# Influence of Heat Treatment on Biological Properties of Chitosan toward Vascular Cells *in vitro*

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**Summary:** The effects of heat treatment of chitosan film on the proliferation of vascular cells were examined *in vitro*. Chitosan film was heat treated at 110 °C *in vacuo* for 1~3 days. Heat treatment slightly improved the hydrophilicity of chitosan, although the cross-linking of chitosan took place after heat treatment. Chitosan films were cytocompatible toward smooth muscle cells (SMCs) and human dermal microvascular endothelial cells (HDMECs) before and after heat treatment of chitosan. In particular, the heat treated chitosan supported the proliferation of both vascular cell types for one day. The growth of HDMECs was greater than that of SMCs on heat treated chitosan film for one day. Heat treated chitosan has intrinsic biological activity which could contribute to a retardation of SMC growth relative to HDMECs.

**Keywords:** biocompatibility; chitosan; endothelial cells; heat treatment; smooth muscle cells

## Introduction

Tissue engineering has accelerated the demand for materials which are tissue compatible, biodegradable, and with mechanical properties closely matched to the target tissues.[1-4] The wide array of tissue engineering applications intensifies the need for biodegradable materials with broad potential.

Chitosan, the partially deacetylated derivative of chitin, may be one such material. Chitosan could be used to present antithrombogenic properties,[5] to stimulate the immune system of the host against viral and bacterial infections,[6] to possess wound-healing properties and favor both soft and hard tissue regeneration.[7,8] In normal conditions of use, clinical tests carried out in order to promote chitosan-based biomaterials do not report any

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inflammatory or allergic reaction following implantation, injection, topical application or ingestion in the human body.[9,10] Its biodegradation leads to the release of aminosugars which can be incorporated into glycosaminoglycan and glycoprotein metabolic pathways, or excreted.[10]

Proper strength in cell adhesion to the scaffold for tissue engineering seems to be required for suitable cell migration and proliferation. It is well known that fibroblasts strongly adhere to the chitosan film[11] and chitosan inhibits cell proliferation although it is not cytotoxic.[12,13] 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, because fibroblasts are recognized to be critical for the wound healing process: they follow inflammatory cells into sites of tissue injury and contribute to wound healing through the synthesis of structural proteins.

However, we previously showed that rigorous dry heat treatment of chitosan changes its biocompatibility in initial cell binding capacity and cell growth rate of human dermal fibroblasts (HDFs).[14] HDFs attached onto the heat treated chitosan film exhibited a more flattened and genuine fibroblastic morphology, while HDFs on the untreated chitosan film exhibited a round morphology. Rigorous heat treatment stimulated the cell growth of HDFs on the heat treated film by 1.5~1.95 fold as opposed to the untreated film. Since heat treated chitosan appears to be a good candidate for soft tissue regeneration, it seemed worthy to investigate possible interactions between chitosan and vascular cells, such as endothelial cells, smooth muscle cells, and fibroblasts.

In this study, as an attempt to investigate the biocompatibility of chitosan for an effective small-diameter vascular graft, we investigated the effect of duration of heat treatment on the biological properties of chitosan films, and evaluated their cell binding capacity and cell proliferation based on an MTT assay towards vascular cell types.

## Experimental

**Materials**. Chitosan from crab shell, whose degree of deacetylation is 85 %, based on amino content, was purchased from Korea Chitosan, Ltd. (Seoul, Korea). 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). Unless otherwise noted, other reagents were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO).

**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 coating cover glass with chitosan solution as follows. A cover glass (12 mm φ) was placed on the bottom of each 24-well culture plate, followed by the covering of each well with 50 μL of sterile chitosan solution put on the cover glass. The solution was then evaporated to dryness at room temperature in a 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 a pre-cooled condition was poured into flat bottomed molds (99 $cm^2$), then quickly frozen at -80℃ for 24 hours, and lyophilized at -5℃ under a vacuum of 0.2 torr for 24 hours.

Crude chitosonium acetate films and scaffolds were heated in an oven at 110℃ *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 a neutral pH.

