Microstructure analysis of calcium phosphate formed in tendon*

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The surface of soft tendon tissue has been modified using calcium phosphate in order for the tendon to directly connect with hard bone and reconstruct an injured ligament. Calcium phosphate was coated onto the tendon in a soaking process using alternating a $CaCl_2$ (200 mM) and a Na_2HPO_4 (120 mM) solution. According to SEM/EDX observations, calcium phosphate was formed, not only on the tendon surface, but also inside the tendon tissue. When the tendon was treated with seven soaking cycles, calcium phosphate was detected between 0–500 μm from the tendon surface. According to TEM observations, the crystal morphology of calcium phosphate depends on the distance from the surface. Hydroxyapatite crystals were observed near the surface, while octa-calcium phosphate crystals could be observed further from the surface, thus at initial soaking. The crystals were formed on collagen fibrils in spaces between the collagen fibrils with the *c*-axes of the crystals aligned parallel with the collagen fibrils. This finding suggests Ca^{2+} ions to interact with the tendon surface, most probably with the carboxyl functional groups of collagen, and subsequently forming nucleation centers for the crystals.

Introduction

In biological hard tissues, minerals are generally oriented along organic matrix materials [1–6]. For example, bone mainly consisting of hydroxyapatite (HAp) and type I collagen, has the *c*-axes of the HAp crystals orientated along the collagen fibrils [4–6], resulting in its high mechanical property.

Tendon mainly consists of type I collagen, and is highly organized with a crimp pattern [7–9]. When an anterior cruciate ligament (ACL) is injured, a popular treatment is its replacement with autograft tendon. However, the initial bonding strength between the autograft tissue and bone is not sufficient [10]. Therefore, an enhancement in the bonding strength between soft and hard tissues is necessary [8–11].

Calcium phosphate compounds such as hydroxyapatite and tri-calcium phosphate have been used as biocompatible and osteoconductive substitutes in orthopedic surgery [12, 13]. In the present paper, tendon was modified by calcium phosphate to enhance the bonding strength between the tendon and bone at the initial stage of rehabilitation training [14]. The tendon modification method was an alternate soaking in a Ca^{2+} and a PO_4^{3-} solution, in which calcium phosphate could be deposited on the tendon surface [11–13]. The microstructure of the modified tendon was observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Animal tests were carried out to show empirically the effectiveness of the tendon modification.

Materials and methods

Calcium phosphate formation in/on tendon

Flexor degitorum longus (FDL) tendons were obtained from mature female Japanese white rabbits and frozen till used. After removing adhering tissue from the tendons,

