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Controlled Release Behavior of Bioactive Molecules from Photo-Reactive Hyaluronic Acid-Alginate Scaffolds

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Abstract : There are three important components in tissue engineering: the cells, signaling factors (cytokines and growth factors), and scaffolds. To obtain finely engineered tissue, all three components should perform their individual functions and be fully integrated with each other. For the past few years, we have studied the characteristics of photo-dimerizable HA (CHA)/alginate (CA) composite materials. CHA/CA complex hydrogels, which were irradiated under UV light and then treated with calcium ions, were found to have good biocompatibility, mechanical properties and water resistance for implantable tissue scaffolds. In this study, we introduced a cell growth factor (basic fibroblast growth factor; bFGF) into the CHA/CA scaffolds and studied its release behavior. We also introduced tetracycline hydrochloride and flurbiprofen into the same scaffolds as model activation factors and evaluated their release behaviors from the scaffolds. The drug release rate from the materials was influenced by various parameters, such as the degree of crosslinking, the crosslinker type, the physico-chemical properties of the drug, and the amount of the drug in the polymer. The results indicated that the negatively charged CHA/CA composite materials showed sustained release behavior and that HA has a particularly strong negative charge, making it attractive toward tetracycline hydrochloride and bFGF, but repulsive toward flurbiprofen.

Keywords : photodimerization, hyaluronic acid, sodium alginate, basic fibroblast growth factor (bFGF), biocompatible scaffold.

Introduction

Tissue engineering, which involves combining cells with polymers to repair damaged tissue or inherently abnormal tissue, is one of the novel enterprising research fields. To successfully culture various cells for the generation of engineered tissue products, there are three important factors to consider, namely the cells, signaling molecules and scaffolds. Each cell has an extracellular matrix (ECM) surrounding it, containing abundant amounts of certain components. The ECM of the chondrocytes in the cartilage contains an abundant amount of type II collagen,[1,2] while that of hard bone tissue is filled with calcified type I collagen and glycosaminoglycan (GAG).[3] It has long been known that bFGF is a potent mitogen for a variety of cells of the osteogenic and chondrogenic lineage, and has the ability to increase the proliferation and inhibit the differentiation of the cells.[4,5] bFGF, which is produced by osteoblasts and stored in the ECM around them,[6,7] has shown stimulatory effects on bone formation[8] and its low-dose administration into culture media, producing the stimulation of endosteal and endochondral bone formation in in vivo experiments.[9-13] Hyaluronic acid (HA) is a water-soluble glycosaminoglycan having *N*-acetyl glucosamine and glucuronic acid as its repeating unit. The biological functions of HA, such as protection, lubrication, separation of cells, transportation, the regulation of cell metabolites, maintenance of the structural integrity on connective tissues and fluid retention in the intercellular matrix, have been well investigated.[14,15] Although HA has all of these benefits, however, it is hydrophilic and water soluble.

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Therefore, we attempted to modify its chemical structure in order to improve its physical properties.[16-20] The structure of sodium alginate (SA, sodium salts of alginic acid) is very similar to that of HA, and alginic acid is a naturally derived linear polysaccharide composed of (1,4)-linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) residues. The sodium salts of alginic acid are often used for a variety of special applications such as cell immobilization, as well as in wound dressings, pharmaceutical excipients, and matrices for drug delivery and it can easily form hydrogels in the presence of multivalent cations (i.e. Ca^{2+}, Al^{3+}) or if there is a high G unit content in its chain.[21-23]

Our experimental concept is that the best scaffold for manufacturing the engineered tissue should resemble the original ECM of the target tissue and show the sustained release behavior of cytokines (signaling molecules) in order to enhance tissue regeneration. However, most of the procedures used to make scaffolds include many tedious steps using toxic organic solvents. Therefore, the introduction of a bio-clean method of fabricating scaffolds would be very useful.[24]

In this study, we attempted to combine bFGF with our photoreactive and ion treated hyaluronic acid/alginate based scaffold[25] using an organic solvent free method. The synthesized CHA hydrogels were modified with a UV crosslinker and blended with photo-dimerizable alginate derivatives (CA). The photoreactive CHA/CA hydrogel scaffolds containing bFGF are predicted to show good biocompatibility and to enhance the growth and differentiation of osteoblast cells. Selected tetracycline hydrochloride and flurbiprofen, which showed biological characteristics as pseudo-cytokines,[26-29] are investigated their controlled-release behaviors compared to that of the bFGF from scaffolds.