**Acetylated chitosan film and scaffold**. To each crude chitosonium acetate film and scaffold in deionized water was added acetic anhydride (1.5 mol/D-glucosamine), and the mixture was allowed to stand at room temperature overnight. The scaffolds and films were neutralized with 0.1 *N* NaOH in 70% ethanol and washed with deionized water until the filtrate reached a neutral pH.

**Characterization of physicochemical properties**. The structural changes in chitosan derivatives were confirmed with a Fourier transform infrared (FT-IR) spectrometer system. FT-IR spectra of chitosan derivatives were measured through a Nicolet 5DX FT-IR spectrophotometer using KBr pellets. The degree of deacetylation of the chitosan scaffold was determined by colloid titration [15] using poly(vinyl potassium sulfate).

**Surface characterization**. Static water contact angles were measured at 25℃ and 60 % relative humidity by the sessile drop method, using a 3 μL water droplet in a telescopic goniometer (model: G1, ERMA Inc.). The telescope, with a magnification power of 23x, was equipped with a protractor of 1 graduation. For each angle reported, at least five measurements on different surface locations were averaged.

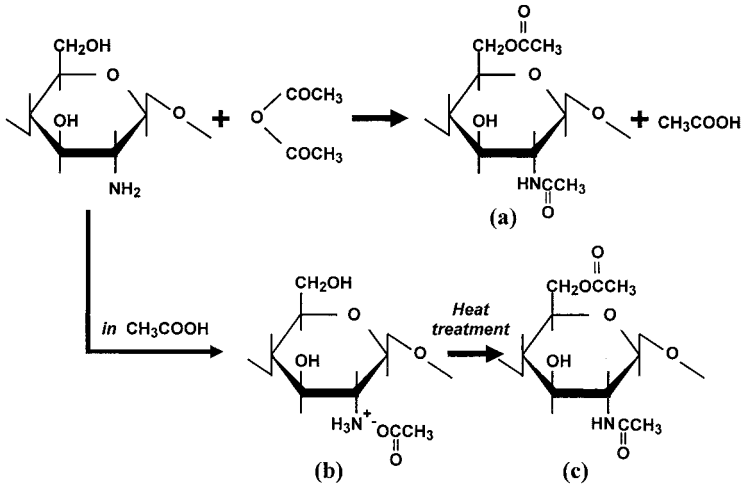


Figure 1. The reaction scheme of the acetylation and heat treatment of chitosan; (a) acetylated chitosan, (b) chitosonium acetate, and (c) heat treated chitosan.

**Isolation of vascular cells 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 in this study. 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.

We have isolated microvascular endothelial cells (HDMECs) from newborn foreskin based on the endothelial cell specific expression of CD105. Subconfluent primary cultures, a mixture of endothelial cells and fibroblasts, were trypsinized and HDMECs were isolated by their selective binding to magnetic beads coupled with an anti-CD105 monoclonal antibody. HDMECs at passage 2 and 6 were used in all experiments in this study.

SMCs were isolated from pig aortas using an adaptation of a published technique.[16,17] In brief, the descending aortas of pigs, after the removal of the endothelium, adventitia, fat, and connective tissue, were cut into multiple small pieces and incubated for 90 min at 37°C in a sterile spinner flask containing an enzymatic dissociation buffer. This buffer contained 0.125 mg/mL elastase, 1.0 mg/mL collagenase (CLS type I, 204 units/mg, Worthington Biochemical Corp., Freehold, NJ, USA), 0.250 mg/mL soybean trypsin inhibitor (type 1-S), and 2.0 mg/mL crystallized bovine serum albumin (BSA, Gibco/Life Technologies, Gaithersburg, MD, USA). The resultant tissue suspension was filtered

through a 100-mm Nitex filter (Tetko, Inc., Briarcliff Manor, NY, USA) and centrifuged at 200 g for 5 min. The pellet was re-suspended in a 199 growth medium (Gibco) supplemented with 20% FBS, 2 mM L-glutamine (Gibco), 100 units/mL penicillin, and 0.1 mg/mL streptomycin. SMCs were maintained in a growth medium containing 20% FBS until the first passage, while all subsequent cultures were grown in the presence of 10% FBS. SMCs between passages 6 and 12 were used in all experiments in this study. The cells were maintained at 37°C in a humidified 5% $CO_2$ atmosphere with fresh medium that was changed every day.

