

In vitro calcium phosphate formation on a natural composite material, bamboo

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A natural self-reinforced composite material, bamboo, is studied for the first time as a biomedical material. Its anatomical structure was investigated and its mechanical properties were measured and compared with those of some common bone-bonding or bone-repairing biomaterials. It is found that, among all kinds of biomaterials, bamboo has the closest modulus of elasticity to human long bone. The cytotoxicity of bamboo was tested using the agar overlay method before and after heat or chemical treatments. The results reveal that ethanol, methanol and toluene can remove toxic leachable components from bamboo to some extent through extraction. After grafting a polymer whose molecule includes poly(ethylene glycol), α,ω -di(aminopropyl)poly(ethylene glycol) 800 on bamboo, bamboo has the ability to form a calcium phosphate coating after being immersed in calcification solution (simulated body fluid and accelerated calcification solution). The characteristics and the morphology of the mineral formed on bamboo were studied by infrared spectroscopy and scanning electron microscopy. © 1997 Elsevier Science Limited. All rights reserved

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Biomaterials now come from all kinds of man-made materials, including polymeric, bioceramic and metallic materials and 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, its modulus of elasticity is still five times higher than that of human bone¹. The low modulus of polymeric materials 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, thus the desire to search for new biomaterials is always rational.

Inspired by accidental events in history, some studies using wood as implant material were done², revealing that adverse cell reactions occurred due to leachable constituents despite functional restoration and painless utilization of the limb. Besides wood, bamboo is a widespread plant family occurring on all continents. Owing to its importance, much work has been done concerning its anatomical structure, chemical composition, various mechanical properties and even from the viewpoint of biomimetics³.

Bamboo is composed of cellulose and hemicellulose (45.3%), lignin (2.5%), polyoses (24.3%) and extractive

(2.6%), as well as some minerals⁴. Compared with wood, bamboo has the following advantages if used as a biomaterial. (1) Structural similarity with human long bone. Bamboo has a gradient structure along the radial direction. The structure of bone shows the same features from the outer cortical to the inner cancellous bone regions. (2) The average modulus of elasticity across the thickness of bamboo culm can be 18 GPa, which is equal to that of human cortical bone. Besides this, the wide span of the distribution of its mechanical properties provides more choices even from one bamboo culm. (3) The longitudinal tensile, bending and compressive strengths of bamboo are higher than those of wood, which is expected for load-bearing bone repairing or replacing biomaterial. (4) In the case of popular and birth wood, bone ingrowth was found in the larger pores², so the porous structure is beneficial to the ingrowth and anchorage of bone. The structure of bamboo has a wide variety of pores (sieve tube, vessel and thin-walled cells) as compared to timber, possibly providing more chances for the ingrowth of bone. (5) Contrary to wood, bamboo contains some silicon in both the inner surface (Pith-ring) and outer surface (Rind) of the bamboo culm, and it is known that silica may play a positive role in the process of bone mineralization⁵; the effects of silica in bioglass and glass-ceramic are also well known^{6–8}. The author's recent work proved that the silica in bamboo has the ability of inducing precipitation of calcium phosphate after removing surface fatty substances using chemical methods.

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Based on the advantages mentioned above, a study was undertaken to evaluate bamboo as a biomaterial in the present work. The cytotoxicity of bamboo was tested using the agar overlay method before and after heat treatment or extraction with some organic solvents (ethanol, methanol and toluene). The results reveal that some organic solvents can remove the cytotoxic components from bamboo.

The generation of a reactive surface apatite layer is a feature shared by all bioactive bone-bonding biomaterials⁹⁻¹², so for the formation of a bone-bonding bamboo *in vivo*, forming an apatite layer on it is a necessary prerequisite. In this work, bamboo was firstly grafted with α,ω -di(aminopropyl)poly(ethylene glycol) 800 (NH₂-PEG-NH₂) and then soaked in two kinds of calcification solutions, accelerated calcification solution (ACS) and simulated body fluid (SBF) at 37°C, the final result being that a continuous layer of calcium phosphate was formed on the surface layer of the bamboo.

