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In situ Sodium Alginate-Hyaluronic Acid Hydrogel Coating Method for Clinical Applications

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Abstract: A novel synthetic method is reported to prepare HAAL hydrogels derived from hyaluronic acid (HA) and sodium alginate (AL) with different compositions (ranging from 1:1 to 1:5) through ionic and covalent crosslinking is reported in this study. The synthesized hydrogels were characterized by FTIR spectroscopy. Surface morphology and equilibrium swelling behavior of the hydrogels were also examined. In an MTT assay, the hydrogel extracts were found to be non-cytotoxic to L929 mouse fibroblasts. The blood compatibility of the synthesized hydrogels was assayed with a platelet adhesion test, and was comparable with that of medical-grade polyurethane (PU, Pellethane®). HAAL hydrogels with recombinant human epidermal growth factor (rh-EGF) exhibited enhanced cell proliferation and adhesion (human L929 fibroblast). These characteristics demonstrate that HAAL hydrogels treated using this *in situ* coating method have potential for usage in various clinical applications.

Keywords: sodium alginate, hyaluronic acid, hydrogel, *in situ* coating, non-toxic, recombinant human epidermal growth factor (rh-EGF).

Introduction

Hydrogels, which have three-dimensional physical structures with great potential for applications in tissue engineering, are considered to be promising biomaterials in biotechnology and medicine due to their extraordinary properties, such as their biocompatibility and structural similarities to macromolecular-based components with minimal inflammatory response, thrombosis, and tissue damage.[1] Hydrogel materials originating from natural polymers have been widely used in medical applications including as wound dressing, drug delivery devices, artificial implants, and contact lenses. *In situ* coating techniques can be used to modify the substrate surface of hydrogels to produce alginate sheets for wound dressing. Coated hydrogel substrates synergistically combine the sample and hydrogel properties potentially provide a much more suitable material for medical applications.[2-4]

Hyaluronic acid (HA), a type of glycosaminoglycuronan, is a linear, high molecular weight polysaccharide consisting of *β-D*-glucuronic acid and 2-acetamido-2-deoxy-*β-D*-glucose units. Similarly, alginate (AL), which is derived from brown algae, is a linear, copolymer of repeating (1→4) *α-L*-guluronate (G) and *β-D*-mannuronate (M) monomeric units. These two well-known natural polymers exhibit outstanding properties for hydrogels of biocompatibility, biodegradability, non-antigenicity, and excellent gel formation. In spite of such advantages, synthesized hydrogels formed by ionic or covalent crosslinking methods have some issues.[5] For example, HA hydrogels show weak mechanical properties, while AL hydrogels show low cellular interaction. Therefore, our ultimate goal was to combine these two types of natural polymers into a novel hybrid hydrogel that would be more effective in medical applications while simultaneously compensating for their shortcomings of the single component gels.

Sodium alginate and hyaluronic acid hydrogels (HAAL) were synthesized through ionic and covalent crosslinking methods using calcium chloride ($CaCl_2$) and adipic dihydrazide (ADH). The potential of HAAL hydrogels for clinical applications was explored through investigations of *in vitro* cytotoxicity, *in vitro* blood compatibility, and cell adhesion and proliferation behavior with hydrogels modified with recombinant human epidermal growth factor (rh-EGF).[6,15,16]

Experimental

Materials. Hyaluronic acid (HA) was purchased from Biorain (Ansan, Korea), and sodium alginate (AL) was purchased from Yakuri Pure Chemicals Co. (Kyoto, Japan). Adipic dihydrazide (ADH), 1-ethyl-3-(3-dimethy(aminopropyl)-carbodiimide)

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hydrochloride (EDC), and calcium chloride ($CaCl_2$) were obtained from TCI (Tokyo Chemical Industry, Tokyo, Japan). Polyurethane (PU) (Pellethane® 2363-80AE) was medical grade. Canine whole blood was obtained from the Animal Blood Bank (Sokcho, Korea) and thromboplastin was purchased from MP Biomedicals, LLC. (Santa Ana, CA, USA). Recombinant human EGF was obtained from Sigma Aldrich Chem. Co. (St. Louis, MO, USA). Deionized water (18.2 MΩ cm resistivity at 25 °C) was used in all experiments.

Synthesis of HAAL Hydrogel Sheets. HA and AL in ratios of 1:1~1:5 were mixed in distilled water (1.5 wt% solution) for 4 h at room temperature. The mixed solution was then spread on a glass plate while maintaining the thickness of 1 mm or less. The glass plate was immersed into a solution of pre-prepared crosslinking agent composed of 0.1 m/l ADH, 0.1 mol/l EDC, and 0.1 mol/l $CaCl_2$ adjusted to pH 6.0 (as shown in Figure 1). The hydrogels could be easily separated from the glass plate after a few minutes, since these types of hydrogels completely crosslinked. Finally, to remove the remaining crosslinking agent, the hydrogel sheets were thoroughly washed with distilled water. Followed drying under vacuum at room temperature overnight, the dried HAAL hydrogel was reswollen, and its properties were evaluated.

