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The controlling biodegradation of chitosan fibers by N-acetylation in vitro and in vivo

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Abstract
Aim In the present study, we investigated the biodegradation of the fibers of chitosan and its acetylated derivatives in vitro and in vivo.
Methods A series of chitosan fibers, with acetylation degrees of 7.7%, 21.6%, 40.9%, 61.2%, 82.5% and 93.4%, were obtained by acetylating chitosan filament with acetic anhydride, and were investigated by FT-IR analysis, elemental analysis and scanning electron microscopy analysis.
Results The in vitro experimental data indicated that the degradation rate of chitosan fiber was strongly dependent on the degree of acetylation, and the degradation rate increased with an enhancement of the acetylation degree of chitosan fibers. In vivo degradation experiment evaluated by light microscopy as well as scanning electron microscopy, was studied by implanting the fibers between the two nerve stumps of the rat sciatic nerve gap (6 months). The findings demonstrated that acetylation degree could influence the degradation rate of chitosan fibers in vivo.
Conclusion These results suggested that acetylated chitosan (chitin) fibers were more biodegradable than chitosan and the biodegradation rate of chitin fiber can be controlled to desirable extent by the variation of acetylation degree.

Introduction

Recently, much attention has been paid to chitin and chitosan as a functional polymer because it has several distinctive biomedical properties such as no nontoxicity, biocompatibility and biodegradability. Chitosan is derived from chitin by deacetylation in the presence of alkali and is composed primarily of glucosamine unit usually with content exceeding 85% [1, 2]. In fact, chitin and chitosan cannot be regarded as a homopolymer. Chitin contains of 2-amino-2-deoxy-D-glucose units usually below 5–10%. However chitosan contain of 2-acetamino-2-deoxy-D-glucose units usually about 5–15% [3]. The chitin sutures resist attack in bile, urine and pancreatic juice, which are problem areas with other absorbable sutures. It has been claimed that wound dressings, made of chitin and chitosan fibers, accelerate the healing of wounds by up to 75% [4–6]. The main advantage of biodegradable materials is the disappearance of these materials from the body as a result of their biodegradation after being implanted in human body. So many kinds of biodegradable materials that possess a variety of biodegradation rate to match the rate of tissue regeneration were required [7–9]. However the natural-based materials, chitosan and chitin, have not gained wide usage in tissue engineering, at least in part, because their biodegradation properties can not be easily tailored to the specific application by proper choice of the chemistry composite.

An investigation of the degradation rate of biomaterials is thus important for their practical application. We have investigated the interaction of Schwann cells with chitosan fibers in vitro [4]. In the present study, we prepared a series of acetylated chitosan fiber by acetylating chitosan filaments to various extents with acetic anhydride of methanol solution, and investigated the biodegradation of chitosan fiber and its acetylated derivatives in vitro and in vivo. It is therefore desirable to develop and produce a chitosan and chitin fiber which having controlling biodegradation rate for using in tissue engineering.

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Materials and methods

Chemicals and reagents

Chitosan was obtained from Nantong Xincheng Biochemical Company (Jiangsu, China). Its degree of deacetylation was 92.3% and its average-molecular weight (M_w) was 2.8×10^4 by measuring viscosity [10]. Egg-white lysozyme (Product Number L-6876: 50,000 units/mg) was purchased from Sigma Chemical Co. (USA). All the other chemicals used in this study were of research purity grade.

Preparation of chitosan fibers

Chitosan fibers were prepared by wet-spinning procedure. The dope was prepared by dissolving 40 g chitosan in 1000 mL of 2% (w/w) aqueous acetic acid solution. A laboratory scale extrusion unit, comprised of a reservoir, a metering pump (2.4 cm^3/rev), and a spinneret (500 holes, 80 μm diameter), was used. By applying nitrogen of pressure 7×10^5 Pa, the polymer was passed through a constant volume metering pump, and then to a stainless steel spinneret, which was immersed in a coagulation bath containing a solution of 7% NaOH and 10% Na_2SO_4. After exiting the coagulation bath, the fiber was advanced into a 1 m-long bath pool containing hot water. The take-up rollers, drawing system, drying rollers and the winding up procedure were as described elsewhere [11]. The filaments were washed and dried by radiant heat.

