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Growth of calcium phosphate on phosphorylated chitin fibres

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Calcium phosphate growth on chitin phosphorylated fibres was studied using scanning electron microscopy and energy dispersive X-ray analysis (SEM, EDX), micro-Fourier transform infrared spectroscopy (FTIR), and solid state magic angle spinning nuclear magnetic resonance (MAS NMR) techniques. The C6 chemical shift positions of ^{13}C MAS NMR in the chitin fibres phosphorylated using urea and H_3PO_4 are obvious indicating that phosphorylation takes place not in the C1 but in the C6 region. Micro-FTIR and ^{31}P MAS NMR suggested that ammonium hydrogen phosphate formed during the phosphorylation procedure. Chitin fibres phosphorylated using urea and H_3PO_4 and then soaked in saturated $Ca(OH)_2$ solution at ambient temperature, which lead to the formation of thin coatings formed by partial hydrolysis of the PO_4 functionalities, were found to stimulate the growth of a calcium phosphate coating on their surfaces after soaking in $1.5 \times$ SBF solution for as little as one day. The thin layer after $Ca(OH)_2$ treatment functioned as a nucleation layer for further calcium phosphate deposition after soaking in $1.5 \times$ SBF solution. EDX-measured Ca : P ratios of the coatings of $Ca(OH)_2$-treated phosphorylated chitin in $1.5 \times$ SBF solution suggested that calcium-deficient apatite was formed.

1. Introduction

Extensive research has been undertaken into organic polymer substrates designed to enhance the growth of calcium phosphate (hydroxyapatite) on their surface, because of the contribution that such investigations can make towards the understanding of biomineralization processes. Research into the stimulation of hydroxyapatite growth on a substrate has employed a number of techniques including a procedure for raising the ionic activity of hydroxyapatite products in the solution containing the substrate to be coated so that stimulation precipitation and the creation of apatite nucleation sites can occur [1], and the surface modification by surface graft polymerization of a phosphorous-containing monomer inducing the deposition of Ca and PO_4 ions in the form of a carbonated hydroxyapatite layer firmly bonded with the materials [2].

We studied a useful method for creating favourable local conditions leading to the nucleation and growth of calcium phosphate on cellulose cotton fibre [3, 4]. This method of phosphorylation and partial hydrolysis of introduced phosphorous-containing functionalities by $Ca(OH)_2$ treatment resulted in hydrolysis products in intimate contact with the substance.

In this present study, chitin fibres were used as the substrate (instead of cellulose) on which to grow calcium phosphate using a similar procedure. Chitin, a polymer of 2-acetamido-2-D-glucose, is the principal constituent of the shells of crabs, lobsters, and other crustaceans. It is also observed in the cell membranes of fungi and various bacteria. Chitin, very similar to cellulose in structure, also reacts in many ways similarly to cellulose, and is also biodegradable and nontoxic. The chitin/hydroxyapatite system has potential use as a virus filter given the adsorptive properties of hydroxyapatite [5] which may allow the attachment of drugs for future treatment of a serious viral disease. Results of the calcium phosphate growth on chitin fibres phosphorylated using the urea/H_3PO_4 method and subsequently soaked in saturated $Ca(OH)_2$ solution for approximately one week and in $1.5 \times$ SBF solution (1.5 times simulated body fluid solution) for various soaking times will be presented. Characterization tests will be carried out using SEM/EDX, micro-FTIR, TF-XRD, ICP and MAS NMR to determine the presence of calcium phosphate on the chitin fibres.

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2. Experimental

2.1. Chemicals

All chemicals used in this study were supplied by Wako Pure Chemical Industries, Ltd. or Katayama Chemicals and used without further purification. Commercial chitin (Beschitin-W) was supplied by Unitika, Ltd. N_2-purged water obtained from distilling water previously passed through an ion-exchange resin was used in the preparation of all aqueous solutions.

The calcium phosphate growth medium used in all cases was $1.5 \times$ SBF (simulated body fluid) solution, a precise description of which is published in earlier reports [3, 4]. This solution is prepared by dissolving NaCl, KCl, $CaCl_2$, $NaHCO_3$, K_2HPO_4 and Na_2SO_4 in distilled water together with Tris$[(CH_2OH)_3CNH_2]$ and HCl which acted as buffering agents keeping the pH of the solution within the range 7.10–7.50 during the soaking procedure.