Infrared (IR) Spectroscopic Measurement. Fourier transform infrared (FTIR) spectra of HA, AL, and HAAL hydrogels (using HAAL-3 as a standard) were obtained to confirm the existence of the expected functional groups. All samples were measured with an infrared spectrophotometer (Bruker IFS-66/S FTIR, Bruker Optics, Germany) using the potassium bromide (KBr) pellet method.

Swelling Behavior. To examine the swelling behavior, hydrogel sheets were cut into 2 cm×2 cm samples, and the dry weight (W_0) was measured at 25 °C. Next, the samples were soaked in distilled water or PBS solution at 37 °C until swelling equilibrium had been reached.[7] The sheets were removed from the soaking solution, excess water was removed with filter paper, and the wet weight (W_s) was measured. The equilibrium swelling ratio (SR) of hydrogel sheets was calculated from the following eq. (1):[8,9]

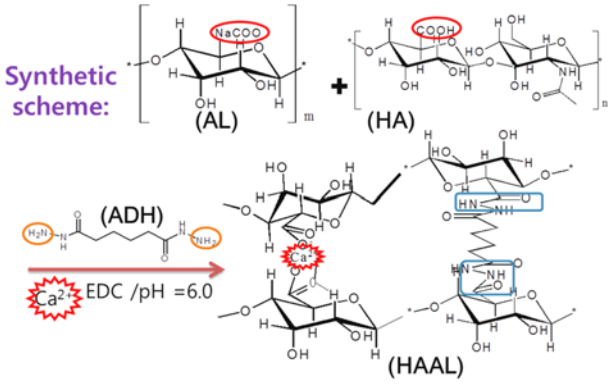

Figure 1. Synthetic scheme for sodium alginate and hyaluronic acid hydrogel sheets.

$$SR\ (\%)=(W_s-W_0)/W_0\times 100\% \quad (1)$$

Surface Morphologies of the Hydrogels. The morphologies of HAAL hydrogels were characterized by scanning electron microscopy (SEM). After swelling, the hydrogels were quickly frozen in liquid nitrogen and then freeze-dried for 48 h to evaporate the water while maintaining the three-dimensional microstructure. After gold-coating, the surface and cross-sectional morphologies of the lyophilized hydrogels were imaged using a Hitachi S-2200 SEM (Tokyo, Japan) at a 15 kV accelerating voltage.

***In vitro* Cytotoxicity Studies-MTT Assay.** Cytotoxicity testing was performed according to the MTT-formazan method.[10-12] Two milliliters of sodium alginate and hyaluronic acid precursor solutions were spread on the surface of a glass plate (4 cm×4 cm). After separating the resulting sheet, the entire sheet was immersed in crosslinking agent solution, then dried and swelled. The swollen HAAL sheets were soaked in pre-sterilized PBS for one day before testing.

L929 mouse fibroblasts cells obtained from the Korean Cell Line Bank were cultivated at 37 °C in an atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin. The culture medium was changed every two days. Following this period, 30, 90, 150, or 300 μL of solution extracted from the hydrogels in PBS were added to the 12-well plate, and each well was supplemented with 3 mL of medium. The seeded cell concentration was adjusted to 2.5×10^4 cells/well. The tissue culture polystyrene (TCPS) plates were placed in a cell incubator for 48 h. After incubation, DMEM (400 μL) and MTT solution (100 μL) were added to each well, followed by an additional hour of incubation under the same conditions (37 °C, 5% CO_2). Finally, MTT reagent and medium were substituted with dimethyl sulfoxide (DMSO, 800 μL) and glycine buffer (100 μL, pH 10.5). After a few seconds of shaking, the color of the solution turned blue-violet. After transferring 100 μL of each solution to a 96-well plate, the absorbance of the MTT solution was measured at 570 nm using an ELISA instrument (Spectra Max M5, Molecular Devices, Sunnyvale, CA, USA).