Experimental

Materials. Hyaluronic acid (HA; molecular weight: 1.7×10^5) from E. Coli was kindly provided by Pacific Chemicals Co. Ltd.(Seoul, Korea). HA was dialyzed using a cellulose membrane tube (Dialysis membrane, cutoff M.W. 3,500; Spectra/Pore® Membrane, Spectrum Laboratories, Inc. CA, USA) with deionized water for 3 days to remove the low molecular weight peptides and lyophilized at -80 °C. Sodium alginate (SA; molecular weight: 4.8×10^4) was purchased from Yacuri Chemicals Co. Ltd. (Tokyo, Japan). HPLC grade *N*,*N*-dimethylformamide (DMF) was purchased from Daejung Chemicals and Metals Co. (Seoul, Korea). Pyridine was purchased from TEDIA (Tokyo, Japan). Cinnamoyl chloride and sodium azide were purchased from Aldrich Chemical Co. (Milwaukee, MI, USA). Tetrabutyl ammonium hydroxide (TBAOH; 12.5% solution in water) was purchased from Merck (New Jersey, USA). Water was purified with Milli Q systems (Millipore Co, MA, USA). All chemicals were of reagent grade and used without further purification.

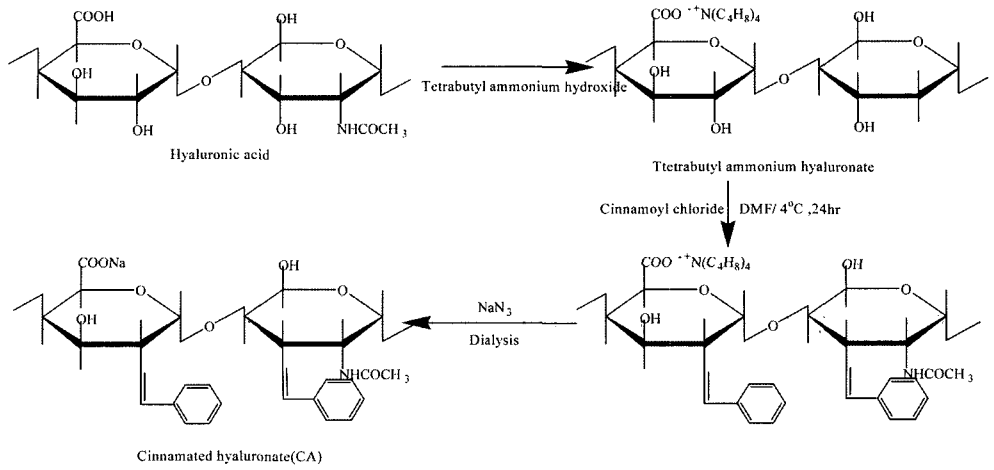
Model Drugs. Flurbiprofen ([±]-2-fluoro-α-methyl-4-biphenyl acetic acid), tetracycline hydrochloride, which are non-steroidal anti-inflammatory drugs used as model drugs for releasing tests, and human recombinant basic fibroblast growth factor (bFGF) expressed in E. Coli. were purchased from Sigma Chemicals Co. (Milwaukee, MI, USA).

Modification of Hyaluronic Acid and Alginate with Cinnamoyl Group.[16] The tetrabutyl ammonium salt of HA (TBA-HA) was obtained by the neutralization of HA with TBAOH and the subsequent lyophilization of the reaction mixture. After dissolving TBA-HA (3 g) in 100 mL of a DMF/pyridine mixed solvent (7:3, v/v), cinnamoyl chloride in DMF solution (30 mL, 2:1 v/v) was slowly added. The mixture was vigorously stirred for 8 hrs at 4 °C under nitrogen gas, and then concentrated under vacuum to obtain high viscous solution. After rinsing this concentrate with a large amount of acetone, and drying under a vacuum for 24 hrs, the reaction products were dialyzed for 3 days in deionized water containing sodium azide (400 mg/L). After freeze-drying, the final products were obtained as shown in Figure 1.

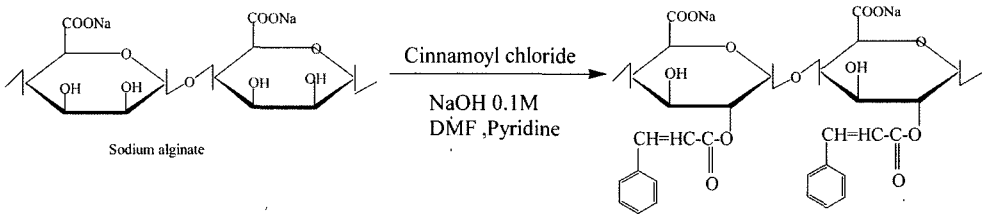
Sodium alginate was dissolved in deionized water and filtered prior to use (3 wt% aqueous solution). DMF/pyridine mixture solution (60 mL, 2:1 v/v) was added to 30 mL of the alginate solution previously made. Forty mililiter of cinnamoyl chloride/DMF solution (1:1, v/v) was slowly dropped into the above solution mixture and kept at 4 °C for 8 hrs. The reaction products were collected by precipitation with excess acetone and dried in a vacuum overnight.