MATERIALS AND METHODS

Anatomy and mechanical properties of bamboo

All the bamboo used in the present work was *Phyllostachys bambusoides*, purchased in the Netherlands (Edo Plant-Holland). The bamboo culm can be divided into two parts, internode and node, having different structures, as shown in Figure 1A. Bamboo's nodes increase its structural rigidity and stability¹³. The internode possesses a gradient structure in the radial direction (Figure 1B), caused by the gradient distribution of vascular bundles. A vascular bundle consists of fibre strands, sieve tubes and vessels, as shown in Figure 1C. The fibre strands play a dominant role in bearing mechanical loads.

In this work, we separated the bamboo culm into several layers and measured the mechanical properties of each layer separately. The plate samples for tensile modulus and tensile strength measured $160 \times 12 \times h$ mm³, h being the thickness, and the working span was 80 mm; the two ends of the tensile sample were sandwiched with thin bamboo plates to avoid stress concentration. Tests were performed on a Hounsfield testing machine at room temperature. The crosshead speed was 2 mm min⁻¹. The samples for compressive strength were prisms measuring $10 \times 10 \times 20$ mm³. The longest dimension was along the longitudinal direction. The crosshead speed in comparison was set at 1 mm min⁻¹.

Cytotoxicity test

The agar diffusion test (48 h incubation) was performed as a cytotoxicity test. Three kinds of bamboo samples were prepared for cytotoxicity. (1) As-received bamboo without any treatment. Firstly, the bamboo internode was sliced into layers 1 mm in thickness, then these layers were cut in squares of 1×1 cm². (2) Sliced thin-layer samples were extracted with several organic solvents at room temperature for 2 days and then in a Soxhlet extractor for 2 days. The organic solvents were 100% ethanol, methanol and toluene. After extraction, the samples were rinsed thoroughly with culture working solution to remove the extraction solvents from bamboo. (3) As-received bamboo was heated at