2.2. Phosphorylation of chitin

The phosphorylation of the chitin samples was carried out following the method described in earlier reports [3, 4]. Twelve sheets (1–2 g) of chitin were placed in a round-bottomed flask equipped with a thermometer, mechanical stirrer, condenser, and N_2 gas inlet tube. 40 g of urea was added to the flask along with 200 ml of dimethyl formamide (DMF). This solution was heated to a temperature of 110 °C at which point a solution of 32 g of 98% H_3PO_4 in 50 ml DMF was added. After further heating, the temperature of the reaction was increased to 155 °C and left to reflux for 1 h. At the end of the refluxing time, the reaction mixture was cooled under flowing N_2 gas. The reaction solution was subsequently filtered off and the fibres thoroughly washed in water to remove any excess phosphoric acid.

2.3. $Ca(OH)_2$-treated phosphorylated chitin

The phosphorylated chitin fibres were soaked in saturated $Ca(OH)_2$ solution (pH = 12.4) for 8 days. The $Ca(OH)_2$ solution was renewed every 4 days. After completion of the soaking period, the $Ca(OH)_2$-treated phosphorylated chitin fibres were washed, filtered and dried under vacuum at 60 °C.

2.4. Soaking in $1.5 \times$ SBF solution

Samples 0.05–0.1 g of $Ca(OH)_2$-treated phosphorylated chitin fibre were placed in a plastic screwtop flask to which 10–20 ml of $1.5 \times$ SBF solution was added. The pH of the chitin/$1.5 \times$ SBF solutions was then measured after the flasks were immersed in a covered water bath thermostatically controlled at 36.5 °C for periods of 1, 2, 4, 6, 9, 10 and 17 days. For soaking periods of greater than one day, the $1.5 \times$ SBF solution was replaced each day and the pH of the old solution measured. After they were soaked for various periods, the chitin fibres were removed from the SBF solution, washed with distilled water, and then dried at 60 °C under vacuum.

2.5. Instrumentation

Scanning electron microscopy and EDX analyses were performed using a Hitachi S-530 scanning electron microscope and a Horiba EMAX-2200 X-ray microanalyser. All micro-FTIR spectra were recorded using samples encased in a transparent KBr matrix on a Jasco Micro-FTIR Jansen Fourier transform infrared spectrometer and the ATR (attenuated total reflectance) technique was also applied due to the difficulty in obtaining a clear spectra of calcium phosphate by the KBr technique. Thin film X-ray diffraction patterns were analysed using a Mac Science MXP3 X-ray diffractometer at 40 kV and 20 mA Cu target with a 1° angle of incidence. All ^{13}C and ^{31}P MAS NMR spectra were recorded on a Bruker MSL-200 NMR spectrometer using a magic angle spinning rate of 3 Hz and referenced to 85% H_3PO_4. Spectra were obtained after an accumulation of 500–3000 transients using pulse delays ranging from 6–50 s. In order to produce spinnable samples, it was necessary to cut the samples into small pieces approximately 2 mm in diameter for efficient packing in the NMR sample holders. The determination of the P content of the chitin fibre was carried out using a Nippon Jarrell-Ash ICAP-1000S ICP-AES instrument. Each phosphorylated chitin sample was dissolved in a mixture of water, H_2O_2 and H_2SO_4 and then diluted prior to the analysis.

3. Results and discussion

3.1. Characterization of the phosphorylated chitin fibres

EDX spectra of the phosphorylated chitin fibres clearly showed that P was present in the chitin fibres. The ICP measured phosphorous content of the phosphorylated chitin was found to be at 2.95 wt % and lower than the phosphorus content of phosphorylated cellulose fibres [4]. During phosphorylation, the chitin fibres may possibly decompose causing a decrease in the phosphorus content. During the reaction, the chitin fibres did not retain their original forms and showed a tendency to become loose in the gel. After drying, their form seemed to be restored and also appeared unchanged from that of the unphosphorylated chitin fibres as shown in Fig. 1 but were somewhat weaker.

