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Improvement of the Biocompatibility of Chitosan Dermal Scaffold by Rigorous Dry Heat Treatment

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Abstract: We have developed a rigorous heat treatment method to improve the biocompatibility of chitosan as a tissue-engineered scaffold. The chitosan scaffold was prepared by the controlled freezing and lyophilizing method using dilute acetic acid and then it was heat-treated at 110 °C *in vacuo* for 1~3 days. To explore changes in the physicochemical properties of the heat-treated scaffold, we analyzed the degree of deacetylation by colloid titration with poly(vinyl potassium sulfate) and the structural changes were analyzed by scanning electron microscopy, Fourier transform infrared (FT-IR) spectroscopy, wide-angle X-ray diffractometry (WAXD), and lysozyme susceptibility. The degree of deacetylation of chitosan scaffolds decreased significantly from 85 to 30% as the heat treatment time increased. FT-IR spectroscopic and WAXD data indicated the formation of amide bonds between the amino groups of chitosan and acetic acids carbonyl group, and of interchain hydrogen bonding between the carbonyl groups in the C-6 residues of chitosan and the *N*-acetyl groups. Our rigorous heat treatment method causes the scaffold to become more susceptible to lysozyme treatment. We performed further examinations of the changes in the biocompatibility of the chitosan scaffold after rigorous heat treatment by measuring the initial cell binding capacity and cell growth rate. Human dermal fibroblasts (HDFs) adhere and spread more effectively to the heat-treated chitosan than to the untreated sample. When the cell growth of the HDFs on the film or the scaffold was analyzed by an MTT assay, we found that rigorous heat treatment stimulated cell growth by 1.5~1.95-fold relative to that of the untreated chitosan. We conclude that the rigorous dry heat treatment process increases the biocompatibility of the chitosan scaffold by decreasing the degree of deacetylation and by increasing cell attachment and growth.

Keywords: chitosan, heat treatment, cytocompatibility, human dermal fibroblast, dermal scaffold.

Introduction

Tissue repair and regeneration requires a complex biological process involving inward migration and proliferation of various types of neighboring cells and the deposition and remodeling of the extracellular matrix secreted by the emigrant cells. If proper biocompatible scaffold is provided, guided cell migration to the wound site and reformation of vascular network may be facilitated.

A number of natural or synthetic polymers have been tested for tissue engineering scaffolds. Their potential usage for tissue scaffold has been evaluated by their biocompatibility mainly based on the cell binding and proliferation capacity as well as cytotoxicity. With this aspect, synthetic biodegradable biomaterials such as PGA, PLA, PLGA, and their derivatives may cause problems *in vivo* since their degradation products are acidic which may create deleterious condition for cell growth and survival.[1,2] Therefore more suitable and biocompatible dermal scaffold overcoming or controlling several technical limitations has to be developed for successful launching of tissue-engineered dermis for wide clinical application.

Chitosan, poly(β-1,4-D-glucosamine), is a partially deacetylated derivative of chitin, primary structural polymer in arthropod exoskeletons. It has been considered as a candidate natural polymer for the scaffold since chitosan shows several advantages over other synthetic biomaterials,[3-8] low cytotoxicity, antimicrobial activity, easy manipulation of stable porous structure by lyophilization, and generation of biologically safe degradation products. Its structural similarity to glycosaminoglycans and its water-attracting capacity

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similarly to the hyaluronic acid also provide the biological basis of its application to the tissue engineering scaffold. Furthermore, chitosan seems to have many advantages for wound healing[3,4] by stimulating hemostasis[9] and by accelerating the tissue regeneration,[10-13] which is facilitated by the fibroblastic synthesis of collagen, the induction of cytokine production,[5] and activation of inflammatory cells in animals.

Proper strength in cell adhesion to the scaffold seems to be required for suitable cell migration and proliferation. It is well known that fibroblasts strongly adhere to the chitosan film[14] and chitosan inhibits the cell proliferation although chitosan is not cytotoxic.[15,16] Those previous reports suggest that fibroblasts may be stuck on the chitosan film, which may inhibit their cell migration and further cell proliferation. This property of chitosan may cause delayed wound healing.