Microscopic Observation of Cell Adhesion on the Hydrogel Surface. The hyaluronic acid and sodium alginate precursor solution was coated on the surface of a cover glass (diameter ~18 mm). Three milliliters of crosslinking agent solution was added to the hydrogel coated glass sample, which was placed in the well of a culture dish. This crosslinking agent solution was substituted with distilled water after about 30 min to remove unreacted crosslinking agent. After drying, the coated cover glass was reswollen. Following UV irradiation for 30 min and cell seeding (5×10^4 cell/well), medium was added. After incubation for 24 or 48 h at 37 °C in a humidified atmosphere containing 5% CO_2, the cell morphology of the adhered L929 cells was observed using an optical microscope (Olympus CK40, Tokyo, Japan) with 200 times mag-

nification.[13,14]

***In vitro* Blood Compatibility: Platelet Adhesion.** HAAL hydrogel precursor solutions were coated on cover slides (diameter ~18 mm) using an electro-spinning method. The glass plates were placed into 12-well tissue culture plates, and the crosslinking agent was then added. Following gelation, the cover slides were washed several times with distilled water. PU (as a control) was dissolved in THF to obtain a 10 wt% concentration. Using the same method described above, the PU solution was coated on cover slides and dried overnight in a vacuum oven. Platelets were obtained by centrifugation of whole blood at 1,000 rpm for 5 min to obtain platelet rich plasma (PRP) and at 3,000 rpm for 10 min to obtain platelet poor plasma (PPP). A platelet concentration of 1×10^5 platelets/mL was prepared by mixing the PRP and PPP.

After UV light sterilization, the coated cover slides were hydrated with PBS for 2 h. Next, PBS was substituted with 0.5 mL of diluted platelet solution, and the tissue culture plates were incubated in a shaking incubator (Daeil Engineering Co. Ltd., Korea) at 37 ºC for 3 h. During this procedure, non-adhesive platelets were counted at a predetermined time interval corresponding to the blood compatibility of hydrogels. After incubation, the glass slides were washed two or three times with PBS, and the adhered platelets were then fixed on the surface of the samples by addition of 2% glutaraldehyde solution for 8 h at 4 ºC. The glass plates were dehydrated in a series of ethanol-water mixtures (30%, 50%, 70%, and 90%). Finally, after freezing with liquid nitrogen, drying overnight, and gold coating, the adhered platelet features were observed by SEM.

Release Profile of rh-EGF from HAAL Hydrogels. For the rh-EGF release study, rh-EGF was dissolved in 1.5 wt% HAAL hydrogel precursor solutions with different ratios of hyaluronic acid to sodium alginate. Briefly, 0.5 mg of rh-EGF was added to 10 mL of HAAL precursor solution. After the mixture had completely dissolved in distilled water after 4 h to generate a homogeneous solution at room temperature, crosslinking agent was added to the hydrogel precursor on glass plate to give rh-EGF-loaded HAAL hydrogels. The rh-EGF-loaded HAAL hydrogels were immersed into 3 mL of phosphate buffer solution (PBS, pH 7.4) at 37 ºC in a water bath with shaking. At specific time intervals, the PBS was removed from the vials and replaced with 3 mL of fresh PBS. The amount of rh-EGF released from the HAAL hydrogel was determined by measurement of the absorbance at 260 nm using an UV spectrometer (S-4100, Sinco, Seoul, Korea).

Promoting Effect of Growth Factor on Fibroblast Proliferation on HAAL Hydrogels. Human L929 fibroblast cells were used to evaluate the effect of rh-EGF-loaded HAAL hydrogels on the promotion of cell proliferation *in vitro*. First, rh-EGF was dissolved in HAAL hydrogel precursor solutions at various concentrations of rh-EGF (5, 25, 50, and 100 μg/mL of precursor solution). Next, 0.05 mL of each solution was coated on the top surface of cover slides 12 mm in diameter. Distilled water was used to wash away remaining crosslinking agents several times. The cover slides were placed into 24 well culture plates, which were sterilized by exposure to UV light for 0.5 h before cell culturing.

L929 cells were seeded onto culture plates (in the appropriate medium) at a density of 10^5 cells/well and were cultivated in an incubator containing 5% CO_2 at 37 ºC for 72 h. During the cultivation period, the cell growth behavior was observed by optical microscopy, and pictures were taken at 100 times magnification. The number of adhered cells was counted, and the cell density on the hydrogel surfaces was calculated to estimate the promotional effect on fibroblast proliferation. Tissue culture polystyrene (TCPS) and HAAL hydrogels without rh-EGF were used as controls.[6,15-17]