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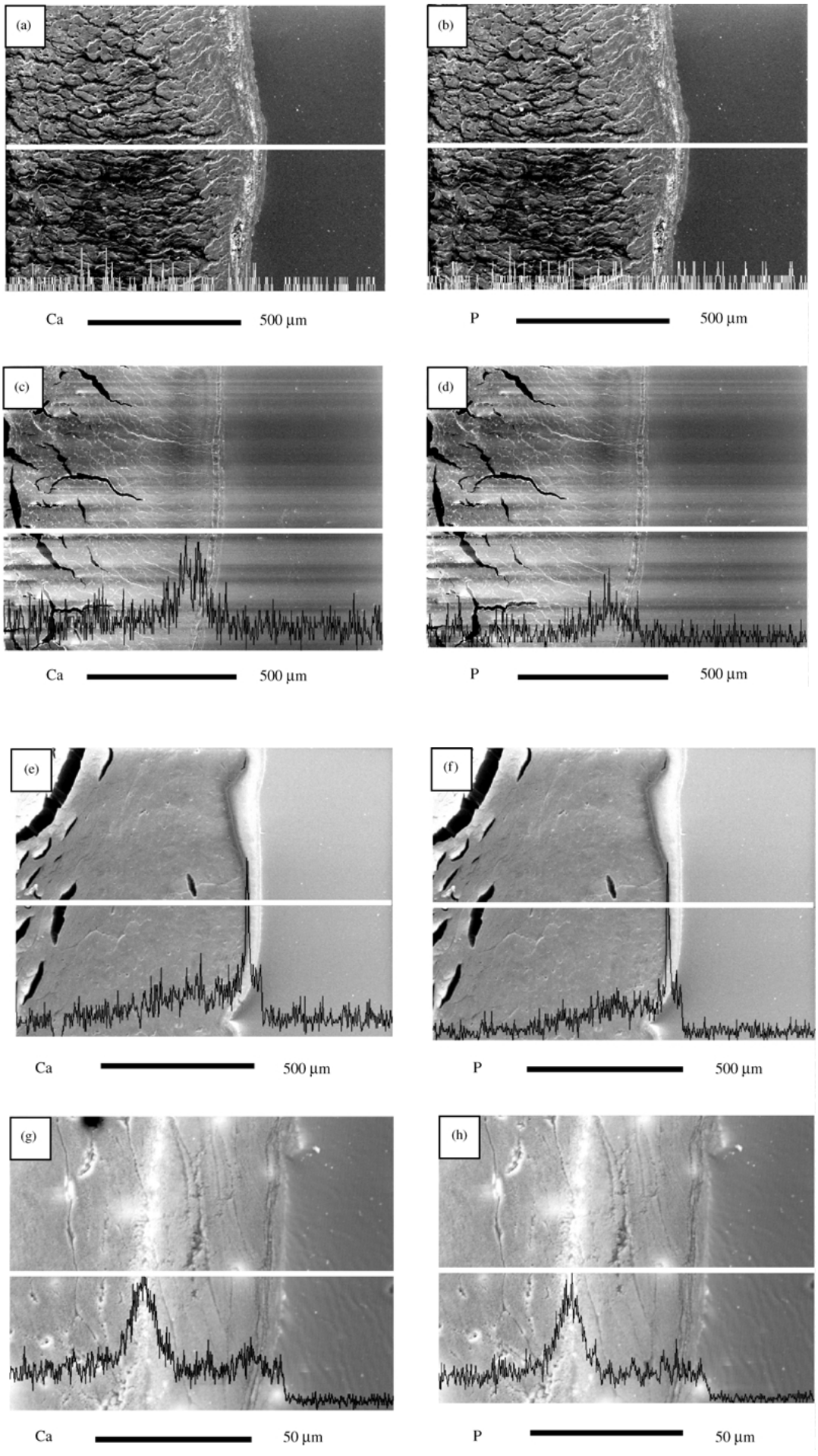

Figure 1 The SEM images of a non-treated tendon (a, b) and tendons treated by alternate soaking: the soaking cycles were respectively 2 (c, d) and 7 (e, f). The left images show the line analyses of calcium (a, c, e, g) and the right images of phosphate (b, d, f, h). The expanded images (g) and (h) correspond to (e) and (f), respectively. Calcium and phosphorus is shown to exist not only at the tendon surface, but also in the inner region.

these were cut into 20-mm-long parts. The formation of calcium phosphate in the tendon was achieved by the alternate soaking method [15–17], as follows: Step 1: tendons were soaked in 50 ml of $CaCl_2$ (200 mM) buffered with Tris–HCl at pH 7.4 for 30 min. And step 2: the tendons were subsequently soaked in 50 ml of Na_2HPO_4 (120 mM) for 30 min.

The two steps were repeated 2–7 times, with the tendons being rinsed with distilled water prior to each step.

Analysis of tendon–calcium phosphate composites

The spatial distribution of calcium phosphate formed in the tendon was analyzed using SEM (JSM5600LV, JEOL Co., Japan) attached with an energy dispersion X-ray analyzer (EDX : JED2200, JEOL). The tendon sample was embedded in an epoxy resin (Quetol 812, Nisshin EM Co., Japan), and then cut into sections perpendicular to the longitudinal axis of the tendon with a diamond cutter. The cross sections were polished with diamond paper, and tungsten was sputtered onto the surface using an ion-sputtering apparatus (ESC101, Elionix Co., Japan) to ensure electroconductivity of the sample. The contents of calcium and phosphorus in the tendon were determined with line scanning analysis.

