linkages between polymer chains can be obtained by the reaction of functional groups of crosslinking agent (vinyl group, $CH_2=CH-$) and HA (hydroxyl group, -OH). The network properties is adjusted by the concentration of the dissolved polymer and the amount of crosslinking agent.[13-16]

Experimental

Materials. HA with different molecular weights (1058 and 1368 kDa) was purchased from Shiseido Company (Tokyo, Japan). Divinyl sulfone (DVS, 97%) was purchased from Sigma-Aldrich (Germany). 2-methyl-1-propanol (99%), ethanol (99.5%), and sodium hydroxide (bead, 98%) were obtained from Samchun Pure Chemical Company (Korea).

Preparation of Microspheres. HA solutions of 0.5 wt% concentration were prepared by dissolving sodium hyaluronate (*Streptococcus*, M_w =1058 kDa, Shiseido Co., Japan) in 0.05 mol/L NaOH for 24 h at room temperature (RT). A pH in the range of 11 to 12 was adjusted by adding 10 mol/L NaOH to the HA solution. Then, the HA solution was placed in a solution hopper attached to the Masterflex L/S tubing pump (Cole Parmer, USA) and fed into a syringe equipped with a 22-gage metal needle with a flat outlet at a flow rate of 0.005 mL/min.[13-16] The distance between the nozzle and the solution was 4 cm. Microbeads were fabricated by supplying compressed air (0.034 MPa) along the HA solution nozzle. The nozzle was enclosed by a delivery tube with a diameter of 6 mm. Microbeads were collected in a solution mixture of 0.2 vol% of DVS in 2-methyl-1-propanol, followed by a stirring process (MST Digital, IKA, Germany) for 24 h at RT. Then, the crosslinked microbeads were screened through a 325 mesh sieve. The microbeads were immersed in distilled water for 0.5 h and ethanol for 0.5 h to remove the residual crosslinker. After removal of an unreacted residual crosslinker, the microbeads were then dried for 2 h at 60 °C in a vacuum of 20 torr. The as-dried microbeads were examined by using SEM (S-3000H, Hitachi, Japan) and optical microscopy (SV-55, Sometech, Korea) to investigate the morphology and the size of the beads.

Hydrogels. The HA hydrogels (HAHs) are biphasic products consisting of the crosslinked beads (1058 kDa) suspended in non-crosslinked HA (1368 kDa) used as a carrier. For gel

preparation, free HAs (20 mg/mL) dissolved in a phosphate buffered saline solution (PBS, NaH_2PO_4) and swollen cross-linked microspheres were homogenized for 1~4 min and then incubated for 24 h.[12] The ratio of the crosslinked HA to the free HA is varied from 65:35 to 95:5. 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. Rheological behavior of HAHs were analyzed with a Thermo Haake RS1 Rheometer (Newington, USA), using a plate and plate geometry with a 1.2 mm gap.[12] 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 measured properties of hydrogels was evaluated by measuring the elastic modulus (G') and viscous modulus (G''). The measured modulus at frequency of 5 Hz for HAHs was compared.[12] In addition, PTF is achieved by observing or touching HAHs using an optical microscope (Pro Camscope, Sometech, Korea) or fingers, respectively.

Swelling Property. The swelling characteristics were measured by immersing weighed samples of dry microspheres for 24 h in PBS. The gels were screened through a 3.0 μm membrane filter (Advantec, Japan). The excess surface water in the swollen gel was removed by blotting and then the swollen gel was weighed. After measuring the weight of the gels, the swelling ratio (S) was determined by using eq. (1),

$$S(\%) = \frac{W_s - W_d}{W_d} \times 100 \qquad (1)$$

Where, W_s and W_d are the weight of the swollen gel and the dry gel, respectively.