Results and Discussion

Structure of HAAL Hydrogels. HA was modified by ADH to introduce amine groups to HA. Thus, a network structure of HAAL hydrogels (as shown in Figure 1) could be formed through chemical crosslinking reactions between HA and AL. In addition to the well-known crosslinking of AL through ionic bonds with divalent cations (Ca^{2+}) and intra- and inter-molecular chains, AL can also be connected by covalent linkages between carboxylic acid groups in alginate chains and the amine groups of ADH (introduced into the modified HA in this study). The crosslinking of hyaluronic acid and sodium alginate was verified with infrared spectroscopy data (Figure 2). HA and AL showed anti-symmetric stretching peaks at 1612 and 1618 cm^{-1}, and a symmetric stretching peak at 1415 cm^{-1} derived from carboxylic groups in the molecular chain (Figure 2(a) and (b)). However, the synthesized HAAL hydrogel had an amide bond (-CO-NH-) in the crosslinked structure formed by covalent linkage, as mentioned above, which showed characteristic amide peaks at 1641 and 1550 cm^{-1} (corresponding to the newly formed amide I and amide II linkages), as shown in Figure 2(c) and (d). The decrease in the absorption peak near 1380 cm^{-1} (C=O stretching of HA) indicated the formation of HA-ADH resulting from reactions between the carboxyl groups of HA and the amine groups of ADH. Additionally, a decrease in the absorption peak near 1460 cm^{-1} (C-N stretching of HA) suggested that the C-N stretching vibration was modulated by intermolecular crosslinking between HA molecules.[18] Amide groups were not observed in either the unmodified HA or AL. Thus, the amide groups formed in HAAL were generated by crosslinking reactions between ADH modified HA and AL.

Equilibrium Swelling of Hydrogels. The equilibrium swelling ratio (Figure 3) of the hydrogels was investigated after soaking in distilled water and PBS. In the case of swelling experiment of HAAL hydrogels in distilled water, all of the HAAL hydrogel samples did not show significant

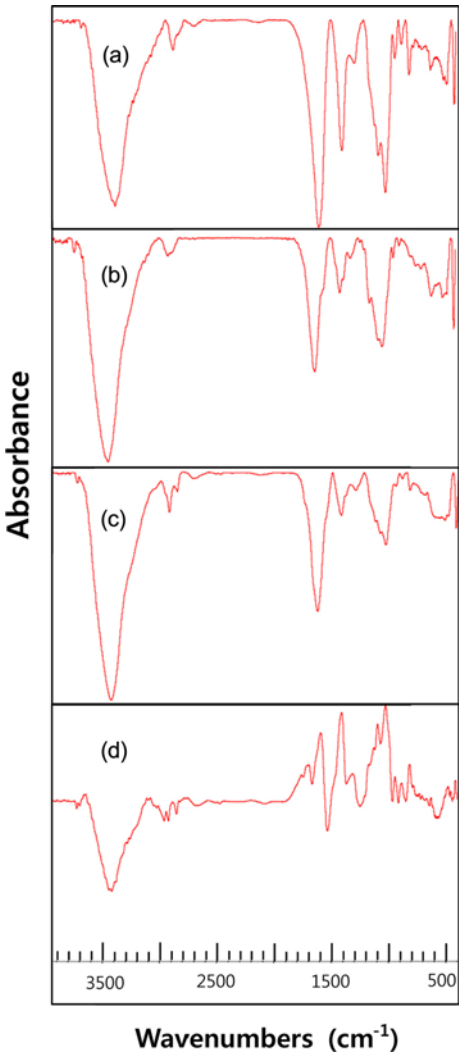

Figure 2. FTIR spectra of (a) sodium alginate, (b) hyaluronic acid, (c) HAAL-3, and (d) subtraction spectrum of HA from HAAL-3.

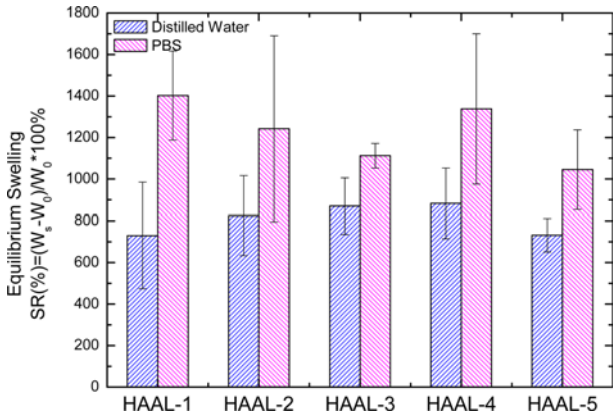

Figure 3. The equilibrium swelling ratio of as-grown hydrogels soaked in distilled water and PBS.

differences in swelling ratio. But, in the case of PBS swelling, swelling behaviors of HAAL hydrogels were decrease according

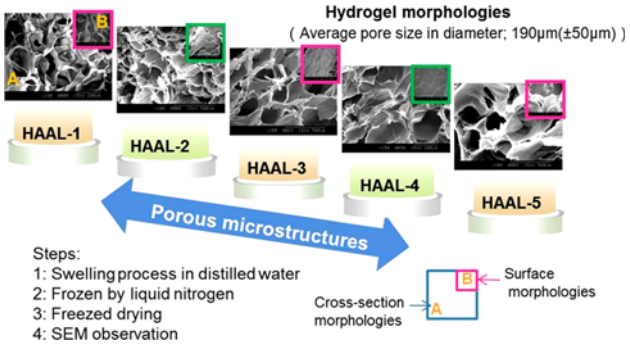

Figure 4. SEM images of different freeze-dried HAAL hydrogels.