The cinnamoylated HA (CHA) and cinnamoylated alginate (CA) were dialyzed using a cellulose membrane tube in deionized water for 3 days to remove the low molecular weight impurities. The chemical modification of cinnamoylated alginate and hyaluronic acid were confirmed by FTIR (Mattson 5000 FTIR, Mattson Instruments Inc., WI, USA), ^{1}H-NMR (Varian Unity Inova, 500 MHz, Germany) and UV spectrometry (UNICAM UV/Vis spectrometer UV 2, USA). The number of cinnamoyl moieties in the chemically modified polysaccharide molecule was quantified by the measuring UV absorbance at 255 nm originated from cinnamoyl moieties in modified HA or SA solutions using the previously determined calibration curve.

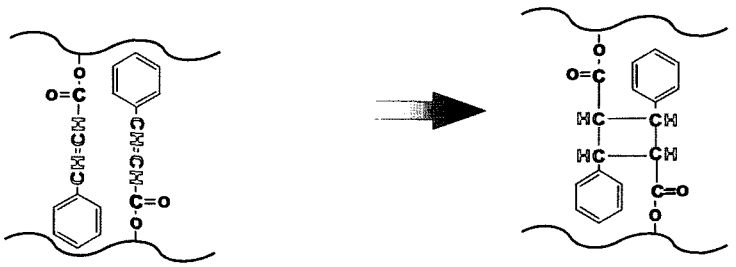
Fabrication of Hyaluronic Acid-Alginate Composite Scaffold. After dialysis, the CHA and CA solutions were blended with various volume ratios (0:10, 2:8, 4:6, 6:4, 8:2, and 10:0) and concentrated using a rotary evaporator (EYELA, Tokyo, Japan). The concentrated CHA/CA solution was poured into 24 well cell culture dishes (used as a mold, Techno Plastic Products AG, Transadingen, Switzerland) and subjected to UV irradiation from a 400 W high-pressure mercury lamp (KIIC-1019, Joil Lighting Industry, Seoul, Korea) as shown in Figure 2. The light intensity was adjusted to 18 mW/cm^2 at 25 °C, and measured with a photometer (UV power pack™, High energy UV radiometer,



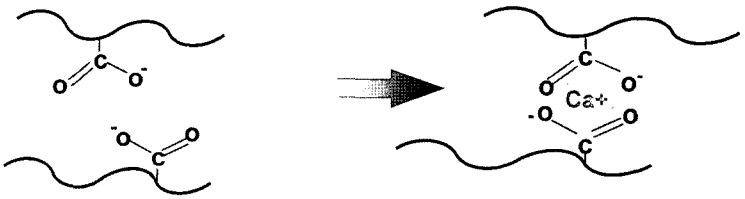
(a) Hyaluronic acid modification with cinnamoyl moiety



(b) Sodium alginate modification with cinnamoyl moiety



(c) Photo-crosslinking of modified HA and SA



(d) Network forming of modified alginate with calcium treatment

Figure 1. The physico-chemical modification scheme of HA, SA, CHA, and CA.

EIT Inc., VA, USA), and the irradiating wavelength (λ>300 nm) was selected by means of cutoff filters (Toshiba UVD33S, Tokyo, Japan). As the photodimerizing reaction proceeded, the viscosity of the polymer solution increased, and this phenomenon was confirmed by measuring the viscosity of the solution using a Brookfield viscometer (RVDVII+, Brookfield Engineering Laboratories, Inc., MA, USA). After irradiation, the water containing gel solution was quickly frozen at different temperatures (-5, -20, -80 °C, liquid nitrogen (N_2)) and lyophilized for 5 days. The lyophilized CHA/CA sponge was immersed into calcium acetate (5 wt% aq. solution) containing sodium azide (500 mg/

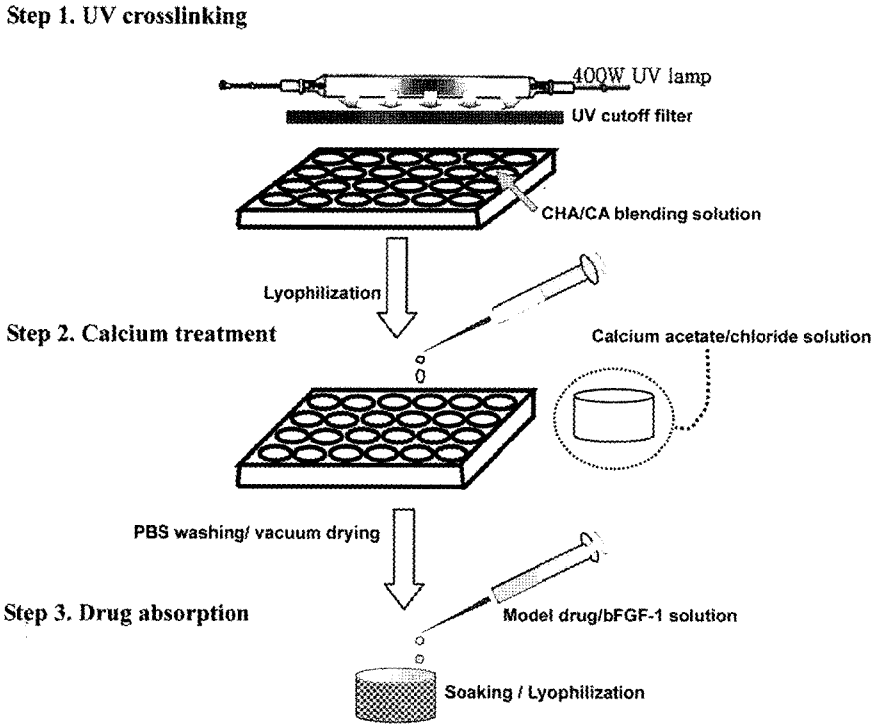


Figure 2. Fabrication of hydrogel scaffolds.

