Calcium phosphate formation induced on silica in bamboo

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The effect of *in vitro* induction of calcium phosphate on bamboo surfaces is reported for the first time. Bamboo is studied for biomaterial application due to its elasticity modulus being closer to human bone than other biomaterials. Following an earlier study of cytotoxicity and precipitation of apatite on ground tissue and vascular bundles of bamboo, the composition and function of the minerals in bamboo, especially silica, are considered in the present work. It is found that in both outer and inner surfaces of bamboo culm, there exists some silica. Bamboo elicits an inert response when soaked directly in calcification solution. After the rind of bamboo is treated with sodium hydroxide solution, the silica underneath can induce precipitation of calcium phosphate in an ambient environment. Furthermore, by subsequent grafting with polyethylene glycol (PEG 1000), calcium phosphate induction of bamboo rind can be improved, depending on the concentration of NaOH solution and treatment time. Heat treatment of bamboo can remove the organic materials around the minerals in bamboo, allowing the calcification behaviour of the silica-containing inorganic phase of bamboo in aqueous solution to be studied.

1. Introduction

Biomaterials are now made of all kinds of man-made materials, including polymeric, bioceramic and metallic materials as well as their composites. However, none of them can serve as perfectly as the living tissues to be replaced. If used as bone repairing or replacing material, metallic implants will cause stress-shielding and bone resorption due to the elasticity mismatch with the surrounding bone. Even for the less rigid titanium, the elasticity modulus is still five times higher than that of human bone. The low modulus of polymeric materials also limits their extensive application in bone reconstruction. For ceramic materials, the poor resistance against fatigue failure and low fracture toughness are not favourable for bone-repairing material.

In the author's previous publication [1], the mechanical properties of bamboo were tested and compared with some common biomaterials where it was found to possess longitudinal mechanical properties close to those of human bone. The cytotoxicity testing of bamboo before and after extraction by some organic solvents, showed that the cytotoxic leachable components in bamboo can be removed to some extent by ethanol and methanol extraction. Besides, apatite-structured ceramic was precipitated on bamboo after bamboo was grafted with α,ω-di(aminopropyl) polyethylene glycol 800 (NH_2-PEG-NH_2). Compared with wood, bamboo has several advantages when used as a biomaterial as summarized in [1]. One of the advantages of bamboo over wood is that bamboo contains some silicon in both inner surface (pith-ring) and outer surface (rind) of the bamboo culm. It is well known that silica plays an important role in bioglass and glass-ceramic in the process of bonding to bone. Glasses based on CaO, SiO_2 may form a silica hydrogel on their surfaces prior to the formation of the apatite layer, because the hydrated silica provides favourable sites for apatite nucleation [2]. The importance of hydrated silica in forming apatite nuclei on silicon single crystals [3, 4] and silica gel [5] has also been reported. It could be of benefit to make full use of the potential of the silica in bamboo.

2. Experimental procedures

2.1. Materials and methods

All the bamboo used in the present work is *Phyllostachys bambusoids*, bought in Holland (Edo-Plant-Holland, The Netherlands). All the samples used were cut from bamboo internode. Polyethylene glycol (PEG Mw = 1000) used was a Fluka Chemie product. Accelerated calcification solution (ACS) was

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developed in this laboratory, its composition being $[Na^+] = 136.8$ mM, $[Cl^-] = 144.5$ mM, $[Ca^{2+}] = 3.87$ mM and $[PO_4^{3-}] = 2.32$ mM. The solution was buffered with 50 mM Tris buffer at pH 7.4 and at room temperature. The composition of the simulated body fluid (SBF) used was: $[Na^+] = 142$ mM, $[K^+] = 5.0$ mM, $[Ca^{2+}] = 2.5$ mM, $[Mg^{2+}] = 1.5$ mM, $[Cl^-] = 147.8$ mM, $[HCO_3^-] = 4.2$ mM, $[HPO_4^{2-}] = 1.0$ mM and $[SO_4^{2-}] = 0.5$ mM.

2.2. Preparation of samples and experimental method

Samples used for the calcification experiment were all extracted with 100% ethanol for 2 weeks at room temperature and then treated with NaOH solution. NaOH solutions were prepared as 5%, 10%, 15% and 20%, the samples were treated for 5, 10, 15 and 20 min for each concentration solution. The NaOH solutions turned light green after immersion, while the samples swelled to some extent. After NaOH treatment, samples were rinsed and dried, then coated with carbon for scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) study, line scanning and mapping analyses. NaOH-treated samples were soaked in ACS and SBF separately. After immersion, samples were rinsed with demineralized water, dried in an oven at 40 °C for several hours, and then coated with carbon and gold for SEM analyses.