**MTT assay**. HDFs, SMCs, and HDMECs were seeded onto the films within 3 $cm^2$ metallic rings at a density of 3~4 × $10^4$ cells/$cm^2$. Cell growth on each surface was observed under a phase contrast microscope (DMIL; Leica, Wetzlar, Germany). MTT assay of the HDF on the chitosan film was performed on the 3 day culture according to the modified MTT colorimetric assay originally described by Mosmann.[18] For MTT assay, 1 mL of the MTT solution was added to each well and incubated for 4 hours at 37 °C. Cells were washed with phosphate and shaken for 30 minutes in order to release cells in scaffolds. For colorimetric analysis, 100 μL of supernatant solution was allocated 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.

Table 1. The effect of heat treatment on the surface characteristics of chitosan.

| Samples | Heat treated time (days) | Deacetylation degrees of chitosan scaffolds | Water contact angles of chitosan films |
|---|---|---|---|
| Chitosan | 0 | 82.5 % | 68.0 ± 1.1 ° |
| | 1 | 45.0 % | 62.9 ±1.0 ° |
| | 3 | 28.7 % | 62.1 ±0.6 ° |
| Acetylated chitosan | - | - | 54.2 ±1.2 ° |

## Results and Discussion

**Characteristics of the heat treated chitosan films and scaffold**. We measured the deacetylation degree of the heat treated scaffold based on the colloid titration assay with PVSK (Table 1). Rigorous heat treatment at 110°C under a vacuum condition is sufficient

enough for chemical modification of the chitosan scaffold leading to a decrease in the deacetylation degree of the chitosan, which was dependent on the duration of the heat treatment. Our rigorous heat treatment can be safely applied for the processing of the chitosan scaffold, without gross morphological changes in the integrity of the scaffold (data not shown). The resulting heat treated films and scaffolds did not dissolve either in dilute acetic acid or in water.

To evaluate whether or not rigorous heat treatment caused changes in the surface characteristics of chitosan scaffolds, we measured the contact angle of the 3-day heat treated films and the untreated chitosan films, instead of the chitosan scaffolds. Table 1 also shows the change of the contact angle of the 1-day and 3-day heat treated films, the untreated chitosan film, and the chemically acetylated chitosan film. Measurement of the contact angles on the chitosan surfaces modified either physically or chemically, gives an indication of the relative hydrophilicity of these surfaces before and after modification. The contact angles on the surfaces of unmodified chitosan and modified chitosan films changed accordingly. The chitosan film chemically acetylated with acetic anhydride

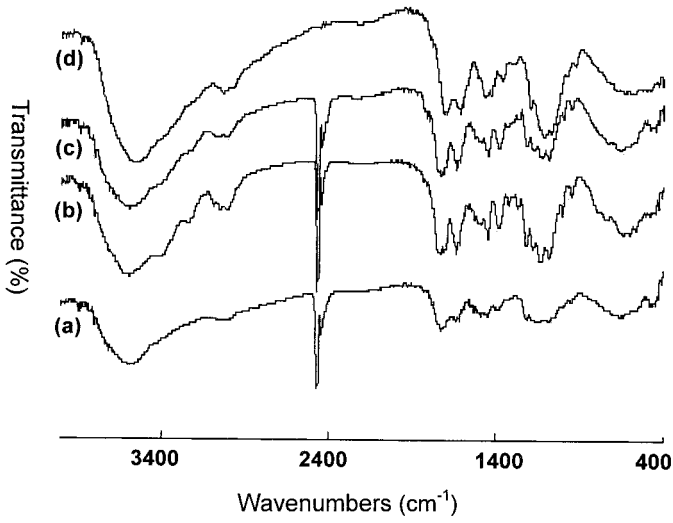


Figure 2. FT-IR spectrum of scaffolds; (a) chitosan, (b) chitin, (c) acetylated chitosan, and (d) 3-day heat treated chitosan.

demonstrated a rather lower contact angle than that found on unmodified chitosan film, implying an improvement of the hydrophilicity. For the physically heat treated chitosan film, the water contact angle slightly decreased before modification. This indicated that while the surface hydrophilicity was greatly enhanced by chemically acetylation of chitosan, the surface hydrophilicity of the physically modified chitosan was little enhanced compared with that of untreated chitosan. These results show that the heat treated films are expected to have improved cytocompatibility, because of having a proper contact angle for water (around 60°) to initiate cell attachment on the substrate.

