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Further studies of calcium phosphate growth on phosphorylated cotton fibres

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Further studies using scanning electron microscopy/energy dispersive X-ray analysis (SEM/EDX), micro-Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS) and solid state magic angle spinning nuclear magnetic resonance (MAS NMR) techniques of calcium phosphate growth on $Ca(OH)_2$-treated urea/H_3PO_3- and urea/H_3PO_4-modified cotton fibres are reported. In the case of the $Ca(OH)_2$-treated urea/H_3PO_3-modified fibres which have been reported in an earlier paper, further experiments subjecting the urea/H_3PO_3-modified cotton to alternative soaking treatment procedures to $Ca(OH)_2$ as well as different calcium phosphate growth media such as the alkaline phosphatase-catalysed hydrolysis of disodium p-nitrophenylphosphate to free phosphate have reaffirmed the importance of the $Ca(OH)_2$ treatment step for the stimulus and growth of calcium phosphate growth on the fibres. Studies on cotton phosphorylated by a slightly different method using urea/H_3PO_4 instead of urea/H_3PO_3 show that a phosphorylated cotton with similar properties to the urea/H_3PO_3-modified fibres can be produced. Soaking of these fibres in saturated $Ca(OH)_2$ solution leads to cotton coated with thin layers of calcium phosphate formed by partial hydrolysis of the PO_4 functionalities in the phosphorylated cotton which are believed to act as nucleation layers for further calcium phosphate deposition when the fibres are subsequently soaked in 1.5 × SBF solution. SEM/EDX studies of the calcium phosphate coatings formed on the $Ca(OH)_2$-treated urea-H_3PO_4 fibres as a function of soaking time in 1.5 × SBF show that coatings deposit and become noticeably thick after approximately 9 days. XPS studies indicated the presence of carbonate species in the calcium phosphate coating deposited. In common with the calcium phosphate coated $Ca(OH)_2$-treated urea/H_3PO_3-modified fibres studied earlier, the average EDX-measured Ca : P ratios of the coatings formed on the $Ca(OH)_2$-treated urea/H_3PO_4 fibres are ~1.60 and give very similar micro-FTIR spectra with evidence of carbonate which suggests that amorphous calcium deficient apatite has deposited.

1. Introduction

Earlier, a study was reported [1] in which the growth of calcium phosphate on cotton modified via treatment with urea/phosphorous acid (H_3PO_3) was described. Studies of the stimulation of growth of calcium phosphate (hydroxyapatite) on pre-treated or specially designed substrates are of potential value in the design of biomaterials which more closely resemble the natural tissues in which they come into contact with. However, another potential use of the cotton/hydroxyapatite system is as a virus filter given the adsorptive properties of hydroxyapatite [2] which may allow the attachment of drugs for any future treatment of serious viral diseases.

In the previous study [1], it was found that amorphous calcium phosphate material could be stimulated to form on cotton fibres phosphorylated by the urea/phosphorous acid method after soaking in saturated $Ca(OH)_2$ for approximately one week. The prior $Ca(OH)_2$ treatment was found to produce highly crystalline clusters lodged in the fibres which were confirmed by micro-FTIR to be calcium phosphite monohydrate ($CaHPO_3 \cdot H_2O$). These are believed to slowly dissolve upon introduction of the cotton samples in 1.5 × SBF (1.5 × concentration of simulated body fluid solution) so elevating the Ca^{2+} ion concentration in the vicinity of the fibres and stimulating calcium phosphate formation.

In the present study, results on the calcium phosphate growth on cotton fibres phosphorylated in a different manner (i.e. by urea/phosphoric acid) from the previous investigation [1] will be presented. In addition, continuing investigations on the urea/phosphorous acid modified cotton system are reported. These studies were carried out in order to see whether the $Ca(OH)_2$ treatment step (necessary for the stimulation of calcium phosphate growth on the fibres) could be avoided since soaking in this highly alkaline medium

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tends to weaken the cotton fibres. An additional study involving the use of a different calcium phosphate growth medium with the aim of gaining more information on the mechanism of calcium phosphate growth on the $Ca(OH)_2$-treated urea/phosphorous acid modified fibres is also reported.

2. Experimental procedures

2.1. Chemicals/solutions

The chemicals used in the study were supplied either by Wako Pure Chemical Industries or Katayama Chemicals and were used without further purification. Ciegal Cotton Wipes (100% cotton) as supplied by Chiyoda Co. Ltd were the source of cotton used in experiments. Water purified by first passing through an ion exchange resin column followed by distillation was used in the preparation of all aqueous solutions.