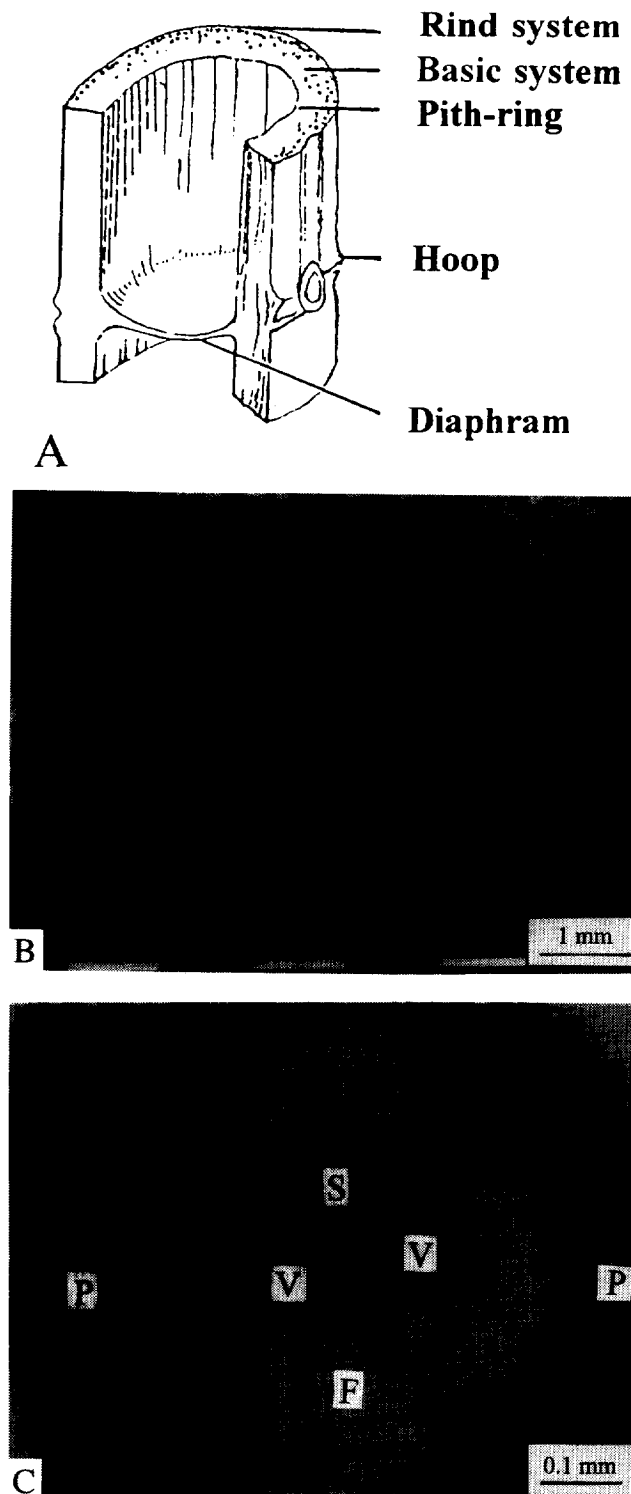


Figure 1 Schematic diagram of bamboo structure (A), bamboo culm (B) and cross-section (C) of the vascular bundle. V, vessels; S, sieve tube; F, fibre strand; P, ground parenchyma tissue around vascular bundle.

180°C for 2 h; its colour turned dark after heat treatment. It was then sliced into 1 mm layers and cut as 1×1 cm² squares.

All samples were sterilized by gamma ray radiation. Human skin fibroblasts were used as test cells. Latex rubber sheets (Hilversume Rubberhandel BV) and ultra-high-molecular-weight polyethylene (Goodfellow, Cambridge, UK) were used as positive and negative controls, respectively.

The cytotoxicity test was performed according to ASTM F813-83 and ISO/TR 7405. The agar medium (0.5 ml) was pipetted on the monolayer and slightly gelled. The test samples were placed on the gel, pressed down lightly and the remaining 1.5 ml was added. Test samples and negative controls were incubated in triplicate, positive controls at least once. The plates were placed in a CO₂ incubator at 37°C and 5% CO₂.

After 48 h, the cell cultures were evaluated for morphology, zone of affected cells around the sample, intracellular granulation or fatty degeneration related to the negative control.

Calcification behaviour of as-received bamboo in ACS and SBF

Two kinds of calcification solution were used: SBF and ACS. The composition of SBF was (mM): Na⁺ 142, K⁺ 5.0, Ca²⁺ 2.5, Mg²⁺ 1.5, Cl⁻ 147.8, HCO₃⁻ 4.2, HPO₄²⁻ 1.0 and SO₄²⁻ 0.5. ACS was developed in this laboratory¹⁴; its composition was (mM): Na⁺ 136.8, Cl⁻ 144.5, Ca²⁺ 3.87 and PO₄³⁻ 2.32. The solution was buffered in 50 mM Tris buffer (pH 7.4) at room temperature.

Bamboo internode was sliced into 1-mm-thick plates, across the axial direction of bamboo culm, then these plates were cut about 10 mm wide. Two groups of samples were soaked in 30 ml ACS or SBF, respectively; there were three samples in each group. The samples soaked in ACS were taken out at day 3, and those in SBF were taken out after 2 weeks. All samples were then rinsed thoroughly with demineralized water, dried and coated with carbon for scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDX).

Grafting with α,ω -di(aminopropyl)poly(ethylene glycol) 800

Bamboo internode was cut into 1-mm-thick plates, then extracted with 100% ethanol at room temperature for 5 days and with toluene for 20 h before grafting. Bamboo samples were dried at 40°C for 2 h and then at 120°C for 2 days. To graft NH₂-PEG-NH₂, bamboo samples (0.23 g) were first treated with a solution of toluene and γ -glycidoxypropyl trimethoxysilane for 8 h, then rinsed with toluene followed by treatment with a 10% solution of NH₂-PEG-NH₂ in dimethyl sulphoxide (DMSO) at 80°C overnight. Finally, the samples were rinsed thoroughly with ethanol and acetone to remove the unreacted NH₂-PEG-NH₂ and dried at 60°C for 1 h.

Untreated bamboo samples were used as control. They were soaked in 20% PEG 1000 solution for 2 h; after thoroughly rinsing with 100% ethanol and distilled water, samples were subjected to the same calcification procedure as the as-received bamboo samples and, after 3 days in ACS and 2 weeks in SBF, samples were taken out and dried. Carbon was coated for SEM analysis.

