Development of the chitosan tube prepared from crab tendon for nerve regeneration

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Development of the chitosan tube prepared from crab tendon for nerve regeneration

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Abstract

Crystalline chitosan was successfully prepared from crab tendons with aligned chitin molecules. Chitosan obtained preserved the tube structure with a suitable size for nerve conduits (t-chitosan tube). The chitosan tubes were compounded with hydroxy apatite (HAp) using an alternate soaking method (t-chitosan/HAp tube) to enhance the strength of the tube walls. HAp crystals formed in the walls of the chitosan tubes, and their c-axis aligned well in parallel with the chitosan molecules. The growth of the HAp crystals was found to occur at the nucleation sites, most probably by forming complexes with amino groups on chitosan mediated by Ca ions. Furthermore, these tubes were treated at 120 °C to prevent from swelling. These treatments preserved well the hollow nature of the tubes. And the mechanical test showed that the force was significantly higher in the t-chitosan/HAp tube when compared to the circular or triangular t-chitosan tube at each strain.

Bridge grafting (15 mm) into the sciatic nerve of SD rats was carried out using the t-chitosan tubes having either a circular or triangular cross-section as well as t-chitosan/HAp tubes (N = 18 in each group). Specimens were taken after two, four, six and eight weeks for histology (N = 3 in each group). And nerve regeneration was evaluated histologically after 12 weeks (N = 6 in each group). The t-chitosan/HAp tubes, demonstrated preferable biodegradation and biocompatibility. In addition to the mechanical properties of the tubes, the results of histological findings suggest that a triangular shape of the tube’s cross-section and HAp coating may benefit nerve regeneration.

Keywords: Crab tendon; Chitosan; Alignment; Apatite; Nerve regeneration; Tendon chitosan tube; Hydroxy-apatite coating; Nerve scaffold

1. Introduction

Crustacean’s shells consist of chitin, calcium carbonates, and proteins [1]. Chitosan is prepared by the N-deacetylation of chitin from the crustacean’s shells. The chitin component is extracted from those shells and is subsequently subject to N-deacetylation to produce chitosan. Thus derived chitosan possesses many useful biological properties such as biocompatibility, biodegradation, wound-healing and anti-bacterial action [2–6]. Therefore, much attention has been paid to develop chitosan-based biomedical materials. Although chitosan, one of the natural polymers, can be used in various shapes, the medical application is limited due to its low mechanical strength [5–9]. HAp was formed on the chitosan tubes using an alternate soaking method [10–12] to enhance the strength of the tube walls. Because chitosan usually turns less soluble in an aqueous solution by heating [13–15], i.e. strengthening the tube walls by reducing swelling in vivo, the tubes were treated at 120 °C. Furthermore, the shape of the tubes was molded to triangular shape to prevent collapse after implantation.

In this study, the microstructure, mechanical property, and biocompatibility of the novel chitosan/HAp tube having a triangular-shape were evaluated. In addition, efficacy as a nerve conduit of these chitosan tubes prepared was examined.
2. Materials and methods

2.1. Preparation of tendon chitosan

The tendon of 15–20 cm in length was taken from the crab, Macrocheira Kaempferi that was selected because of its comparatively large size. The crab’s tendons were washed with distilled water, and treated in a 4 wt% NaOH aqueous solution at 100 °C for 4 h to eliminate included proteins. Subsequently, the samples were treated in ethanol of 95 wt% at 95 °C for 8 h. The samples obtained are referred ‘tendon chitin’. Furthermore, the samples were deacetylated with a 50 wt% NaOH aqueous solution at 100 °C for 8 h in nitrogen atmosphere. This deacetylation process was repeated three times to ensure complete deacetylation. The samples obtained were rinsed repeatedly with distilled water to remove any excess NaOH. The final samples are named tendon chitosan (t-chitosan). Although the crab tendon is a flat shape, the tendon chitosan has a slightly-flattened hollow-tube structure with a diameter in the order of 1–5 mm.

2.2. HAp formation in/on the chitosan tubes

HAp binding to t-chitosan was performed by an alternate soaking method [10–12] as follows. Step 1: t-chitosan was soaked in 50 ml of 200 mM CaCl2 in Tris–HCl (pH 7.4) for 15 min. Step 2: It was subsequently soaked in 50 ml of 120 mM Na2HPO4 for 15 min. These two steps were repeated 15 times, where t-chitosan was rinsed in distilled water to remove any excess NaOH. The final samples are named tendon chitosan/HAp. Although the t-chitosan tubes were treated 15 cycles, the amounts of HAp increased to 57.1 ± 0.2 wt%.