to the increase of AL composition in HAAL hydrogels (except in HAAL-4). And the overall swelling ratios in PBS was higher than those in water, probably due to the ionic nature of the hydrogels. When HAAL hydrogels are placed in the PBS solution, the Na^+ ions contained in PBS were substituted with Ca^{2+} ions which are binding with COO^- groups in the AL. As a result, the increased electrostatic repulsion among negatively charged COO^- groups causes the chain relaxation and enhances the swelling ratio comparing with distilled water.[19-21]

Morphologies of Hydrogels. After freeze-drying, synthesized hydrogels with different molar ratios of HA and AL were observed to have connected, porous structures, as shown in the SEM images in Figure 4, which resembled the structures of other hydrogel composed of natural macromolecules. The surfaces of the HAAL hydrogels were porous and appeared smooth, while cross-sections of HAAL hydrogels revealed high porosity throughout the longitudinal section, with pore sizes ranging from 130 to 240 μm in diameter.

Cell Biocompatibility of Hydrogels – MTT assay. Different surface compositions and morphologies of biomaterials can regulate cell growth, proliferation, and differentiation. In this study, cell compatibility and the cell adhesion behavior of HAAL hydrogels were investigated using L929 mouse fibroblasts. The MTT assay is commonly used to evaluate the *in vitro* cytotoxicity of biomaterials. Figure 5 shows results of the MTT assay with the 1%, 3%, 5%, and 10% hydrogel extracts immersed in PBS solution, which affected L929 fibroblast cell viability after 48 h incubation. As the concentration of the extract increased, the cell viability of adherent cells on the hydrogel surface decreased. However, compared with the control sample (TCPS), the viability of cells on the various extracts was above 80% of the control, which indicated that the hydrogel extracts were not cytotoxic according to the GB/T 16886.5-2003 (ISO 10993-5:1999), as samples are considered non-cytotoxic as long as sample cell viability is higher than 75%.[10-12]

Microscopic Observation of Cell Adhesion on HAAL Surfaces. The morphology of cells adhered to the hydrogel surface was used to directly assess cell adhesion behavior.

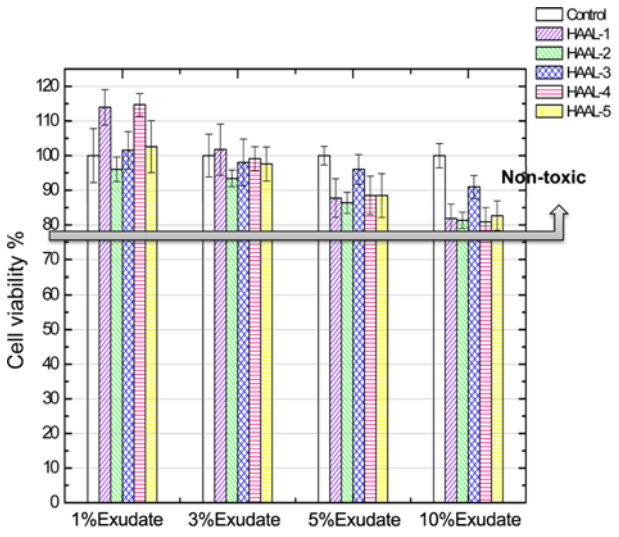

Figure 5. Comparison of L929 mouse fibroblast cell viability after treatment by 1%, 3%, 5%, 10% concentration exudates of hydrogels.

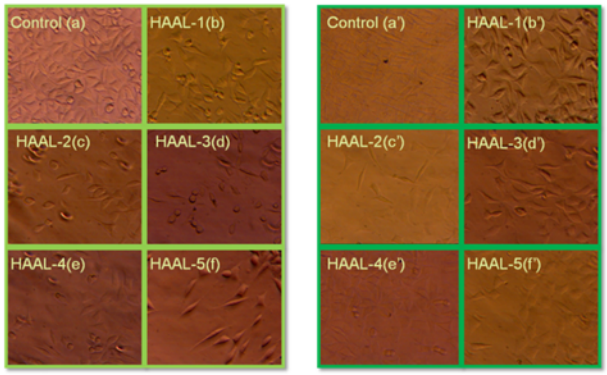

Figure 6. Optical images of L929 mouse fibroblast cell adhesion behavior on various hydrogels for 24 h (a-f) and 48 h (a'-f') incubation, magnified 200 times.