To further enhance the calcification ability, bamboo samples after NaOH treatment were grafted with polymer via the following procedure: 12 g polyethylene glycol (PEG Mw = 1000) and 4 g NaOH were put in 88 g distilled water for half an hour to make the grafting solution; bamboo samples (0.68 g) were put in this solution for 20 min, then rinsed thoroughly with demineralized water. After the above process, bamboo samples were soaked in $CaCl_2$ (3.3%) solution for 1 h. After grafting, samples were rinsed with demineralized water and soaked in calcification solution for 12 h, then rinsed, dried and coated with carbon or gold for SEM observation.

To study the calcification function of the minerals in bamboo, bamboo was burnt in a fire or furnace at 580 °C for 12 h. The remnants were rinsed with distilled water to remove the ash, then dried in an oven at 40 °C before being studied by IR, SEM and EDX. After soaking the powders in calcification solutions, the ceramic formed on the bamboo remnant was analysed with SEM-EDX and IR.

2.3. Apparatus and techniques

A DuPont 951 Thermogravimetric Analyser (TGA) was used to measure the bamboo compositions. Scanning electron microscopy equipment (Philips SEM 525, Holland) was used, connected with energy dispersive X-ray apparatus (EDX) for spectrum, line scanning and mapping analyses. A dispersive infrared spectrophotometer (Perkin Elmer 783) and KBr pellets were used to characterize the bamboo mineral. A programmed furnace (Nabertherm®, Germany) was used for burning bamboo, its maximum temperature being 1100 °C.

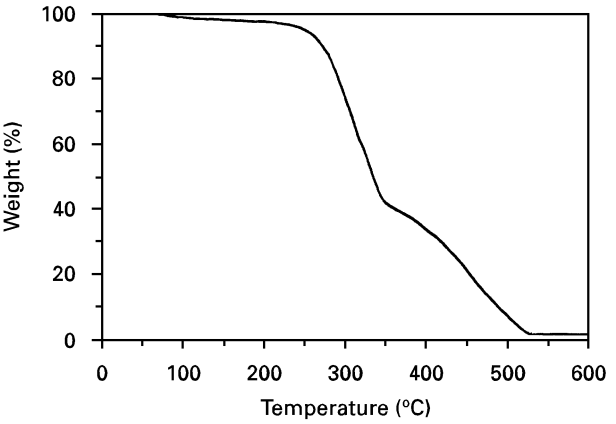


Figure 1 TGA result of natural bamboo.

3. Results and discussions

3.1. Minerals in bamboo

Bamboo culm consists of the rind, basic and vascular systems. The rind system is composed of epidermis, hyperdermis and cortex which are of small, thick-walled and well packed cells, and functions mainly as an outer wall to protect the culm tissue. The epidermis is the outermost layer of bamboo culm. The epidermal cells are arranged compactly with rare breaks in their continuity. The bamboo epidermis typically contains long cells and two kinds of short cells, silica cells and cork cells. The short cells frequently occur together in pairs. The silica cells are almost completely filled with SiO_2 [6]. The cork cells have suberized walls and contain solid organic materials; the process of the bamboo cell wall being impregnated by fatty substances is called cutinization and suberization. The inner surface of the bamboo culm is called the "pith-ring", and is composed of 8–15 layers of short and square thick-walled cells which are arranged compactly. Their cell walls thicken as bamboo grows and subsequently become so-called "stone cells", a kind of cell whose walls are extremely thick and hard. After secondary deposits or more specifically, lignification and cutinization, their lumens finally disappear.

Bamboo culm was cut in a direction across the thickness and a piece was tested by thermogravimetric analyser (TGA). Fig. 1 displays the result indicating that the weight of the sample changed rapidly above 230 °C, and from about 550 °C remained constant. Obviously the organic components in bamboo decompose over this temperature range. Finally, a residue of about 2.7% weight, consisting of minerals, is left, which is in agreement with reports in the literature [6].

A piece of bamboo culm, including outer rind and inner pith-ring, was placed in a furnace at 580 °C for 12 h, so that all the organic components in the bamboo would be burnt. The remnant was rinsed and coated with carbon for examination with SEM-EDX. Fig. 2 shows that the minerals in bamboo are composed of Zn, Mg, Si, P, S, Cl, K and Ca. When bamboo is considered as a biomaterial, Si, P and Ca are of special importance.

