In-vitro apatite formation on phosphorylated bamboo

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Natural self-reinforced composite, bamboo, was surface modified by phosphorylation with urea–H_3PO_4 and NaOH–H_3PO_4 methods; then precalcification was performed by immersing samples in saturated $Ca(OH)_2$ solution. After that, calcium phosphate can be formed on the surface of bamboo samples in calcification media: simulated body fluid (1.5 SBF) and accelerated calcification solution (ACS). Experimental results reveal that pre-calcification is an inevitable step for the formation of calcium phosphate. The calcium phosphate formed in 1.5 SBF was identified by thin-film X-ray diffraction as apatite which was not well crystallized. Compared with the urea–H_3PO_4 method, the NaOH–H_3PO_4 method has the advantages of quicker and continuous apatite formation and stronger adhesive between apatite and bamboo.

1. Introduction

Many kinds of material have been used as bone-repairing or bone-replacing materials, including ceramic (hydroxyapatite, alumina, glass–ceramic, etc.), metallic (Ti–6Al–4V, stainless steel, etc.) and polymeric materials (polyactive, polyurethane, etc.) as well as their composites (polyethylene–hydroxyapatite). Although some of them have been successfully used in bone repair or replacement, none of them can serve as perfectly as the living tissues to be replaced. Thus the effort of searching for novel biomaterials has never stopped.

Some natural materials have found biological applications, especially cellulose [1] and collagen [2]. As bone substitute materials, wood has also been previously studied [3]. No literature was found on the application of bamboo as a biomaterial. Wood and certainly bamboo possess mechanical properties close to those of human bone [4], especially the elastic modulus in the direction of fibres, which is of importance for bone substitute or bone-repairing biomaterials. As a potential biomaterial, besides the mechanical properties, the tissue biocompatibility of bamboo has to be studied. Considering the biocompatibility result of wood in the literature [3] and the similarity of the chemical components of wood and bamboo, direct implantation without any treatment is not feasible. The possible ways to improve the biocompatibility of bamboo are firstly to remove the toxic components from bamboo by chemical treatment and/or secondly to form a bioactive ceramic coating on bamboo surface layer which can enhance bone bonding and shield off the bamboo components. Some results of the first method were reported in the present authors' previous work [4]. In the present paper, a surface modification method which results in formation of a ceramic coating on bamboo at room temperature is reported. Phosphorylation of cotton fibre was originally used for making cation exchange material for Ca^{2+} [5] and then was successfully used for growing calcium phosphates by Mucalo *et al.* [6]. A similar phosphorylation by the urea–phosphoric acid (H_3PO_4) method was used in the present study to modify the bamboo surface, and an improved phosphorylation method (sodium hydroxide (NaOH)–H_3PO_4) was found, which leads to quicker and continuous apatite layer formation.

2. Experimental procedure

2.1. Samples, solutions and chemicals

All the bamboo used in the present work is *Phyllostachys bambusoids*, grown in Japan and bought in Holland (Edo-Plant-Holland, The Netherlands). All the samples used were cut from bamboo internode, sliced into sections 1mm thick and then extracted with 100% ethanol for 2 weeks at room temperature.

The calcification media used in this work were simulated body fluid (1.5 SBF) [7] and accelerated calcification solution (ACS) which was developed in this laboratory [8]. The ion concentrations of 1.5 SBF and ACS are listed in Table I.

Orthophosphoric acid (H_3PO_4) (purity 99%) is a product of Fluka Chemical Company, urea is from Merck Company. Saturated $Ca(OH)_2$ solution was made by dissolving $Ca(OH)_2$ (Merck Company) in

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TABLE I Ion concentrations of 1.5 SBF and ACS

	Ion concentration (mM)							
	Na^+	K^+	Ca^{2+}	Mg^{2+}	HCO_3^-	Cl^-	HPO_4^{2-}	SO_4^{2-}
1.5 SBF	213.0	7.5	3.8	2.3	6.3	223.0	1.5	0.75
ACS	136.8	4.64	3.87	—	—	144.5	2.32	—

deionized water and then filtered by filter paper, so that a clear saturated solution was obtained.