Acetylation of chitosan fibers

Seven portions (0.2 g) of the chitosan filament were immersed in 150 mL 5% (w/w) acetic anhydride of methanol solution. The acetylation treatment was carried out at 25 °C. The degree of acetylation of chitosan was controlled by the varied reaction time. After 10 min, 20 min, 30 min, 40 min, 60 min, 90 min, 120 min, the filaments were taken out and rinsed with distilled water to remove exceeding anhydride solution respectively. The acetylated chitosan fibers were treated with 10 mL 1 mol/L aqueous NaOH solution overnight to remove O-acetylation. Finally, the fibers were washed with distilled water and dried. The acetylation degree was calculated from the N/C ratio, as obtained by elemental analysis.

In vitro degradation

The filament of chitosan and its different acetylated derivatives of a known weight (0.1 g) were immersed in 2 mL of 100 μmol/L sodium phosphate buffer (pH 7.4) containing lysozyme (4 mg/mL) at 37 °C. The lysozyme solution was replaced with fresh enzyme solution everyday. After determined intervals of time the fibers were taken out from the lysozyme solution, rinsed with distilled water, dried under vacuum and weighed. The degradation rate of samples was then expressed as the percentage (W/W) of degraded fraction, as calculated with the following formula:

$$\eta\ (\%) = (W_1 - W_2)/W_1 \times 100$$

where η was weight percentage of degraded fraction (%); W_1 was the original weight of fibers (g); and W_2 was the weight of fibers after degradation (g). Three samples were determined at very time point. So the values are denoted as means ±SD for three determinations.

In vivo degradation test

For in vivo degradation examination, chitosan fibers of three acetylation degrees, namely 7.7%, 61.2% and 93.4%, were used. Fibers were cut into 10-mm-long segments, formed a bundle (about 1000 fibers), irradiated using 20 kGy $_{60}$Co and soaked in sterile saline 30 min prior to use.

Eighteen male sprague-Dawley (SD) rats, weighing 180–200 g, were used for testing biodegradability of chitosan fibers. Animals were from the Experimental Animal Center of Nantong University, and all animal tests were carried out in accordance to the "NIH Guidelines for the Care and Use of Laboratory Animals". The rats were randomly divided into three groups, i.e. a Ch-7.7 group, a Ch-61.2 group and a Ch-93.4 group, for implanting the three kinds of fibers mentioned above, respectively. Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The left sciatic nerve was exposed under aseptic condition, and an 8-mm segment was excised at the center of the thigh. The bundle of chitosan fibers was interposed between the two nerve stumps, with its two ends fastened to the epineuria of nerve stumps using 9–0 nylon sutures. Incisions were closed using 4–0 sutures. Animals were routinely housed following surgical procedures.

All rats were sacrificed 6 months postoperatively. After being anesthetized by an overdose of sodium pentobarbital, the implanted bundle of fibers including its surrounding tissue was dissected out and fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer, washed in water, dehydrated in a series of ethanol and embedded in paraffin. Additionally, for a comparison of the three kinds of fibers before implantation, chitosan fibers of each acetylation degree, that were formed in bundles and not implanted, were embedded in paraffin following a similar procedure as mentioned above. Transverse sections (5 μm) of the mid-bundle were prepared and stained with hematoxilin and eosin for microscopic observation.

Results and discussion

Wet-spinning procedure and acetylation of chitosan fibers

The experiment showed that the chitosan solution was easy to spin through the aforementioned wet spinning system, and good quality fibers were obtained as early reported [12, 13]. The yarn count of resultant fibers by above method was 1.86 dtex; its fiber tenacity was 1.9 cN/dtex and it had an elongation at break of 10.3%.