The spreading behavior of L929 cells (presenting as large and spindle-shaped on the HAAL hydrogel surface) was monitored by optical microscopy using glass templates coated with HAAL. After incubation for 24 h, fewer cells were adhered to the HAAL hydrogel surface compared with the control (Figure 6). L929 cells on the hydrogel showed a linear or round morphology. In contrast, large numbers of cells that exhibited a spindle morphology adhered to the control sample (TCPS). Over time, the number of adhered cells increased with incubation time, and the morphologies were also almost the same as those of the control. Although the number of attached cells was less than that of the control, the cells grew well, and growth continued to spread on the surface of the hydrogel. The adhered cell density data demonstrated a similar result (Table I). Therefore, the results of the MTT assays and the morphologies of the adhered cell both indicate that HAAL hydrogels provide excellent L929 fibroblast cell viability, although proliferation was not quite as good as with TCPS. This phenomenon might be attributable to two effects. First, the surface of the hydrogels changed from the acidic environment (-COOH) of HA and AL to a neutral environment (-CO-NH-) through crosslinking reactions, suggesting that cells preferred to adhere to uncharged surfaces. Second, the hydrogel raw materials (HA and AL) are biologically compatible with low cytotoxicities, which implies a suitable environment for cell growth.[13,14]

Table I. L929 Mouse Fibroblast Cell Density on the Surface of Hydrogels Over 48 h

Sample	Cell Density (Cell/cm^2)	
	24 h Incubation	48 h Incubation
Control	2.22×10^4	3.58×10^4
HAAL-1	1.87×10^4	2.17×10^4
HAAL-2	1.67×10^4	1.81×10^4
HAAL-3	1.50×10^4	2.14×10^4
HAAL-4	1.69×10^4	2.22×10^4
HAAL-5	1.52×10^4	2.67×10^4

HAAL-1 was loosely crosslinked comparing to HAAL-5, because of lack of second crosslinking effect by Ca^{2+} ion occurring in alginate moiety in HAAL-1 sample. On the contrary, HAAL-5 had firmly crosslinked structure owing to the high probability for second crosslinking reactions through Ca^{2+} ion in HAAL-5 (more AL moiety comparing to HAAL-1) sample. The tensile strength measured with HAAL-1 and HAAL-5 was 13.5 and 56.5 MPa respectively using Instron. So HAAL-5 sample showed more rigid surface than HAAL-1 and cell adhesion density on HAAL-5 surface after 48 h was superior to that of HAAL-1 (Table I). In general case, the cell adhesion behavior on hydrogel surface was inferior owing to its soft/smooth surface morphology and highly hydrated state of surface. Therefore increment of surface rigidity (based with the crosslinking degree of HAAL) shows superior cell adhesion behavior. Such phenomenon was also reported in D.D Kim's result.[22] So, we can conclude that the surface rigidity of HAAL samples is deeply related with the cell adhesion characteristic on their surface and second crosslinking reaction with Ca^{2+} ion between AL molecular chains in different HAAL samples is also important factor for enhancing their mechanical properties and surface rigidity.

Blood Compatibility of Hydrogels. Plasma proteins can absorb onto the surfaces of biomaterials in contact with blood, provoking platelet adhesion, which leads to release of ADP and ATP from adherent platelets, thereby inducing further platelet aggregation to form a thrombus.[23] Therefore, evaluation of platelet adhesion is an important step in evaluating the blood compatibility of a biomaterial. In this study, medical-grade polyurethane (PU), which is already used in clinical applications, was used as a control. As shown in Figure

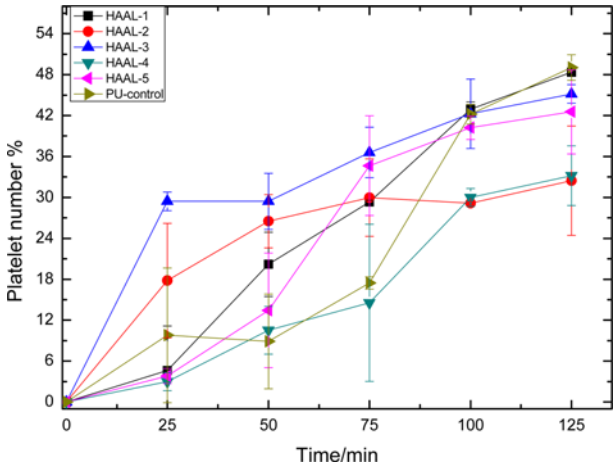

Figure 7. Platelet adhesion behavior on PU and HAAL hydrogel surfaces coated on glass plates.

7, the amount of adhered platelets on the HAAL hydrogel surface was slightly lower than the PU control during a 125-minute incubation. Compared with PU, HAAL-2 and HAAL-3 showed fast platelet adhesion within 25 min, while HAAL-1, HAAL-4, and HAAL-5 demonstrated significant adhesion after 50 min. The morphologies of the adhered platelets as examined by SEM showed that all of the platelets were attached to the surfaces by discrete filopodia and maintained their round shape (Figure 8). Most of the platelets appeared to coagulate, with few spread along the surface. The highly magnified SEM images showed clumps of multiple platelets, which indicated that the platelets did not favor the HAAL hydrogel surfaces.