2.2. Phosphorylation of bamboo

Phosphorylation of bamboo with the urea–H_3PO_4 method was carried out similarly to the methods in [6, 9], the only difference was that phosphoric acid was used instead of phosphorous acid. It has been reported that, for cotton fibres, the overall quality and uniformity of the coatings formed on urea–H_3PO_4-treated fibres are superior to those formed on urea–H_3PO_3-treated fibres [7]. The reaction procedure and the phenomena were almost the same as in the case of these cotton fibres. 1.54 g of bamboo samples were placed in a round-bottomed flask equipped with a thermometer, mechanical stirrer, condenser and N_2 gas inlet tube. 40 g of urea were subsequently added to the flask and dissolved in 500 ml of dimethyl formamide (DMF); the bamboo–urea–DMF was then heated to 130 °C upon which a solution of 27g of H_3PO_4 in 100 ml of DMF was added. Then the solution was heated to the boiling point of DMF and in reflux for 0.5 h. After phosphorylation and thorough drying, the bamboo samples shrunk in size and turned darker.

An improved phosphorylation technique was based on the following procedure. Extracted bamboo samples (thin plate, 1 mm in thickness) were treated with NaOH solution (10%) for 10 min and then rinsed with demineralized water and 100% ethanol. After this treatment, samples were heated in a 7.8% solution of H_3PO_4 in DMF up to the boiling point of DMF (154 °C). After 30 min reaction, the solution turned opaque and foamed slightly; bamboo samples swell greatly. The heavily swollen bamboo samples were rinsed thoroughly with demineralized water to remove the unreacted H_3PO_4.

2.3. Precalcification of the phosphorylated bamboo

Phosphorylated bamboo samples obtained with the urea–H_3PO_4 method were soaked in saturated $Ca(OH)_2$ solution (pH 12.5) in closed plastic bottles for 6 days. Phosphorylated bamboo samples from the NaOH–H_3PO_4 method were soaked in saturated $Ca(OH)_2$ solutions for 4 days. After such precalcification process, the samples were thoroughly rinsed with demineralized water and dried. Carbon or Au was coated on samples for scanning electron microscopy (SEM) and energy-dispersive X-ray analysis (EDXA) studies.

2.4. Growth of apatite

Samples of precalcified or unprecalcified phosphorylated bamboo were put into a beaker to which 30 ml of 1.5 SBF or ACS was added. The beakers were put in a covered water bath which was kept at 37 °C. Samples were taken out at 2, 4, 7 and 14 days and then rinsed and dried. The calcium phosphate formed on the surface of bamboo sample was characterized by thin-film XRD and EDXA.

2.5. Instrumentation

SEM and EDXA were performed using a Philips scanning electron microscope 525 (Eindhoven, The Netherlands). An infrared spectrophotometer (Perkin–Elmer 783) was used to characterize the composition change of bamboo samples by the modification treatments. A reciprocal water bath shaker (New Bronswick Scientific Co., Inc., USA) was used for the process of apatite formation. Scanning electron micrographs of samples were usually obtained from Au-coated specimens.

3. Results and discussion

3.1. Preliminary studies of as-received bamboo and unprecalcified phosphorylated bamboo soaked in calcification solution

Fig. 1a shows the cross-section of as-received bamboo in which the gradient distribution of vascular bundles (Fig. 1b) along the radial direction is shown; the part which bears the most outer mechanical loads is composed of bast fibres, as shown in the solid dark areas in Fig. 1b. Fig. 2a is the infrared (IR) spectrum of as-received bamboo; IR spectra of bamboo samples after NaOH solution and later H_3PO_4 treatment are shown in Figs 2b and c.

As-received bamboo samples were directly soaked in ACS for 3 days or in 1.5 SBF for 2 weeks; there was no apatite formed on bamboo surface; this indicates that as-received bamboo is inert in calcification media. After phosphorylation by urea–H_3PO_4 or NaOH–H_3PO_4 techniques, the bamboo samples obviously shrunk. Soaking these kind of samples in calcification media did not result in apatite formation. If phosphorylated samples were soaked in ACS for 2.5 days, SEM–EDXA revealed a Ca to P ratio of 1.57 on the surface, although no visible apatite had formed.