L) for the additional ionic crosslinking of the CA components in the mixture and the in situ ion exchange from TBA^+ to Na^+ of the CHA component in the mixture. The polymer scaffolds were washed more than 3 times with deionized water and dried in a vacuum oven for 5 hrs. The dried scaffolds were soaked in 10 wt% solutions of flurbiprofen and tetracycline hydrochloride in methanol until they did not show volume change owing to swelling. bFGF in purified water (1 mg/mL) was diluted with 99 mL deionized water and used for the loading bFGF onto the synthesized scaffold (Figure 2). The drug-loading efficiency was confirmed by measuring the UV absorbance of the remaining solution of the drug and growth factor.

Porosity Determination of CHA/CA Composite Scaffolds. The theoretical mass of the solid scaffold (W_T) was determined by calculating the air-dried CHA/CA sponge density. The mass of our scaffolds was determined by weighing the manufactured CHA/CA sponges (W_S). In this way, the void volume could be calculated utilizing the following eq. (1)

$$\varepsilon = \left[1 - \frac{W_S}{W_T}\right] \times 100 \quad (1)$$

Gel Fraction and Swelling Behavior of CHA/CA Scaffolds. The CHA/CA scaffolds with various compositions were cut into samples with dimensions of $1 \times 1 \times 1$ cm. These cubic type scaffolds were immersed in tris-phosphate buffered saline (pH 7.4) at 37 °C and obtained disc-shaped swollen gels were weighed (W_o). The obtained gels were weighed again after drying under a vacuum (W_g). The gel fraction (GF) was calculated using the following eq. (2).

$$GF = \frac{W_g}{W_o} \times 100 \quad (2)$$

Porous Structure Observation by SEM. Porous scaffolds were prepared as specimens of SEM. After sputter coating for 5 min, the specimens were examined using scanning electron microscopy (SEM; Hitachi S-2400, Hitachi, Tokyo, Japan).

In vitro Dug Release Behavior. Immersing the drug (tetracycline, flurbiprofen and bFGF) loaded scaffold cubes in tris-phosphate buffered saline (pH 7.4) at 37 °C, the amount of the released drug was measured by UV spectroscopy at predetermined time intervals.

Degradation of CHA/CA Scaffolds. The scaffold cubes were incubated in phosphate buffered saline (pH 7.4) at 37 °C. The weight changes of the samples were measured everyday for 2 months in order to confirm their biodegradability.

Results and Discussion

Modification of Hyaluronic Acid-Alginate Blend with Cinnamoyl Group. Hyaluronic acid (HA) is a very attractive

biocompatible material, in spite of its range of applications being limited by its poor mechanical properties. Therefore, a number of researchers have tried to modify its properties by chemical or mechanical methods. Physico-chemically modified HA may significantly differ from native HA, and most modified HA derivatives retain the biocompatibility and other biological properties of HA. The chemical modification of HA generally involves the modification of the carboxyl groups and hydroxyl groups.[18-20] We tried to modify HA by introducing a photo-dimerizable cinnamoyl moiety. To accomplish this, the H^+ ion of the HA carboxyl group was exchanged with the TBA^+ ion using a previously reported method.[16,17] The formation of the TBA salts of HA was confirmed by ^{1}H-NMR and FTIR. The TBA^+ ion shows a signal at 3.2 ppm in the ^{1}H-NMR spectrum and doublet absorbance peaks at 2800~3000 cm^{-1} and around 1500 cm^{-1} in the FTIR spectrum. The TBA salt of HA in DMF can react with cinnamoyl chloride without any side reactions. The substituted cinnamoyl moieties were detected by ^{1}H-NMR (signal expressions at 7.5 and 8.2 ppm) and the UV absorbance peak of the -C=C- bond. HA and SA modified with the cinnamoyl moiety can form tetracyclic dimers under irradiation with UV light at a wavelength of >300 nm, as shown in Figure 1(c) (If the UV light source contains short radiation wavelength under 300 nm, the cinnamoyl moiety cannot form the tetracyclic dimers). The numbers of cinnamoyl moieties incorporated into the HA and SA molecules (degree of modification; DM) are shown in Table I. In the case where SA and HA were reacted with cinnamoyl chloride under the same conditions, the reaction rate of SA was faster than that of HA, because of the smaller molecular weight and superior mobility of the SA molecule. Therefore, under the same reaction conditions, SA had a large DM (shown in Table I). If the modification reaction of HA and SA was not sufficient, the photo-dimerized CHA/CA blends did not show sufficiently improved mechanical properties compared to those of the unmodified HA or SA. Finally, the found effective DM for best mechanical properties of engineered tissue scaffolds were 10 mol% DM in both CHA and CA. After blending CHA and CA, they were photo-dimerized with UV lights to cause interpenetrating network structure formation. The modified hydrogels were randomly photo-dimerized and then lyophilized to form sponge structure. Sponge type scaffolds obtained were carried out by calcium acetate treatment for additional crosslinking. In the case of blending CHA and CA, the composite materials with various ratio show improved gel properties compared to that of CHA alone.