To evaluate whether rigorous heat treatment caused changes in the chemical properties of chitosan scaffolds or not, we analyzed FT-IR spectra of the 3-day heat treated and untreated chitosan scaffolds (Figure 2). The heat treated chitosan scaffold (d) showed a different FT-IR spectrum compared to the chemically acetylated chitosan scaffold (c), and chitin powder as a control (b). After heat treatment of chitosan, there was a new, very small shoulder at 1730 $cm^{-1}$, and the peak intensities at 1550 $cm^{-1}$, and 1420 $cm^{-1}$, get stronger than that of the untreated group. The increase of the peak intensities at 1550 $cm^{-1}$ due to amide II suggest that the amide bond between chitosan and acetic acid may be formed, and new carboxyl groups at 1730 $cm^{-1}$ may be formed due to the acetylation on hydroxyl groups in the C-6 residue of the carbohydrate unit. The peak due to the secondary amine group at 1420 $cm^{-1}$ in Fig. 2 (d), gets stronger than that of the untreated group and the chemically acetylated chitosan. There seems to be an inter-chain cross-link between hydroxyl groups in the C-6 residue of the carbohydrate unit and the free amine of the un-reacted chitosan.

It has been reported that exposure to high temperatures can change the properties of chitosan and it is expected that rigorous heat treatment may lead to a cross-link of chitosonium acetate, the ionic complex between chitosan and acetic acid.[20,21] In particular, Kumbar et al.[19] suggested that the cross-linking of chitosan took place in the free amine group by heat treatment of chitosan. Our result was in accordance with the report of Ritthidej et al.[20], excepting their result of non-solubility in aqueous dilute acid. These results suggest that not only may the amide bond between chitosan and acetic acid be formed, but new carboxyl groups may be formed due to the acetylation between carboxyl groups in the chitosonium acetate complex as a reaction intermediate (Fig 1 (a)). Therefore we concluded that there were amide formations between chitosan and acetic acid, and an inter-chain cross-link formation after heat treatment.

**Evaluation of the biocompatibility of the rigorously heat treated chitosan films.** We examined whether heat treatment changed the properties of vascular cell attachment and growth on the chitosan film, and the morphological change of HDMECs that adhered onto each film. Fig. 3 shows light microscopy photographs of HDMECs on chitosan films after 1 day and 3 days of incubation. The HDMECs attached well to all the test surfaces without significant differences and their morphologies were normal.

On day 3, an MTT-test was performed to quantify the cell viability. The results, which were derived from the average of three independent experiments, were shown in Figure 4. At day 1, heat treated chitosan film was observed that retained a 45 % deacetylation degree, and a 152 % and 164 % higher HDFs and HDMECs, respectively, growth than the untreated film. This improved cell growth on heat treated chitosan films varied according to the cell types after day 1 of heat treatment time. HDFs showed a gradual decrease in their cell growth with the increase in the duration of heat treatment. At day 3,