Gas Chromatography (GC). DVS reacts with the primary alcohol groups in the HA backbone. The crosslinked microspheres were cleaned in water and ethanol to eliminate an unreacted residual crosslinker. The presence of DVS 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).[12,14]

Results and Discussion

HA microspheres were fabricated by using a modified spray method.[13-17] The experimental procedure was described else-
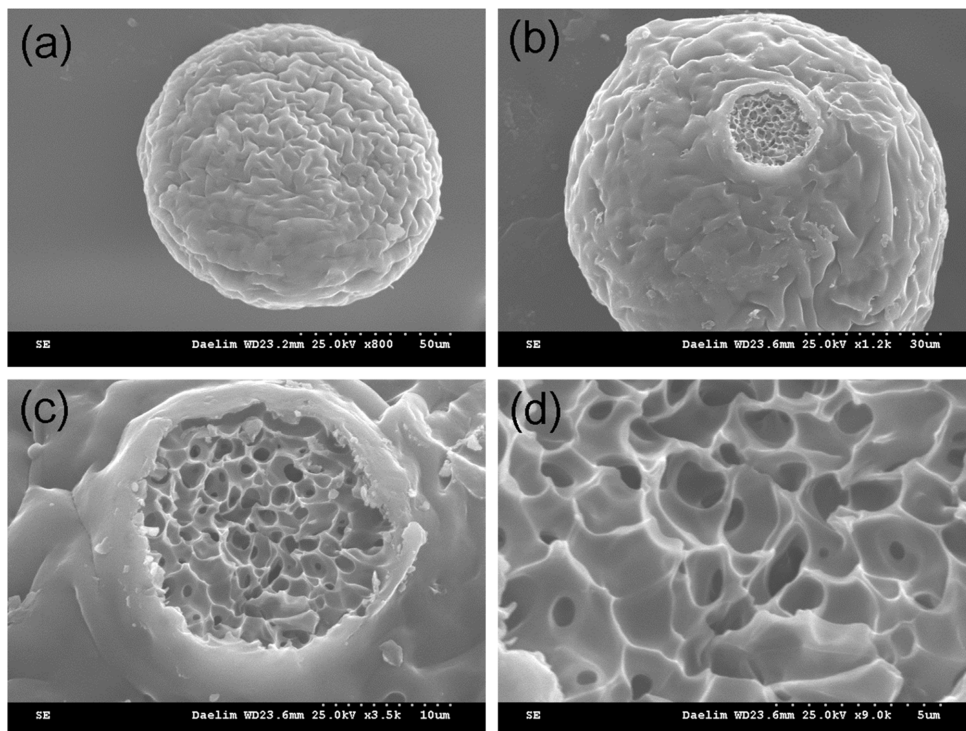

Figure 1. SEM images of ((a)-(d)) HA microspheres crosslinked for 24 h. Note that (b)~(d) images exhibit porous network inside the HA microspheres.

where.[13,17] The size of the microspheres prepared under the same condition was reported to be in the range of 100 to 140 μm because the crosslinked microspheres were screened through a 200 mesh sieve (75 μm).[13] However, the average diameter of HA microspheres was in the range of 60 to 100±4 μm and swelling rate of 1000% probably due to the use of a 325 mesh sieve (45 μm). Morphologically the microspheres were white colored spheres having a smooth surface, as shown in Figure 1. Although no pores on the surface of the spheres were visible, pores inside the microspheres were easily seen. The microspheres were a 3-dimensional porous network structure channeled with 2~4±0.5 μm pores, as shown in Figure 1(d). The HA hydrogels were prepared by immersing the microspheres in PBS solution. They can be used as the tissue membrane to release of entrapped drug under a certain condition in a controlled manner. It could be used to encapsulate and cultivate cells inside the gel, where the network will act as a semipermeable membrane allowing only growth factors to enter to aid the growth of cells.[13]

Microspheres were crosslinked with DVS, followed by cleaning in ethanol and distilled water. Covalent linkages between polymer chains can be obtained by the reaction of functional groups of a DVS crosslinking agent (vinyl group) and HA (hydroxyl group).[11-23] The presence of crosslinker's residue in the HA hydrogels after cleaning was evaluated by GC.[12] The residual crosslinker peak located at 1.18 min (Figure 2), corresponding to DVS, was not detected after cleaning, suggesting that the unreacted residual crosslinker was successfully removed.