Structures of CHA/CA Composite Scaffold. Porous CHA/CA composite scaffolds were prepared from the swollen hydrogels by the freeze-drying method. As the freezing rate of the swollen hydrogel increased, the size of the porogens (i.e. ice crystals) that were formed was reduced, and the amount of porogens increased as the water content of the hydrogel increased. Therefore, the pore sizes of the manufactured scaffold decreased with increasing freezing rate (Table I and Figure 3). The effect of the water content of the hydrogel on the pore size of the scaffolds is shown in Figure 4, and the effect of the calcium ion source on the shapes of the pores in the scaffold is shown in Figure 5. In the case where calcium chloride was used as the source of divalent ions, the shapes of the pores in the scaffold were of the random sphere type, whereas when calcium acetate was used, the shapes of the pore were of the regular oval type.

Physicochemical Properties of Photo-Reactive CHA/CA Composite Scaffold. Because of the high water solubility of natural HA, polymer scaffolds made from this material have poor mechanical properties. By blending CHA and CA, we can improve the mechanical properties of the scaffolds in the culturing media. SA is known to form a network structure with divalent ions such as calcium or aluminum ions. Therefore, calcium treatment can support and reinforce the structure of photo dimerized CHA/CA scaffolds.

The swelling ratio of the photo-dimerized CHA/CA scaffold was smaller than that of the HA scaffold which was not supported by alginate (Figure 6). As the porosity of the scaffolds increased, their pore size decreased and their mechanical properties improved. The CHA/CA scaffold also shows pH responsive swelling behaviors owing to the pH sensitiv-

Table I. Gel Fraction, Degree of Modification, and Porosity of Prepared Scaffolds

	GF (%)	DM (mol%)	Porosity (ε)				
			-5 °C	-20 °C	-40 °C	-80 °C	Liquid N_2
CHA	65.3±5.44	11.8±2.34	91.4±3.24	79.2±3.12	65.2±2.83	23.3±4.99	13.5±2.98
CA	86.4±1.65	9.5±1.55	85.5±1.89	72.2±2.56	59.1±0.45	27.3±1.45	10.8±2.26
CHA/CA (4:1)	71.4±3.67	11.3±2.18	87.9±2.56	76.5±2.89	67.4±1.52	27.7±3.80	13.9±1.76
CHA/CA (3:2)	71.1±3.74	10.9±2.02	84.4±4.23	70.9±1.93	69.5±1.91	29.1±1.39	12.9±0.97
CHA/CA (1:1)	77.8±1.63	10.7±1.95	82.1±1.04	68.6±1.78	59.3±0.69	31.3±5.12	13.3±0.48
CHA/CA (2:3)	79.6±1.25	10.5±1.87	83.6±0.99	63.7±0.99	58.7±0.97	36.3±1.95	11.0±0.93
CHA/CA (1:4)	82.1±1.39	9.7±1.79	83.5±1.02	64.3±0.83	53.2±0.56	35.6±2.21	11.6±0.34