The microstructures of the tendon–calcium phosphate composites were observed with TEM (JEM-2000EXII, JEOL) and an electron diffraction technique with an accelerated electron voltage of 100 kV. The samples used

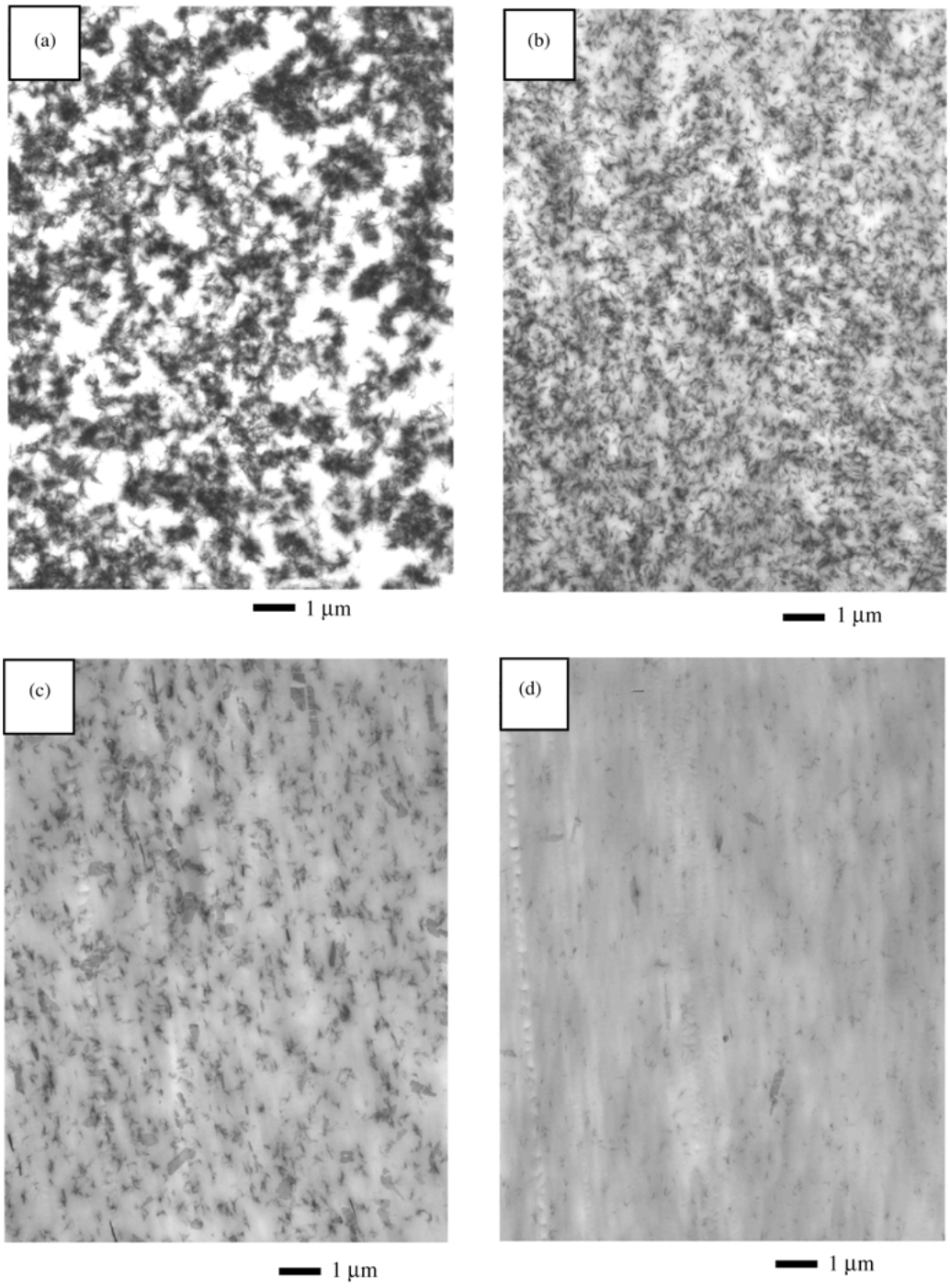

Figure 2 TEM images of tendons modified with calcium phosphate (seven soaking cycles). The images are observed parallel to the longitudinal axis of the tendon. The distance from the tendon surface increases in the order of (a)–(d). The crystal density gradually decreases with the distance from the surface.

were small sections of about $1 \times 1 \times 2\,mm^3$ and embedded in epoxy resin, which were cut into thin sections perpendicular to or parallel to the longitudinal axis of a tendon using an ultra-microtome with a diamond knife (Sumi knife 45°, Sumitomo Electric Industries).