3.2. Silica in bamboo

SEM-EDX study reveals that most silicon existed on the outer and inner surfaces of the bamboo culm,

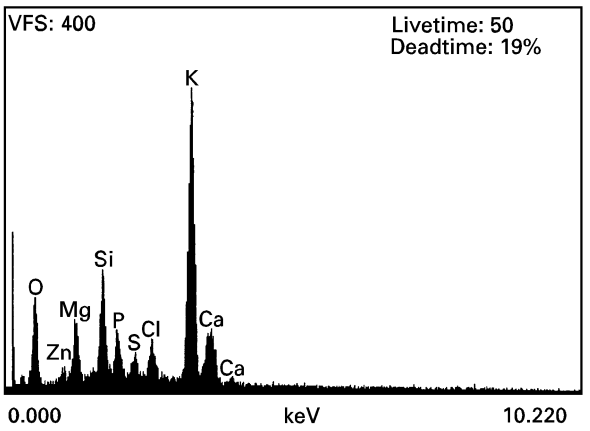


Figure 2 SEM-EDX spectrum of the remnant of bamboo after burning at 580 °C in a furnace for 12 h.

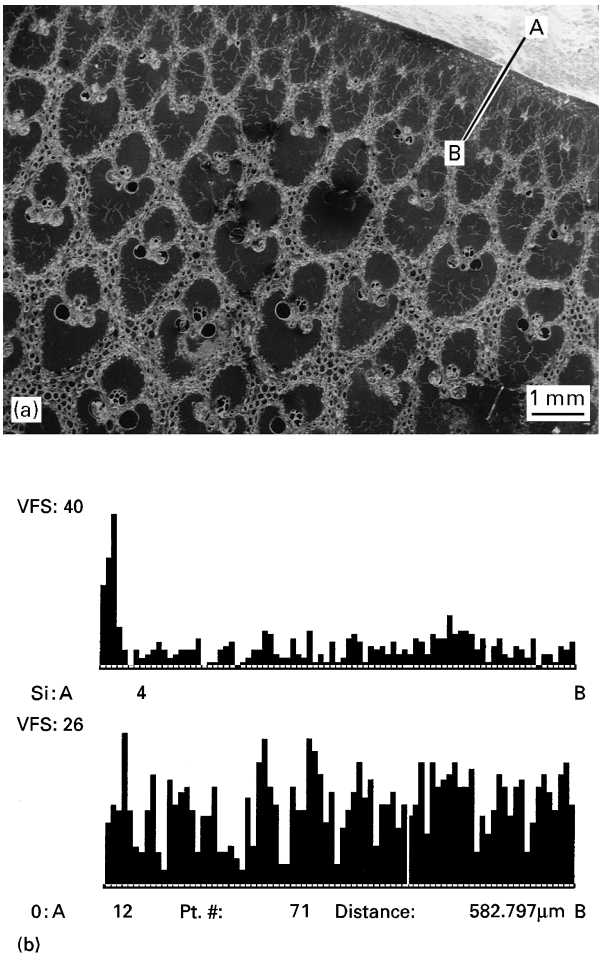


Figure 3 (a) Part of the cross-section of bamboo culm, and (b) line-scanning spectrum of a path from A to B.

namely the rind and more specifically, the epidermis and pith-ring. Fig. 3 shows (a) a cross-section of bamboo culm and (b) the line scanning spectrum of a path across its outer surface. The outermost layer of bamboo has the highest Si concentration. For the inner surface, the pith-ring, we find a similar situation.

When bamboo culm was soaked in 10% NaOH solution for 20 min, some of the surface fatty substance on the bamboo was etched off, as indicated in Fig. 4, which is an SEM photograph of the bamboo rind. It is clear that some globular structures

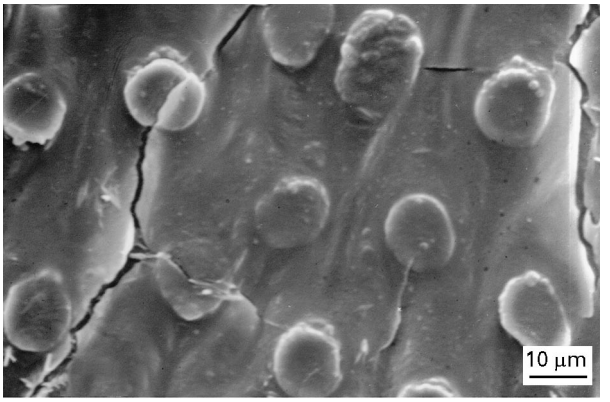


Figure 4 SEM photograph of the bamboo rind after soaking in 10% NaOH solution for 20 min.

appeared, the silicon concentration in these spheres being much higher than in the area around them, as shown in Fig. 5, the microanalytical X-ray mapping. We suppose that these spheres consist mainly of silica. Silica only existed on a surface thin layer as indicated in Fig. 6, in which the central area of the silica-rich layer was peeled off; no silicon can be detected there by EDX. In the bamboo powder burnt in a fire these spheres are embedded in a matrix sheet (Fig. 7). However, in the furnace-burnt powder separate spheres can be seen because the matrix which held the silica spheres was burnt out. The geometric morphology of these silica-rich spheres, from SEM observation, may be both spherical or round plate (ellipsoid). The cracks in Fig. 4 were caused by dehydration; some cracks split the covering of the sphere.