Inagaki *et al.* [9] have reported that phosphorylation of cotton fibres leads to the incorporation of phosphite groups into the material. Because more

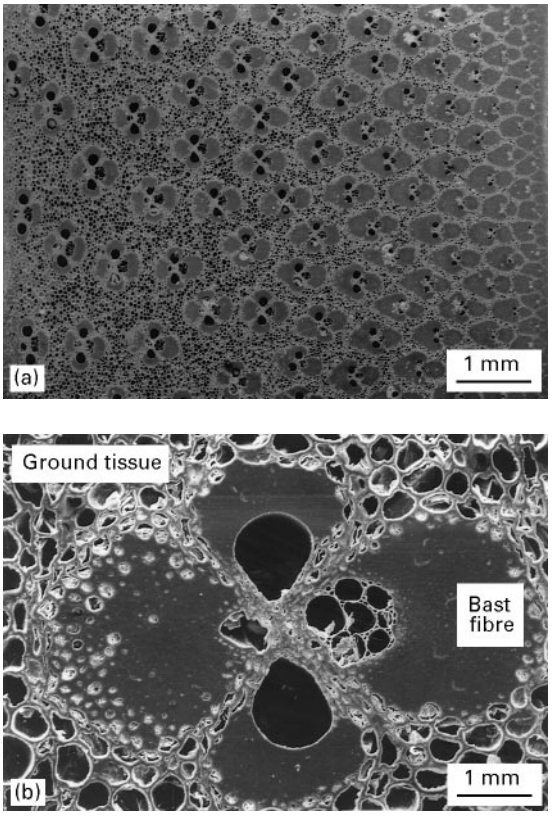


Figure 1 SEM images showing (a) the cross-section of bamboo culm and (b) a vascular bundle in (a).

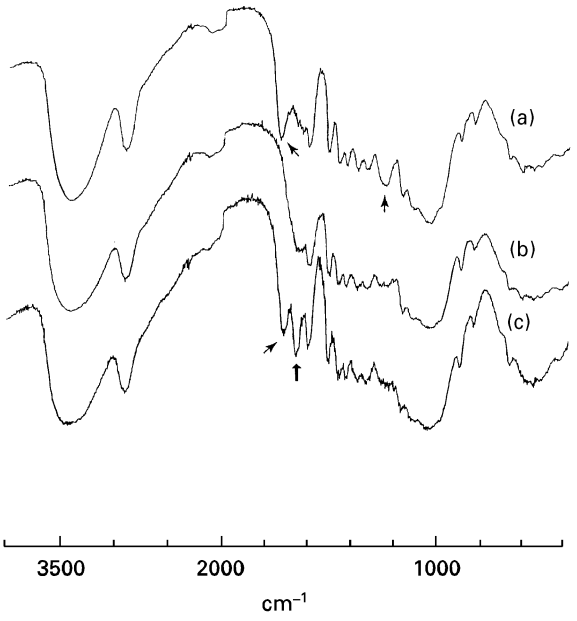


Figure 2 IR spectra of (a) as-received bamboo, (b) bamboo after 10% NaOH treatment for 10 min (in this spectrum, the peaks at 1730 and 1250 cm^{-1} in (a) disappeared) and (c) after NaOH and later H_3PO_4 treatment (a peak appeared at 1660 cm^{-1}).

than 90% of the components of cotton fibre is cellulose, it may be assumed the phosphite groups were incorporated in cellulose. Bamboo is mainly composed of cellulose and lignin, cellulose existing in both parenthyma tissue and the bast fibres in vascular bundles. After phosphorylation, phosphite groups can be incorporated with both bast fibres and the thin-walled tissue, giving bamboo cation exchange properties. Some Ca^{2+} in calcification media can be absorbed on bamboo but apparently not enough to induce the precipitation of calcium phosphate, even in ACS in which the Ca^{2+} concentration is higher than those in human body. Thus precalcification of the bamboo samples after phosphorylation becomes necessary.

3.2. Precalcification of bamboo after phosphorylation

Different from the situation of cotton fibres [6], no calcium phosphate clusters were observed on the $Ca(OH)_2$-solution-treated bamboo samples which were phosphorylated by urea–H_3PO_4, as shown in Fig. 3.

For the samples phosphorylated in air environment by the NaOH–H_3PO_4 method, no calcium phosphate clusters could be observed by SEM after soaking in saturated $Ca(OH)_2$ solution for 4 days; EDXAs analyses revealed that the Ca to P ratio was 5.65 on sample surface. The Ca^{2+} ion concentration on bamboo surface increased with time, as shown in Fig. 4. Also after soaking phosphorylated samples in $CaCl_2$ solution for 2 days, the surface Ca to P ratio as determined by EDXA can reach 2.4.