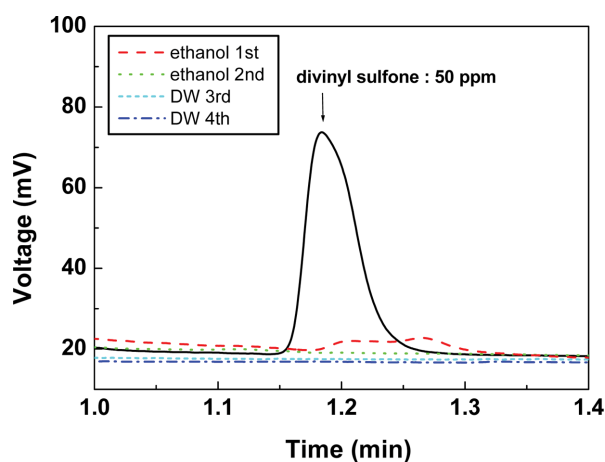The biocompatibilities of HA hydrogels, such as cytotox-

icity, genotoxicity (*in vitro* chromosome aberration test, reverse mutation assay, *in vivo* micronucleus test), skin sensitization, intradermal reactivity, and pyrogenicity, were previously evaluated and determined to be suitable for soft tissue augmentation due to the absence of abnormal clinical signs.[14,15] There was no difference between the HA hydrogels and negative control mean scores because skin reaction such as erythema or oedema was not observed after injection. In addition, the HA hydrogels had no subchronic systemic toxicity, indicating that the implants were excellent in biological synthesis and transplantation as evidenced by non-capsule reaction and disappearance of inflammatory cells.[16] It was reported that the implants of HA hydrogels are clinically safe and effective.

Since the filling capacity of a dermal filler is known to be dependent on its water uptake capacity, the swelling ratio was examined. It is suggested that the highest water uptake indicates 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.[12,13] It was evident that the expansion capacity of HA hydrogels rose with increasing the amount of pure HA and was inversely proportional to the crosslinking degree.[12,13,17] The swelling ratios of the uncrosslinked HA having the molecular weight of 1368 kDa and the crosslinked HA microsphere (molecular weight of 1058 kDa) were 4000% and 1000%, respectively.[12,13] The swelling ratio may decrease with increasing the concentration of crosslinked HA due to an increased number of coiled HA chain interactions.[12-17] A 3-dimensional network of HA hydrogels is easily formed when crosslinks between the HA chains are introduced. A strong gel has a high elasticity, meaning that the response to deformation is mainly elastic. Uncrosslinked HA is completely metabolized in a few days after injection.[2-4]

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 duration. Elastic modulus (G') of HA hydrogels was evaluated as a function of the volume fraction of the crosslinked HA microsphere to the uncrosslinked HA in the range of 65:35 to 95:5. Among the commercial fillers, Restylane is biphasic products consisting of crosslinked particles (75%) suspended in uncrosslinked HA (25%) used as a carrier. In the present study, microspheres made from 1058 kDa HA were blended with 1368 kDa HA in different ratios, as depicted in Figure 3. Microspheres were synthesized after the consumption of the vinyl groups by the crosslinker of DVS.

Figure 2. GC graphs of divinyl sulfone (50 ppm) and HA microspheres cleaned by ethanol and distilled water. Note that the microspheres were crosslinked by divinyl sulfone for 24 h.