Ogawa[17] and Toffey[18] *et al*. reported that chitosan acetate film is converted into chitin by heat treatment undergoing cure process.[19] Thus heat treatment may modify the degree of chitosan deacetylation at the scaffold state. In this study, as an attempt to improve biocompatibility of the chitosan scaffold for tissue engineered artificial dermis, we applied rigorous heat treatment *in vacuo* to the chitosan film and chitosan sponge, analyzed their physicochemical properties by SEM, FT-IR, and WAXD, and evaluated their cell binding capacity and cell proliferation based on MTT assay.

Experimental

Materials. Chitosan from crab shell, whose degree of deacetylation is 85% based on the amino content, was purchased from Korea Chitosan Ltd. (Seoul, Korea). Bovine type I atelocollagen acidic solution was purchased from Nitta collagen (Japan). 3-[4,5-Dimethylthiazol-2-yl]-2,5-dipheyltetrazolium bromide (MTT) was purchased from Sigma Aldrich (St Louis, MI, USA). Dulbecco's modified Eagle's minimal essential medium (DMEM), ethylenediaminetetracetic acid (EDTA), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Deionized water was obtained with a Milli-Q water filter system from Millipore Corporation (Bedford, MA, USA).

Preparation of Chitosan Scaffolds and Dry Heat Treatment. Chitosan was dissolved in 1% acetic acid to give 1.5 w/v% solutions. Chitosonium acetate films were prepared by covering the cover glass (12 mm ϕ) in 24 culture plate, followed by solvent evaporation and drying at room temperature in biological laminar flow hood for 2 weeks. In order to prepare chitosonium acetate scaffolds, thirty-five grams of 1.5 w/v % chitosonium solution under pre-cooled condition was poured into flat bottomed molds (99 cm^2), then quickly froze at -80 °C for 24 hrs, and lyophilized at -55 °C under a vacuum of 0.2 torr for 24 hrs.

Crude chitosonium acetate films and scaffolds were heated in the oven at 110 °C under *in vacuo* for 1, 2, and 3 days. After the heat treatment, the scaffolds and films were neutralized with 0.1 N NaOH in 70% ethanol and washed with deionized water until the filtrate reached neutral pH.

Characterization of Physicochemical Properties. The structural changes in chitosan derivatives were confirmed with Fourier transform infrared (FT-IR), and a wide-angle X-ray diffractometer (WAXD). Infrared spectra of chitosan derivatives were measured through a Nicolet 5DX FT-IR spectrophotometer using KBr pellet. WAXD were recorded with the reflection mode using nickel-filtered CuKα radiation produced by a Rigaku Denky X-ray diffractometer (Model RAD-C) operated at 30 kV, 30 mA. The scan rate was 5 ° (2θ)/min.

Degree of deacetylation of chitosan scaffold was determined by colloid titration[23] using poly(vinyl potassium sulfate) (PVSK). Briefly, chitosan solution was prepared by dissolving 0.5 g chitosan in 5% acetic acid to reach a final volume 100 g. 1 g of chitosan solution was exactly quantified and then added in 30 mL of distilled water with mixing enough 1/400 N PVSK solution. Titration was carried out by dropping 1/400 N PVSK solution with 2~3 drops of 0.1 % toluidine blue as an indicator during agitation on a magnetic stirrer.

$$\text{Degree of deacetylation (\%)} = \frac{X/161}{X/161 + Y/203} \times 100$$

$X = 1/400 \times 1/1000 \times f \times 161 \times V$

$Y = 0.5 \times 1/100 - X$

f: 1/400 N PVSK solution factor

V: Titration volume (mL) of 1/400 N PVSK solution

Isolation of Human Dermal Fibroblasts and Cell Culture. Primary human dermal fibroblasts (HDFs) were obtained from newborn foreskin by digestion in 1% trypsin/EDTA and cells at passage 2 and 6 were used. HDFs were cultured in Dulbecco's modified Eagle's medium (DMEM : F12 = 3 : 1) supplemented with 10% FBS and 50 U/mL of penicillin and streptomycin. The cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere with fresh medium changed every day.

