Preparation of Acetylated Chitosan Sponges (Chitin Sponges)

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ABSTRACT: Acetylated chitosan sponges (chitin sponges) were prepared according to acetylation time (25, 50, 75, and 100 h). As the acetylation time increased, the degree of acetylation increased, and a 75-h acetylation time produced the highest degree of acetylation (DA). The surface morphologies of samples were examined by scanning electron microscopy. Sponge samples were shown by a water uptake ability test to have higher water absorption abilities. An *in vitro* biodegradation test showed that sponges with a higher DA were more susceptible to lysozyme hydrolysis. Acetylated chitosan sponges were further shown by an *in vitro* fibroblast proliferation test to have a higher degree of cell viability on increasing the DA, with 75 h exhibiting the maximum effect. The results showed that the wound healing effect of chitosan sponges can be controlled by the DA. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 117: 1618–1623, 2010

Key words: chitosan; chitin; degree of acetylation; sponge; wound healing

INTRODUCTION

Wound dressing, defined as restoration of the continuity of living tissue, is an integrated response of several cell types to injury.[1] An ideal wound dressing should protect from bacterial infection, provide moisture, adequate healing environment, and be biocompatible.[2–4] Recently, there has been substantial interest in the use of sponges as a wound healing material within the pharmaceutical, biomedical, and tissue engineering industries. In particular, sponges based on polysaccharides such as alginate, chitin, and chitosan are commonly studied because of their low toxicity, favorable mechanical properties, and capacity for bioresorption of constituent materials.[5]

Chitin is one of the most abundant organic materials in nature and can be easily prepared from the shells of crab, shrimp, and squid pens. Chitin and its derivatives possess a high degree of availability, biodegradability, and biocompatibility and therefore are widely applied, such as in cosmetics, food and health supplements, agriculture, and the biotechnology and pharmaceutical industries.[6] Chitin's monomeric unit, *N*-acetylglucosamine is present in hyaluronic acid, a compound important in wound healing.[7] Chitin may possess tissue cell growth functions, acting as a favorable scaffold for cell attachment and proliferation. This promotes rapid dermal regeneration and accelerated wound.[8]

Many types of chitin-based materials have been adapted to applications related to wound dressing.[6,9–11] However, these applications are limited because of the low solubility of chitin in most common organic solvents.[9] Concentrated acids are known to be the most effective solvents for chitin but lead to side effects such as chain hydrolysis, removal of residual solvents, and excess toxicity. Therefore, the chemical structure of chitin has been modified to overcome this undesirable characteristic,[6,9,12,13] including in this study in which a chitin sponge is prepared using chitosan.

Chitosan (β-1,4-linked-2-amino-2-deoxy-D-glucopyranose) is a polysaccharide synthesized from deacetylated chitin and holds considerable promise in the field of biomedical research. It possesses a high degree of biodegradability, biocompatibility, and nontoxicity, allowing its widespread application in wound healing.[8] Many research have been performed regarding chitosan sponges; however, exactly how the DA of chitosan or chitin is related to wound healing is unclear. In addition, few studies can be found regarding chitin sponges synthesized by the acetylation of chitosan. In this study, chitosan sponges (chitin sponges) acetylated for various lengths of time were prepared and assessed for their ability to accelerate wound healing using a fibroblast cell culture.

Jung A Ko and Bum Keun Kim contributed equally to this work.