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.[6] In contrast, larger needles (26~27 gage) are required for the extrusion of firmer gels (1400~1800 Pa). The G' value of commercial soft skin filler, Restylane Skinbooster, is determined to be 175 Pa.[12] The G' values of HA fillers having different ratios of DVS crosslinked HA microspheres to pure HA, as shown in Figure 3, are between 211 and 702 Pa. The fillers having the
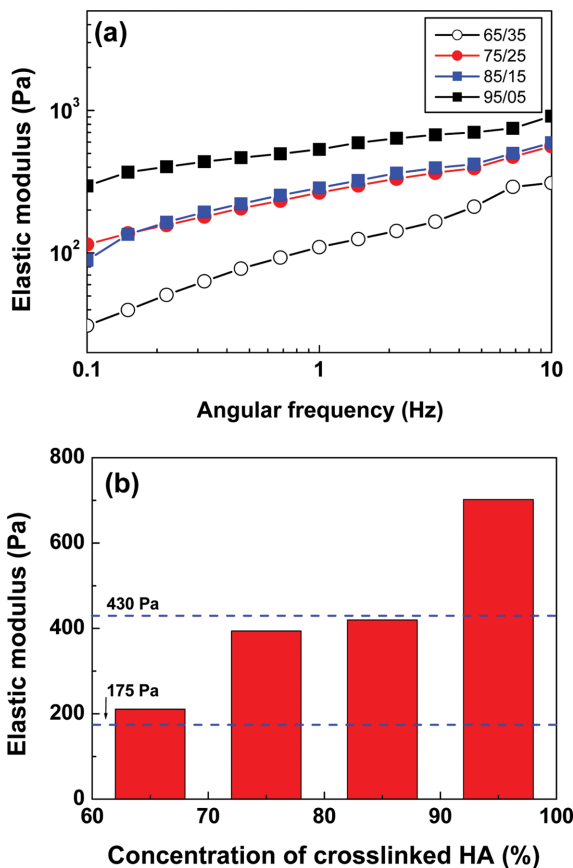
ratio of the crosslinked to the uncrosslinked HA between 65:35 and 85:25 showed the G' values in the range of 175 to 430 Pa, can be extruded through the 29~30-gage needle allowing for ease of injection, which can be applicable to the treatment of non-severe defects and generally for thinner and softer skin rather than the correction of deep folds and skin deformation.[4] The content of DVS crosslinked HA microsphere is in the range of 65 to 85, which is similar to Restylane products (75%) commercially widely used in the market.[7,24] The elastic moduli of the as-prepared fillers (211~402 Pa) were even stiffer than that of Restylane products (175 Pa).[13]

Unlike the monophasic fillers, the commercial Restylane fillers show PTF when touching gels with hands. The biphasic fillers prepared in the present study consist of crosslinked micro-sized gel particles suspended in free HA. Our previous studies revealed that the crosslinked nanoparticles of the dermal fillers increased the flowability dramatically, which was detrimental to the fillers. The nanoparticles contain a less-accessible crosslinked polymer structure, thus reducing polymer chain interactions and thereby lowering the viscosity. Viscous modulus (G'') is also called loss modulus because it describes the energy that is lost as viscous dissipation. The G'' value is a measure of the flow (rheological) properties for the fillers. Although G'' of 72 Pa was observed for the fillers composing of 65:35, as depicted in Figure 4, the rest of fillers showed higher G'' values higher than 128 Pa. The G'' values of the as-prepared fillers (128 to 166 Pa) are similar to those of Restylane (119 Pa) and Perlane (125 Pa), which are categorized in the medium-viscosity and medium-elasticity group.[25] No appreciable flowability is detected. In addition, PTF is suc-
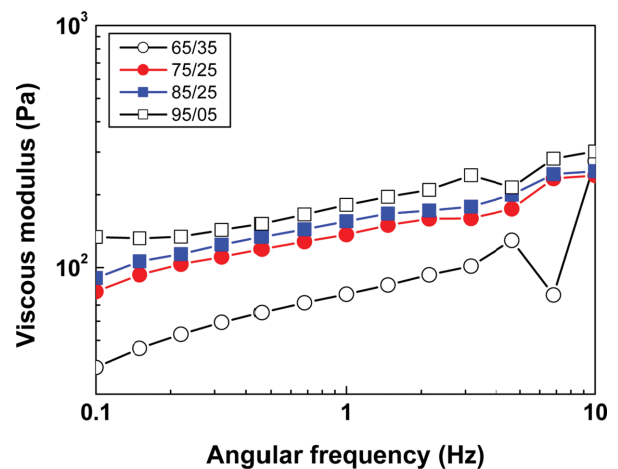

Figure 3. (a) Elastic modulus as a function of angular frequency at 25 °C; (b) the comparison data of dermal fillers. Note that the region of the dotted line in (b) represents the easy delivery of the fillers through a 29~30 gage needle.