The main calcium phosphate growth medium utilized in these studies was 1.5 × SBF (a solution with ion concentrations 1.5 times that of *s*imulated *b*ody *f*luid). The details of its preparation are supplied in an earlier publication [3]. The solution is basically prepared by the dissolution of NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, Na_2SO_4, and K_2HPO_4 in distilled water to which buffering agents "TRIS" ($(CH_2OH)_3CNH_2$) and HCl are co-added which usually keep the pH of the solution to within a range of pH 7.20–7.60 during soaking experiments. A variant of this calcium phosphate growth medium referred to as "Ca-P-TRIS solution" was prepared by mixing only 25 ml of $0.4\ \mathrm{mol\,l^{-1}}$ TRIS, 25 ml of $0.36\ \mathrm{mol\,l^{-1}}$ HCl, 15 ml of $0.02\ \mathrm{mol\,l^{-1}}$ K_2HPO_4, and 15 ml of $0.05\ \mathrm{mol\,l^{-1}}$ of $CaCl_2$ in a 200 ml volumetric flask and making up to the mark with distilled water. A second growth medium used in soaking experiments involved the well known alkaline-phosphatase-catalysed reaction of p-nitrophenyl phosphate (PNP) to p-nitrophenol and inorganic phosphate which has been used by the authors in an earlier reported system involving growth of calcium phosphate on ion exchange resins presaturated with Ca^{2+} ions [3].

2.2. Phosphorylation of cotton

The experimental procedure for the phosphorylation of cotton using the urea/H_3PO_3 method has already been described in an earlier report [1]. Phosphorylation of cotton using the urea/H_3PO_4 method was carried out in the following manner: 2–3 cotton wipes (4–6 g) cut into 10–20 pieces were placed into 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 200–250 ml of DMF. This solution was heated under N_2 with mechanical stirring up to a temperature of 120 °C at which point a solution of approximately 32.0 g of 85% H_3PO_4 in 50–100 ml of DMF was added. After further heating, the temperature of the reaction mixture increased to 155 °C and was left to reflux for 1 h. Foaminess was noticed in the reaction mixture during reflux. At the end of the refluxing time, the reaction mixture was left to cool under the N_2 gas flow. The reaction solution was subsequently filtered off and the fibres washed thoroughly in water to rinse out excess phosphoric acid. The fibres were then dried at 40–60 °C under vacuum before being subjected to SEM/EDX and other instrumental analyses.

2.3. Determination of phosphorous content in cotton samples

200 mg each of urea/H_3PO_3- and urea/H_3PO_4-modified cotton samples were dissolved in 3 ml of water, 2 ml of H_2O_2 and 5 ml of H_2SO_4. Dissolution which was violent was effected in 10 min after which each sample was diluted to 100 ml with water. The P content of both samples was then analysed on a Nippon-Jarrell-Ash ICAP-1000S ICP-AES instrument using the 213.618 nm line for phosphorous. Three determinations of P content were made from each solution prepared and the results averaged.

2.4. $Ca(OH)_2$ soaking of phosphorylated cotton samples

The procedure for soaking the urea/H_3PO_3-modified cotton in saturated $Ca(OH)_2$ solution has already been described in an earlier publication [1]. The procedure used for the urea/H_3PO_4-modified cotton was identical. Samples were soaked in sealed plastic flasks for approximately one week in saturated $Ca(OH)_2$ solution (pH = 12.4) with a change in soaking solution every 4 days. After 4 days, the pH had decreased to values in the range 10–12 while the second soaking generally produced little change in pH. Upon completion of the soaking period, the samples were subsequently filtered, rinsed thoroughly with distilled water and dried under vacuum in a 40–60 °C oven.

2.5. Soaking experiments

2.5.1. Experiments using the 1.5 × SBF growth medium

Soaking of cotton samples was similar to that reported in an earlier study [1]. 0.04–0.2 g of the cotton samples were placed into plastic screwtop flasks and 10–20 ml of 1.5 × SBF added. The pH of the cotton/1.5 × SBF solutions was then measured with the flasks being subsequently immersed in a thermostatically controlled 36.5 °C covered water bath for periods of 1–17 days. For soaking periods of >1 day, the 1.5 × SBF solution was replaced each day after pH measurement of the old solution. Just prior to reimmersion in the water bath, the pH of the freshly added solution was also measured. After conclusion of the soaking periods, all cotton samples were filtered off, washed with distilled water and dried in a vacuum heater at 40–60 °C.

Prior to soaking, some cotton samples not subjected to treatment in $Ca(OH)_2$ were subjected to various pre-treatments such as soaking in NaOH solution, saturation with Ca^{2+} ions, etc. These will be more fully discussed in the Results and Discussion section when they arise (see later).