To elucidate the relationship between calcium phosphate crystallites and collagen matrices in more detail, tendons were stained with heavy metals. The tendons were fixed at 4 °C for 2 h in a mixture solution of glutaraldehyde, acrolein and paraformaldehyde with cacodylate buffer, and subsequently embedded in epoxy resin after being soaked in a 1% osmium tetroxide solution. Thin sections were stained with a 3% lead citrate solution and a 4% uranylacetate solution for 5 min, respectively.

In vivo experiments

Beagles (weight 12–14 kg) were anesthetized using intravenous barbiturates and maintained on inhalation anesthesia (Sevofrane and oxygen). The long digital extensor (EDL) tendons were treated according to alternate soaking process with a soaking time of 3 min for each solution, and five cycles. The resulting EDL tendons were inserted into a hole (ϕ 2.6 mm) of the tibia.

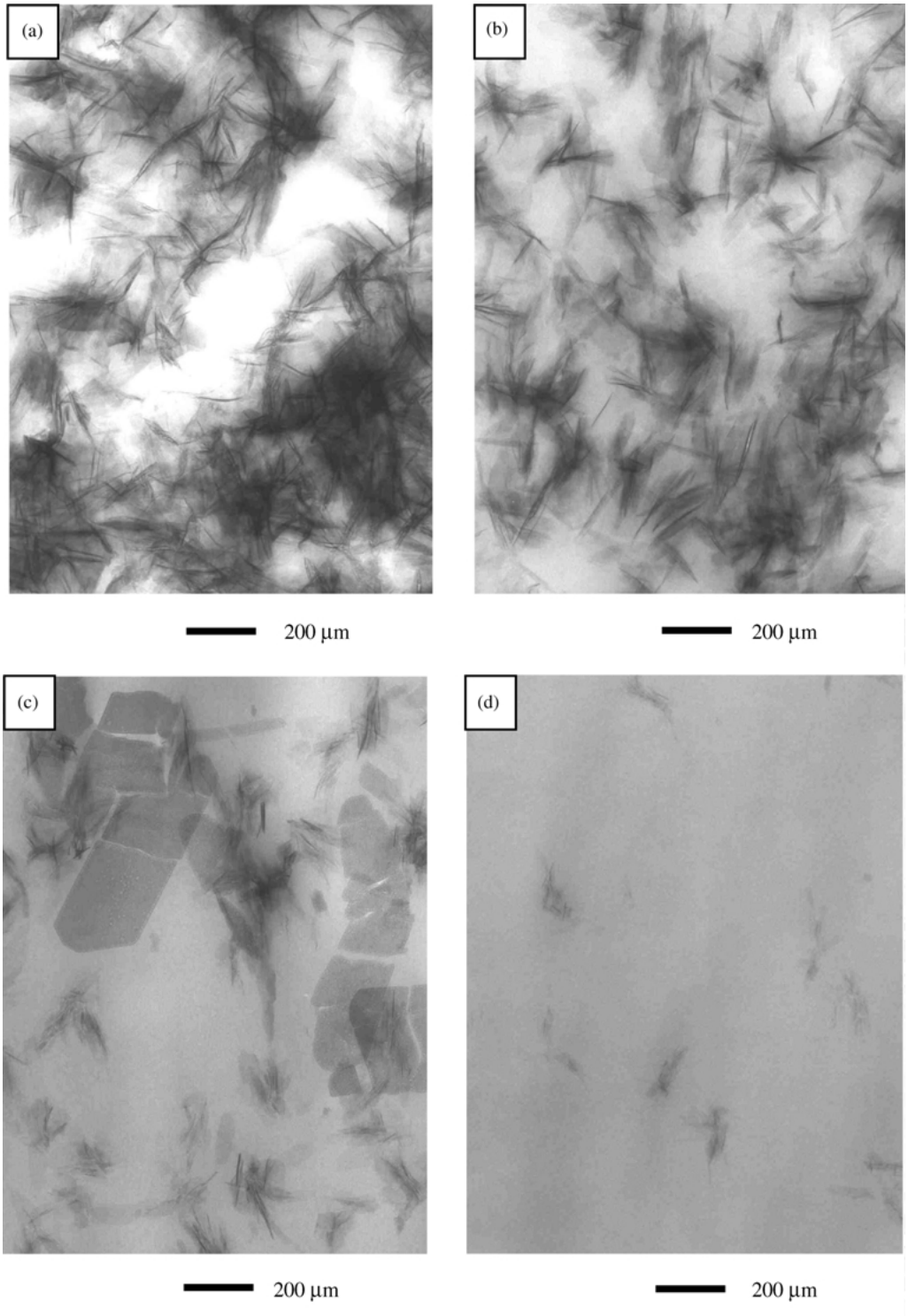

Figure 3 Extended TEM images corresponding to Fig. 2(a)–(d).

EDL tendons without calcium phosphate treatment were used as control. Four weeks after implantation, the sections were taken out and fixed in 10% formalin. The sections were then embedded in resin using standard procedures. Thin sections were subsequently prepared and stained with hematoxylin and eosin for observation under a light microscope (BX-50; Olympus, Promarketing Inc., Japan).

Results and discussion

The distribution of calcium phosphate in tendon

Fig. 1 shows SEM images of tendons including line analyses of Ca and P. Calcium and phosphorus are not visible in an untreated tendon (Fig. 1(a) and (b)), while both elements exist, not only on the surface, but also inside a treated tendon (Fig. 1(c)–(f)). The concentration of calcium and phosphorus are found relatively high near the surface of the tendon, and gradually decreases inside the tendon. The thickness of the calcium phosphate layer increases with soaking time and soaking duration, for example, about 250 μm after two soaking cycles (Fig. 1(c)–(d)) and 500 μm after 7 cycles (Fig. 1(e)–(f)). Fig. 1(g) and (h) are expanded images of Fig. 1(e) and (f), respectively. The element intensity peaks were found around 50 μm from the surface, as the calcium and phosphate ions (electrostatically) adsorbed at the surface may be partly rinsed out when washed by distilled water.

TEM observations of calcium phosphate in tendon