The bamboo cell wall can be impregnated by fatty substances, mineral substances and lignin; the corresponding phenomena are referred to as cutinization and suberization, mineralization and lignification. Because of the chemical nature and the peripheral position in the bamboo culm, the fatty substance is considered to be effective in reducing transpiration and protecting bamboo from the leaching effects of rain.

A piece of bamboo cut from a bamboo internode and burnt in fire until a flame no longer occurred results in a solid black remnant that, according to EDX analysis, included C, O, Mg, Si, P, K and Ca. The organic part of the bamboo turned out to be charcoal after burning in a fire, but small particles of SiO_2 and CaO agglomerated to form bigger individual mineral particles. If bamboo was burnt in a furnace at 580 °C for 12 h, all organic components are consumed, no bamboo charcoal is left, but there is still some oxygen which comes from oxides such as SiO_2, CaO, etc. The transmission IR spectrum of the furnace-burnt bamboo powder is illustrated in Fig. 8; the peak at 475 cm^{-1} is Si–O bending [7], lending further support to the existence of SiO_2 in bamboo.

3.3. Formation of apatite on bamboo rind (silica-rich layer)

After treatment with NaOH solution, which removes the surface fatty substance covering the silica-rich layer, bamboo rind was soaked in ACS and SBF. After

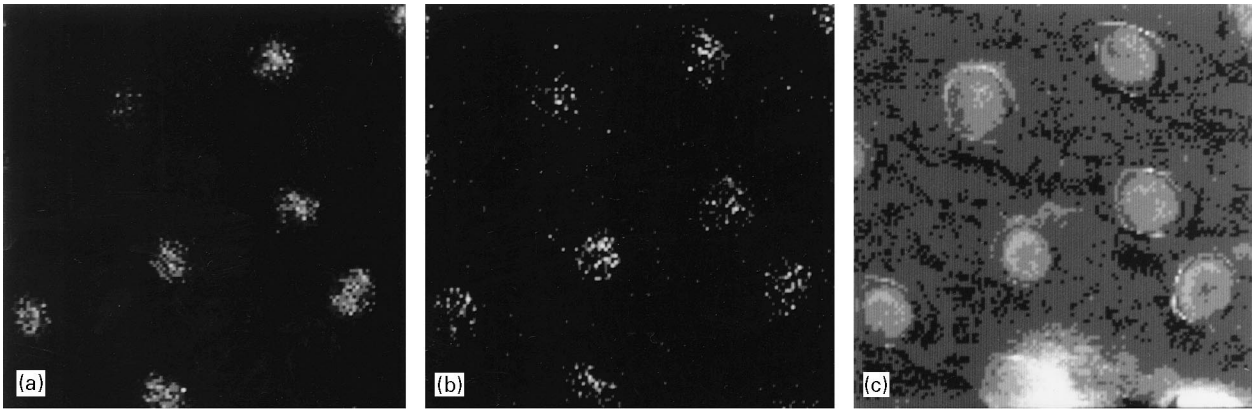


Figure 5 Microanalytical X-ray mapping showing the higher concentration of Si and O in the spheres than in the surrounding area (a) Si; (b) O; (c) SEM image.

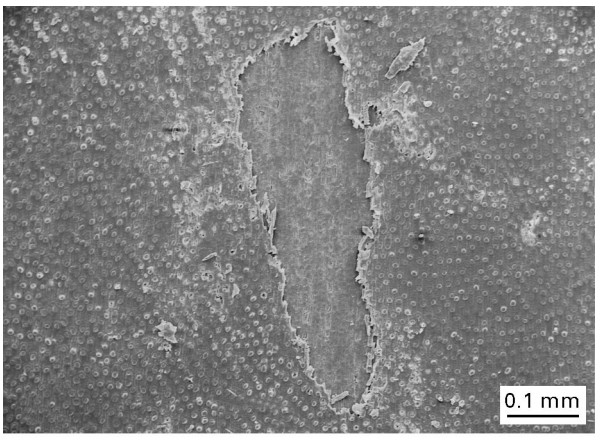


Figure 6 SEM photograph of bamboo rind showing silica existing in the form of globular particles embedded in a surface layer.