Formation of a continuous layer of calcium phosphate

Grafted bamboo samples were cut into 1 × 3 × *h* mm³ (*h* is the bamboo culm thickness) pieces and put into 30 ml of ACS or SBF, in a sealed polystyrene disposable

beaker, and shaken in a water bath at 37°C. Samples in ACS were taken out at 1 and 2 days; samples in SBF were taken out at 2 weeks. After carefully rinsing in distilled water and drying, the samples were carbon coated for SEM observation. Some precipitate on the surface of the samples was scratched off for infrared analysis.

RESULTS AND DISCUSSION

Corresponding to the gradient structure of the bamboo internode, the mechanical properties of bamboo show a non-linear gradient along the radial direction. The distributions of tensile modulus and strength are shown in Figure 2. Figure 3 shows a typical load-displacement curve for bamboo in a compressive test. It indicates that bamboo, different from the isotropic brittle materials, does not break abruptly, the drop from the maximum load being caused by the buckling of the bamboo fibres. After buckling, the load can be maintained at a lower value. Mean values of strength and modulus, averaged from three samples, are listed in Table 1. The mechanical properties of human long bone¹⁵ and some common biomaterials are also listed¹⁶⁻¹⁸ for comparison.

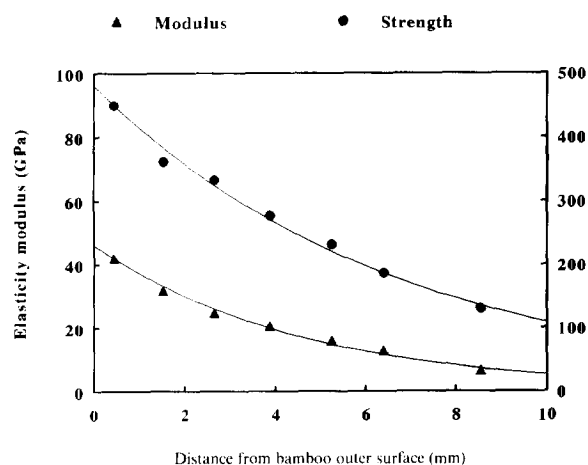


Figure 2 The gradient of changing trends in bamboo's mechanical properties.

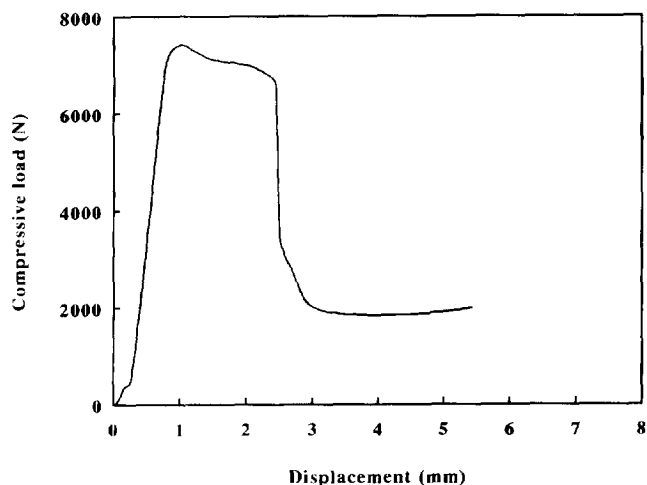


Figure 3 Load-displacement curve of bamboo in the compressive process.

Table 1 Comparison of the mechanical properties of human bones, some common biomaterials and bamboo

	Direction of test	Modulus of elasticity (GPa)	Tensile strength (MPa)	Compressive strength (MPa)
Leg bones ¹⁵				
Femur	Longitudinal	17.2	121	167
Tibia	Longitudinal	18.1	140	159
Fibula	Longitudinal	18.6	146	123
Arm bones ¹⁵				
Humerus	Longitudinal	17.2	130	132
Radius	Longitudinal	18.6	149	114
Ulna	Longitudinal	18.0	148	117
Bamboo	Longitudinal	18.4	237	105
Calcium phosphate ¹⁶		40–117	—	294
Titanium ¹⁷		115	340	—
Hydroxyapatite ¹⁸		90	120	40–500

Table 2 Cytotoxicity test results for bamboo before and after heat treatment or chemical extraction