2.5.2. Experiments using alkaline phosphatase/PNP growth medium

Approximately 0.026 g of $Ca(OH)_2$-treated urea/H_3PO_3 modified cotton was added to a solution of 0.03 mol l^{-1} disodium p-nitrophenylphosphate (PNP) prepared in borax buffer as the solvent. The pH of this solution was 10.02. Following equilibration (in a thermostatically controlled water bath) at 36.5 °C of the PNP/cotton solution in a sealed plastic flask, 12 µl (300 units) of alkaline phosphatase (*Sigma Chemical Co.* orthophosphoric monoester phosphohydrolase [alkaline optimum], type VII-S from bovine intestinal mucosa) was injected via a micropipette into the solution upon which point the solution instantly began to take on a yellow colour. The solution was left immersed in the 36.5 °C water bath for a period of 3 days after which time the pH was observed to have fallen to 8.59. To confirm 100% conversion of the PNP to p-nitrophenol, UV analysis of the diluted filtrate from the sample was conducted in the 210–360 nm region where a broad band due to PNP (310 nm) occurs. The disappearance of this band and replacement by the typical UV spectrum of p-nitrophenol was taken as confirmation that 100% conversion of PNP to p-nitrophenol had occurred.

2.6. Instrumentation

Scanning electron microscopy (SEM) and EDX analyses were performed using a Hitachi S-530 scanning electron microscope and a Horiba EMAX-2200 X-ray microanalyser. EDX analyses (using the ZAF correction) were obtained off C-coated samples using CaF_2 and GaP standard spectra included with the EDX software. Scanning electron micrographs of cotton samples were usually obtained from sputter Pt coated specimens. All Micro-FTIR cotton spectra were recorded as samples encased in a transparent KBr matrix on a JASCO Micro-FTIR Jansen Fourier transform infrared spectrometer. All ^{31}P and ^{13}C MAS NMR spectra were recorded on a Bruker MSL-200 NMR spectrometer using a magic angle spinning rate of 3 kHz and referencing to 85% H_3PO_4 (^{31}P) and solid adamantane (^{13}C). Spectra were usually obtained after an accumulation of 400–1900 transients using pulse delays (D_0) ranging from 5–30 s. In order to produce spinnable samples, it was necessary to cut the cotton samples into small pieces approximately 2 mm in diameter for efficient packing in the NMR sample holders. Kaolin powder was used as a packing agent in the NMR sample tube. Cotton samples for XPS analysis were mounted on stubs with double-sided sticky tape together with gold foil as a reference. X-ray photoelectron spectra wide scans were recorded from 0–600 eV using AlK_α radiation on a Kratos XSAM 800 X-ray photoelectron spectrometer. Narrow scans over the C1s (300–280 eV) and Au4f (95–80 eV) regions were also acquired. C1s narrow scans were later band-fitted with three to four 80% Gaussian–20% Lorentzian sub-bands of 1.7–2.0 eV bandwidth.

3. Results and discussion

3.1. Characterization of the phosphorylated fibres

3.1.1. Urea/H_3PO_3-modified fibres (P content, ^{13}C MAS NMR)

EDX, XPS, ^{31}P MAS NMR, and micro-FTIR analyses of the urea/H_3PO_3-modified cotton fibres have already been discussed in an earlier study [1]. The ICP-measured phosphorous content of the digested fibres was found to be high at (10.3 ± 0.2) wt%. The ^{13}C MAS NMR spectra observed from the urea/H_3PO_3-modified cotton samples (Fig. 1a) were found to differ only slightly from that of unphosphorylated cotton (Fig. 1b). The only change detected was a slight splitting in the peak assigned to "C1" by previous work [4] in the repeating D-glucose unit of cellulose. "C1" is the anomeric carbon in the D-glucose unit which is linked to the "C4" hydroxy of the next D-glucose unit in cellulose. It is likely that phosphorylation takes place at this carbon so breaking