3.3. Growth of apatite

Although there was no visible precipitation of calcium phosphate formed on the phosphorylated bamboo

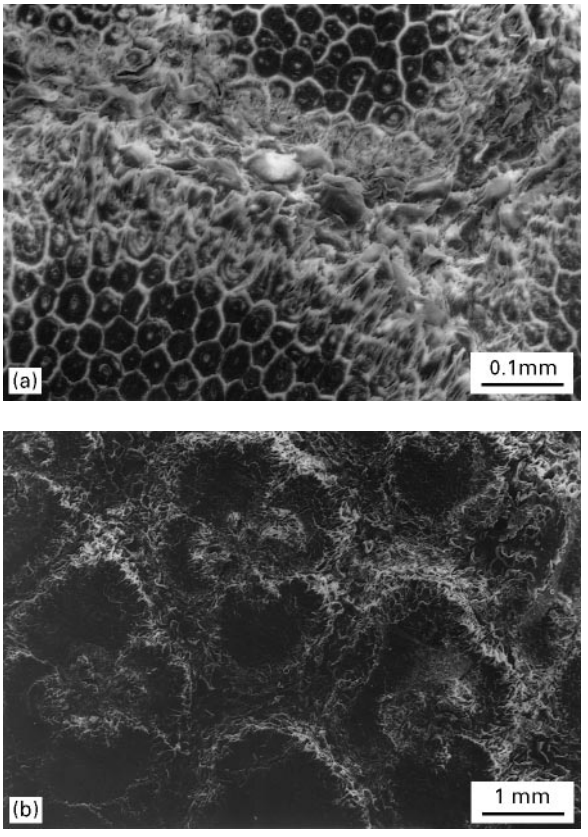


Figure 3 SEM images showing the surfaces of bamboo samples after (a) phosphorylation by urea–H_3PO_4 and precalcification by $Ca(OH)_2$ for 6 days and (b) phosphorylation by NaOH–H_3PO_4 and precalcification by $Ca(OH)_2$ for 4 days.

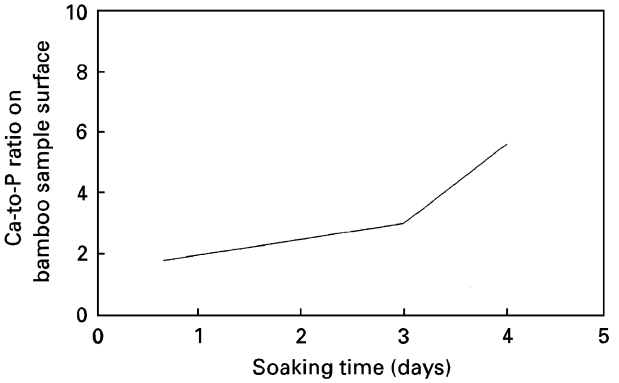


Figure 4 Changing trend of Ca to P ratio on the surface of bamboo samples after precalcification, as detected by SEM–EDXA.

samples after precalcification, a high concentration of Ca^{2+} was collected at the surface; the result is that, after soaking in phosphorylated $Ca(OH)_2$-treated bamboo samples in ACS or SBF, apatite can be formed on bamboo surface. The morphology and the structure of the apatite layers formed on the samples treated by urea–H_3PO_4 or NaOH–H_3PO_4 were different. Fig. 5 contains a group of SEM images showing the process of apatite formation on bamboo samples phosphorylated by urea–H_3PO_4 and pre-calcified by $Ca(OH)_2$ for 6 days, and later in 1.5 SBF for 2 days–2 weeks. The features of the apatite formation elicited in Fig. 5 can be expressed as follows.

1. The apatite is not a uniform continuous layer.
2. The locally continuous layer of apatite is easy to break; some of the apatite formed on bast fibres has already fallen down (Fig. 5e), which indicates that the adhesive between apatite layer and bast fibre is not strong.
3. The bast fibre area from where apatite broke off did not show renewed formation of apatite (Figs 5g and h).