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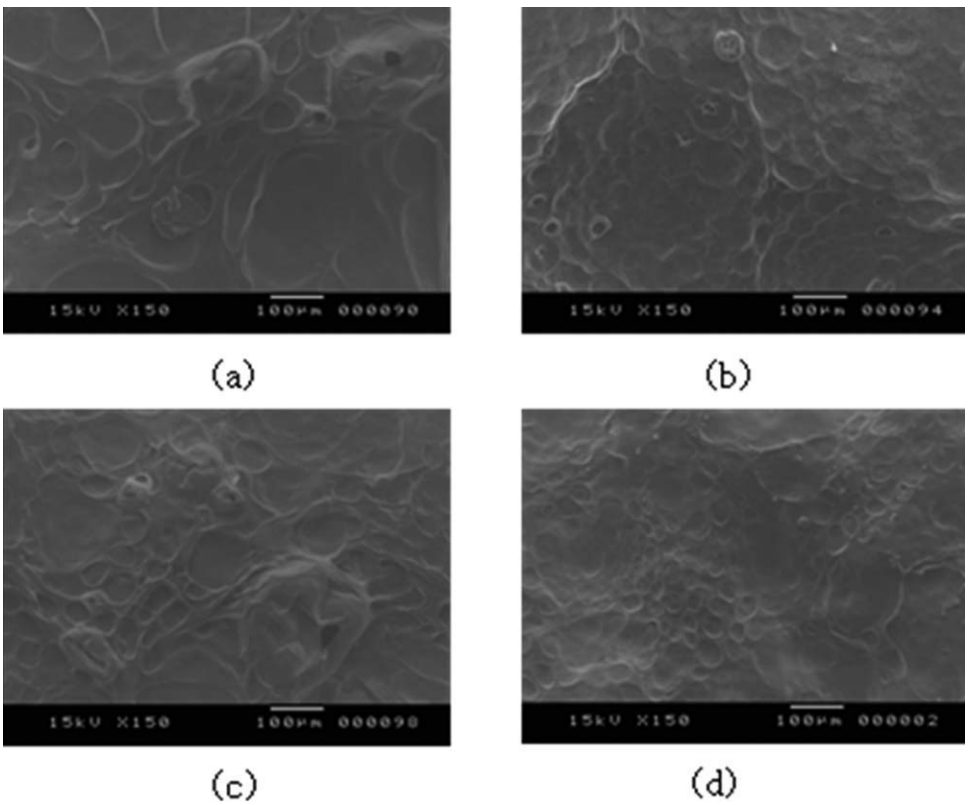

Figure 1 Surface morphologies of acetylated chitosan sponges according to acetylation times of (a) 25 h, (b) 50 h, (c) 75 h, and (d) 100 h.

MATERIALS AND METHODS

Materials

Chitosan (degree of acetylation, DA; 22%) was provided by Biotech Co., Ltd. (Mokpo, Korea). Acetic anhydride and lactic acid were purchased from Duksan pure Chemical Co., Ltd. (Ansan, Korea). Tween 20 was purchased from Showa Co., Ltd. (Totyo, Japan). HeLa cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (HyClone Laboratories, Logan, UT), 2 m*M* L-glutamine, 50 U·mL penicillin and 50 μg/mL streptomycin.

Preparation of acetylated chitosan sponges (chitin sponges)

A 3% chitosan solution containing 1% lactic acid was prepared. The volumetric ratios of chitosan solution:acetic anhydride : ethanol : Tween 20 were 20 : 10 : 3 : 1. Tween20 was used to improve the accessibility of reaction of acetic anhydride with chitosan. The solution was mixed in a falcon tube and vortexed for 30 s, followed by pouring into a Petri dish. The ready hydrogels were then dipped into acetic anhydride solution. The reaction was stopped after various lengths of time (25, 50, 75, and 100 h) by 5% NaOH solution. To remove any remaining acetic anhydride, hydrogels were dipped into 95% EtOH solution for 1 d and then washed with distilled water and freeze dried.

Determination of degree of deacetylation by ^{13}C NMR

The ^{13}C CP/MAS NMR spectrum was recorded at 100.62 MHz by a Bruker MSL 400P spectrometer with a spinning rate of 5 kHz. The deconvoluted intensities (areas) of the CH_3 resonance, along with all the chitosan peaks in the spectrum of the mixture, were obtained with a contact time of 1 ms. The results allowed the amount of the carbon observed in the spectrum to be estimated, which was then compared with the chemical analysis data.

The DA of the samples was determined according to Raymond et al.[14] from ^{13}C CP/MAS NMR spectra acquired (contact time of 1 ms and relaxation delay 5 s). Chemical shifts are quoted in ppm from tetramethylsilane (TMS).

Scanning electron microscopy

The surface morphology of freeze-dried sponges was determined by scanning electron microscopy (SEM; JSM-5310LV Scanning Microscope, Tokyo, Japan) at 25 kV. Samples were mounted on an

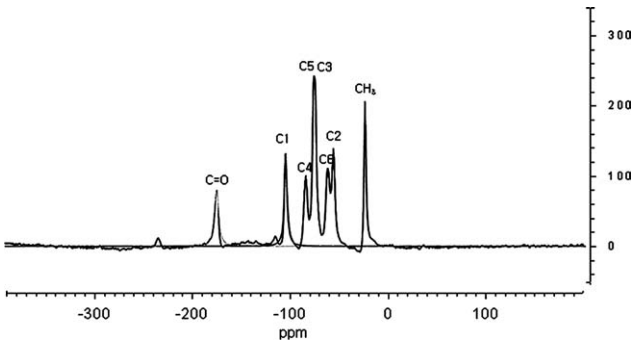

Figure 2 Solid state ^{13}C NMR spectra of acetylated chitosan sponges (chitin sponge).

aluminum mount and sputtered with gold palladium and coated with gold.