The Effect of rh-EGF in HAAL on Cells. The release profile of rh-EGF incorporated in HAAL hydrogels could be measured by UV spectroscopy, since the rh-EGF protein contains tyrosine, phenylalanine, and tryptophan residues, which absorb at 260 nm and an isoelectric point at pH 4.60.

As shown in Figure 9, most of the loaded rh-EGF (total loading amount: 100 μg) was released from the hydrogels over 4 days. Over the first 12 h, the release rates of HAAL-1 and HAAL-5 were very slow, but the rates increased thereafter. During this process, HAAL-1 loaded with rh-EGF had a longer

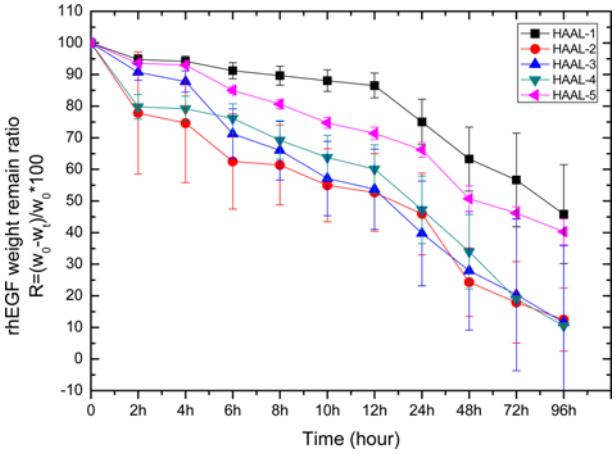

Figure 9. Release behavior of recombinant human epidermal growth factor (rh-EGF) from HAAL hydrogels in phosphate-buffered saline (pH=7.4) at 37 °C in a water bath with shaking.

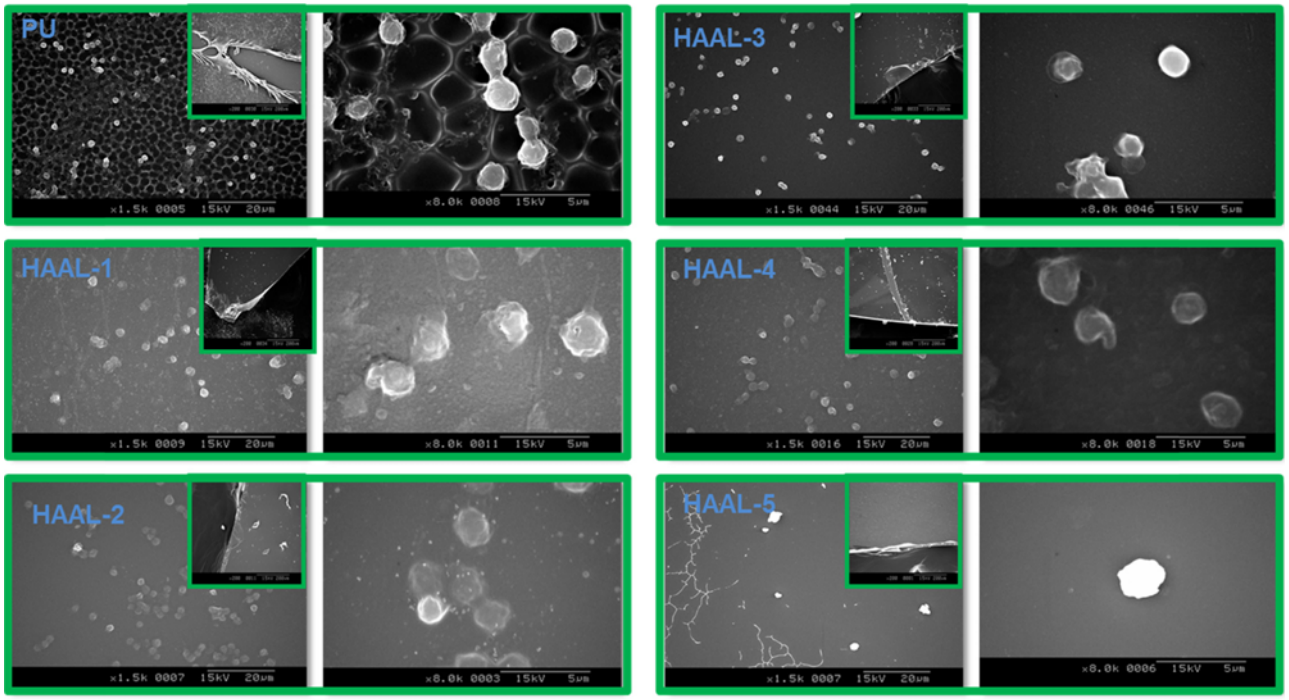

Figure 8. SEM images for adhered platelet on PU and HAAL hydrogels coated on glass plates surfaces.