However, for the same period of soaking time (2 days in 1.5 SBF), samples which were phosphorylated

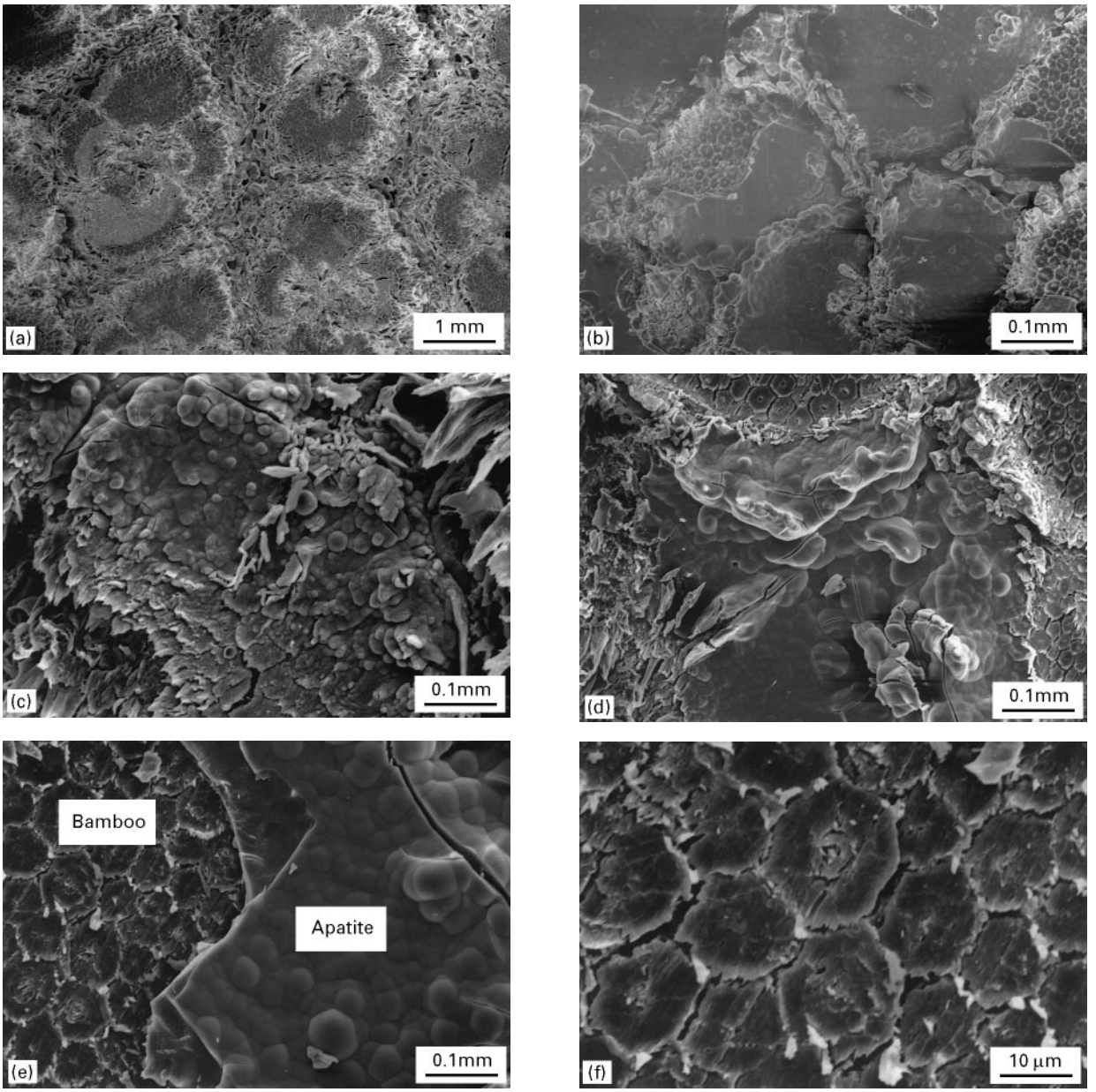
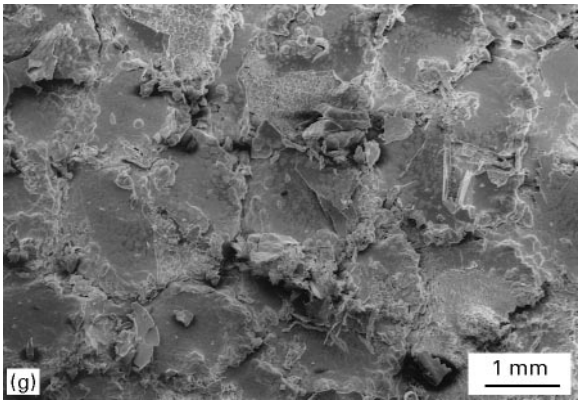


Figure 5 SEM images showing the apatite formed on bamboo after phosphorylation by urea–H_3PO_4, $Ca(OH)_2$ treatment and then (a)–(f) soaking in 1.5 SBF for 2 days ((a), (b) cross-sections of bamboo sample; (c) nucleation on bast fibre; (d) central area in vascular bundle; (e) apatite plate, part of which has already fallen down; (f) naked bast fibres) and (g), (h) soaking in 1.5 SBF for 2 weeks (some parts are still uncovered).