The degree of acetylation of chitosan fiber was affected by the acetylation condition such as reaction time. So we got a series of fibers with acetylation degrees of 7.7%, 21.6%, 40.9%, 61.2%, 82.5% and 93.4% by acetylating chitosan filament with acetic anhydride.

FT-IR spectra of the chitosan and chitin fiber

Figure 1 shows FT-IR spectra of chitosan fiber and chitin (degree of acetylation: 93.4%) fiber respectively. In Fig. 1a, the characteristic absorptions were displayed at 1658.3 cm^{-1} and 1595.3 cm^{-1} attributable to amide bands 1and 2, which indicated the amine groups of chitosan [14]. In Fig. 2b, amide bands 1and 2 were at 1659.6 cm^{-1} and 1624.1 cm^{-1}, while the C=O absorptions of N-acetyl groups appeared at 1559.9 cm^{-1}. These data indicated that the chitosan fiber has been acetylated.

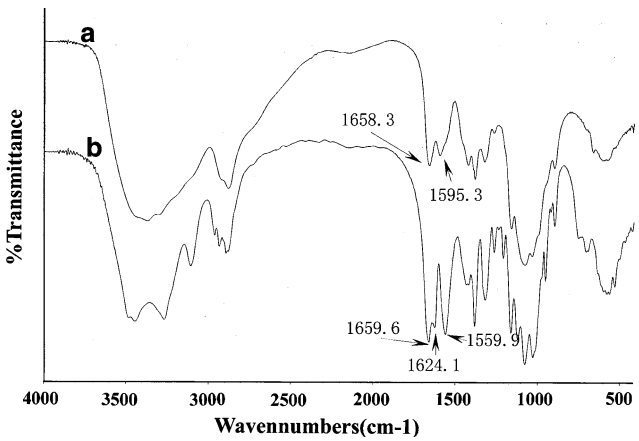

Fig. 1 FT-IR spectra of (**a**) chitosan fiber (**b**) acetylated chitosan fiber (its degree of acetylation was 93.4)

Scanning electron microscopic morphology

The microstructure of a fiber has a prominent influence on its degradation rate, cell proliferation, function, and migration in tissue engineering. To observe and confirm the microstructure, the chiotsan fiber (ch-7.7) and chitin fiber (ch-93.4) prepared by acetylation was investigated by scanning electron microscopic (SEM) (Fig. 2). According to the SEM micrographs, the surfaces of chitosan fiber and chitin fiber all exhibited smoothness and no difference could be discerned between them and less shrinkage was observed in chitin fiber. It indicated that the acetyl reaction did not change the fiber's microstructure and surface morphology.

In vitro degradation

The chitosan fibers and some acetylated chitosan fibers with different degree of acetylation were degraded by lysozyme solution. The degree of acetylation of original chitosan fiber were 7.7%, 21.6%, 40.9%, 61.2%, 82.5% and 93.4% respectively. Accordingly these fibers were named Ch-7.7, Ch-21.6, Ch-40.9, Ch-61.2, Ch-82.5 and Ch-93.4, respectively. Figure 3 showed the degradation of these fibers in vitro. The data demonstrated that chitosan is practically not biodegradable in marked contrast to its acetylated derivatives, and that its acetylated derivatives were more degradable than chitosan. After 5 days, the degradable percentage of Ch-7.7, Ch-21.6, Ch-40.9, Ch-61.2 and Ch-82.5 were 2.9%, 28.9%, 36.4%, 40.2% and 60.2% respectively, while the Ch-93.4 was completely degradable. The degradation of chitosan and its acetylated derivatives means that they were completely degradated into their aminoglucose unit or may be degradated into