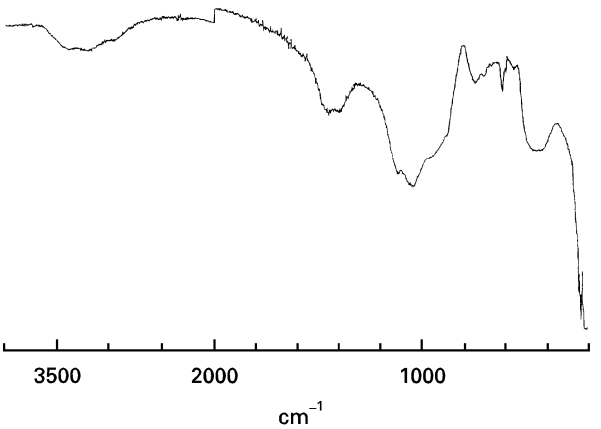


Figure 8 Transmission IR spectrum of furnace burnt bamboo powder, in which the characteristic peak of Si–O bending is shown.

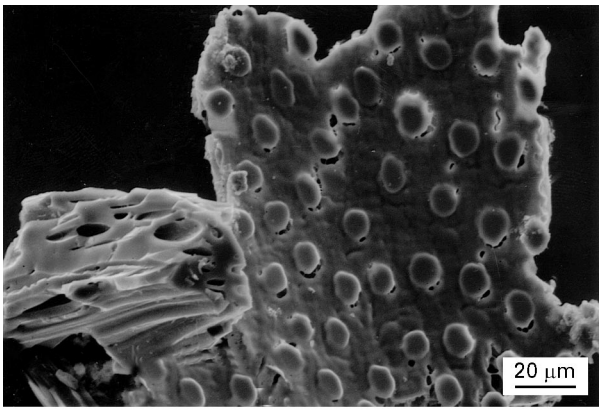


Figure 7 Fire-burnt remnant of bamboo showing a piece of the silica-rich layer.

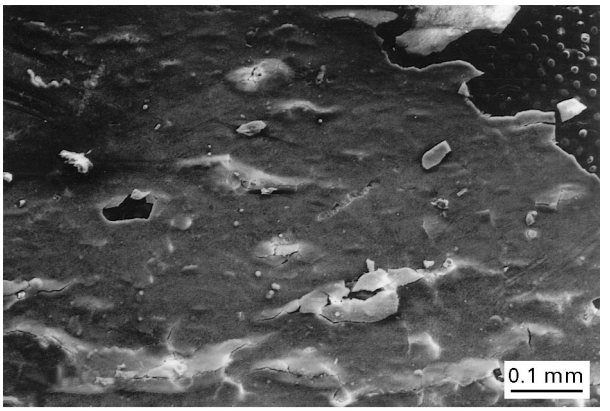


Figure 9 Scanning electron micrograph of a continuous layer of Ca/P mineral formed on the bamboo rind after 10% NaOH solution treatment.

2 days, a continuous layer of Ca/P mineral was formed on the samples soaked in ACS, as shown in Fig. 9, with a Ca/P ratio, as detected by EDX, of 1.35. The IR spectrum of this Ca/P mineral is shown in Fig. 10a, in comparison with that of commercial hydroxyapatite, Fig. 10b. It can deduced that the Ca/P mineral is a kind of apatite-structured calcium phosphate containing some carbonate, which may come from the CO_2 in air. For comparison, commercial crystal quartz was also soaked in ACS for 2 days, but no Ca/P mineral could be found.

To enhance the bioactive ability, grafting with a bioactive polymer, for instance PEG 1000, is an appropriate method. The concentration of NaOH solution and treatment time has an influence on the bioactivity ability of the bamboo rind after grafting, as verified by the following experiment. Bamboo samples were treated with 5% and 10% NaOH solution for

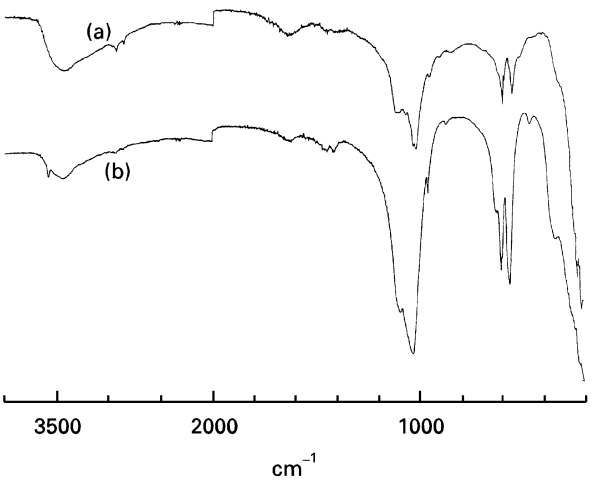


Figure 10 IR spectrum of (a) the calcium phosphate formed on bamboo after NaOH treatment and (b) commercial hydroxyapatite without sintering.