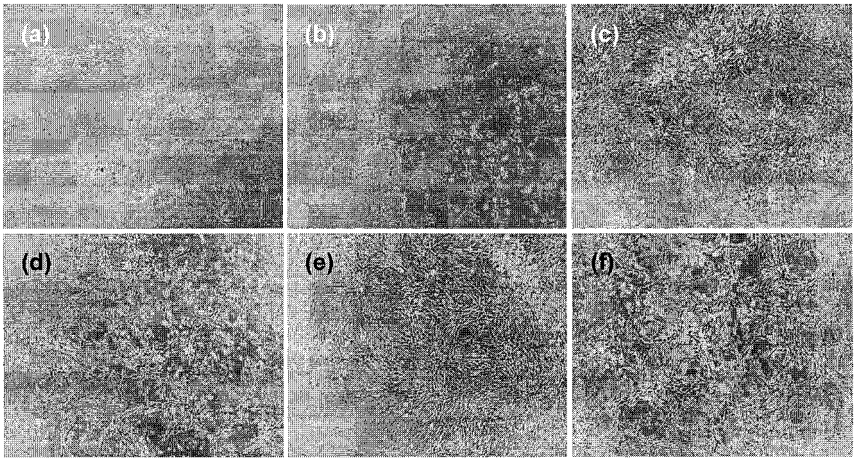


Figure 3. Phase contrast morphology of HDMECs on the 1-day and 3-day heat treated films or on the untreated chitosan films; (a) chitosan film at day 1, (b) 1-day heat treated chitosan film at day 1, (c) 3-day heat treated chitosan film at day 1, (d) chitosan film at day 3, (e) 1-day heat treated chitosan film at day 3, (f) 3-day heat treated chitosan film at day 3.

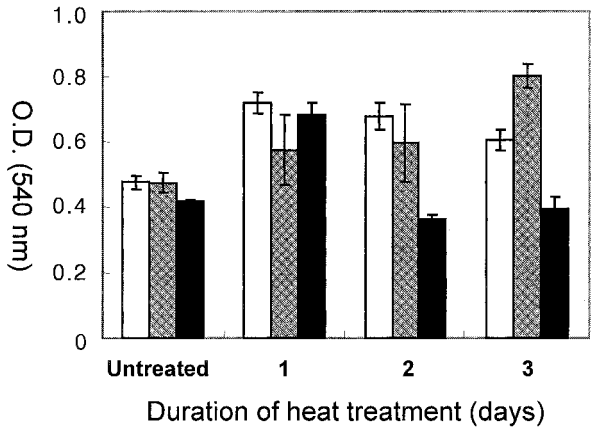


Figure 4. Effect of heat treatment time on cytocompatibility of the chitosan on HDF (white), SMC (dotted), and HDMEC (black). HDFs, SMC, and HDMEC were seeded onto the films at a density of $3\times 10^4$ cells/cm$^2$, $4\times 10^4$ cells/cm$^2$, $3\times 10^4$ cells/cm$^2$, respectively. MTT assay was performed at day 3 culture. Each column represents the mean value ±SD (n=3).

heat treated chitosan film that retained a 28.7 % deacetylation degree and a 127 % higher HDFs growth than the untreated film was observed. On the contrary, SMCs showed gradual increase in their cell growth with increase in the duration of heat treatment.

Interestingly, HDMECs were different from the above cells. HDMECs growths significantly decreased with an increase in heat treatment time after an initial significant increment (163% higher) for 1 day of heat treatment time. Although we observed a slight difference in surface hydrophilicity after heat treatment on chitosan, there was a significant difference in cell growth between vascular cells. They seem to need a proper number of amine groups in order to grow HDMECs on a substrate, but not hydrophilicity. The amine groups might first adhere some protein, such as serum or cytokines, onto a substrate that induces the adhesion of endothelial cells. Mori et al.[26] reported that there were no different cell growth rates between chitin, chitosan and its chemical derivatives on the proliferation of cultured endothelial cells. However, we observed that the results for the viability of growth of endothelial cells on the heat treated chitosan film was 163% higher compared to the un-treated film. Further exploration of the mechanisms that result in different cell growth rates between those two are worth investigating.

Incomplete endothelialization and SMCs hyperplasia are two major problems contributing to the poor performance of existing small-diameter vascular grafts.[23-25] Complete

endothelialization specifically prevents the migration of SMCs to the graft lumen, and restrains SMCs hyperplasia. Improvements in long-term performance of these devices might be attained through the use of structural materials with specific biological activities. Chupa et al.[22] reported that chitosan supported proliferation of SMCs and vascular endothelial cells, but also retarded SMCs growth to a greater extent than endothelial growth. Our results showed that 1-day heat treated chitosan supported the proliferation of both vascular cells types; the growth of HDMECs was far greater than that of SMCs on 1-day heat treated chitosan film. This result suggests that the scaffold material may have intrinsic biological activity that could contribute to a retardation of SMCs growth relative to endothelial cells.

In summary, our results suggest that modification of physicochemical properties of the chitosan attributed by a 1-day rigorous heat treatment probably provides a better environment for the cell attachment and selective growth of vascular cells. The heat treated chitosan have significant potential for a tissue engineered small-diameter vascular graft, which can modulate the activities of vascular cells.

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