MTT Assay. HDFs were seeded onto films within 3 cm^2 metallic rings and onto scaffolds at 3×10^4 cells/cm^2 and 1.6×10^5 cells/scaffolds, respectively. Cell growth was observed under phase contrast microscope (DMIL; Leica, Wetzlar, Germany). MTT assay of the HDF on the chitosan film was performed at 3 day culture and MTT assay on the scaffold was performed at 14 day culture according to the modified MTT colorimetric assay originally described by Mosmann.[20] For MTT assay, 1 mL of the MTT solution was added to each well and incubated for 4 hrs at 37 °C. Cells were washed with phosphate buffered saline (PBS) and were lysed with the buffer (EtOH : DMSO = 1 : 1) while shaking for 30 min in order to release cells in scaffolds. For colorimetric analysis, 100 μL of supernatant solution was aliquoted into a 96 well and the optical density (OD) at 540 nm was measured

by a Bio-Rad model 450 microplate reader (Richmond, CA, USA). All data were presented as mean values of three independent experiments.

Scanning Electron Microscopy (SEM). Chitosan and heat treated chitosan scaffolds were washed with PBS and cells adhered to scaffolds were fixed with 1% glutaraldehyde for 1 hr at room temperature, followed by 24 hrs incubation at 4 °C for gross visualization of cells in the scaffolds. The scaffolds were dehydrated by immersion in a series of aqueous solutions with increasing alcohol content for 15 min each and freeze-dried under the same condition as described earlier. The samples were critically point dried and coated with an ultra-thin gold layer (100 Å). A scanning electron microscope (Jeol, Model JSM-35CF) was used to image the samples.

***In vitro* Biodegradability of Scaffolds.** Degradability of the chitosan scaffolds and the heat treated chitosan scaffolds were determined by mass change of scaffolds after their incubation in 1.5 mL PBS (pH 7.4) containing 50 μg/mL of lysozyme (58,000 U/mg, Sigma L-6876) from white egg and 0.5 mL of FBS while shaking at 37 °C. After incubation for several time intervals, the scaffold was carefully withdrawn, repeatedly washed with PBS, and freeze-dried under the same condition as described earlier. The extent of the *in vitro* degradation was calculated as the percentage of weight difference of the dry scaffolds before and after hydrolysis with the lysozyme solution.

Results and Discussion

SEM Image of the Heat Treated Chitosan Scaffold. SEM surface morphologies of the chitosan scaffold and the heat treated chitosan scaffold are shown in Figure 1. The chitosan scaffold showed even and regular pores ranging in size from 80 to 150 μm (Figure 1(a)), which may be a suitable framework for the cell seeding. Heat treatment for 3 days under vacuum at 110 °C did not cause major change in the pore size and its integrity (Figure 1(b)). In considering that porosity, mean pore size, and orientation of porous matrix are important elements for biologic activity of the scaffold with open-pored structures,[21] our rigorous heat treatment can be safely applied for the processing of the chitosan scaffold. The resulting heat treated scaffold did not dissolve in dilute acetic acid as well as in water.

Change in Physicochemical Properties of the Chitosan Scaffold by Rigorous Heat Treatment. It has been reported that exposure to high temperatures can change the properties of chitosan and expected that the rigorous heat treatment could lead to crosslink of chitosonium acetate, the ionic complex between chitosan and acetic acid.[18,22,24]

We measured deacetylation degree of the heat treated scaffold based on the colloid titration assay with PVSK (Table I). Rigorous heat treatment for 1 day caused decrease in the deacetylation degree of the chitosan scaffolds to 45% comparing to 85% deacetylation of the untreated chitosan scaffold. Heat treatment for 3 days further decreased the deacetylation degree to 28.7%. Therefore our result suggests that rigorous heat treatment at 110 °C under the vacuum condition is sufficient enough for chemical modification of the chitosan scaffold leading to the decrease in the deacetylation degree of the chitosan, which was dependent on the duration of heat treatment.