5 and 20 min, respectively, then grafted with PEG 1000. After immersion in ACS for 12 h, samples were taken out and examined by SEM (Fig. 11a–d). From these photographs the influence of NaOH solution treatment on the formation of calcium phosphate is clear. Of the four different experimental conditions, the sample treated with 10% NaOH for 5 min induces the most calcium phosphate precipitation. The higher concentration NaOH solutions dissolve the fatty substance quickly, but if the treatment time is too long, some silica particles embedded in the bamboo dissolve, which retards the formation of calcium phosphate. Less Ca/P mineral was formed in Fig. 11d than in Fig. 11c. Instead of on the silica, PEG 1000 was grafted on to the organic components of bamboo: cellulose and lignin, as explained by the following reactions:

$$\text{Cellulose–OH} + \text{NaOH} \rightarrow \text{Cellulose–O}^{-}\text{Na}^{+} + \text{H}_2\text{O}$$

$$\text{PEG–OH} + \text{NaOH} \rightarrow \text{PEG–O}^{-}\text{Na}^{+} + \text{H}_2\text{O}$$

$$\text{Cellulose–O}^{-}\text{Na}^{+}$$

$$+ \text{PEG–O}^{-}\text{Na}^{+}\underline{\text{Ca}^{2+}}\text{–Cellulose–O–Ca–PEG}$$

After grafting, Ca^{2+} will bridge PEG with cellulose or lignin in bamboo. Besides the Ca^{2+} grafted on to the bamboo, the PEG molecule is capable of chelating Ca^{2+} [8, 9], which also contributes to the formation of a calcium phosphate layer.

The mechanism of the silica induction effect in bamboo can be explained as follows: bamboo cells take in silicon from the earth through water conduction. Thus the formation of silica in bamboo takes place in the presence of water, suggesting that the silica composition in bamboo is something like a silica gel. It can be assumed that, after removal of the fatty covering substance, at least one layer of hydrated silica (rich in silanol groups, SiOH) can be found on the silica-rich spheres. This silica hydrogel layer may play an essential role in providing the sites for apatite nuclei [2].

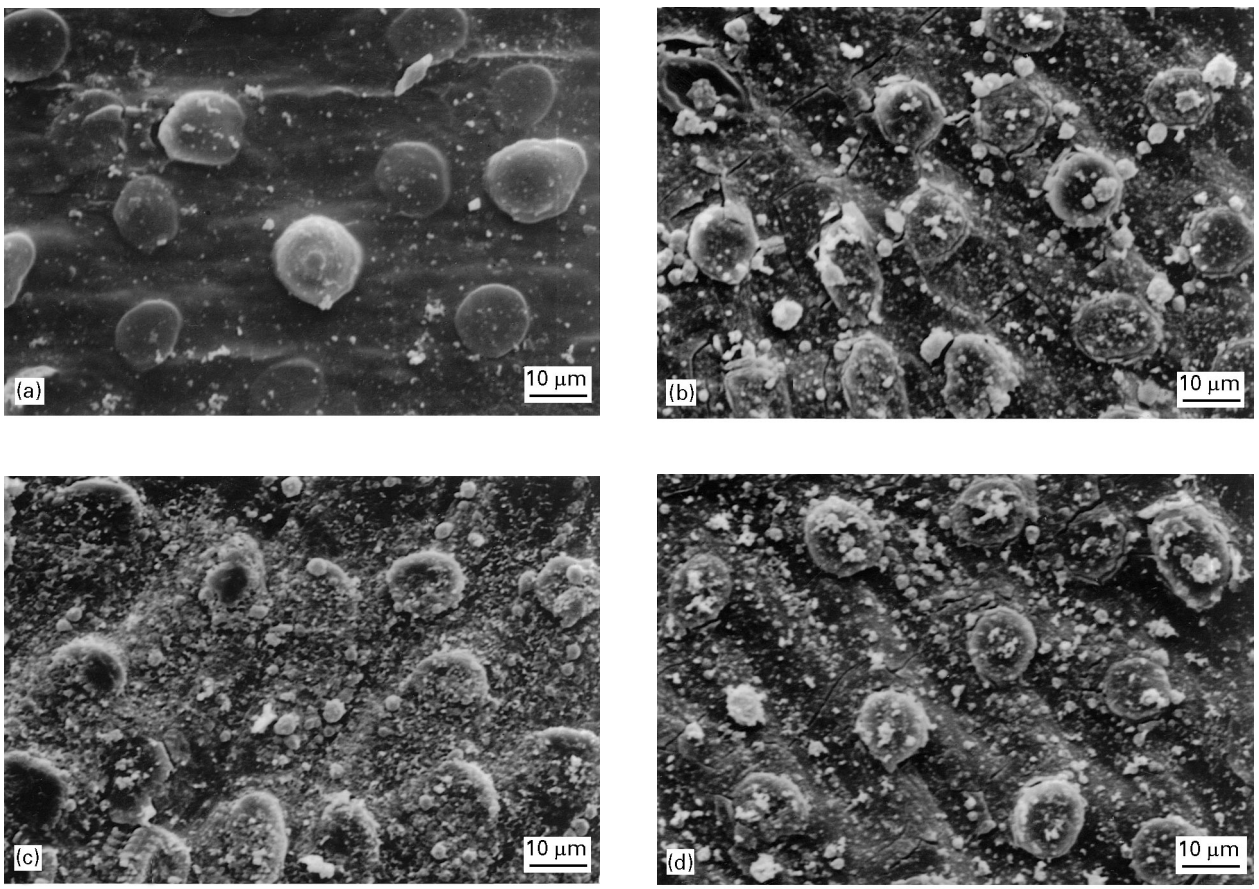
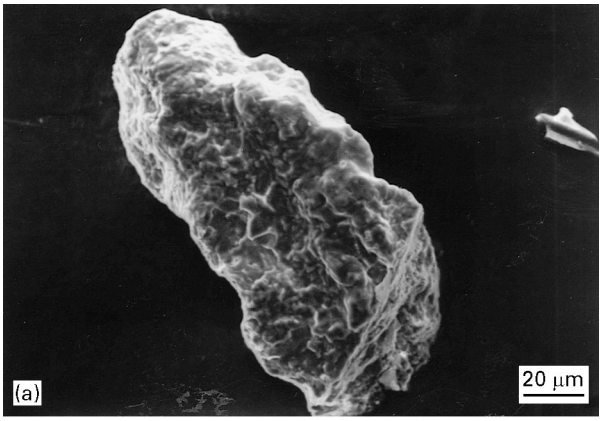