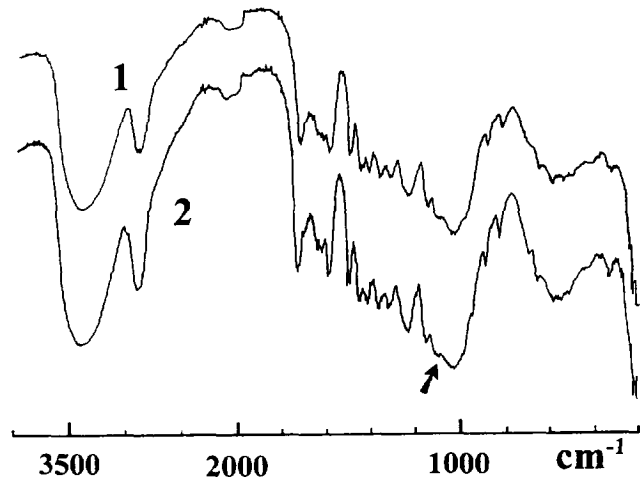
Material	As-received bamboo	Ethanol treated	Methanol treated	Toluene treated	Heated at 180°
Result	Fail	Pass	Retest	Retest	Fail

In *Table 1*, the most notable observation is that the modulus of elasticity of bamboo is equal to that of human bone. The compressive buckling strength of bamboo is somewhat lower than that of bone, the tensile strength being considerably higher. From the viewpoint of bone replacement materials, the longitudinal mechanical properties make bamboo probably the most suitable biomaterial to be found in nature.

The results of the cytotoxicity test are summarized in *Table 2*, revealing that the as-received bamboo sample (untreated) and the samples after heat treatment at 180°C were cytotoxic. The heat treatment cannot remove the cytotoxic leachable components effectively, even though some polymerization processes may occur and the chemical components may change to some extent. Extraction with organic solvents has significant effects. After extraction in ethanol and rinsing in culture medium, bamboo is not cytotoxic in the agar overlay test. However, for the samples extracted with methanol and toluene, the results were 'retest', which means that samples had mild reactivity with cells. Therefore, ethanol is suggested to be the recommended extracting solvent.

After soaking in ACS for 3 days or in SBF for 2 weeks, there was no calcium phosphate formed on bamboo detectable by SEM-EDX, which means that as-received bamboo is inert in calcification media. Furthermore, for the bamboo samples directly soaked in 20% PEG 1000 solution, only a few separate particles of Ca/P mineral could be observed after 3 days in ACS, which means that the bamboo is still inert.

After grafting, the dry weight of the samples increased by about 20%. The IR spectra also show the difference before and after grafting polymer. *Figure 4* shows the IR spectra of bamboo as-received (*Figure 4a*) and after grafting with NH₂-PEG-NH₂ (*Figure 4b*). The peaks at 955 and 710 cm⁻¹ in *Figure 4b* are due to the grafting; the peak at 1100 cm⁻¹ is from CH₂-O-CH₂, which can only come from PEG.

**Figure 4** IR spectra of bamboo. 1, Before grafting (as-received). 2, After grafting with NH₂-PEG-NH₂.

After being soaked in ACS for 1 day, some mineral deposit could be observed on both the cross-section and longitudinal section of bamboo samples. However, the situation was obviously different between fibre strands and ground parenchyma tissue (as shown in *Figure 5*). The ceramic grew on parenchyma tissue (thin-walled cells) much more than on fibre strands. The SEM-EDX spectrum showed that the ceramic consisted mainly of calcium, phosphorus and oxygen, as shown in *Figure 6*. *Figure 7(1)* shows the IR spectrum of the mineral formed on a bamboo cross-section in ACS after 3 days. Peaks attributable to HPO₄²⁻ (868 cm⁻¹), PO₄³⁻ (1030, 1080 cm⁻¹) and CO₃²⁻ (1400–1500 cm⁻¹) could be observed. For comparison, the IR spectrum of commercial hydroxyapatite (HA) is also given in *Figure 7(2)*. The mineral is apparently carbonate-containing calcium phosphate.