Fig. 2 shows TEM images of the tendon soaked seven cycles; the images have been taken parallel to the longitudinal axis of the tendon, with positions at some distance from the surface in the order (a)–(d). Calcium phosphate crystallites have also been formed in the tendon, but their density gradually decreases with distance from the surface. Fig. 3(a)–(d) are extended images of Fig. 2(a)–(d), respectively.

The morphology of the crystallites varied with distance from the surface. Near the surface (Fig. 3(a) and (b)), aggregates of small flaky crystallites are found, and each crystallite is rather thin, approximately 5 nm, as can be seen in Fig. 4(a).

At the inner region, the crystal structure was assigned to octa-calcium phosphate (OCP) as the (1 0 0) lattice fringes of the crystallite measures 1.86 nm, as shown in Fig. 4(b). The OCP is known as a pre-existing compound for the formation of biological hydroxyapatite [18–20]. Therefore, this result may suggest that the initial formation of OCP particles cause the subsequent growth of HAp crystallites, acting as the nucleation center. And subsequently, the HAp crystallite should contain the lamella particles of OCP at its center. In general, the OCP and HAp crystallites form at respectively pH = 4–6 and pH > 6. Consequently, three possible reasons exist why the OCP crystallites precipitate spontaneously although the pH of the reaction solution has been controled at pH = 7.4–9.0. First, due to the chemical reaction of Na_2HPO_4 and $CaCl_2$, HCl is produced and the pH of the inner region of the tendon becomes locally pH < 6. However, the HAp crystallites formed at the surface region, rinsed with distilled water and immersed in fresh Na_2HPO_4 (pH = 9.0) and $CaCl_2$ (pH = 7.4) solutions. The second reason is related to the isoelectric point of collagen, that is, about pH = 9. At pH < 9, collagen fibers are positively charged, and anions PO_4^{3-} are then able to pass through the tendon matrix, but cations Ca^{2+} electrostatically repulsed [21, 22]. Therefore, the concentration of PO_4^{3-} ions has been found relatively higher than Ca^{2+} ions in the tendon, resulting in the formation of OCP inside the tendon. The third reason is that various organic materials often act as inhibitor in the HAp formation [21–25]. In all cases, related to the alternate soaking method, the chemical conditions in the tendon matrix varied with the reaction process.