Fig. 2 gives the micro-FTIR spectra of the unphosphorylated chitin (a) and phosphorylated chitin (b). The typical chitin band at 1000 cm^{-1} band due to C=O stretching on the unphosphorylated chitin was overlapped by the P–O stretching band at 1050 cm^{-1} from PO_4 groups chemically bonded to the chitin. The presence of a small peak at 800 cm^{-1} may also be attributed to the P–O bending band after phosphorylation of the chitin fibre. The presence of observable peaks at 3200 cm^{-1} and 1000 cm^{-1} may be due to the formation of ammonium hydrogen phosphate. The amide I band at about 1655 cm^{-1} and amide II band at about 1555 cm^{-1} gives the amino content in chitin. A broad 3000–3200 cm^{-1} band due to N–H and O–H stretching with small shoulders at about

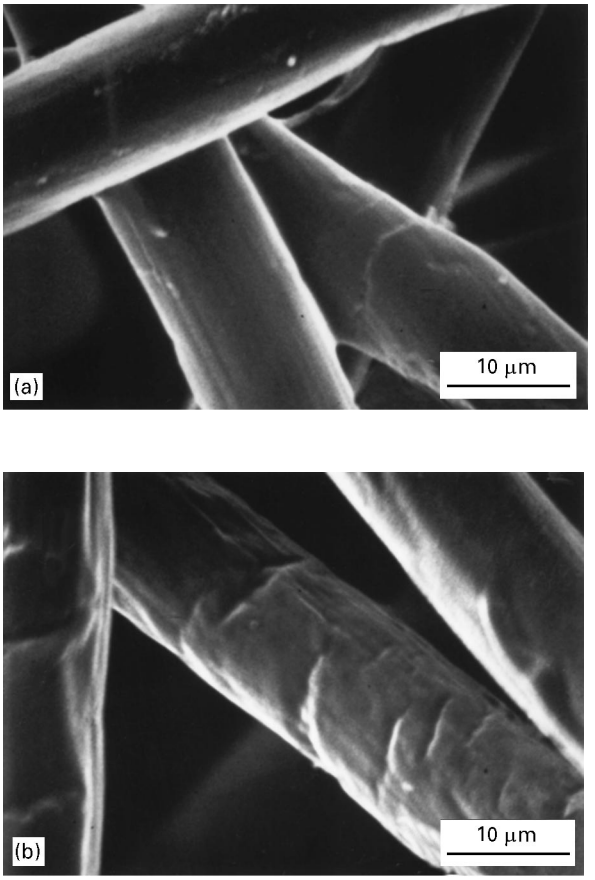

Figure 1 Scanning electron micrographs of (a) unphosphorylated chitin (as-received) fibres and (b) phosphorylated chitin fibres.

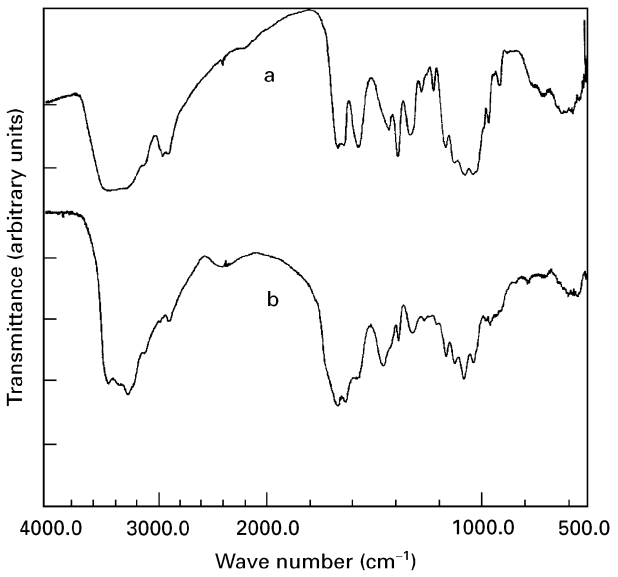

Figure 2 Micro-FTIR spectra of (a) unphosphorylated chitin (as-received) fibres and (b) phosphorylated chitin fibre.