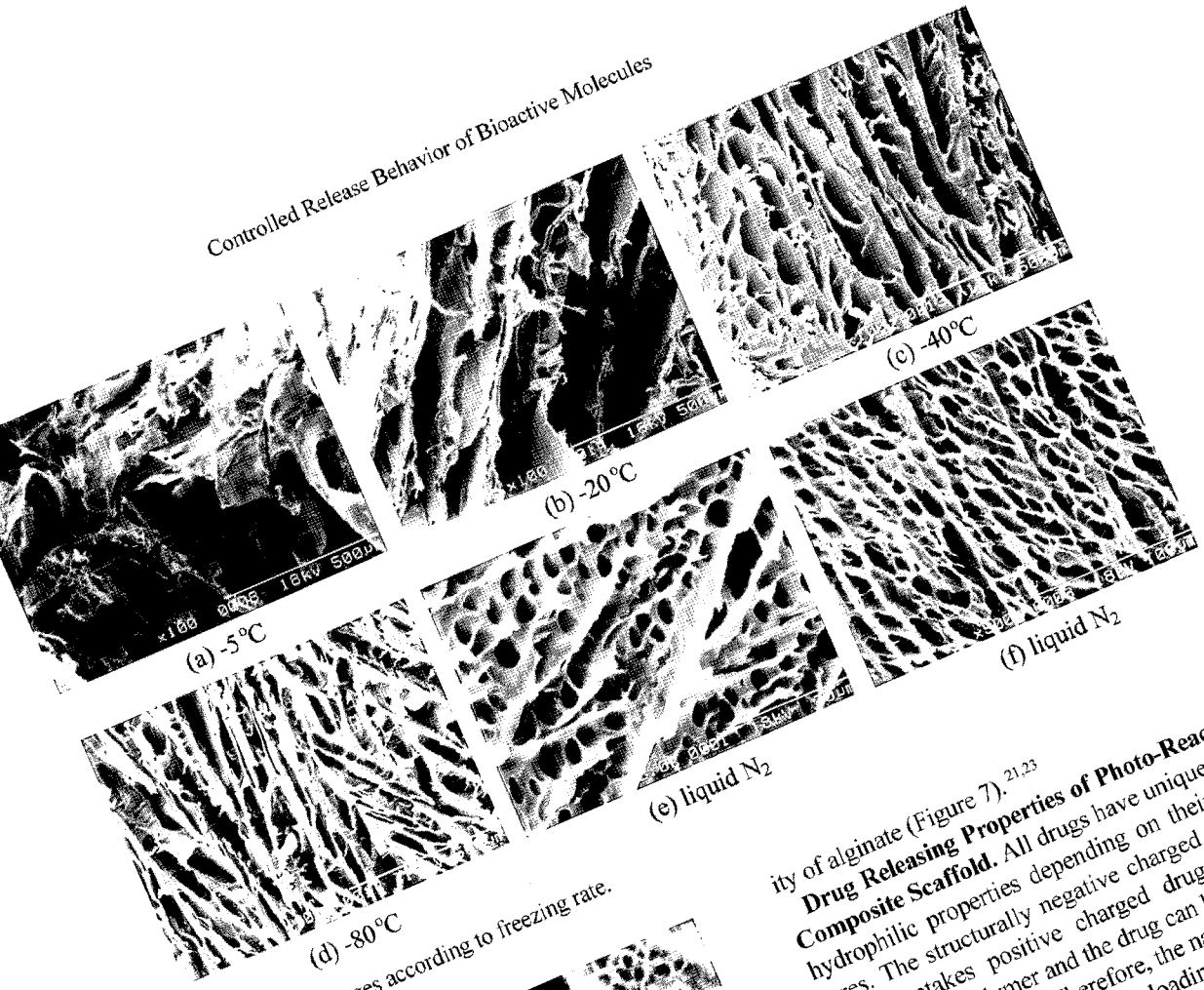


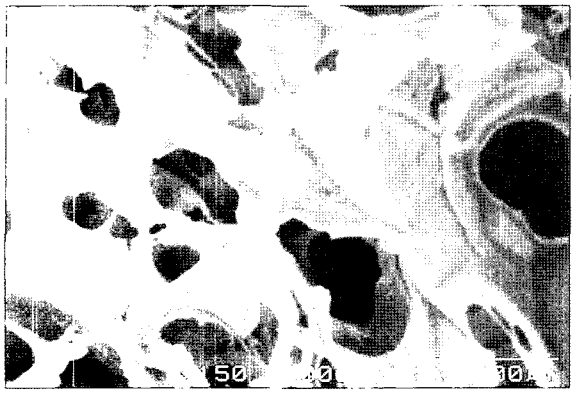
Figure 3. Pore size changes according to freezing rate.

Figure 4. Pore size changes according to water content of hydrogel.

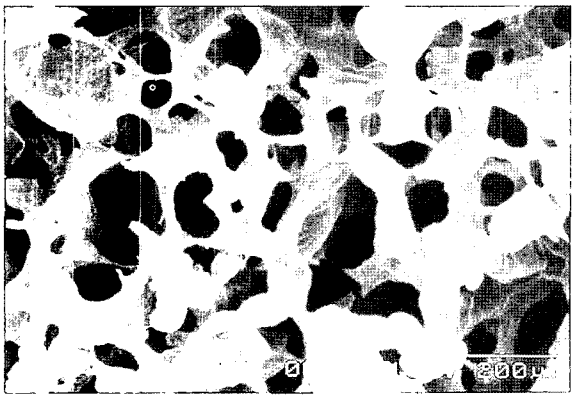
ity of alginate (Figure 7).[21,23]