Fig. 5 shows the TEM image of the tendon modified and stained with the direction of view perpendicular to

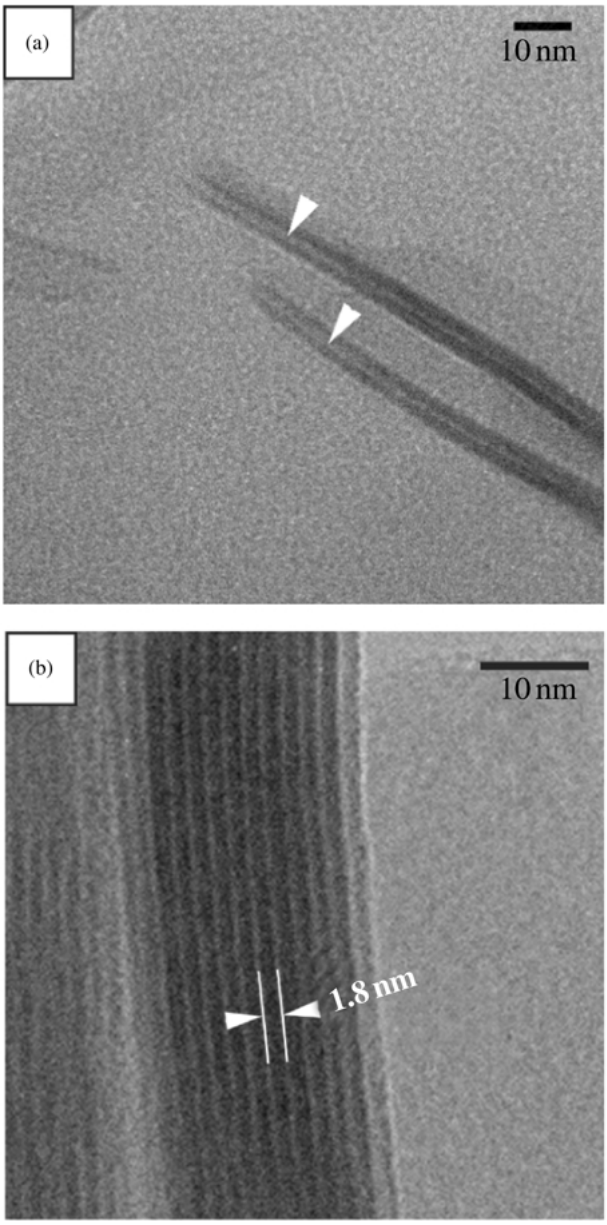

Figure 4 Further expanded TEM images of surface and inner region of modified tendon: (a) corresponding to Fig. 3(b) and (b) to Fig. 3(c). The crystallites are approximately 5 nm in thickness near the surface. At the inner region, the crystal structure was assigned to OCP as the (1 0 0) lattice fringes of the crystallite measures 1.86 nm.

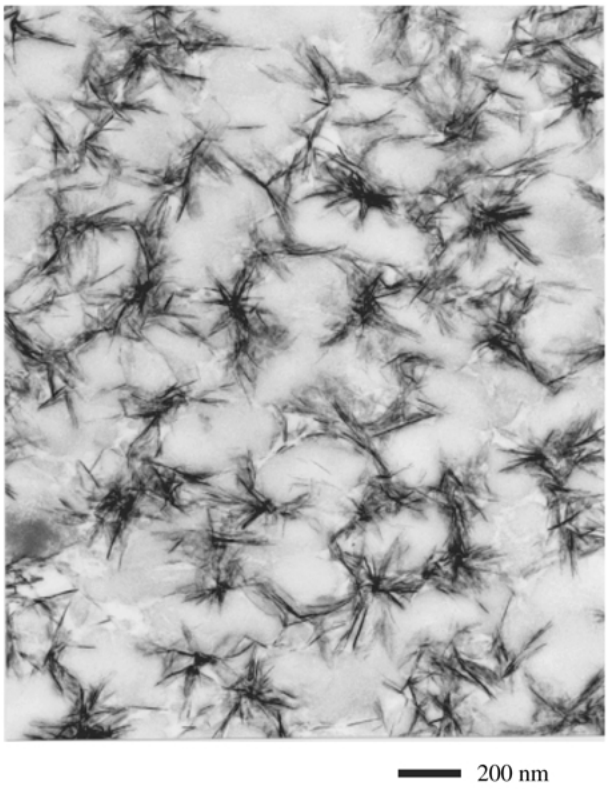

Figure 5 TEM images of the tendon stained with heavy metals. This image is obtained from the direction perpendicular to the longitudinal axis of the tendon.