3106 cm^{-1} band is observed for the unphosphorylated chitin. After phosphorylation, observable bands appear in this region which may be due to the crystallization of a small amount of hydrogen phosphate.

^{13}C and ^{31}P MAS NMR spectra revealed the conformational behaviour of unphosphorylated and phosphorylated chitin as shown in Fig. 3 and showed some differences from the case of cellulose [3, 4]. In

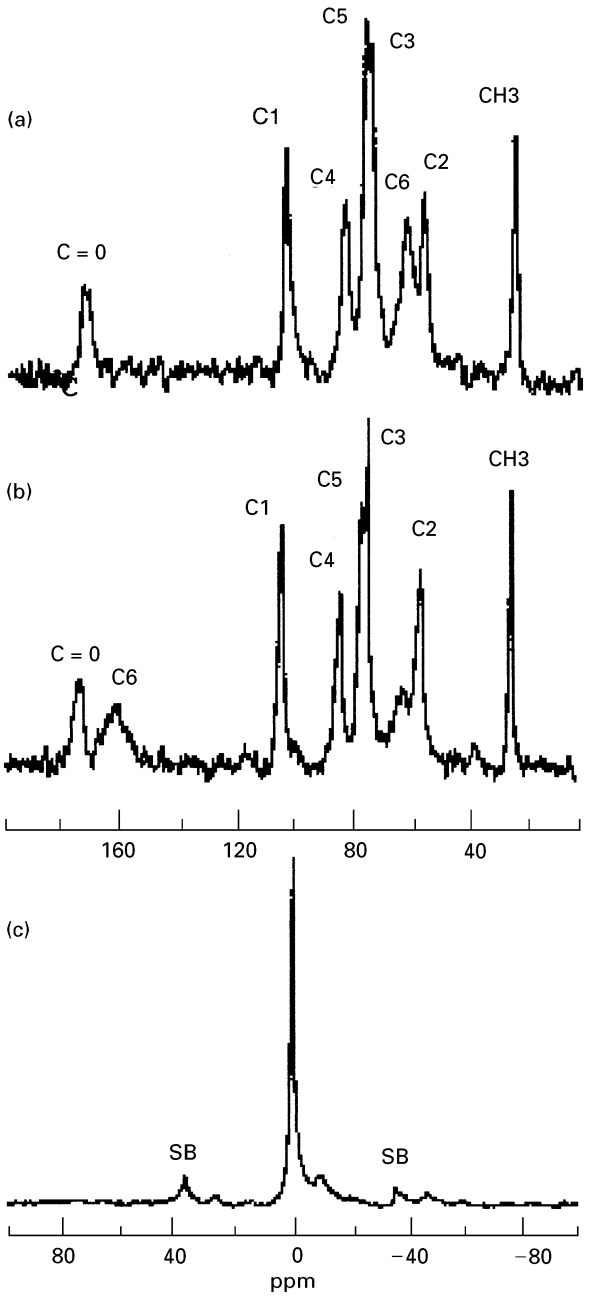

Figure 3 Solid state MAS NMR spectra of chitin samples: (a) ^{13}C NMR spectrum of unphosphorylated chitin; (b) ^{13}C NMR spectrum of phosphorylated chitin; and (c) ^{31}P NMR spectrum of phosphorylated chitin.

the ^{13}C MAS NMR spectrum of phosphorylated chitin, a slight splitting in the peak assigned to C1 was detected suggesting that phosphorylation of the chitin fibre took place at C1 similar to the C4 thus partially breaking down the cellulose structure [3, 4]. In the case of chitin, all carbon signals of the chitin are well resolved resulting in high quality resolution (Fig. 3a). The C-6 chemical shift position in phosphorylated chitin is obvious (shift to higher p.p.m. near C=O) indicating that phosphorylation takes place in this region (Fig. 3b). The sharp peak produced in the phosphorylation could also indicate that more than one type of PO_4 group is present in the phosphorylated chitin. Fig. 3c shows the ^{31}P MAS NMR spectrum of the phosphorylated chitin. The ^{31}P MAS NMR spectrum of the phosphorylated chitin