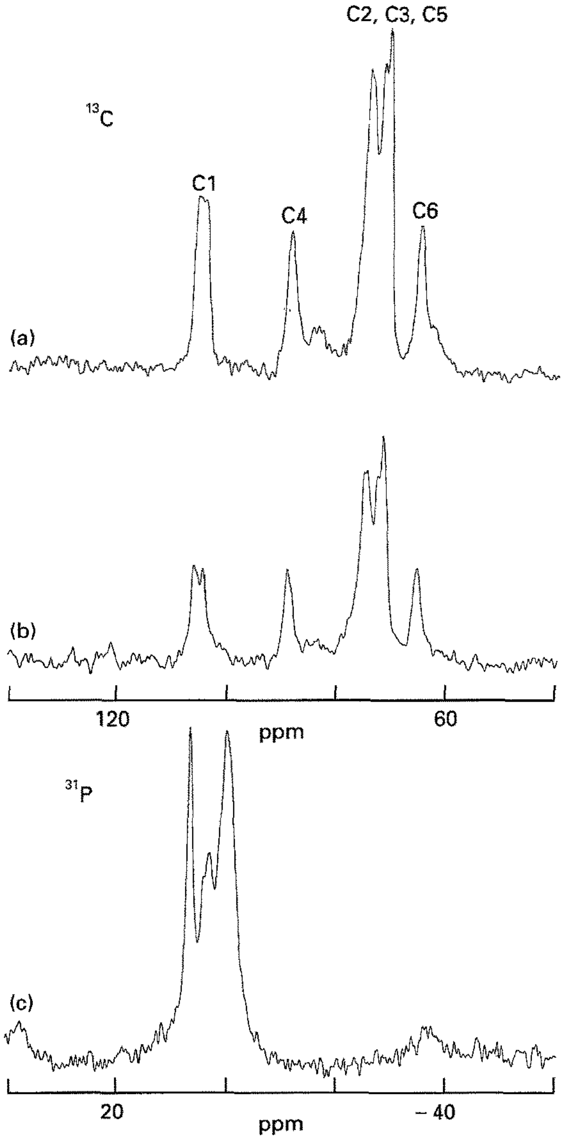

Figure 1 Solid state MAS NMR spectra of cotton samples: (a) ^{13}C NMR spectrum of unphosphorylated cotton (400 transients, $D_0 = 20$ s); (b) ^{13}C NMR spectrum of urea/H_3PO_3-modified cotton (500 transients, $D_0 = 6$ s); and (c) ^{31}P NMR spectrum of urea/H_3PO_4-modified cotton (500 transients, $D_0 = 5$ s).

down the cellulose structure partially. The perturbation to the ^{13}C NMR signal of "C1" is probably greater than that at "C4" where after detachment of the D-glucose unit, an OH group might possibly enter.

3.1.2. Urea/H_3PO_4-modified fibres (SEM/EDX, P content, micro-FTIR, ^{13}C, ^{31}P MAS NMR)

EDX spectra of the urea/H_3PO_4-modified cotton fibres clearly showed that P was present in the fibres. The ICP-measured phosphorous content of the urea/H_3PO_4-modified fibres was found to be much lower at (4.06 ± 0.13) wt% than that measured for the urea/H_3PO_3-modified cotton. This lower phosphorous content was reflected in the physical characteristics of the urea/H_3PO_4-modified fibres which showed less of a tendency than the urea/H_3PO_3-modified cotton fibres to gel when water was added. After drying, the urea/H_3PO_4-modified fibres were relatively soft, similar to the unmodified cotton wipes whereas the urea/H_3PO_3-modified cotton fibres upon drying became extremely tough and difficult to pull apart. The appearance of the urea/H_3PO_4-modified fibres under the electron microscope also appeared unchanged from that of unmodified cotton fibres (see Fig. 2a and 2b). Urea/H_3PO_3-modified cotton fibres, on the other hand, were more agglomerated and contorted in appearance after phosphorylation [1].

Micro-FTIR spectra of unmodified cotton (Fig. 3a) and urea/H_3PO_4-modified cotton (Fig. 3b) confirmed that chemical modification of the fibres had taken place. Broadening of the typical cellulose bands was observed at $\sim 1000\ \text{cm}^{-1}$ which corresponds to the ν(PO) stretching region. The broadening can thus be attributed to the ν(PO) stretching bands from PO_4 groups chemically bonded to the cotton cellulose structure. In addition, considerable broadening of the bands in the ν(OH) stretching region was observed indicating the presence of hydrogen-bonding OH groups from the PO_4 functionalities bonded to the cotton.

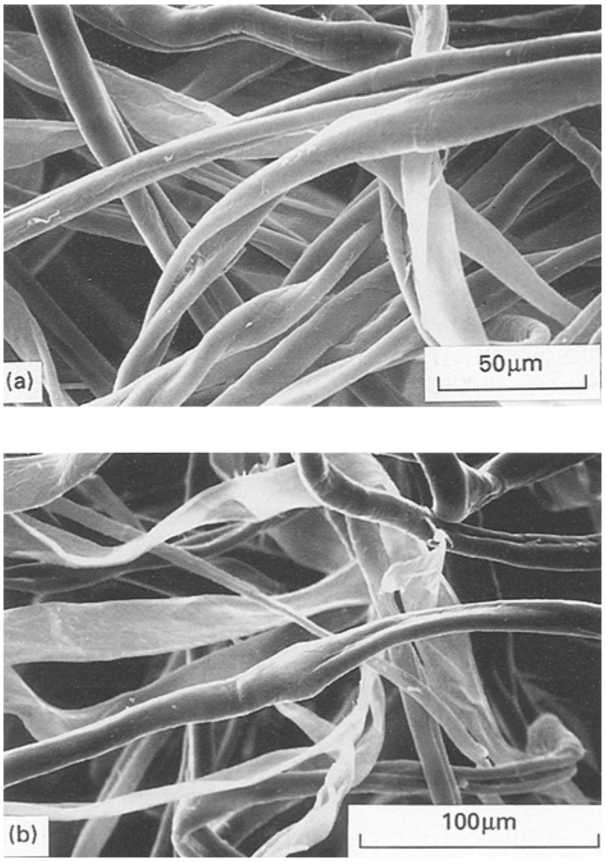

Figure 2 Scanning electron micrographs of (a) unphosphorylated (as-received) cotton fibres and (b) urea/H_3PO_4-modified cotton fibres.