To further evaluate whether rigorous heat treatment caused changes in the chemical properties of the chitosan scaffold or not, we analyzed FT-IR spectra of the heat treated for 3

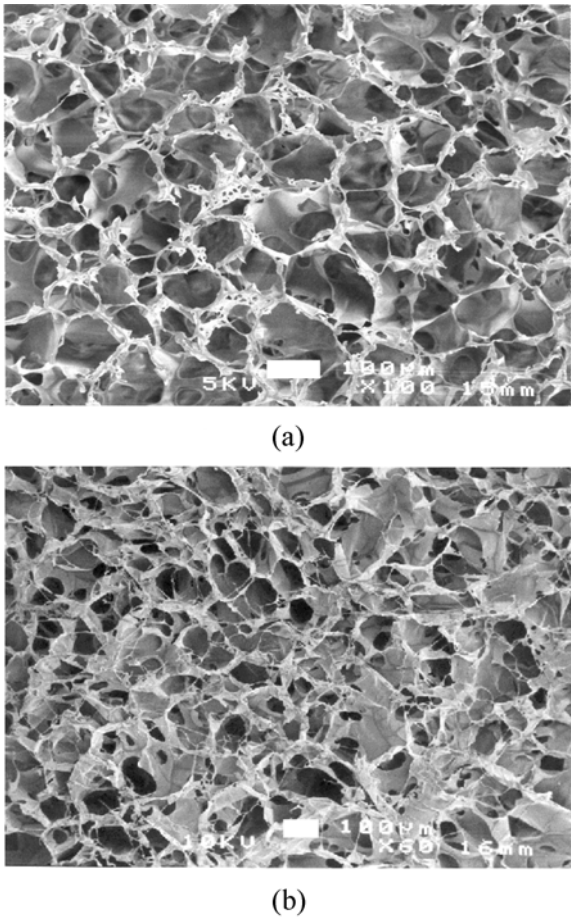

Figure 1. SEM image of the surface of dermal scaffolds. (a) Chitosan and (b) heat treated chitosan. Scale bar = 100 μm. Original magnification × 60.

Table I. The Effect of Heat Treatment on the Degree of Deacetylation of Chitosan

Scaffolds	Heat Treated Time (days)	Deacetylation Degree (%)
Chitosan	0	82.5
	1	45.0
	3	28.7

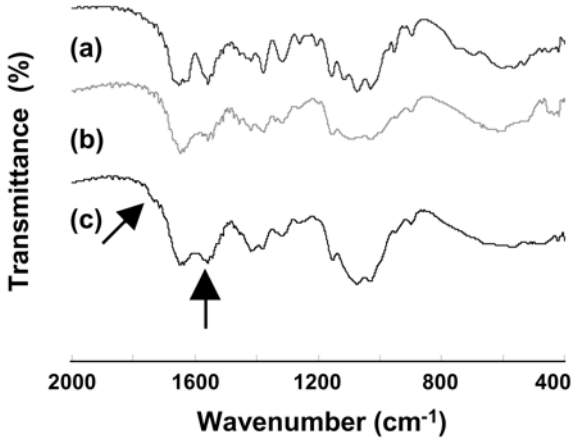

Figure 2. FT-IR spectrum of dermal scaffolds. (a) Chitin, (b) chitosan, and (c) heat treated chitosan for 3 days.

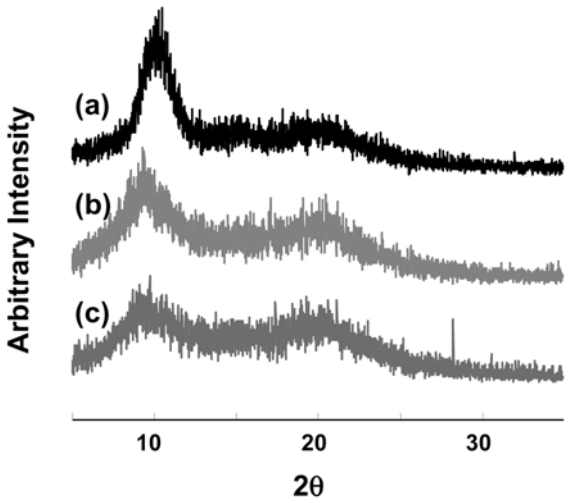

Figure 3. WAXD patterns of dermal scaffolds. (a) Chitosan, heat treated chitosan for (b) 1 day, and (c) 3 days.