the longitudinal axis of the tendon. The area of light gray is collagen fibrils as it has 100–200 nm diameter and a periodicity of 67 nm in the direction of view parallel to the longitudinal axis of the tendon (indicated in the next paragraph). HAp crystallites being formed in spaces between the collagen fibrils can be seen. Spaces formed between collagen molecules [26, 27], having a diameter of 1.12 nm, are too small for HAp/OCP crystallite formation. The OCP crystallites were transformed into the HAp structure during the staining process, although the morphology was kept.

Fig. 6 shows the TEM images observed parallel to the longitudinal axis of the tendon together with the electron diffraction pattern. The HAp crystallites and the collagen fibrils have been detected using electronic heavy metal ion staining. The collagen fibrils showed a periodicity of 67 nm, corresponding to collagen bands. The HAp crystallites formed two aggregates sized, respectively $150 \times 20\,\text{nm}^2$ and $250 \times 80\,\text{nm}^2$. Fig. 6(b) is extended image of Fig. 6(a), the inset in Fig. 6(b) shows a diffraction pattern taken from the circle area of TEM image, in which four arrows indicate two pairs of crescent shaped diffractions: each pair is ascribed to (0 0 2) and (0 0 4) of HAp. The *c*-axes of the HAp crystallites aligned along the collagen fibrils, although the HAp crystallites formed in less confined spaces did not align. The inset in Fig. 6(a) shows the diffraction pattern taken from the whole region of the TEM image. The arrows show a pair of crescent-shaped diffractions and are ascribed to HAp (0 0 2). Indicating that the *c*-axes of the HAp crystallites have been aligned parallel to the collagen fibrils. This alignment was found stronger at the inner region of the tendon tissue.

In many mineralized tissue materials such as bone, dentin and shell, mineral crystallites are ordered more or less under the influence of organic matrices. A similar alignment of HAp crystallites along collagen is observed *in vitro* without cells present [28–30]. And suggesting that nucleation of HAp crystallites occurred with