In vitro water absorption test

The water absorption capacities of sponges were determined by swelling the samples in phosphate-buffered saline (PBS, pH 7.4) at room temperature. The weighed sample was placed in media for the required period of time, after which the percentage water absorption of the samples was calculated by the following formula:

$$E_{sw} = [(W_e - W_o)/W_o]$$

Where E_{sw} is the swelling ratio (g water/g sample), W_e is the weight of the samples at equilibrium water absorption, and W_o is the initial weight of the samples. Each swelling experiment was repeated three times, and the average value was taken as the percentage water absorption.

In vitro biodegradation test

Studies on the biodegradation of the samples were conducted *in vitro* by incubating the samples in PBS (pH 7.4) with 350 μg/mL of egg white lysozyme kept at 37°C. After various lengths of time (25, 50, 75, and 100 h), the samples were taken out of the incubation medium, washed with distilled water, dried, and weighed. The extent of degradation was expressed as the percentage dry weight of the mass remaining versus the initial weight. The acquired data were expressed as the mean ± standard deviation ($n = 3$).

In vitro cell culture

For cell culture test, specimens were glued to the center of a 60-mm dish. After incubation in Dulbecco's modified eagle medium (DMEM) for 5 d, the samples were removed, and Hela fibroblasts were seeded evenly at a frequency of 5–6 × 10^4 cells per well. The cell culture was maintained in a humidified incubator at 37°C with 5% CO_2. After 2 days of culture, the media were removed, and cells of each sample were photographed using an inverted light microscope.

To count the number of cells, cell samples were diluted in Trypan Blue (gibco BRL) dye exclusion medium and were carefully and continuously filled the hemocytometer chamber. Cells were left undisturbed for 1–2 min at room temperature and then counted under the microscope.

TABLE I
Determination of the DA Value According to Acetylation Time by ^{13}C NMR

Acetylation Time (h)	Degree of Acetylation (%)
25	82
50	88
75	92
100	86

RESULTS AND DISCUSSION

Preparation of acetylated chitosan sponges (chitin sponges)

Figure 1 shows the surface morphologies of the acetylated chitosan sponges based on varying acetylation times. The results show that the surface of the sponges becomes more dense as the acetylation time increases. These results reveal that the surface morphology of acetylated chitosan sponges can be modified by altering the DA. This might be due to structural adjustment between acetyl groups and other regions of the chitosan.

Determination of DA

The DA of chitosan is one of the critical factors in its wound healing activity. Among the techniques already used to determine the DA of chitin and chitosan, ^{13}C NMR spectroscopy is one of the most powerful, allowing a direct determination of the DA of both soluble and nonsoluble samples, and sensitive.[15] Therefore, ^{13}C NMR was used to measure the

TABLE II
Water Absorption Abilities of Acetylated Chitosan Sponges According to Acetylation Times of (A) 25 h, (B) 50 h, (C) 75 h, and (D) 100 h

Sample	Water Absorption (g water/g sample)
A	13.6 ± 1.3
B	13.4 ± 0.7
C	10.6 ± 3.9
D	13.2 ± 0.4

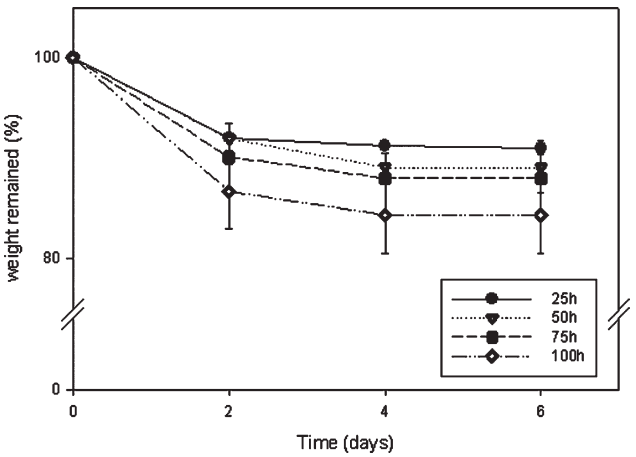

Figure 3 *In vitro* biodegradation of acetylated chitosan sponges according to acetylation time in PBS lysozyme solution.