release period such that after 4 days, approximately 45% of the rh-EGF remained within the hydrogel. On the other hand, HAAL-2, HAAL-3, and HAAL-4 showed different release trends, with about 90% of the rh-EGF released over 4 days. These phenomena appear to be related to the crosslinking density. In the case of a high crosslinking density (HAAL-5), the release rate of rh-EGF was suppressed to up to half of the rate of HAAL-2, HAAL-3, and HAAL-4. In contrast, a loose hydrogel (HAAL-1) network could not control the rh-EGF release rate sufficiently as compared with the tight 3D network structure of HAAL-2, HAAL-3, and HAAL-4. This is due to the electrostatic interactions between rh-EGF and the unreacted carboxylic groups in HAAL, which had a low crosslinking density (high swelling ratio).

The promotion of fibroblast proliferation on rh-EGF-loaded HAAL hydrogels was examined. Three tests were used to investigate the enhancement of cell growth on HAAL hydrogels loaded with rh-EGF and to calculate the adhered cell density on rh-EGF-loaded HAAL hydrogels.

Figure 10 presents the cell viability results for HAAL hydrogels containing different amounts of rh-EGF by the MTT assay. All of the HAAL hydrogels with 5 μg of rh-EGF (loaded with 0.05 mL of 100 μg/mL rh-EGF solution) showed much higher cell viability than HAAL samples which contained less rh-EGF (less than 5 μg of rh-EGF, 5 μg/mL, 25 μg/mL, 50 μg/mL).

To investigate the effects of EGF loading amount with one type of HAAL hydrogel, cell attachment behavior on HAAL-1 was observed by optical microscopy on 12 mm glass templates coated with HAAL-1 containing different concentrations of rh-EGF.

As can be seen from the results in Figure 10, the adhered cell viability(which corresponded with cell adhesion density) on HAAL loaded with rh-EGF became incrementally higher as the amount of loaded rh-EGF increased. Within 1 h of cell seeding, most of the cells had not attached to the surface of hydrogels loaded with 2.5 and 5 μg rh-EGF, except for the control sample (TCPS). A large amount of cells adhered and showed a spread morphology on the surface of HAAL loaded with 2.5 μg rh-EGF (about 3 times the number of adhered cells compared with control). However, the HAAL loaded with 5 μg rh-EGF did not show cell adhesion exceeding that of HAAL loaded with 2.5 μg rh-EGF even after 48 h of cultivation under the same conditions (data not shown).

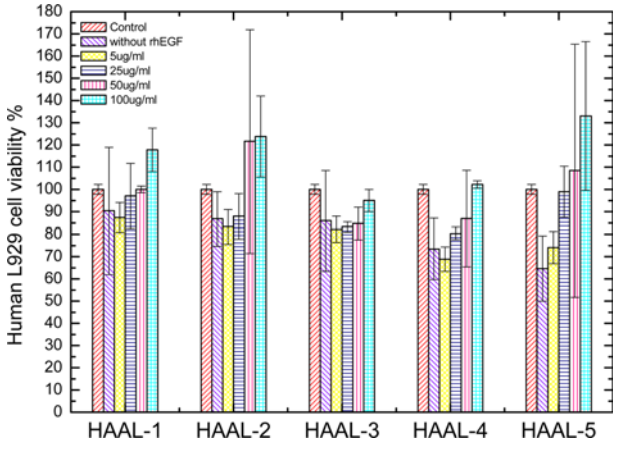

Figure 10. Human L929 cell viability on the surfaces of hydrogels without and with different concentrations of rh-EGF after 72 h of incubation.

Conclusions

HAAL hydrogels were successfully produced *in situ* by coupling reactions and ionic interactions using adipic dihydrazide (ADH) and calcium chloride as cross linking agents, respectively. The equilibrium swelling, interconnected porous structures, and pore sizes were dependent upon crosslinking density. The swelling ratios were higher in PBS than in distilled water due to the salt effect of PBS. In addition, cell culture experiments showed that HAAL hydrogels were non-toxic and that L929 mouse fibroblasts cells adhered to the HAAL hydrogels. HAAL hydrogels loaded with rh-EGF provided a controlled release rate of the growth factor and enhanced human L929 cell proliferation and adhesion behavior. In tests with blood, hydrogels showed good blood compatibility, as the level of adhered platelets was the same as with medical-grade PU.

These results indicate that the HAAL hydrogels display outstanding cell and blood compatibility, and as such, they have potential for use in clinical applications such as matrices for wound healing, vehicles for drug delivery, and as scaffolds for tissue engineering.

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