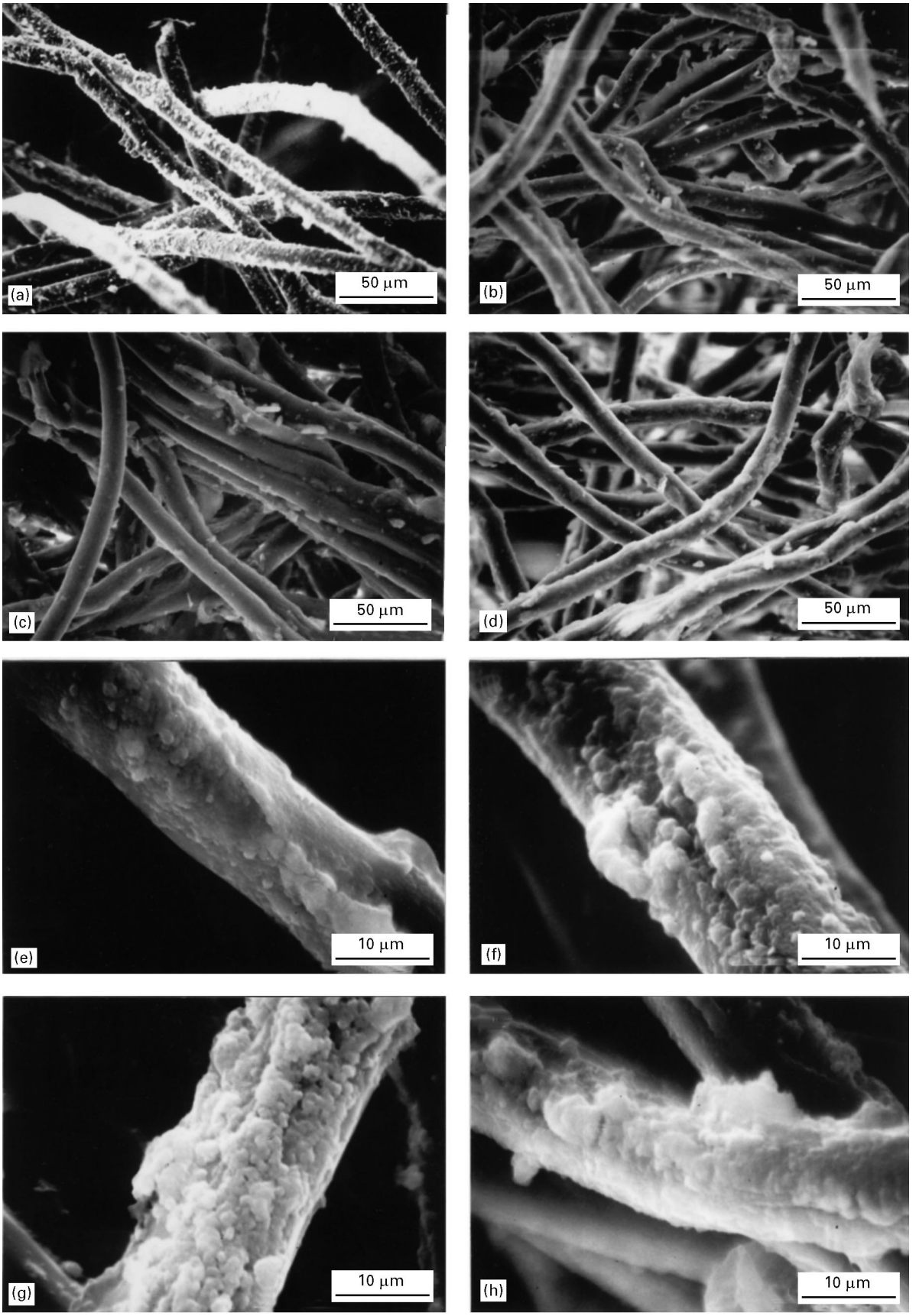

Figure 4 Scanning electron micrographs of sample of (a) phosphorylated chitin fibres after soaking in saturated $Ca(OH)_2$ (pH = 12.4) for one week at room temperature and after soaking in 1.5 × SBF solution at 36.5 °C for (b) 1 day, (c) 2 days, (d) 4 days, (e) 6 days, (f) 9 days, (g) 10 days, and (h) 17 days.

fibres showed an intense and narrow peak at 0.91 p.p.m. indicating that the PO_4 functionalities are attached near the C-6 position. In addition, the presence of a small peak at -9.9 p.p.m. indicates that another compound is formed which is believed to be ammonium hydrogen phosphate. Spinning sidebands (marked by SB) are also observed which are caused by an insufficient magic angle spinning rate, however, this

result is not serious for the present purpose, because such pairs are easily discriminated and are not overlapped with any important signal.