Figure 11 SEM images illustrating bamboo rind after different NaOH solution treatments, then grafted with PEG 1000 and soaked in ACS for 12 h: (a) 5% NaOH for 5 min; (b) 5% NaOH for 20 min; (c) 10% NaOH for 5 min; (d) 10% NaOH for 20 min.



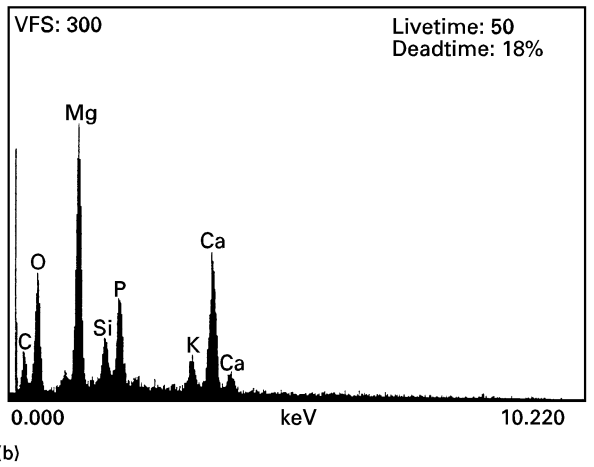


Figure 12 (a) Scanning electron micrograph of remnant mineral particle after burning bamboo in fire; (b) its SEM-EDX spectrum.

3.4. Formation of apatite on bamboo burnt powder

To avoid the influence of the organic components on the formation of apatite, bamboo was burnt in a fire or a furnace at 580 °C overnight. Fig. 12a shows a fire-burnt powder (mineral) particle of bamboo and its EDX spectrum (Fig. 12b), which indicates that the mineral is composed of O, Mg, Si, P, K and Ca. The fire-burnt bamboo powder, after being soaked in ACS for 1 day, showed some Ca/P mineral absorbtion. Increasing the soaking time increased the amount of Ca/P mineral. After 3 days of immersion, no silica could be detected by EDX, proving that all the silica-rich particles were covered by Ca/P minerals. Fig. 13 shows the crystal plates of the calcium phosphate formed on fire-burnt bamboo powders. Extensive SEM observations revealed that calcium phosphate first formed a dense covering of the particle, then this ceramic layer induced further well-crystallized plates.