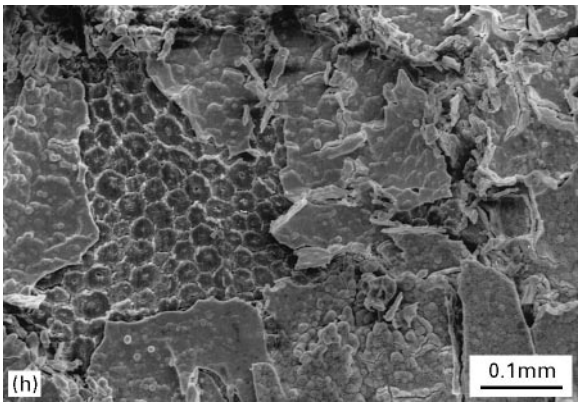
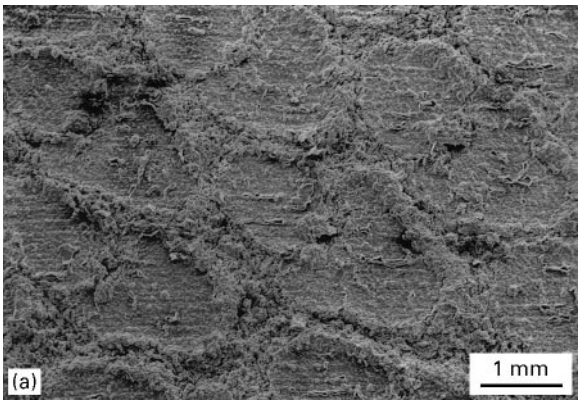
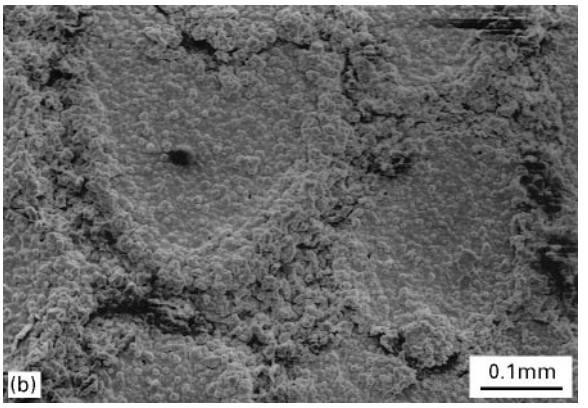
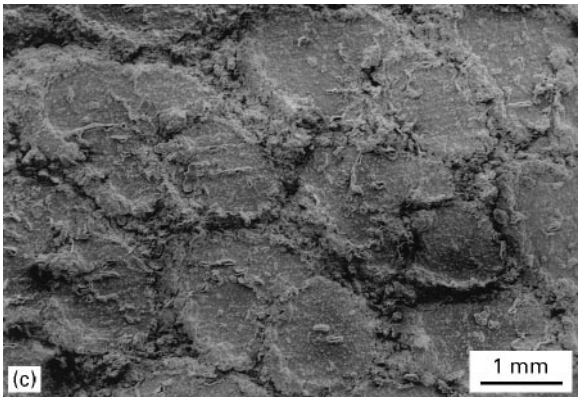
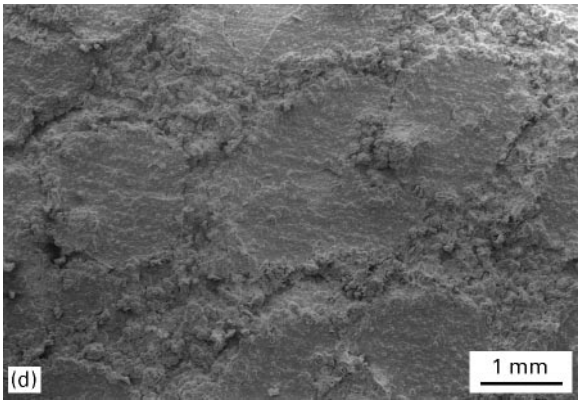


Figure 5 (Continued).