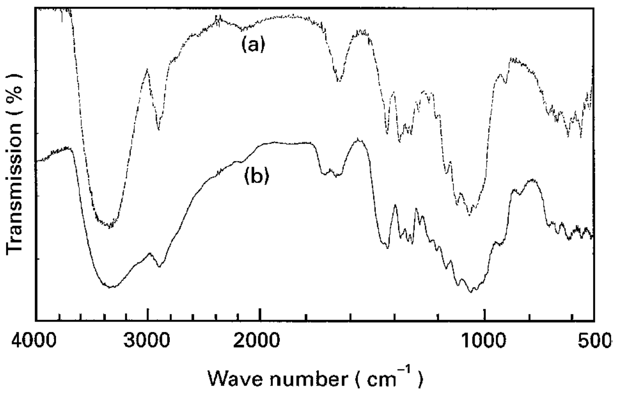

Figure 3 Micro-FTIR spectra of (a) unphosphorylated (as-received) cotton fibres and (b) urea/H_3PO_4-modified cotton fibres.

^{13}C MAS NMR spectra of the urea/H_3PO_4-modified cotton fibres were almost identical to those recorded for the urea/H_3PO_3-modified cotton. Only a slight splitting in the "C1" peak of the D-glucose repeating unit was observed which suggests that the introduction of P-containing moieties into the cellulose structure by the urea/H_3PO_4 method has occurred in a similar fashion to that achieved by the urea/H_3PO_3 method of phosphorylation. The ^{31}P MAS NMR spectrum of the urea/H_3PO_4-modified fibres is shown in Fig. 1c. A relatively broad peak at around − 0.1 ppm with a shoulder at 3.0 ppm was observed in addition to a sharp peak at 6.4 ppm. The broad peak with a shoulder exhibits the chemical shift expected for ^{31}P in PO_4 functionalities, i.e. near 0 ppm. This is very different from the ^{31}P MAS NMR spectrum of the urea/H_3PO_3-modified cotton which exhibited the typical spinning sideband patterns characteristic of shielding anisotropy in the phosphite functional group [1]. The appearance of the shoulder on the broad peak in the spectrum of the urea/H_3PO_4-modified cotton sample could indicate that more than one type of PO_4 group is present in the phosphorylated cotton, although it is difficult to tell from ^{13}C MAS NMR spectra which position on the D-glucose repeating unit of cellulose (aside from the "C1" carbon) has been phosphorylated. The sharp peak at 6.4 ppm is thought to be a P-containing solution species due to its narrowness relative to the other observed peaks. Solution species might be observed in a solid state NMR spectrum if entrained moisture is still present in the sample at the time of spectral acquisition. Evidence for the peak being a solution species was demonstrated by its non-reproducibility. In later spectra, after the cotton sample had been stored in the kaolinite packing material for some time (kaolinite probably acting as a desiccant), the sharp peak was not observed.

3.2. Soaking experiments in various calcium phosphate growth media

A summary of soaking experiments involving both urea/H_3PO_3- and urea/H_3PO_4-modified cotton is given in Table I. Observations, comments and EDX-measured Ca:P ratios of these experiments are summarized in Table II.

3.2.1. Urea/H_3PO_3-modified cotton systems

Further experiments involving different soaking methods and the use of alternative calcium phosphate growth media were conducted on the urea/H_3PO_3-modified cotton system with the aim of gathering information that might lead to a clearer understanding of the mechanism by which calcium phosphate is deposited. An additional aim was to ascertain whether the $Ca(OH)_2$ treatment step prior to soaking of the samples in the 1.5 × SBF calcium phosphate growth medium (for deposition of calcium phosphate) could be avoided as the alkaline treatment of the cotton tends to weaken the fibres.

$Ca(OH)_2$-treated urea/H_3PO_3-modified cotton fibres/PNP/alkaline phosphatase calcium phosphate growth medium. Fig. 4 is an electron micrograph of $Ca(OH)_2$-treated urea/H_3PO_3-modified cotton after soaking for 3 days in the alkaline phosphatase-catalysed reaction medium as described in Table I (PO3COT-AP). It was evident that calcium phosphate coatings had formed in this system which were similar in appearance to those formed in 1.5 × SBF solution. However, there were some areas of the sample examined where the calcium phosphate coating was either sparse or non-existent. In other areas, a large amount of loose material that charged brightly under the electron beam and which seemed to be residing below the top fibres of the samples was found to be Ca-rich suggesting that calcium carbonate had formed as well as calcium phosphate. The average Ca:P ratio exhibited by coating layers as measured by EDX was found to be 1.33 (±17%) indicating Ca-deficient apatite had formed. In general, the observation of calcium phosphate coatings in this sample is significant in that the sole source of Ca^{2+} ion in the soaking solution is from the cotton itself and probably originates from the dissolution of the calcium phosphite clusters. This lends support to the hypothesis that these clusters act as a stimulus for calcium phosphate formation on the cotton fibres.