2.3. Preparation of the triangular tubes for in vivo experiments

A stainless bar having an equilateral triangular-shaped cross section with each side 2.1 mm long was inserted into the t-chitosan/HAp tubes. Then the tubes were thermally annealed in an air-circulating oven at 120 °C for 24 h. As these tubes shrunk to the shape of the stainless bar by establishing hydrogen bonds between chitosan molecules through this annealing, the tubes permanently formed a triangular shape. These tubes were then soaked in phosphate buffer solution (PBS), and sterilized by autoclaving at 120 °C for 20 min (Fig. 1).

2.4. Characterization methods

The composition of organic and inorganic compounds in the tendon chitosan was determined by thermogravimetric analysis (TGA) and differential thermal analysis (DTA) (TG8120, Rigaku Co., Japan). Samples were cut and dried in vacuum, placed in a platinum cup, the weight was adjusted to 5 ± 0.2 mg, and Al2O3 powder was placed in the other cup serving as a reference. The measurements were carried out in air between 25 and 1200 °C at a heating rate of 20 °C/min.

The crystal structure was determined with X-ray diffraction (XRD) (PW1700, Philips Ltd, USA) using Cu Kα radiation generated at 40 kV and 50 mA; the range of diffraction angle 2θ was 5–40°. The molecular alignment in the samples was observed with a polarized optical microscope (POM) (Labophot-pol, Nikon Co., Japan) with crossed nicols by rotating the sample stage. Fourier-transformed infrared diffuse reflectance spectra (FT-IR) (Spectrum 2000, Perkin–Elmer Co., USA) were determined under nitrogen atmosphere, after samples were dried in vacuum and mixed with a potassium bromide (KBr)
Compressive strength testing of tendon chitosan tubes was carried out on a TM TA-XT2i (Eko Co., Tokyo, Japan) at a cross-head speed of 0.1 mm/s. Ten pieces of wet samples of circular or triangular t-chitosan tube, or triangular t-chitosan/HAp tube were prepared by sprinkling water splay for test. The applied force at each transformation ratio of the samples corresponded to a strain measured ranging from 0.01 to 0.4. The variances among these groups were evaluated by the Bartlett test and differences by means of one-way analysis of variance (one-way ANOVA). Thereafter, statistical significances were evaluated according to the multiple comparison Scheffé’s $F$ test. The level of significance was $p < 0.05$.

The tubes were cut into blocks perpendicular or parallel to the longitudinal axis of tendons (1 $\times$ 2 $\times$ 2 mM$^3$ in size), and embedded in epoxy resin. The microstructure of t-chitosan/HAp was observed by transmission electron microscopy (TEM, JEM-2000EXII, JEOL Co., Japan) and an electron diffraction technique with 100 kV accelerated electron voltage.

Expansion ratios were measured as follow. The t-chitosan and t-chitosan/HAp were dried up in an air-circulating oven at 80–160°C for 1 h, and the expansion ratios of the samples were determined in PBS (pH 7.4) at 25°C. Where the percentage is calculated by the formula of (sample width/t-chitosan or t-chitosan/HAp width before drying) $\times$ 100. The changes in length and width were measured at 0.5–48 h after immersion in PBS.

2.5. Implantation of a chitosan tube into a nerve defect

Male Sprague–Dawley (SD) rats weighing 180 g were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The right sciatic nerve was exposed and a section of 10 mm in length excised at the center of the thigh. Bridge grafting of 15 mm length into the nerve gap was then carried out using 8-0 monofilament nylon to connect the chitosan tube surface and nerve ends. T-chitosan tubes, respectively, with a circular or triangular cross-section, as well as t-chitosan/HAp was used for implantation. Each experimental group consisted of 18 rats.

Three rats in each group were sacrificed after, respectively, two, four, six and eight weeks of implantation by an intraperitoneal injection with a high dose of sodium pentobarbital (250 mg/kg body weight). Specimens were taken from the middle 1/3 part of the grafted tube in each group for a transverse section. After being fixed in 2.5 vol% glutaraldehyde and subsequently in 2.0 vol% osmium tetroxide, the specimens were embedded in Epon 812 resin. Thin sections were prepared and stained with toluidine blue for use in light microscopy (LM, BH-2, Olympus Co., Tokyo, Japan). Ultrathin sections were prepared for transmission electron microscopy (TEM, Hitachi H-600; Hitachi Co., Tokyo, Japan).