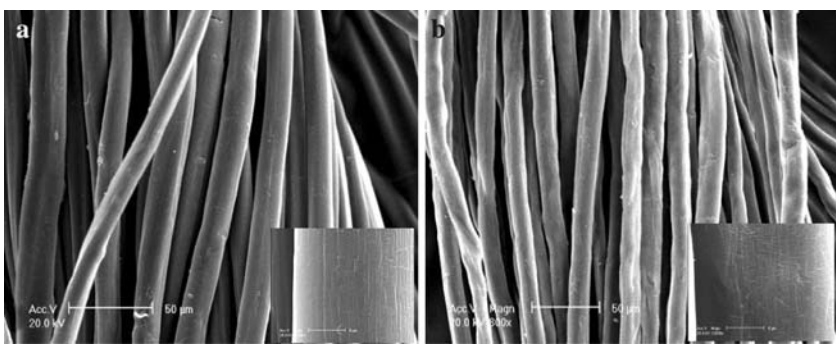

Fig. 2 SEM photos of Ch-7.7 fiber (**a**) and Ch-93.4 fiber (**b**) Scale bar = 50 μm, 5 μm

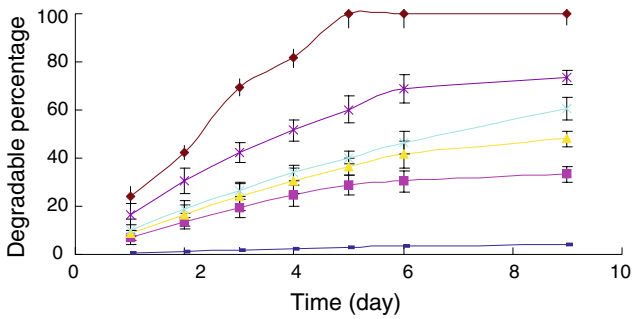

Fig. 3 In vitro degradation of fiber of chitosan and its acetylated derivatives in 4 mg/mL lysozyme in 100 μmol/L sodium phosphate buffer (pH 7.4) at 37 °C. The values are means ± SD of three determinations. ▬, Ch-7.7 (chitosan); ■, Ch-21.6; ▲, Ch-40.9; ×,Ch-61.2; *, Ch-82.5 and ●, Ch-93.4

chitosan oligosaccharides or chitin oligosaccharides with variable length, which were all water-soluble [15]. The experimental data clearly suggested that the degradation rate of chitosan fiber was strongly dependent on the degree of acetylation, and that the degradation rate increased with an enhancement of the acetylation degree of chitosan fibers. So the degradation rate of chitosan fiber can be controlled by adjusting degree of acetylation.

In vivo degradation

The implantated chitosan fibers with the acetylation degrees 7.7%, 61.2% and 93.4% demonstrated no obvious difference both macroscopically and microscopically. Macroscopically, the Ch-7.7 chitosan fiber bundle was embedded in a thin capsule of connective tissue with internal fibers being visible and stiff in nature, while the Ch-93.4 group showed no apparently visible chitosan fibers but a rod of muscle-like tissue between the nerve stumps. Furthermore, the tissue rod was rather soft and indistinguishable from the surrounding tissue. The Ch-61.2 group, however, was observed to be at an intermediary level with less visible fibers than the Ch-7.7 group. Obvious differences were also found microscopically. The Ch-7.7 chitosan fiber was stained red by eosin and dense in structure (Fig. 4a, b), whereas the Ch-61.2 chitosan fiber showed a different appearance, being semitransparent and less dense (Fig. 4c, d). However, the Ch-93.4 fiber became not only semitransparent but also loose with a sponge-like structure (Fig. 4e, f). Moreover, the Ch-93.4 fibers were smaller in diameter and the interface of the fiber and surrounding tissue tended to be unclear. These results demonstrated that the Ch-93.4 chitosan fiber could be degraded most in vivo