days and untreated chitosan scaffold (Figure 2). The representative absorption peak in all samples are assigned to amide I (1654 cm^{-1}), amide II (1550 cm^{-1}), and amide III (1495 cm^{-1}). The heat treated chitosan derivatives (c) showed different FT-IR spectrum comparing to the untreated (b) and the chitin control (a). New but small shoulder appears at 1730 cm^{-1} (noted as single arrow) and the peak intensities of amide II at 1550 cm^{-1} (noted as arrowhead) and C-O group at 1070 cm^{-1}, gets stronger than the untreated group. These results suggest that the amide bond between chitosan and acetic acid may be formed, and new carboxyl groups may be formed due to the acetylation between carboxyl groups in chitosonium acetate complex, reaction intermediate, and hydroxyl groups in the C-6 residue of carbohydrate unit. Since the absorption due to free carbonyl groups appears at around 1750 cm^{-1}, the shift to 1730 cm^{-1} might be due to the interchain hydrogen bond formation between new carboxyl groups in the C-6 residue of carbohydrate unit and new amide groups after the rigid heat treatment, involving the unreacted free amine.

To examine whether rigorous heat treatment caused the change in the crystallinity of the chitosan scaffold or not, we measured the WAXD of the heat treated for 3 days and untreated chitosan scaffold (Figure 3). There are two major reflections at 20° and 10.6° of 2θ from the untreated chitosan scaffold. However, the reflections at 10.6° from the heat treated chitosan scaffold were decreased according to heat treatment time. Decrease in the crystallinity of the heat treated chitosan scaffolds was considered to be due to the deformation of the strong hydrogen bond in the chitosan backbone. These results were in accordance with the report of Ritthidej *et al*.,[22] except their non-solubility in aqueous dilute acid.

In the case of the heat treated chitosan scaffolds, in addition to the peak observed at 10.6°, two new small peaks appeared at 15° and 23°. This lead to the formation of hydrogen bonds between carbonyl groups in C-6 residue of chitosan with *N*-acetyl groups in chitosan, as evidenced by FT-IR. This result is similar to Kumber *et al*.,[24] who expected that the rigorous heat treatment could lead to crosslink of chitosonium acetate, the ionic complex between chitosan and acetic acid.

Therefore our data indicated that there were the amide formation between chitosan and acetic acid, and the interchain hydrogen bond formation between new amide groups and carboxyl groups in the C-6 residue of carbohydrate unit, involving the amine groups of chitosan was occurred.

Change in *in vitro* Biodegradability of Scaffolds. To evaluate whether rigorous heat treatment affect biodegradation rate of the scaffolds, the scaffolds were incubated with lysozyme derived from the egg white and the weight loss was measured. The percent weight loss of the scaffolds is presented as a function of degradation time (Figure 4). The *in vitro* enzymatic degradation rates of the heat treated chitosan derivatives were faster than that of chemically acetylated chitosan scaffolds (data not shown) as well as chitosan itself. Rigorous heat treatment of the chitosan scaffold result in faster degradation of the scaffold, which was best contrasted at 28 days incubation.

Depolymerization of chitosan occur by enzymatic hydrolysis, by acid hydrolysis, and by an oxidative-reductive depolymerization reaction.

Increase in the biodegradability by lysozymatic hydrolysis of the heat treated chitosan scaffolds was considered to be due to the decrease in the deacetylation degree of the chitosan. Only lysozyme in serum in the human body can degrade chitin and its *N*-deacetylated product, chitosan. The *in vitro* degradation rates of chitosan increase strongly with decreasing the degree of *N*-deacetylation of chitosan.[25]

Since depolymerization of chitosan may also occur by acid

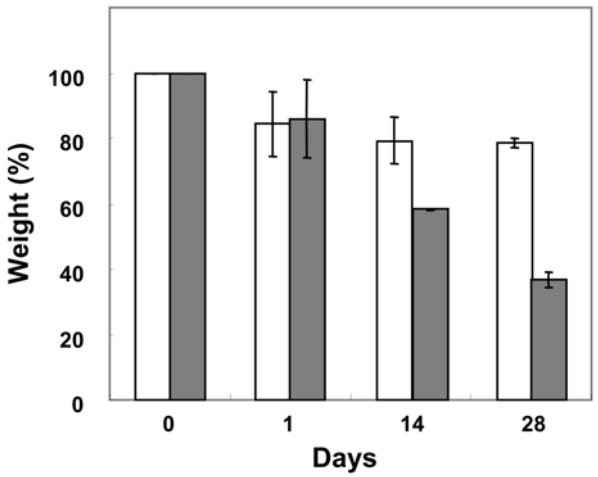

Figure 4. Comparison of biodegradation of the heat treated for 3 days and untreated chitosan scaffold by lysozyme hydrolysis. The scaffold was incubated with 1.5 mL of lysozyme solution (50 μg of lysozyme / mL of PBS (pH 7.4)) and 0.5 mL of FBS at 37 °C for indicated time. Each column represents the mean value ±SD (n=4).