Other urea/H_3PO_3-modified cotton soaking experiments. A series of experiments in which urea/H_3PO_3-modified cotton was soaked in various media was carried out in an attempt to stimulate calcium phosphate growth on the fibres not previously subjected to the $Ca(OH)_2$ pre-treatment (see Tables I and II).

One experiment (PO3COT-CAPTRIS, see Table I) involved the use of a calcium phosphate growth solution (i.e. "Ca-P TRIS" solution) which was simpler in a compositional sense than 1.5 × SBF solution in that only Ca^{2+} and HPO_4^{2-} ions were present (together with the TRIS buffer components). The rationale for the use of this solution was that 1.5 × SBF contains among other cations, Mg^{2+} which has been reported to be an inhibitor to calcium phosphate nucleation in other systems [5]. After soaking of the as-received urea/H_3PO_3-modified cotton in "Ca-P-TRIS" growth

TABLE I Description of soaking experiments involving urea/H_3PO_3-modified ("PO3COT") and urea/H_3PO_4-modified ("PO4COT") cotton samples

Sample name	Description of sample/soaking experiment
PO3COT-AP	0.0255 g urea/H_3PO_3-modified cotton/0.03 mol l^{-1} Na_2PNP in borax buffer/12 µl alkaline phosphatase soaked in 36.5 °C water bath for 3 days
PO3COT-NAOH	0.587 g of urea/H_3PO_3-modified cotton soaked in 1 mol l^{-1} NaOH for 1 day (at ambient temperature)
PO3COT-CAPTRIS	0.428 g of urea/H_3PO_3-modified cotton soaked 10 days in "Ca-P TRIS" solution at 36.5 °C
PO3COT-CAPPPT	0.615 g of urea/H_3PO_3-modified cotton soaked 1 day in precipitated calcium phosphate solution at ambient temperature
PO3COT-CAPPPT*CAPTRIS	PO3COT-CAPPPT sample soaked 9 days in "Ca-P TRIS" solution at 36.5 °C
PO3COT-NAOH*CAPTRIS	0.202 g of PO3COT-NAOH sample soaked 10 days in "Ca-P TRIS" solution at 36.5 °C
PO3COT-NAOH*1.5 × SBF	0.161 g of PO3COT-NAOH sample soaked 10 days in 1.5 × SBF solution at 36.5 °C
PO4COT	Urea/H_3PO_4-modified cotton
PO4COT-CASAT	Urea/H_3PO_4-modified cotton (0.367 g) saturated with Ca^{2+} ion by stirring in 1 mol l^{-1} $Ca(NO_3)_2$ for 25 h at ambient temperature
PO4COT-1.5 × SBF	0.147 g of urea/H_3PO_4-modified cotton soaked as-received in 1.5 × SBF solution for 10 days at 36.5 °C
PO4COT-CASAT*1.5 × SBF	PO4COT-CASAT sample (0.121 g) soaked in 1.5 × SBF solution for 10 days at 36.5 °C
PO4COT-CAOH	$Ca(OH)_2$-soaked urea/H_3PO_4-modified cotton (~1 week soak) at ambient temperature
PO4COT-CAOH*1.5 × SBF*n*D	$Ca(OH)_2$-soaked urea/H_3PO_4-modified cotton soaked for *n* days in 1.5 × SBF solution at 36.5 °C (n = 1–10, 12,17)

TABLE II Observations and EDX-measured Ca:P ratios from experiments involving urea/H_3PO_3 and urea/H_3PO_4-modified cotton as described in Table I