The furnace-burnt powders had different characteristics, looking more like ash, rather than the black particles of the fire-burnt remnants. Their composition is shown in Fig. 2 and Zn, S and Cl can be detected after complete burning. After immersing the furnace-burnt remnant in ACS for 12 h, some Ca/P mineral was found, as shown in Fig. 14. Only in the

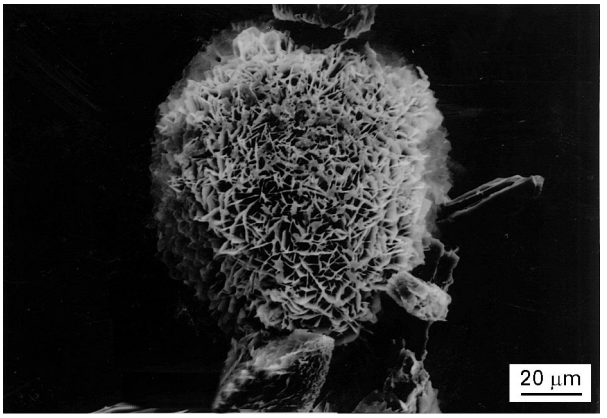
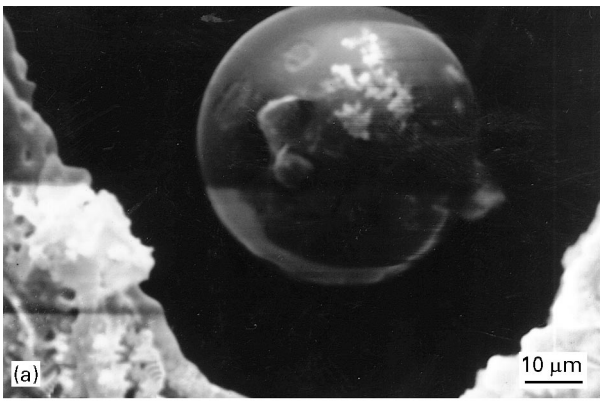


Figure 13 SEM image illustrating the crystal plates of the Ca/P mineral formed on the fire-burnt bamboo remnant.



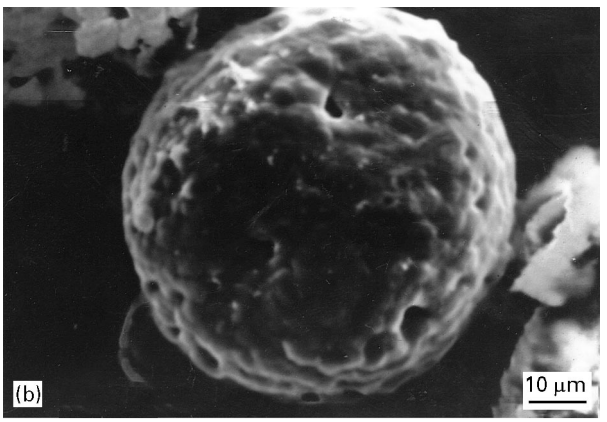


Figure 14 SEM images of silica-rich sphere in the furnace-burnt remnant of bamboo soaked in ACS for (a) 12 h, some Ca/P mineral formed; (b) 3 days, the whole sphere was covered by calcium phosphate.

furnace-burnt remnant were single silica spheres observed, as in Fig. 14a, with diameters of about 23 μm. After immersion in ACS for 1 day, some calcium phosphate was found on the surface of the silica-rich spheres. After 3 days, as shown in Fig. 14b, some silica-rich spheres were covered completely by Ca/P minerals, preventing Si from being detected in the sphere by EDX.

The results above show that the minerals in bamboo, especially silica, can induce apatite formation in a supersaturated calcification solution, after the natural polymer which covers silica is removed through

burning. The silica-rich layer provides bamboo with a natural composite structure: natural cellulosic-lignin sandwiched by two surfaces of quasi-silica gel.

4. Conclusions

Bamboo contains some minerals, most of them existing in the outer surface (rind) and inner surface (pith-ring). If the covering fatty substance in bamboo rind was removed by NaOH solution, a silica-rich layer appeared on the outer surface of the bamboo culm, containing many separate silica particles. This silica-rich layer induced the formation of calcium phosphate in a supersaturated calcification solution. Grafting with PEG 1000 enhanced the bioactivity of NaOH-treated bamboo. There exists an optimal combination of the concentration of NaOH solution and the treatment time. Burning bamboo in a fire or in a furnace at 580 °C for 12 h to remove the organic components resulted in burnt powders which also induced the precipitation of calcium phosphate ceramic. In fire-burnt remnants, embedded silica-rich spheres were observed, but separate silica sphere were only to be found in furnace-burnt remnants. As these burnt powders were relatively rich in silica and contained inorganic materials, their calcification shows the importance of silica for the observed bioactivity of bamboo.

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