On the grafted bamboo samples, a continuous layer of ceramic was formed in SBF. *Figure 8A* shows the longitudinal section of bamboo, indicating that the



Figure 5 Scanning electron photograph of the ceramic formed on the cross-section of bamboo after grafting with $\text{NH}_2\text{-PEG-NH}_2$ and then soaking in ACS for 1 day.

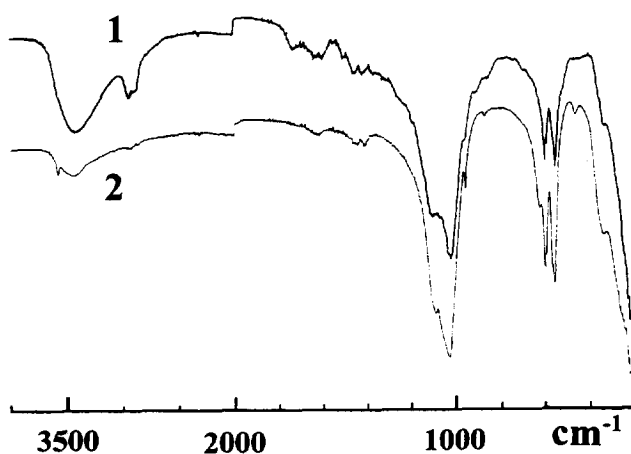


Figure 7 IR spectra of: 1, the ceramic formed on bamboo cross-section after immersion in ACS for 3 days; 2, commercial HA (non-sintered).

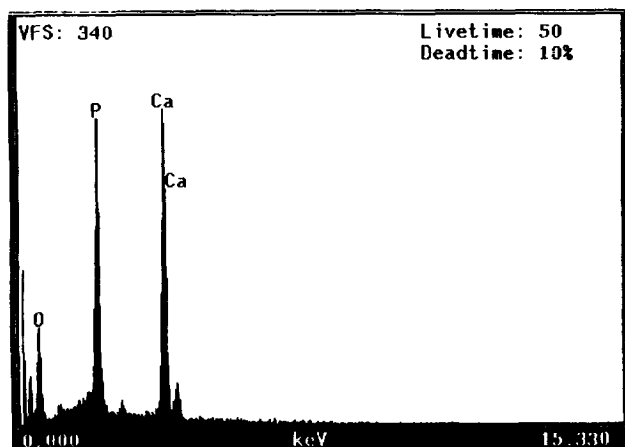


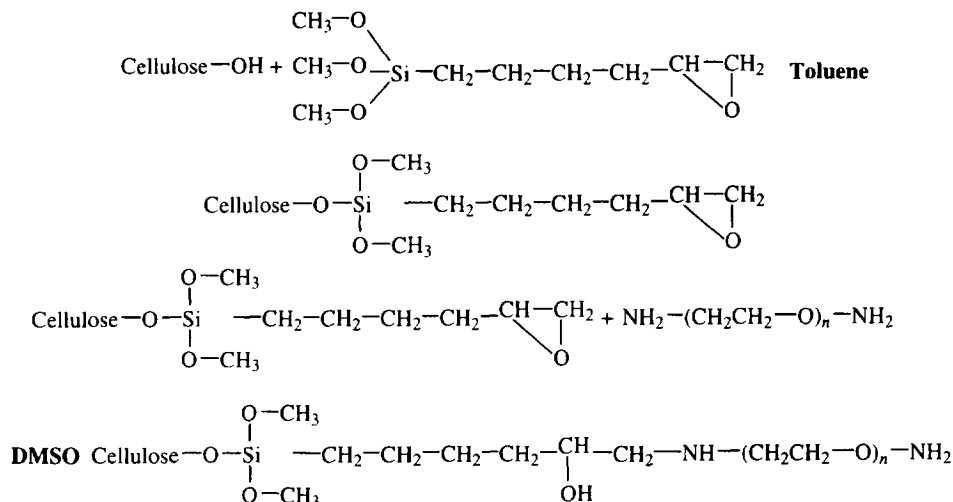
Figure 6 SEM-EDX spectrum of the ceramic layer formed on a bamboo sample after grafting with $\text{NH}_2\text{-PEG-NH}_2$ and soaking in ACS for 1 day.

mineral was formed on both fibre strands and thin-walled cells. *Figure 8B* shows that the ceramic densely covered the entire cross-section of bamboo; even its surface has a very complicated morphology. *Figure 8C* shows the ceramic morphology on bamboo fibres in

the vascular bundle, where apparently it was more difficult for the mineral to precipitate from ACS. *Figure 8D* shows the thin-walled cells covered with ceramic, from which it can be concluded that the mineral crystal even grew inside the chambers of cells. This is important since the bamboo's porous structure may induce the ingrowth of bone. SEM-EDX analyses revealed that the ceramic includes calcium, phosphorus and oxygen (*Figure 9*), identifying it as calcium phosphate.