Drug Releasing Properties of Photo-Reactive CHA/CA Composite Scaffold. All drugs have unique hydrophobic or hydrophilic properties depending on their chemical structures. The structurally negative charged CHA/CA scaffold easily uptakes positive charged drugs. The ionic bond between the polymer and the drug can help to retain the drug within the scaffolds. Therefore, the natural characteristics of the drug can affect their drug loading efficiency in the scaffolds (Table II). Both flurbiprofen and tetracycline hydrochloride showed good loading efficiency in the CHA/CA scaffolds. Flurbiprofen exhibits more affinity toward CA than CHA and shows greater drug-material complex forming ability in CHA/CA blends. Tetracycline hydrochloride is more hydrophilic than flurbiprofen, and tetracycline hydrochloride shows greater affinity toward CHA than CA because CHA is more hydrophilic than CA. Likewise, the CHA/CA scaffolds can uptake more tetracycline hydrochloride, and also the CHA or CA only materials. bFGF shows a similar loading tendency to that of tetracycline hydrochloride, and also shows the same loading tendency in CHA materials. The chemical properties of the activating molecules affected loading efficiency, and those scaffolds containing negcharged model drugs showed sustained release behavior. The release profile of flurbiprofen from CHA materials faster than that from CA materials. The release rate of cycline hydrochloride and bFGF from CA are those from CHA materials (Figure 8). Based results, we would expect the bFGF incorporated CHA/CA scaffolds to show sustained release

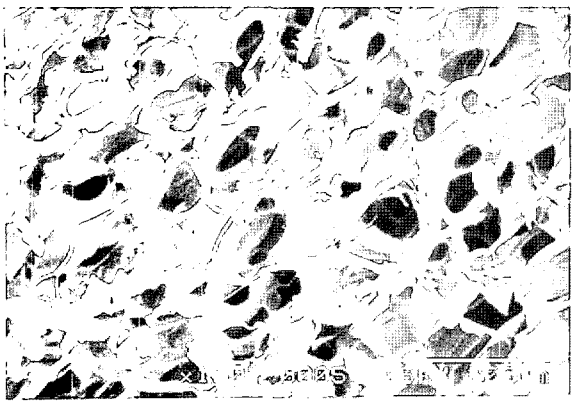
Degradation Profile of CHA/CA Comp



(a) Before calcium ion treatment



(b) Treated with $CaCl_2$ (0.1%)



(c) Treated with Calcium acetate (0.1%)

Figure 5. Pore shape changes depending on ion source of calcium.

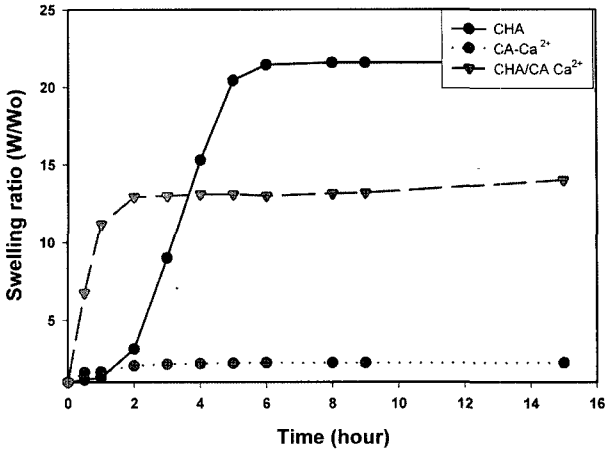


Figure 6. Swelling behavior of various hydrogel scaffolds.

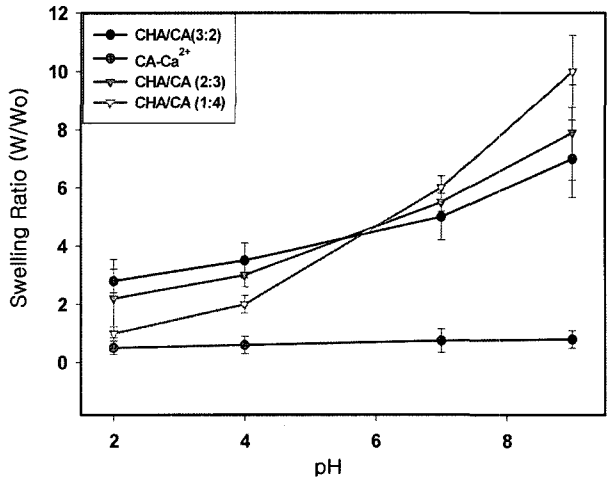


Figure 7. Swelling behavior of scaffolds according to pH change.

The various CHA/CA blended scaffolds were incubated in phosphate buffered saline (pH 7.4) at 37 °C and the weight changes were measured at predetermined time intervals (Figure 9). The mass of the CHA/CA scaffolds slowly decreased and their shape was maintained in the early stages. After 2 months, the decrease ratio in the weight of CHA10 was 30.1±5% and the change in the weight during incubation decreased significantly as the proportion of alginate in the CHA/CA blend increased (4:1, 3:2, 1:1, 2:3, 1:4).