In six rats from each group, specimens were harvested from the grafted tubes for histological examination, as described above. Furthermore, the nerves at 10 mm distance from the distal anastomosed site of the tube (distal nerve) were harvested and embedded in Epon 812 resin for histological analysis. Thin transverse sections of the distal nerves were stained with toluidine blue, causing the myelin of the myelinated axons to stain blue. Photographs of the whole section were taken (as described above) to determine the area of distal nerve and total area of myelinated axons including myelin. Analysis was carried out using the Scion Imaging software. The percentage axon area was calculated as total area of myelinated axons/the area of distal nerve $\times$ 100%. Differences among the experimental and control groups were determined and evaluated for statistical significance according to the multiple comparison Fisher’s protected least significant difference procedure (level of significance was $p < 0.05$).

3. Results and discussion

3.1. Composition of tendon chitosan

The TG-DTA curves of the tendon chitosan showed that weight loss took place in both temperature ranges of 40–200 and 200–800°C. Correspondingly, a broad endothermic peak was observed around 80°C, assigned to the evaporation of water, while two exothermic peaks were found at 330 and around 535°C, and ascribed to the thermal decomposition of chitosan. As no further weight change was found beyond 800°C in the TGA curves, the organic component in the samples was determined from the weight loss between 200 and 800°C, and the inorganic component from the residues at 800°C. The results are shown in Table 1. The original crab tendon consists of 57.6 wt% of organic components and 42.4 wt% of inorganic components. The organic component of tendon chitin, treated with a diluted NaOH solution and ethanol, decreased to 42.1 wt%, resulting from the elimination of alkaline- and ethanol-soluble organic components like proteins and fats. The inorganic component in the tendon chitosan was

| Sample name     | Organic components$^a$ (wt%) | Inorganic components$^b$ (wt%) |
|-----------------|------------------------------|--------------------------------|
| Crab tendon     | 57.6 ± 2.4                   | 42.4 ± 2.4                     |
| Tendon chitin   | 42.1 ± 3.1                   | 57.9 ± 3.1                     |
| Tendon chitosan | 99.5 ± 0.3                   | 0.5 ± 0.3                      |

$^a$ Weight loss at 200–800°C.

$^b$ Residues at 800°C.
intensively reduced to 0.5 wt% after treating with 50 wt% of NaOH solution.

3.2. Crystalline structures of chitosan and inorganic phase

Fig. 2 shows XRD patterns of (a) crab tendon treated at 800 °C for 3 h, (b) crab tendon, (c) tendon chitin and (d) tendon chitosan. The XRD peaks of the crab tendon (Fig. 2a) have been assigned to both β-tricalcium phosphate (β-TCP, filled circles) and hydroxyapatite (HAp, filled squares). These results indicate that an inorganic component in the crab tendon is calcium phosphate. Giraudguille described that the inorganic component of crustacean’s shells is mainly calcium carbonate with calcite crystal structure [1]. In contrast, the inorganic component of the crab tendon involves calcium phosphate. Fig. 2b is the X-ray diffraction profile of the crab tendon before the heat treatment, showing two peaks, i.e. around 10 and 20°. Both peaks were ascribed to the crystalline alignment of chitin/chitosan molecules [16–18]. Calcium phosphate in the crab tendon has a very low crystallinity, as the peaks of calcium phosphate was not detected. Fig. 2c is the tendon chitin, showing peaks around 32° were ascribed to 211, 300 and 202 of HAp. Taking the results of the samples (a) and (b) into account, this finding indicates that calcium phosphate in the crab tendon changes from amorphous to crystallite HAp during the treatment in 4 wt% of NaOH aqueous solution at 100 °C, since HAp is stable in alkaline solutions and at high temperature. In the tendon chitosan (Fig. 2d), no peaks ascribed to the HAp phase were detected, but the crystalline structure of chitosan was found, being in good agreement with the results of the TG-DTA measurement. Although calcium phosphate was there when treated with 4 wt% NaOH, almost inorganic components was removed when treated with 50 wt% NaOH. The reason of this phenomenon may be that even though HAp is stable in alkaline solutions and at high temperature, it is resolved in such a high dose of alkaline solution as 50 wt% NaOH.