Figure 4. Viscous modulus as a function of angular frequency at 25 °C.
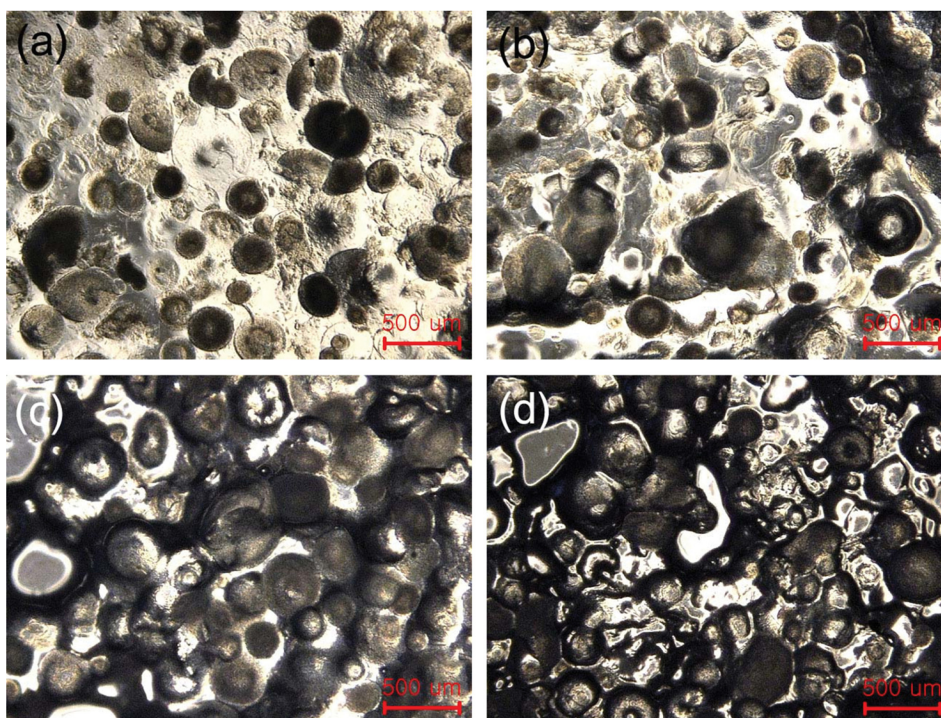
Figure 5. Optical photographs of the fillers having the volume fraction of uncrosslinked HA microspheres: (a) 65%; (b) 75%; (c) 85%; (d) 95%, respectively.

cessfully achieved by observing or touching HAHs using an optical microscope or fingers due to the presence of crosslinked gel particles (300±30 μm), as displayed in Figure 5. As the volume fraction of crosslinked HA microspheres increased, the particle density rose as expected. It can be concluded that the dermal fillers possessing the tailored elasticity and PTF are achieved due to the inclusion of microspheres crosslinked with DVS.

Conclusions

Rheological properties of HA dermal fillers prepared by DVS crosslinked HA microspheres having the volume fractions of 65 to 95 are characterized by using SEM, optical microscopy, and rheometer. The average diameter of HA microspheres is in the range of 60 to 100 μm with a 3-D porous network structure channeled with 2 to 4 μm. The HA fillers consist of gel particles (300 μm in size) of stabilized HA (1058 kDa) suspended in uncrosslinked HA (1368 kDa). The deformable fillers, which can be extruded through the finest needles (29~30 gage), are found. PTF is also sensed for the dermal fillers due to the presence of gel particles.

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