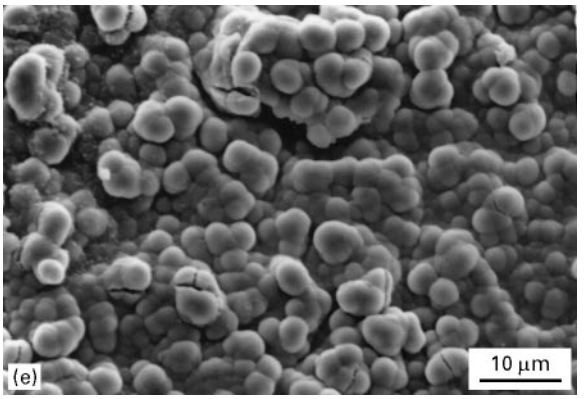


Figure 6 Scanning electron micrographs of $NaOH–H_3PO_4$ phosphorylated bamboo sample after $Ca(OH)_2$ treatment and then (a), (b) soaking in 1.5 SBF for 2 days ((a) cross-section of bamboo culm; (b) high magnification of (a)) (c) soaking in 1.5 SBF for 4 days and (d) soaking in 1.5 SBF for 7 days. (e) Detail of the apatite layer.

by the $NaOH–H_3PO_4$ method show an entirely different result. An apatite layer, composed of numerous spheres, as shown in Fig. 6a, covered the whole area of the samples. Fig. 6b shows a higher magnification of this apatite layer, which was formed on the area of bast fibres. The apatite adhered to bamboo so strongly that even high-speed air could not blow it off. Comparing Fig. 5a and Fig. 6b, which are at the same

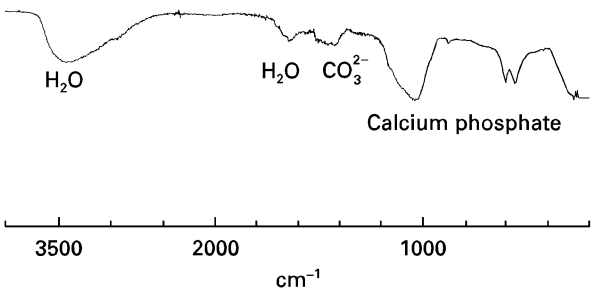


Figure 7 IR spectrum of the apatite formed on phosphorylated bamboo ($NaOH–H_3PO_4$) and then precalcified with $Ca(OH)_2$ solution for 4 days and soaked in 1.5 SBF for 2 weeks.

magnification, Fig. 5a reveals that the areas among bast fibres (thin-walled cells among vascular bundles, and the central part in vascular bundles) were mostly covered by calcium phosphate while, on bast fibres, only a little calcium phosphate can be observed. However, bamboo samples phosphorylated by the $NaOH–H_3PO_4$ method were completely covered by a continuous apatite layer. It seems that, for urea–H_3PO_4-treated samples, apatite that first formed on some spot spread over the surrounding area around but, for $NaOH–H_3PO_4$-treated samples, apatite nucleated on both bast fibre and thin-walled cells, which can be indirectly proved by the fact that the apatite layer cannot easily be blown off by high-speed air. With increase in the soaking time, the apatite was obviously not changed, as shown in Figs 6c and d, which are SEM images of the samples after 7 days in 1.5 SBF. Fig. 6e shows the detailed morphology of the apatite.

3.4. Characterization of the apatite formed on phosphorylated bamboo

The precipitate formed on bamboo sample surface was scratched off for IR analysis; the IR spectrum is shown in Fig. 7. Three characteristic peaks from phosphate-associated vibrational bands at 1031, 601 and 563 cm^{-1} were observed. The peak at 1424.6 cm^{-1} is from carbonate. The absence of a sharp peak at 3500–3600 cm^{-1} due to apatite hydroxy groups suggested that the precipitate is amorphous. There also appears, however, to be water as evidenced by the broad (OH banding)–(OH) stretching peak at 3388 cm^{-1} and the peak at 1654 cm^{-1} due to the bending mode of water.

The thin-film XRD spectrum is shown in Fig. 8a and gives evidence that the calcium phosphate is apatite although it is partly amorphous; assignments of the main peaks in the patterns and spectra were based on the data published in [10]. As a reference, the thin-film XRD spectrum of as-received bamboo is also shown in Fig. 8b.

4. Mechanism of apatite formation on phosphorylated bamboo

Natural bamboo is inert in calcification solution and phosphorylation is one way to improve its bioactivity.