Fig. 3 shows the FT-IR spectra of tendon chitin (a), and tendon chitosan prepared by deacetylation repetition of three times (b) and five times (c). As for the tendon chitin (Fig. 3a), two absorption bands were observed around 1665 and 1543 cm⁻¹; they were, respectively, assigned to amide I (C=O) and amide II (–NH) groups. Regarding the tendon chitosan (Fig. 3b and c), the absorption bands of the amide I and amide II groups decreased with the repetition time of deacetylation treatment, while the peak of amino group (–NH₂, 1595 cm⁻¹) increased correspondingly. Both amide groups could not be detected at all after five times of deacetylation treatments, indicating that chitin perfectly transferred into chitosan upon the deacetylation treatments (almost 100% of deacetylation rate). Although relationship of deacetylation and bioactivity of chitosan films is discussed [8], because t-chitosan has a tubular configuration its degradation speed in vivo is not affected by deacetylation rate only. Instead, its fibrous structure is expected to enhance the absorption speed after implantation.

3.3. Molecular alignment and mechanical property

Fig. 4a shows optical microscope image of crab tendon, a fibrous structure was found parallel along the longitudinal axis of the crab tendon. Fig. 4b shows polarized image, it disappeared perfectly at 45° rotation. These results indicate that the chitin molecules were aligned well. Fig. 4c and d shows tendon chitosan, a similar phenomenon was observed. Thus the removal of protein, calcium phosphate

Fig. 3. FT-IR spectra of tendon chitin (a), tendon chitosan deacetylated three times (b), and tendon chitosan deacetylated five times (c).
and amide groups through a strong treatment in 50 wt% of NaOH solution at 100 °C had no influence on the aligned structure of chitosan molecules.

The relationship between the strain and force is shown in Fig. 5. The force is shown to be significantly higher in the t-chitosan/HAp tube group when compared to the circular or triangular t-chitosan tube group at each strain.

3.4. TEM observation of HAp formed in the t-chitosan

The TEM observation was done for both the inner and outer surface of the t-chitosan/HAp tube and shown in Fig. 6. Fig. 6a is a cut section perpendicular to the longitudinal axis of the tendon. In t-chitosan were formed HAp crystals, which were scattered widely in the t-chitosan tube walls. Fig. 6b is a cut section parallel to the longitudinal axis of the tendon together with an electron diffraction pattern. A large number of aggregations of HAp crystals were found in the t-chitosan tube walls. The arrow in the TEM image shows the fiber direction of chitosan, and the HAp aggregations aligned along the chitosan fibers. These results show that HAp crystals are formed not only on both surfaces of the t-chitosan tube but inside of the t-chitosan when prepared by the technique of alternate soaking method. Because HAp formed by this method has low crystallinity, it is easily resolved to Ca\(^{2+}\) and PO\(_4^{3-}\). The inset in Fig. 6b shows an electron diffraction pattern taken from the circle area of the TEM image. The four arrows indicate two pairs of crescent-shaped diffractions, each pair is ascribed to 002 and 004 of HAp. This diffraction pattern indicates that the c-axis of HAp crystals align along the long axis of the aggregate. It is thus concluded that the c-axis of HAp crystals tend to align along the chitosan fibers. As the crescent angle is tight, it is assumed that the interaction between chitosan and HAp is strong.

Generally, chitosan is characterized by forming chitosan–metal complexes in which metal ions coordinate with amino groups of chitosan [19–23]. The structure of chitosan–Cu complexes has been suggested that the complexes have two OH\(^-\) and one −NH\(_2\) as ligands, and the fourth site could be occupied by H\(_2\)O or −OH on chitosan (C3). This structure is transformed by a change in pH and a pair of anions [20–22]. The complex formation activity of the Ca ions bound to chitosan is weaker than that of transitional metal ions at pH 7.4 [23]. Therefore, the coordination bonds of chitosan–Ca complexes are probably formed with two Cl\(^-\), one −NH\(_2\) and
one H$_2$O or one –OH on chitosan (C3) as ligands in a CaCl$_2$ solution, which follows the growth of HAp crystals from Ca to restrict a growth direction. When the c-axis of HAp is parallel to that of chitosan, a close relationship is found between the adjacent Ca ions of HAp ($d_{Ca} = 0.344 \times 3 = 1.03$ nm) and adjacent amino groups of chitosan (1.03 nm) [24]. Therefore, it is probable that several amino groups are involved in the formation of HAp crystals.