3.2. Soaking in $Ca(OH)_2$ solution

Phosphorylated chitin soaked in $Ca(OH)_2$ solution appeared to be covered with a thin yet observable coating (Fig. 4a). When EDX analysis over a large area was carried out, a Ca : P ratio of 1.78 was obtained indicating a large amount of Ca in the sample, which can be either attributed to $Ca(OH)_2$ or $CaCO_3$. High magnification EDX analyses of the coated fibres gave a Ca : P ratio of 1.29. It has been speculated that at pH > 6.3, octacalcium phosphate (OCP) is the preferred precursor for supersaturation in solutions containing Ca^{2+} and HPO_4^{2-} ions. It is assumed that $Ca(OH)_2$ has partially hydrolysed the chitin-PO_4 groups to produce perhaps OCP as an initial phase that is then rapidly transformed into calcium-deficient apatite. In contrast, phosphorylated chitin fibres not subjected to the $Ca(OH)_2$ treatment did not exhibit calcium phosphate growth upon immersion in 1.5 × SBF solution.

3.3. Soaking in SBF solution

Fig. 4 shows SEM photos of the $Ca(OH)_2$-treated phosphorylated chitin surface after immersion in 1.5 × SBF solution as a function of soaking time in days. Soaking of the $Ca(OH)_2$-treated phosphorylated chitin fibres in 1.5 × SBF solution was found to lead to the deposition of a calcium phosphate layer. It is believed that the thin coatings of calcium phosphate material on the fibres produced by partial hydrolysis of the chitin PO_4 functionalities during soaking in saturated $Ca(OH)_2$ solution act as a nucleation layer upon which the calcium phosphate can grow from the 1.5 × SBF solution. In general, the growth of calcium phosphate from 1.5 × SBF solution begins immediately after 1 day of soaking. This suggests that clusters have partially dissolved upon introduction of the chitin into the 1.5 × SBF solution. The growth of the calcium phosphate layer after soaking for 1–6 days appears to proceed by nucleation on the existing coating in the form of circular flakes which then grow in number and size on the surface. After soaking for 9–17 days, a thicker coating was observed.

Fig. 5 illustrates the EDX measured Ca : P ratio for the calcium phosphate coating as a function of soaking time in 1.5 × SBF solution. Curve (a) shows that the sample has higher Ca : P ratios over a large area which suggests Ca-enrichment due to the presence of calcium carbonate or calcium hydroxide. Curve (b), however, should be interpreted as the characteristic Ca : P ratios of the actual coatings deposited on individual fibres, ranging from 1.29 to 1.55, suggesting Ca-deficient apatite.

Fig. 6 presents the micro-FTIR spectrum of the coating material grown on the chitin fibre after 9 days soaking in 1.5 × SBF solution. Characteristic phosphate associated bands at 1031, 601 and 563 cm^{-1} are observed. The absence of a sharp peak at 3500–

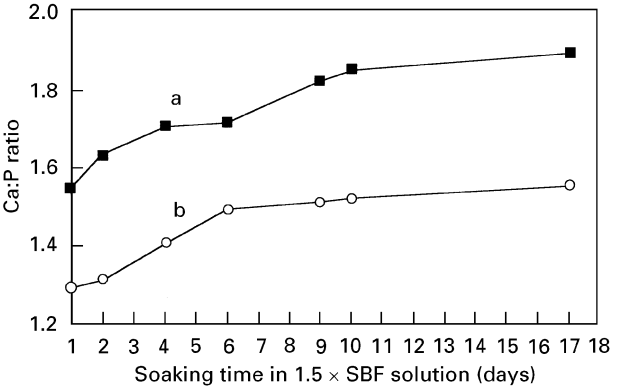

Figure 5 Plot of EDX-measured Ca : P ratios for calcium phosphate coatings soaked in $Ca(OH)_2$ and 1.5 × SBF solution at 36.5 °C. (a) Ca : P ratios over a large area; and (b) Ca : P ratios measured for individual coating at high magnification. All Ca : P ratios are averaged over 10 individual measurements on 10 different areas of samples.