As mentioned before, without any chemical modification or functionalization, bamboo is inert in calcification solutions (ACS and SBF). Only after the grafting of PEG does bamboo have the ability to form a calcium phosphate layer *in vitro*. This can be explained as follows: the major chemical components of bamboo are cellulose and lignin; both have OH in their molecules. In the grafting process, the reactions shown in *Scheme 1* occur.

After grafting, PEG was covalently bound to bamboo. It is well known that PEG has the ability to facilitate calcification, because the polyether soft segment polymer in PEG will cause metal-ion chelation¹⁹⁻²². Hamon *et al.*²³ concluded that a helical arrangement of the PEG backbone resulted in inwardly-directed oxygen atoms, allowing complexa-



Scheme 1

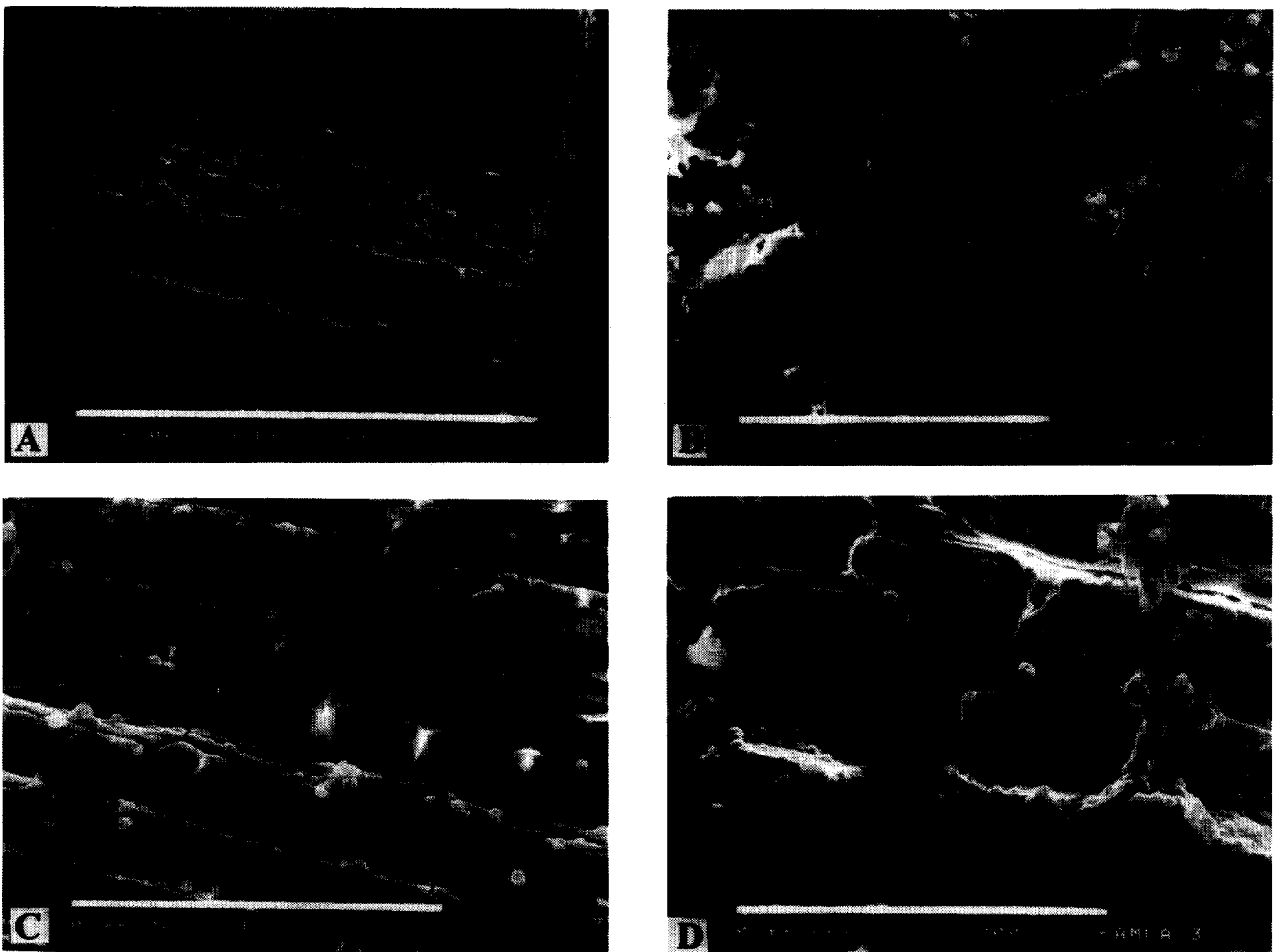


Figure 8 Scanning electron photographs of the ceramic formed on bamboo in SBF for 2 weeks after grafting with $\text{NH}_2\text{-PEG-NH}_2$. **A**, The longitudinal section of bamboo. **B**, Cross-section of bamboo. **C**, Longitudinal photo of fibre. **D**, Longitudinal photo of the parenchyma tissue (thin-walled cells).

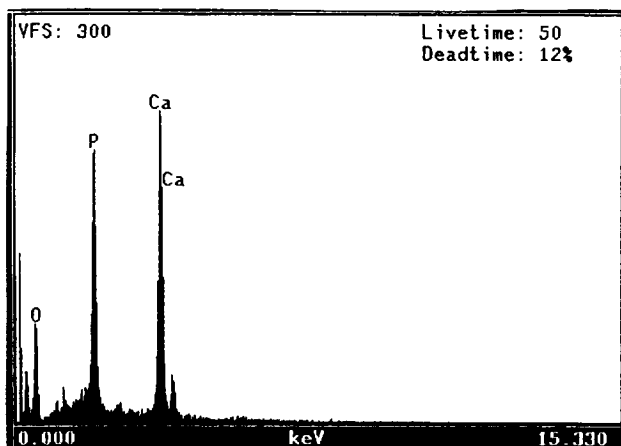


Figure 9 SEM-EDX spectrum of the ceramic layer formed on a bamboo sample after grafting with $\text{NH}_2\text{-PEG-NH}_2$ and soaking in SBF for 2 weeks.

tion between cations and the polymer. Because of the Ca^{2+} complexing ability of the PEG segment, the Ca^{2+} concentration within the grafted PEG layer on the bamboo surface would be higher than that in solution. It is well known that if the ion activity product $[\text{Ca}^{2+}][\text{HPO}_4^{2-}]$ is higher than a certain constant, inhomogeneous nucleation will occur. Thus,