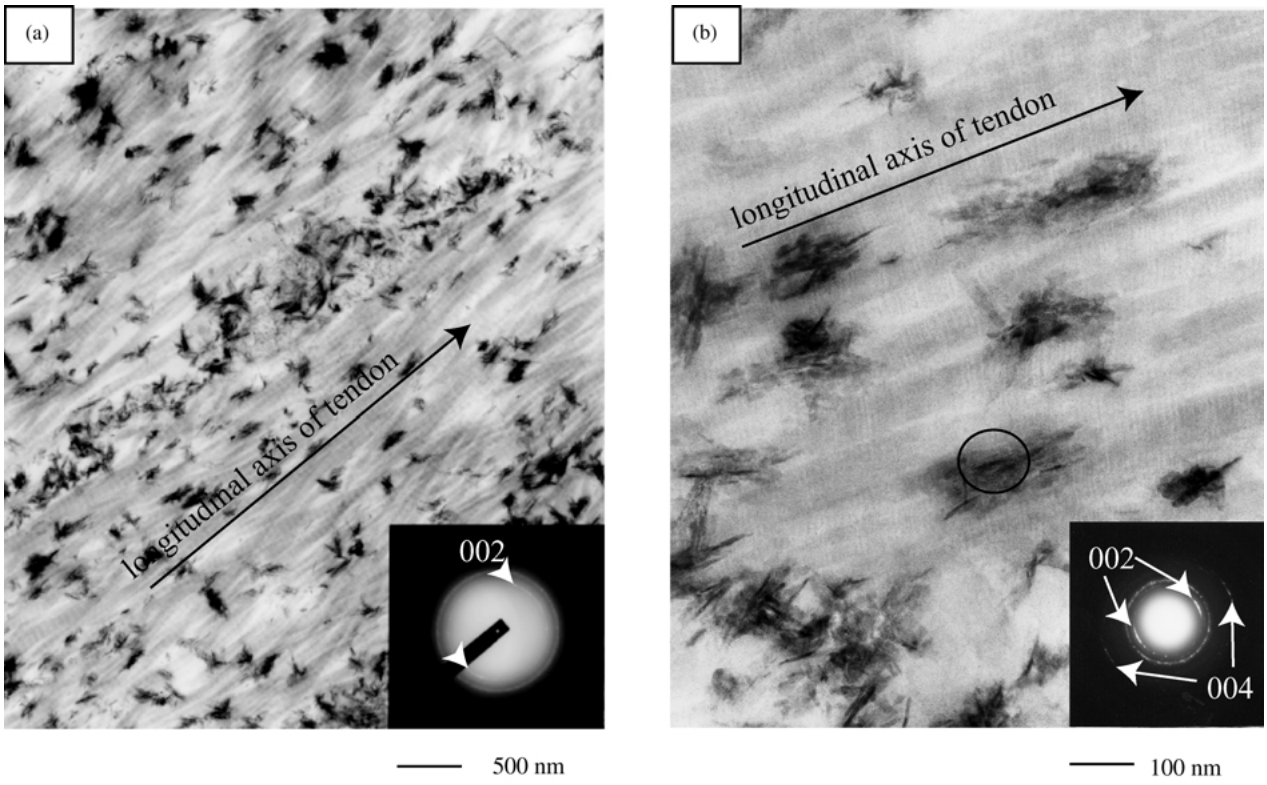

Figure 6 The TEM images observed parallel to the longitudinal axis of the tendon stained with heavy metals with the inset being an electron diffraction pattern. Black particles are HAp crystallites, and gray fibrils are collagen with periodicity of 67 nm. The arrows show a pair of crescent-shaped diffraction patterns.

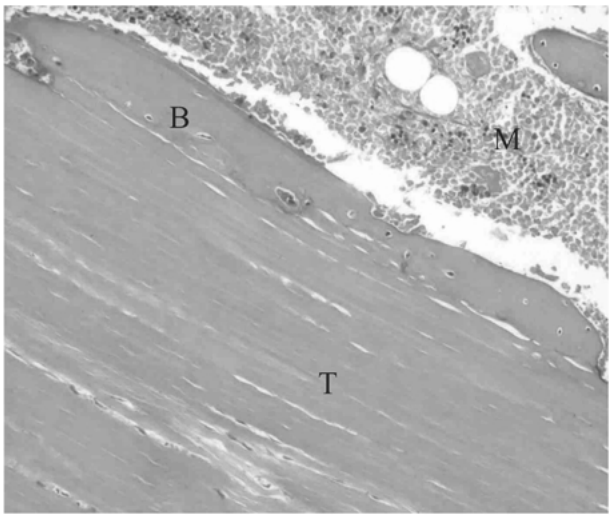

Figure 7 Histological sections of the calcium phosphate treated tendon after implantation at tibia of beagle. The section is a longitudinal section obtained 4 weeks after implantation. (T, tendon; B, newly formed bone; M, bone marrow).

functional groups of collagen, especially carboxyl [23, 24, 28] groups are suggested to control the direction of the subsequent crystal growth.

In vivo experiments

Tendons modified with calcium phosphate were implanted into tibia of beagles, and histological reactions were elucidated. After 4 weeks of implantation, modified tendon directly bound to bone through new bone formation (Fig. 7). However, tendon not treated with calcium phosphate bound with the intervening connective soft tissue. These results suggested that chemical treatment of tendon by the alternate soaking method improved osteo-conduction at the tendon–bone interface.

Conclusions

Tendons were modified with a calcium phosphate coating using the alternate soaking method. Nano-crystals of calcium phosphate were formed at the surface and inner region of the tendons. According to TEM observations, HAp and OCP crystallites were found, respectively, at the surface and inner region of tendons. The crystallites formed were strongly aligned parallel to the collagen fibrils. From *in vivo* animal tests, the chemical modification of tendon with calcium phosphate was shown to improve the interfacial bonding between tendon and other soft tissued. And it is therefore expected that this technique is applicable to the medical treatment of ligaments.

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