exact value of DA. According to Figure 2, peaks describe the methyl-C atom of the *N*-acetyl group, carbon atoms of the D-glucopyranosyl ring, and C=O signal. The DA was calculated from the integral of methyl carbon divided by the summation integrals of carbon atoms of the D-glucopyranosyl ring (C1–C6 atoms), and the values are shown in Table I. The results show that the longer the acetylation time, the higher the DA, except at 100 h, at which the DA values decreased.

Water absorption ability

The water absorption ability of various acetylated chitosan sponges were examined by water absorption test in phosphate-buffer saline (PBS) solution. Table II shows the equilibrium water absorption

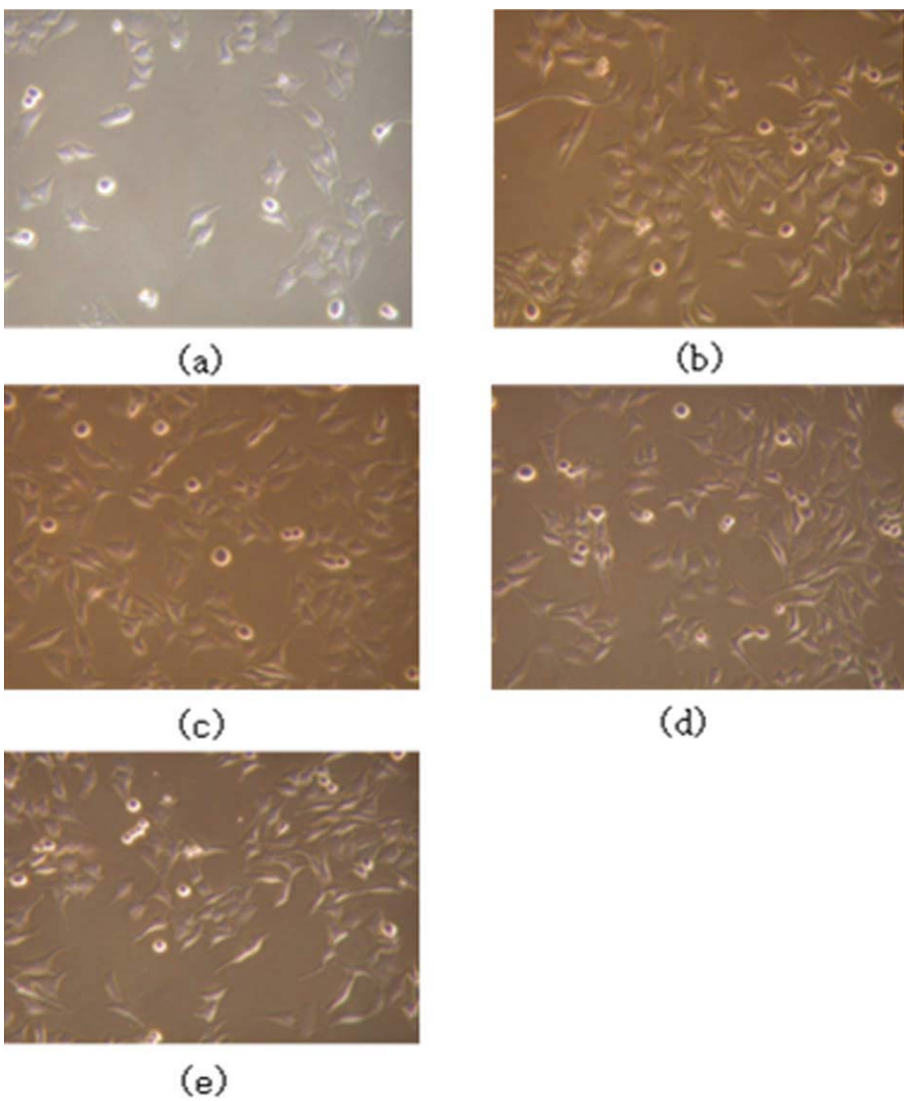

Figure 4 Photographs of HeLa fibroblasts cultured in wells without (a) or with acetylated chitosan sponges according to acetyaltion times of (b) 25 h, (c) 50 h, (d) 75 h, and (e) 100 h; circle one means dead cell and rod-like one means viable cell. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