nucleation most probably occurred on the bamboo surface, where a higher Ca^{2+} concentration was available. After bamboo was soaked in the calcification solution, Ca^{2+} ions from the solution could first be chelated onto the surfaces by the PEG molecules, and this increased the Ca^{2+} concentration and thus the ion activity product $[\text{Ca}^{2+}][\text{HPO}_4^{2-}]$. Consequently, calcium phosphate will precipitate onto the surfaces of bamboo. Due to the chemical bond between PEG and bamboo, and chelation between PEG and Ca^{2+} , we expect the bonding strength between calcium phosphate and bamboo to be strong enough for practical application. Further experiments are planned to verify this.

CONCLUSIONS

As a natural self-reinforced composite, the mechanical properties of bamboo, especially those in the longitudinal direction, are closer to those of human bone than those of wood. Bamboo has a gradient structure and correspondingly a gradient distribution of mechanical properties along the radial direction. Ethanol, methanol and toluene can remove cytotoxic leachable components to some extent from bamboo, as verified in an agar overlay cytotoxicity

test. When PEG in the form of α,ω -di(aminopropyl)poly(ethylene glycol) 800 (NH₂-PEG-NH₂) was grafted onto bamboo, it enabled the bamboo to form a continuous layer of calcium phosphate *in vitro*, in calcification solutions.

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