Sample name	Ca:P (±% error)	Observations/comments
PO3COT-AP	1.33 (17)	Calcium phosphate coating observed (nonuniform)
PO3COT-NAOH	not applicable	Na detected by EDX analysis
PO3COT-CAPTRIS	0.44 (18)	No calcium phosphate formation observed
PO3COT-CAPPPT	0.48 (13)	No evidence of entrapped calcium phosphate precipitate
PO3COT-CAPPPT*CAPTRIS	0.35 (11)	No calcium phosphate formation observed
PO3COT-NAOH*CAPTRIS	0.57 (32)	No calcium phosphate formation observed
PO3COT-NAOH*1.5 × SBF	0.19 (32)	No calcium phosphate formation observed
PO4COT	not applicable	Significantly intense P signal detected in EDX analysis
PO4COT-CASAT	0.80 (9)	High Ca^{2+} ion uptake noted
PO4COT-1.5 × SBF	0.80 (31)	No calcium phosphate observed, Na, Mg, K, CaCl detected by EDX analysis
PO4COT-CASAT*1.5 × SBF	0.87 (17)	No calcium phosphate formation observed
PO4COT-CAOH	1.28 (14)	Thin calcium phosphate-containing coating observed. Ca-rich specks scattered on fibre coatings also in abundance
PO4COT-CAOH*1.5 × SBF1D	1.20 (12)	Nascent calcium phosphate coating observed, Ca:P ratio low
PO4COT-CAOH*1.5 × SBF2D	1.53 (26)	Calcium phosphate formation observed, Ca:P ratios variable
PO4COT-CAOH*1.5 × SBF3D	1.39 (11)	Thicker calcium phosphate coatings observed
PO4COT-CAOH*1.5 × SBF4D	1.46 (12)	Calcium phosphate coatings observed (similar in appearance to 3D sample)
PO4COT-CAOH*1.5 × SBF5D	1.59 (25)	Calcium phosphate coatings observed (similar in appearance to 3D sample)
PO4COT-CAOH*1.5 × SBF6D	1.84 (28)	Calcium phosphate coatings observed (similar in appearance to 3D sample)
PO4COT-CAOH*1.5 × SBF7D	1.46 (9)	Calcium phosphate coatings observed (similar in appearance to 3D sample)
PO4COT-CAOH*1.5 × SBF8D	1.32 (23)	Calcium phosphate coatings observed (similar in appearance to 3D sample)
PO4COT-CAOH*1.5 × SBF9D	1.64 (38)	Much thicker calcium phosphate coatings observed
PO4COT-CAOH*1.5 × SBF10D	1.56 (24)	Much thicker calcium phosphate coatings observed
PO4COT-CAOH*1.5 × SBF12D	1.58 (15)	Fibres encased in very thick coatings of calcium phosphate
PO4COT-CAOH*1.5 × SBF17D	1.59 (13)	Fibres encased in thick coatings of calcium phosphate with cracks in the coating evident

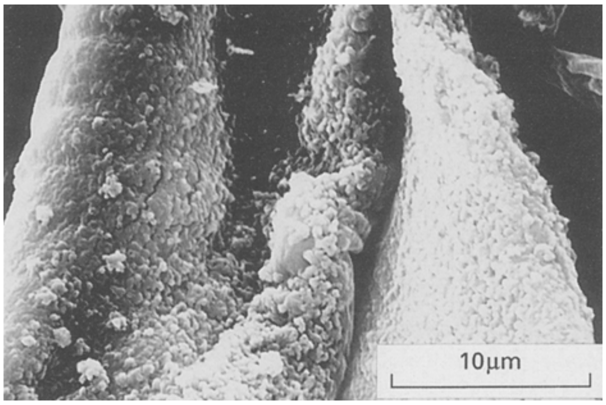

Figure 4 Scanning electron micrograph of platinum coated $Ca(OH)_2$-treated urea/H_3PO_3-modified cotton fibres which had been soaked for 3 days at 36.5 °C in a borax buffer solution (pH ~ 10) of 0.03 mol l^{-1} disodium p-nitrophenylphosphate (Na_2PNP) to which 12 μl of alkaline phosphatase had been added. pH was observed to decrease to 8.59 at the end of this soaking period.

medium for 10 days, the EDX-measured Ca:P ratio was found to be 0.44 ± 18% (see Table II) which is twice as high as the Ca:P value (0.22) reported for as-received urea/H_3PO_3-modified cotton soaked in 1.5 × SBF solution for 10 days [1]. However, no calcium phosphate growth was observed on the fibres even though it was evident that Ca^{2+} ion uptake had been enhanced. Thus the urea/H_3PO_3-modified fibres not subjected to $Ca(OH)_2$ treatment behave in the same way in different calcium phosphate soaking media.

Another experiment involved the soaking of urea/H_3PO_3-modified cotton in 1 mol l^{-1} NaOH for 1 day (sample PO3COT-NAOH, see Table I). It was reasoned that alkaline treatment may enhance Ca^{2+} ion uptake. On the basis of experience gained from $Ca(OH)_2$ treatment of the urea/H_3PO_3-modified cotton, soaking of phosphorylated fibres in alkali solution is known to lead to hydrolysis of the phosphite functionalities. In the case of treatment with NaOH, however, the expected hydrolysis product (sodium phosphite) is soluble in water so that no precipitation of insoluble salts such as calcium phosphite is possible. EDX analysis of the NaOH-soaked sample (PO3COT-NAOH, see Table II) showed uptake of Na^+ ion which would be expected from ion exchange of H^+ for Na^+. However, subsequent soaking of the NaOH-treated fibres in either 1.5 × SBF (sample PO3COT-NAOH*1.5 × SBF) or "Ca-P-TRIS" (sample PO3COT-NAOH*CAPTRIS) calcium phosphate growth medium for 10 days failed to lead to any deposition of calcium phosphate at all (see Table II). The comparative soaking experiment in 1.5 × SBF and "Ca-P-TRIS" growth media, served, however, to confirm that the Ca^{2+} ion uptake of the urea/H_3PO_3-modified cotton is greater in "Ca-P-TRIS" solution than in 1.5 × SBF solution on the basis of the EDX-measured Ca:P ratios (see Table II).