### 3.5. Expansion of t-chitosan/HAp composites

Fig. 7 shows the expansion ratios of t-chitosan and t-chitosan/HAp in the transverse direction as a function of time. Upon drying, the chitosan molecules formed inter-molecular hydrogen bonds, causing their aligned structure to shrink. When t-chitosan and t-chitosan/HAp were dried at 80–160°C for 1 h, the sizes of t-chitosan and t-chitosan/HAp shrunk to about 50 and 60%, respectively. This difference in the constriction ratios was probably resulted from filling up of the inner space of t-chitosan with HAp in the case of t-chitosan/HAp. When immersed further in PBS, these samples reversibly expanded in the transverse direction without a clear change in the longitudinal direction. The expansion in the transverse direction was clearly observed within four hours after immersion in PBS but not significantly later. The size did not recover to the original one probably due to the formation of a bundle with inter-molecular hydrogen bonds of chitosan [23,24]. Since chitosan molecules form rigid chains due to intra-molecular hydrogen bonds, no changes in the length of t-chitosan were found even by a thermal or alkaline treatment in our study. From these results, we conclude that the heat treatment is effective to prevent the tubes from swelling in vivo.

### 3.6. Histological observations of the grafted tubes

Reactive cell infiltration was observed both inside and outside the t-chitosan tube 2–4 weeks after implantation (Fig. 8A). Many cracks occurred through the surface of the t-chitosan tube, with the tube wall becoming fragmented from the surface. And many macrophages were phagocytizing this debris of the tube (Fig. 8B). In the inner space of the tube abundant newly vessels were formed. This active cell infiltration gradually disappeared after six weeks, and nerve generation was observed instead. In the t-chitosan/HAp tube group reactive cell infiltration was not so prominent, and the number or the size of myelinated axons regenerating through the tube was largest after eight weeks. Due to coverage of the chitosan with HAp crystals the area where chitosan is exposed to cause inflammatory reaction decreases. As a result, inflammatory cell infiltration accompanying with phagocytosis of the tube wall by macrophages may be prevented.

![Fig. 6. The TEM images of t-chitosan/HAp with diffraction patterns. (a) and (b) are images of perpendicular and parallel to the longitudinal axis of tendons, respectively.](image)

![Fig. 7. The expansion ratios as a function of soaking time. (●), (□) and (●) indicate 80, 120 and 160°C heat treatment for 1 h, respectively. The open symbols indicate the samples without apatite treatment.](image)
The percentage of axon area represents the total mass of regenerated nerve tissue, and is an indicative parameter of both the number of nerve sprouting and the degree of maturation of the regenerated nerves. The percentage of axon area in the t-chitosan/HAp tube group was found significantly higher than in the circular or triangular t-chitosan tube group (Fig. 9). These results suggest that the inner space of the t-chitosan/HAp has increased rapidly after completion of bridging of the regenerating nerve tissue, followed by nerve regeneration over the distal anastomosed site.

In addition to the mechanical properties of the tubes, the results of histological findings suggest that a triangular shape of the tube’s cross-section and HAp coating may benefit nerve regeneration. Because collapse of an unfilled circular conduit is a major block to nerve regeneration in tubulization, the property of the chitosan tube that can be molded into various configurations and compounded with HAp has effectiveness to enhance nerve regeneration.