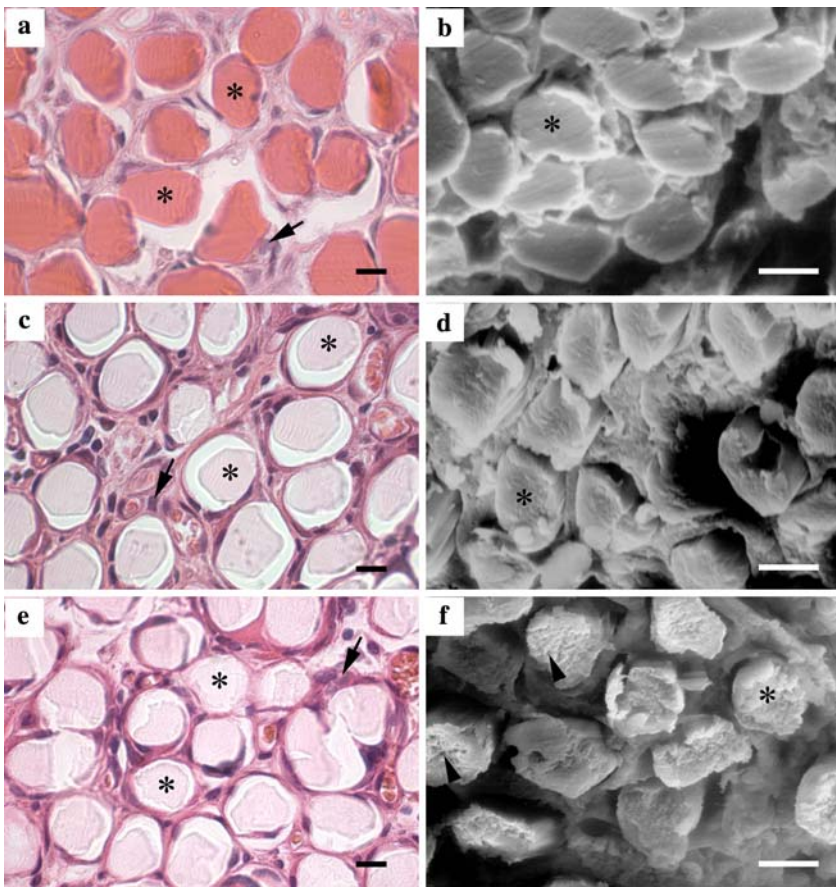

Fig. 4 Micrographs showing transverse sections of mid-implant of chitosan fibers stained by hematoxilin and eosin (HE) 6 months post-implantation. (**a**, **c, e**) Representative micrographs of the Ch-7.7, Ch-61.2 and Ch-93.4 fibers, respective. (**b**, **d**, **f**) are magnification of boxed areas in **a**, **c** and **e**, respective. Asterisks indicated Chitosan fibers. Arrows in **e** and **f** marked the indistinguishable interfaces between fibers and surrounding tissue. Scale bar = 10 μm

among the three groups, while the Ch-7.7 chitosan fiber least. This indicates that the acetylation degree does influence the in vivo degradation of chitosan fibers, and higher acetylation degree predetermines higher degradation rate. On the other hand, macrophage-like cells distributed extensively among chitosan fibers in any of the three groups, this indicates that macrophages may play a role in degradation of chitosan fibers since some biomaterial particulates are taken up by macrophages via phagocytosis [16].

Conclusion

Our experimental result in vitro and in vivo revealed that acetylated chitosan fiber is more biodegradable than chitosan. It is possibly because that N-acetylated chitosan is mainly depolymerized enzymatically by lysozyme, and not by other enzymes or other depolymerization mechanisms. The enzyme biodegrades the polysaccharide by hydrolyzing the glycosidic bonds. In addition, the lysozyme, released from phagocytic cells including macrophaged and neutrophils, will be available for the degradation of chitin and chitosan. [17]. These phenomena proposed that the biodegradability of chitosan fiber should be very slow, which arose a problem on the possibility of long reservation in the body. Our experimental results clearly suggested that this drawback of chitosan fiber can be solved by its chemical derivatives such as N-acetylation and the biodegradation rate of chitosan fiber can be controlled to some extent by the variation in the degree of acetylation.

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