behavior of acetylated chitosan sponges. The acetylation time of chitosan sponges had no significant effect on the water absorption ability. However, the samples generally had higher water absorption abilities, implying that they were adequate for absorption of exudates. Chitosan sponges subjected to 75 h of acetylation time had the highest DA but lowest water absorption ability. Tomihata and Ikada[16] showed the water content of films of chitin and its deacetylated derivatives. They found that deacetylation leads to increased water content of film because of a higher number of amine groups, which are more hydrophilic than acetamide. These results reveal that the DA of chitosan sponges could potentially affect the accumulation of body fluid on the wound area by controlling the absorption of exudates.

In vitro biodegradation test

Biodegradability is one of the most important requirements of wound healing agents. In general, polysaccharides such as chitin and chitosan are degraded by enzymatic hydrolysis. They exhibit no appreciable degradation when brought into contact with aqueous, neutral media containing no enzymes at room temperature.[16] The *in vitro* degradation of acetylated chitosan sponges with 350 ug/mL lysozyme is shown in Figure 3. Samples subjected to acetylation for 25 h were degraded the least, and degradation increased as acetylation time increased.

The higher the DA, the more susceptible it is to lysozyme hydrolysis. Lysozyme hydrolyzes the β-1,4 glycosidic bonds between *N*-acetyl-D-glucosamine and D-glucosamine in chitosan. Therefore, the lack of *N*-acetylglucosamine sequences crucial as a substrate to be recognized by lysozyme is responsible for the slow degradation of chitosan with low DA. Cho et al.[9] showed that water soluble chitin had higher susceptibility to lysozyme than chitosan because of its higher DA. Tomihata and Ikada[16] also showed that chitin film was degraded rapidly *in vitro* compared with other deacetylated derivatives. Although the sample acetylated for 100 h had a lower DA than that of the 75 h acetylated sample, it showed the highest sensitivity to lysozyme, indicating another dominant factor for enzymatic degradation in addition to the DA.

In vitro cell culture

The results of cell growth influenced by different acetylation times are shown in Figures 4 and 5. The acetylated chitosan sponge had more profound effects on fibroblast proliferation compared with that of chitosan (without acetylated), and the extent of cellular proliferation implied that the chitosan sponge promoted cellular adaptability. Taken together, cell viability increases as the DA is increased.

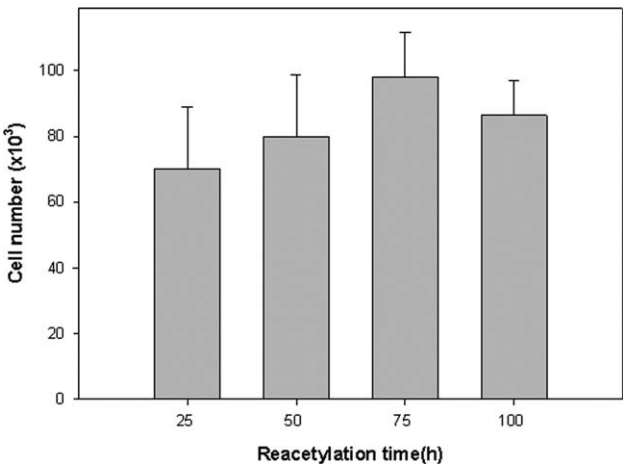

Figure 5 Counting of viable HeLa fibroblast cells according to acetylation time.

As seen in Table I, the sponges acetylated for 75 h showed the highest DA and the highest cell viability. These results show that the wound healing effect of chitin sponges can be controlled by the DA.

CONCLUSION

In this research, acetylated chitosan sponges were prepared according to acetylation time for the improvement of wound dressing. The exact of DA was measured by ^{13}C NMR. As the DA was increased, the surface of the sponges became denser. These results reveal that the surface morphology of acetylated chitosan sponges can be modified by altering the DA. The samples showed higher water absorption abilities, implying that all samples were adequate for absorption of exudates. Furthermore, the biodegradation of acetylated chitosan sponges increased along with acetylation time. Acetylated chitosan sponges had positive effects on fibroblast proliferation, and the chitosan sponges acetylated for 75 h exhibited the maximum effect. These results show that the wound healing effect of chitosan can be controlled by the DA.

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