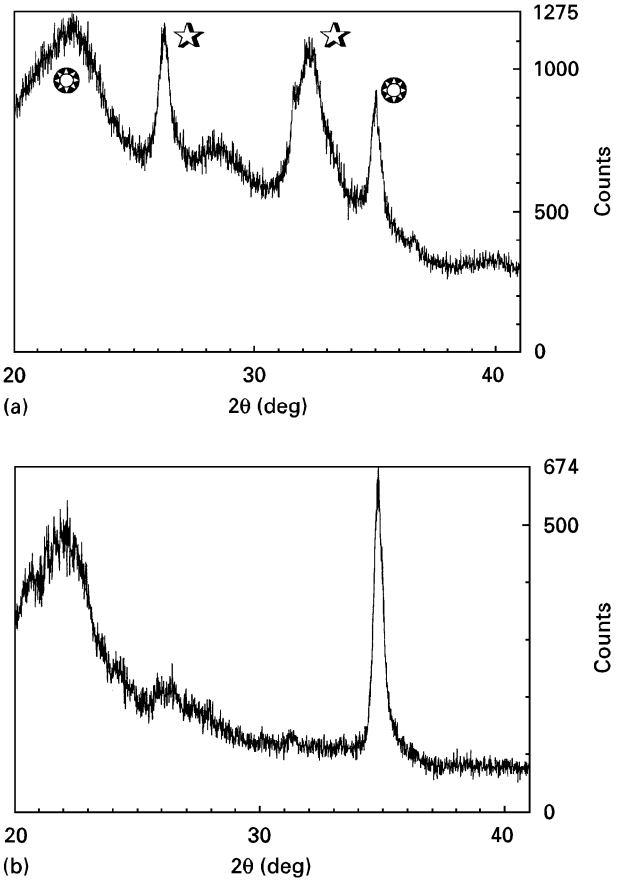


Figure 8 Thin film XRD spectra of (a) the apatite formed on $NaOH–H_3PO_4$ phosphorylated bamboo (❂), apatite; (☆), bamboo and (b) as received bamboo as a reference .

The phosphate groups incorporated in bamboo by phosphorylation are thought to have a role in the nucleation of mineral crystals. A similar process occurs in biomineralization. In general, biominerals are formed by the precipitation of calcium carbonate, calcium phosphate and other minerals within polymeric tissue matrices. It is thought that the organic polymer tissue is the key to the microstructural control found in many bio-organisms. The polymer matrix is formed extracellularly and consists of two components: an underlying collagen sheet and individually attached macromolecules. All these macromolecules contain acidic groups, such as phosphate groups on proteins found in bone and teeth [11]. *In-vivo* studies suggest that nucleation is initiated by the adsorption of cations on to the functional sites of acidic macromolecules [12] which promotes the formation of critical nuclei.

NaOH treatment will break the van der Waals and hydrogen bond between cellulose molecules fibres in bamboo, which causes more cellulose molecules to become exposed to the H_3PO_4.

On both urea–H_3PO_4 and $NaOH–H_3PO_4$ phosphorylated $Ca(OH)_2$-treated samples, no visible calcium phosphate clusters were found. Thus the apatite precipitation was not induced by a source of solid dissolvable calcium phosphate formed at a high pH, differing from the case of cotton fibres [6, 13]. This may be due to the compositional and anatomical differences between cotton fibre and bamboo.

After phosphorylation, phosphate groups were incorporated into the surface of bamboo samples; thus, during the consequent soaking process of precalcification, the Ca^{2+} concentration on the surface of bamboo sample was increased to a higher level. It is well known that, if the ion activity product $[Ca^{2+}][HPO_4^{2-}]$ is higher than a certain constant, inhomogeneous nucleation will occur. Thus, apatite formation on bamboo suggested a different mechanism from those reported on cotton fibres.

5. Conclusions

Without phosphorylation, natural bamboo is inert in calcification media. Phosphorylation can be performed to bamboo through two ways: urea–H_3PO_4 or NaOH–H_3PO_4 reactions. Precalcification with saturated $Ca(OH)_2$ solution is inevitable before forming apatite in 1.5 SBF. The NaOH–H_3PO_4 phosphorylation method is superior to the urea–H_3PO_4 method in increasing the number of nuclear sites, the speed of apatite formation and the adhesive strength of ceramic coating to bamboo substrate.

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