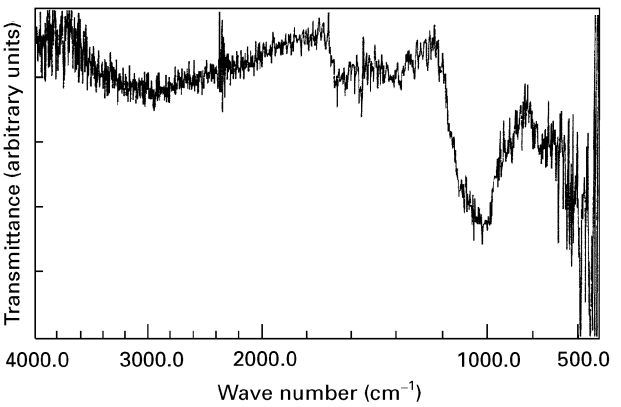

Figure 6 Micro-FTIR spectrum of phosphorylated chitin fibre soaked in $Ca(OH)_2$ for 1 week and 1.5 × SBF solution for 9 days.

3600 cm^{-1} due to apatite hydroxyl groups suggests that the coating is amorphous in nature. Although characterization of the calcium phosphate coating by micro-FTIR in the early stages of growth (after 1–6 days of soaking in 1.5 × SBF solution) was difficult given the thinness of the coatings generated, on the surface of the chitin the spectrum was acquired by the ATR (attenuated total reflectance) method. The coated chitin fibre has a poor characteristic such that it more easily breaks than the cellulose fibre coated in a similar manner [3, 4].

The present study has demonstrated that the technique of phosphorylation and partial hydrolysis of functionalities by $Ca(OH)_2$ treatment to create hydrolysis products in intimate contact with the substrate has been found to be a useful method for creating favourable local conditions leading to the nucleation and growth of calcium phosphate.

4. Conclusions

Calcium phosphate growth on phosphorylated chitin fibres was studied. Chitin fibres phosphorylated using urea and H_3PO_4 and then soaked in saturated $Ca(OH)_2$ solution at ambient temperature, which

leads to the formation of thin coatings formed by partial hydrolysis of PO_4 functionalities, were found to stimulate the growth of a calcium phosphate coating on their surfaces after soaking in $1.5 \times$ SBF solution for as little as 1 day. The thin layer after $Ca(OH)_2$ treatment functioned as a nucleation layer for further calcium phosphate deposition after soaking in $1.5 \times$ SBF solution. A small amount of ammonium phosphate hydrate was also formed after phosphorylation, which may be due to the decomposition of the chitin fibre during the phosphorylation procedure.

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References

1. P. LI, C. OTSUKI, T. KOKUBO, K. NAKANISHI, N. SOGA, K. NAKAMURA and T. YAMAMURO, *J. Mater. Sci. Mater. Med.* **4** (1993) 127.
2. O. N. TRETINNIKOV, K. KATO, Y. IKADA, *J. Biomed. Mater. Res.* **28** (1994) 1365.
3. M. R. MUCALO, Y. YOKOGAWA, M. TORIYAMA, T. SUZUKI, Y. KAWAMOTO, F. NAGATA and K. NISHIZAWA, *J. Mater. Sci. Mater. Med.* **6** (1995) 597.
4. M. R. MUCALO, Y. YOKOGAWA, T. SUZUKI, Y. KAWAMOTO, F. NAGATA and K. NISHIZAWA, *ibid.* **6** (1995) 658.
5. H. AOKI, in "Medical applications of hydroxyapatite" (Ishiyaku EuroAmerica Inc. Tokyo, St. Louis, 1994) p. 144.

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