hydrolysis and by an oxidative-reductive depolymerization reaction, this data indicates that the chitosonium acetate scaffold probably was degraded during heat treatment, although overall porous matrix structure of heat treated chitosan scaffold is well preserved based on the SEM morphology. This result may further implicate that implant of the heat treated scaffold may be degraded much faster within the body fluid and wound tissue than the untreated.

Changes in the Biocompatibility of the Chitosan Films and Scaffolds by Rigorous Heat Treatment. We examined whether heat treatment changed the properties of cell attachment and growth on the chitosan film and the scaffold, and the morphological change of HDFs adhered onto each the films and the scaffolds (Figure 5).

HDF attached onto the heat treated chitosan film showed more flattened and genuine fibroblastic morphology similar to that of the gelatin film. However, HDFs on the untreated chitosan film showed round morphology. After 3 day culture, HDFs on the untreated chitosan scaffold became extended but did not grow. However HDFs on the scaffold, heat treated for 3 days, fully spread on the surface and cell numbers increased. This result suggests that modification of physicochemical properties of the chitosan scaffold attributed by rigorous heat treatment probably provide better environment for the cell attachment and growth of HDF. Our results may be interpreted consistently with the reports of Denuziere *et al.*,[15] who showed that chitosan is cytostatic to HDFs.

To further analyze the relationship between degree of deacetylation and stimulating capacity of cell growth on the scaffold, both of which were obtained after rigorous heat treatment, we compared degree of HDF growth cultured on the chitosan scaffolds with different degree of deacetylation. At 1 day heat treated scaffold which retain 45% deacetylation degree, 150% higher cell growth than the untreated was observed. This result suggests that 45% deacetylation of the chitosan scaffold (medium degree of deacetylation) may provide the most optimum condition for proper cell attachment and cell migration. If cells attach to the surface too firmly,