Table II. Drug Loading Efficiency

Composition Drug (mg)/Matrix (g)	CHA	CA	CHA/CA Blend				
			4:1	3:2	1:1	2:3	1:4
Flurbiprofen	0.6±0.16	0.9±0.59	0.9±0.39	1.1±0.45	1.2±0.28	1.1±0.23	1.4±0.34
Tetracycline*HCl	1.5±0.98	0.2±0.26	2.4±0.76	2.8±0.97	2.3±0.48	2.7±0.85	2.6±0.56
Basic FGF-1*	52.2±4.02	11.8±9.81	44.6±3.19	45.1±2.71	39.7±1.40	35.2±4.78	15.2±1.56

*Unit (μg of cytokine/ g of matrix).

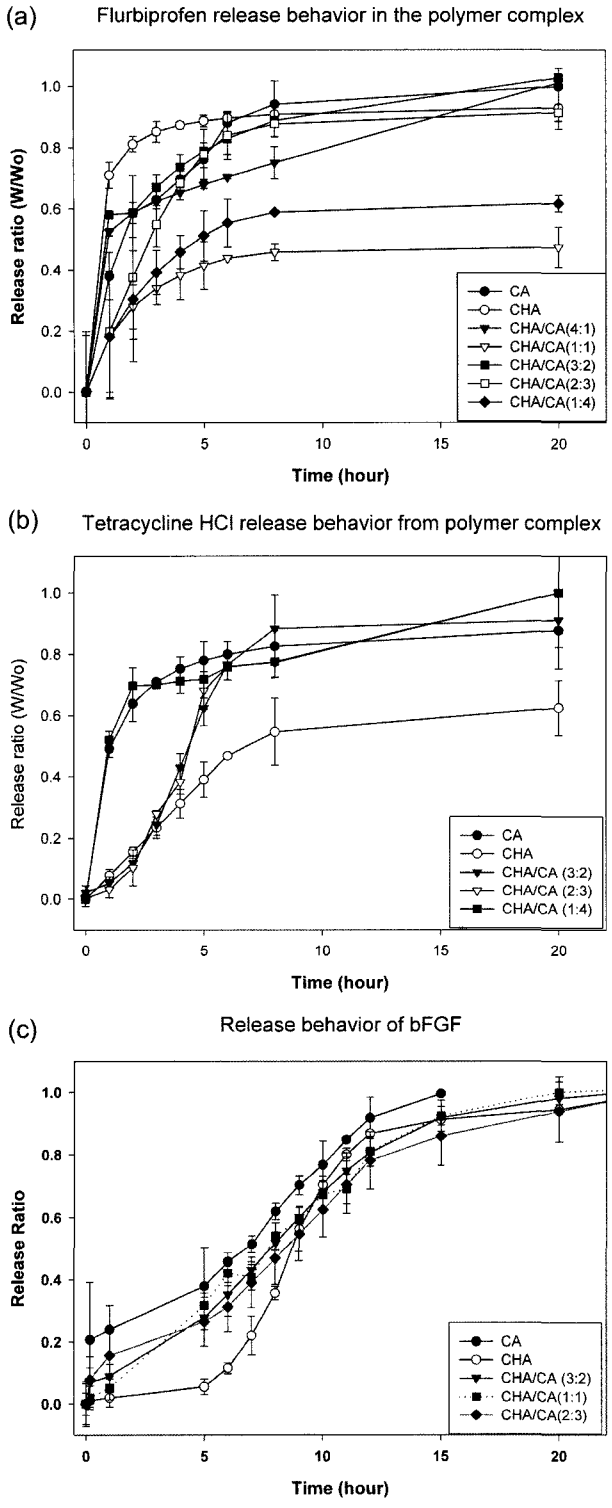


Figure 8. Release profiles of bioactive regents from various CHA-CA scaffolds.

Conclusions

In this paper, we studied the release behavior of hydrophilic model drugs and cytokines from modified HA/alginate (CHA/CA) blends. UV cross-linked and calcium treated CHA/CA blends were made by the hydrogel lyophilization method. The use of a soaking procedure was proposed to load the drugs and cytokines into the CHA/CA blend scaffolds. The CHA/CA blend scaffolds containing activating molecules showed sustained release behaviors. In conclusion, we proposed a valid method of designing specific scaffolds for targeting tissues incorporating a similar matrix and cell function.

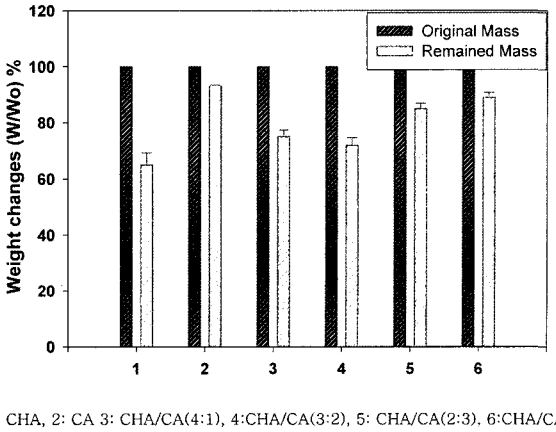


Figure 9. In vitro degradation profiles of various CHA-CA scaffolds.

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