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References

[1] M.M. Giraudguille, Fine-structure of the chitin protein system in the crab cuticle, Tissue Cell 16 (1984) 75–92.
[2] A. Chenite, C. Chaput, D. Wang, C. Combes, M.D. Buschmann, C.D. Hoemann, J.C. Leroux, B.L. Atkinson, F. Binette, A. Selmani, Novel injectable neutral solutions of chitosan form biodegradable gels in situ, Biomaterials 21 (2000) 2155–2161.
[3] B. Ouattara, R.E. Simard, G. Pette, A. Bégin, R.A. Holley, Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan, Microbiology 62 (2000) 139–148.
[4] K. Kofuji, T. Ito, Y. Murata, S. Kawashima, The controlled release of a drug from biodegradable chitosan gel beads, Chem. Pharm. Bull. 48 (2000) 579–581.
[5] M. Sato, M. Maeda, H. Kurosawa, Y. Inoue, Y. Yamauchi, H. Iwase, Reconstruction of rabbit Achilles tendon with three bioabsorbable materials: histological and biomechanical studies, J. Orthop. Sci. 5 (2000) 256–267.
[6] F.L. Mi, S.S. Shyu, Y.B. Wu, S.T. Lee, J.Y. Shyong, R.N. Huang, Fabrication and characterization of a sponge-like asymmetric chitosan membrane as a wound dressing, Biomaterials 22 (2001) 165–173.
[7] I. Yamaguchi, K. Tokuchi, H. Fukuzaki, Y. Koyama, K. Takakuda, H. Momma, J. Tanaka, Preparation and microstructure analysis of chitosan/hydroxyapatite nanocomposites, J. Biomed. Mater. Res. 55 (2001) 20–27.
[8] K. Tomihata, Y. Ikada, In vitro and in vivo degradation of films of chitin and its deacetylated derivatives, Biomaterials 18 (1997) 567–575.
[9] J.Z. Knaul, S.M. Hudson, K.A.M. Creber, Improved mechanical properties of chitosan fibers, J. Appl. Polym. Sci. 72 (1999) 1721–1732.
[10] T. Taguchi, A. Kishida, M. Akashi, Apatite formation on/in hydrogel matrices using an alternate soaking process, Chem. Lett. 8 (1998) 711–712.
[11] T. Taguchi, A. Kishida, M. Akashi, Apatite formation on/in hydrogel matrices using an alternate soaking process. II. Effect of swelling ratios of poly(vinyl alcohol) hydrogel matrices on apatite formation, J. Biomater. Sci. Polym. Ed. 10 (1999) 331–339.

[12] T. Taguchi, A. Kishida, M. Akashi, Apatite formation on/in hydrogel matrices using an alternate soaking process. III. Effect of physicochemical factors on apatite formation on/in poly(vinyl alcohol) hydrogel matrices, J. Biomater. Sci. Polym. Ed. 10 (1999) 795–804.

[13] Y.L. Lee, E. Khor, C.E. Ling, Effects of dry heat and saturated steam on the physical properties of chitosan, J. Biomed. Mater. Res. (Appl. Biomater.) 48 (1999) 111–116.

[14] L.Y. Lim, L.S.C. Wan, Treatment of chitosan films, Drug. Dev. Ind. Pharm. 21 (1995) 839–846.

[15] S.B. Rao, C.P. Sharma, Sterilization of chitosan, J. Biomater. Appl. 10 (1995) 136–143.

[16] K. Okuyama, K. Noguchi, T. Miyazawa, T. Yui, K. Ogawa, Molecular and crystal structure of hydrated chitosan, Macromolecules 30 (1997) 5849–5855.

[17] N. Cartier, A. Domard, H. Chanzy, Single crystals of chitosan, Int. J. Biol. Macromol. 12 (1990) 289–294.

[18] K. Ogawa, S. Hirano, T. Miyazaki, T. Yui, T. Watanabe, New polymorph of chitosan, Macromolecules 17 (1984) 973–975.

[19] K. Ogawa, K. Oka, X-ray study of chitosan–transition metal complexes, Chem. Mater. 5 (1993) 726–728.

[20] A. Domard, pH and c.d. measurements on a fully deacetylated chitosan: application to Cu^{II}–polymer interactions, Int. J. Biol. Macromol. 9 (1987) 98–104.

[21] K. Inoue, Y. Baba, K. Yoshizuka, Adsorption of metal ions on chitosan and crosslinked copper(II)-complexed chitosan, Bull. Chem. Soc. Jpn. 66 (1993) 2915–2921.

[22] N.C. Braier, R.A. Jishi, Density functional studies of Cu^{2+} and Ni^{2+} binding to chitosan, J. Mol. Struct. (Theochem) 499 (2000) 51–55.

[23] N. Nishi, Y. Maekita, S. Nishimura, O. Hasegawa, S. Tokura, Highly phosphorylated derivatives of chitin, partially deacetylated chitin and chitosan as new function polymers: metal binding property of the insolubilized materials, Int. J. Biol. Macromol. 9 (1987) 109–114.

[24] K. Okuyama, K. Noguchi, Y. Hanafusa, K. Osawa, K. Ogawa, Structural study of anhydrous tendon chitosan obtained via chitosan/acetic acid complex, Int. J. Biol. Macromol. 26 (1999) 285–293.