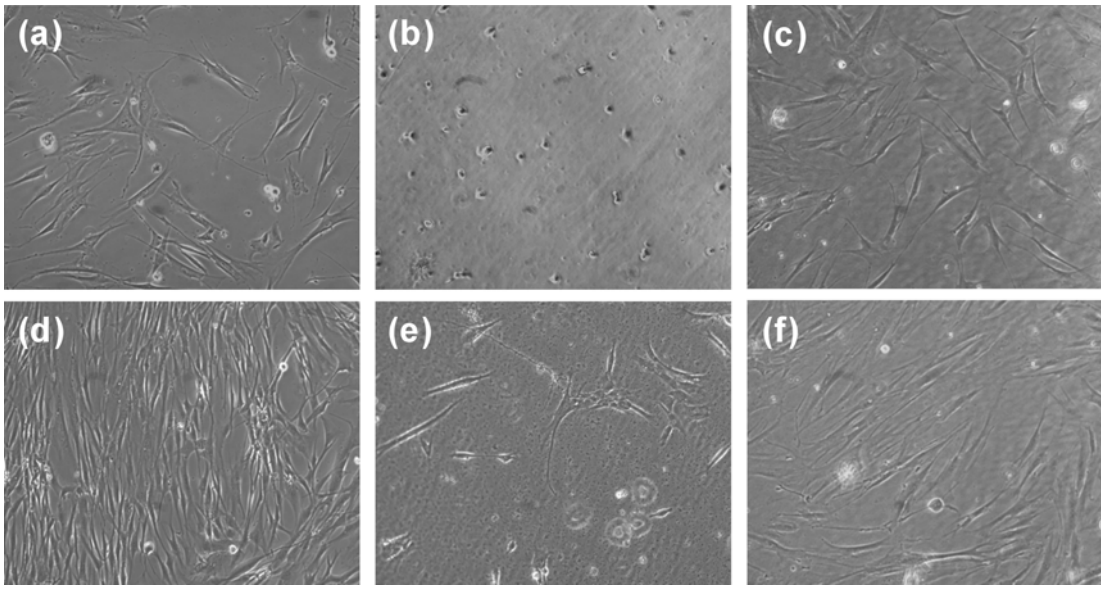

Figure 5. Phase contrast morphology of human dermal fibroblasts on the heat treated for 3 days or the untreated chitosan films. (a) Gelatin coated control culture at day 1, (b) chitosan film at day 1, (c) heat treated chitosan film at day 1, (d) gelatin coated control culture at day 3, (e) chitosan film at day 3, and (f) heat treated chitosan film at day 3. Magnification × 100.

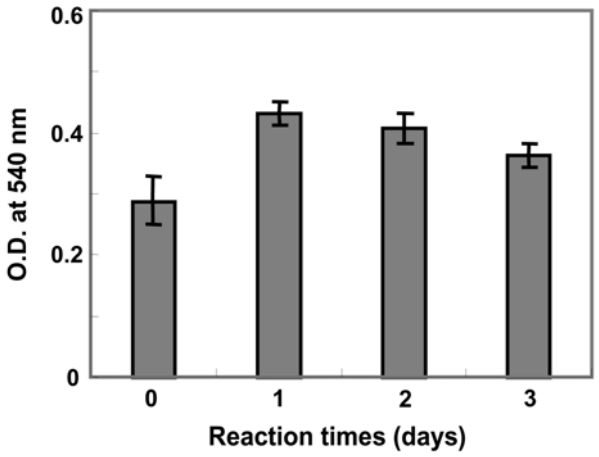

Figure 6. Effect of heat treatment time on cytocompatibility of the chitosan. HDFs were seeded onto the films at a density of 3×10^4 cells/cm^2. MTT assay was performed at day 3 culture. Each column represents the mean value ± SD (n=3).

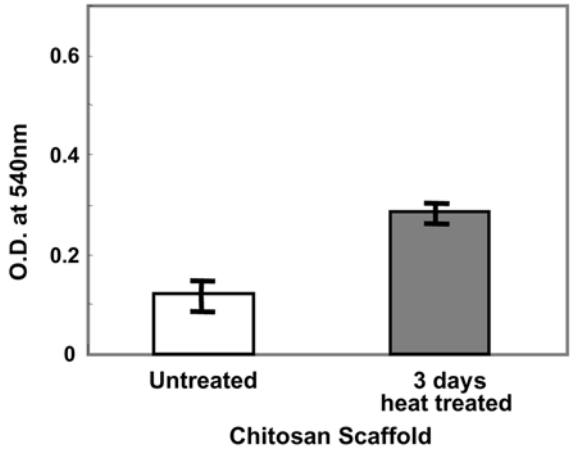

Figure 7. Comparison of cell growth of the heat treated for 3 days or untreated chitosan scaffold. HDFs were seeded in the scaffolds at a density of 1.6×10^5 cells/scaffold. Scaffolds by MTT assay performed at day 14 culture. Open column: untreated scaffolds, closed column: heat treated scaffolds. Each column represents the mean value ±SD (n=3).

those cells can not move and proliferate at all. Therefore 45% deacetylation may create suitable electrostatic microenvironment satisfying both requirement for the cell attachment and migration.

We further evaluated the cell growth capacity of HDFs seeded in the 3 day heat treated chitosan scaffold (Figure 7). Again, HDFs growth in the scaffold was 132% higher than the untreated scaffold. In summary, our results suggest that free amine of chitosan may play a key role in the regulation of cell adhesion and proliferation and can be readily controlled by the duration of rigorous heat treatment.

Conclusions

In this study, we developed rigorous heat treatment method for the modification of physicochemical properties of the chitosan scaffold, which result in decrease in deacetylation of the chitosan without gross changes in the integrity of the scaffold. We confirmed that our heat treatment method strongly improved the biocompatibility of the chitosan film and the scaffold based on the increase in initial cell attachment as well as cell growth and we also suggest that this improvement in biocompatibility seems to be contributed by the decrease in deacetylation degree of the chitosan scaffold upon rigorous heat treatment.

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