The failure of the NaOH-treated cotton fibre soaking experiments reinforced the importance of the $Ca(OH)_2$ treatment process for producing insoluble (i.e. at the high pH existing in the hydrolysis medium) layers and clusters of calcium phosphite on the cotton for the stimulation and/or nucleation of calcium phosphate growth on the fibres when soaked in 1.5 × SBF solution. This view is reinforced by another experiment in which an attempt was made to create nucleation sites for calcium phosphate on the urea/H_3PO_3-modified cotton (see Table I). Soaking of the as-received urea/H_3PO_3-modified cotton fibres in a precipitated calcium phosphate solution for one day (sample PO3COT-CAPPT) followed by a 10-day soaking in "Ca-P-TRIS" calcium phosphate growth media (sample PCOT-CAPPT*CAPTRIS) failed to lead to the formation of calcium phosphate on the fibres (see Table II).

3.3. Urea/H_3PO_4-modified cotton systems

3.3.1. Initial attempts at calcium phosphate formation on the as-received phosphorylated fibres

After confirming by EDX and Micro-FTIR that the urea/H_3PO_4 method had led to chemical incorporation of PO_4 in the cellulose structure of the cotton, it was desired to ascertain whether fibres phosphorylated by this method behaved in a different manner from fibres phosphorylated by the urea/H_3PO_3 method. Thus the urea/H_3PO_4-modified fibres were subjected to an identical battery of soaking tests that the urea/H_3PO_3-modified cotton was subjected to in a previous study [1] before $Ca(OH)_2$ treatment was carried out.

Urea/H_3PO_4-modified cotton was initially soaked as received in 1.5 × SBF solution for a period of 10 days (sample PO4COT-1.5 × SBF, see Table I). Although SEM clearly showed no evidence of calcium phosphate growth on the fibres, EDX analysis revealed that Na, Mg, K, Ca and Cl had been taken up by the fibres. This is almost identical behaviour (i.e. as an ion exchange material) to the urea/H_3PO_3-modified fibre when soaked directly in 1.5 × SBF solution without any intervening $Ca(OH)_2$ treatment. The difference in the samples, however, could be seen in the comparison of the Ca:P ratios measured at 0.80 ± 9% (see Table II) which is more than 3.5 times larger than that measured for the urea/H_3PO_3-modified cotton fibres after soaking an equivalent period in 1.5 × SBF. The high Ca^{2+} ion uptake of H_3PO_4-modified cotton fibres had been reported as long ago as 1948 by Jurgens *et al.* [6].

Saturation of the urea/H_3PO_4-modified cotton with Ca^{2+} ion by stirring in 1 $mol\,l^{-1}$ $Ca(NO_3)_2$ for 25 h (sample PO4COT-CASAT, see Table I) gave a sample with a Ca:P ratio of 0.80 (see Table II) which is higher than the value observed (0.64) for urea/H_3PO_3-modified cotton subjected to saturation with Ca^{2+} ion [1]. However, subsequent soaking of the Ca^{2+} ion-saturated sample in 1.5 × SBF solution (sample PO4COT-CASAT*1.5 × SBF) failed to lead to calcium phosphate formation on the fibres. The negative results from these experiments (PO4COT-1.5 × SBF and PO4COT-CASAT*1.5 × SBF) indicates that the urea/H_3PO_4-modified fibres behave very similarly to the urea/H_3PO_3-modified cotton fibres in not being capable *per se* of nucleating calcium phosphate formation upon immersion in 1.5 × SBF solution.

3.4. $Ca(OH)_2$ treatment of urea/H_3PO_4-modified cotton fibres

A scanning electron micrograph of $Ca(OH)_2$-treated urea/H_3PO_4-modified cotton (PO4COT-CAOH, see Tables I and II) is illustrated in Fig. 5a. All fibres appeared to be covered with a thin yet observable coating upon which speckles of solid were strewn. When EDX analysis over a large area (at 50 × magnification) was carried out, a Ca:P ratio of 1.86 was obtained indicating that 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 variable Ca:P ratios ranging from 1.03 to 1.47 (average 1.28 ± 14%, see Table II). It is likely that this coating consists of calcium-deficient apatite. It has been calculated that at pH > 6.3, OCP (octacalcium phosphate) is the preferred precursor for supersaturation in solutions containing Ca^{2+} and HPO_4^{2-} ions [7]. Attempts to characterize the deposited material by micro-FTIR failed due to the thinness of the coating. It is assumed that $Ca(OH)_2$ has partially hydrolysed the cotton-PO_4 groups to initially produce perhaps OCP as an initial phase that has then been rapidly transformed to calcium deficient apatite. This is a somewhat different observation from that observed for $Ca(OH)_2$ treatment of the urea/H_3PO_3-modified cotton fibres where the more easily identifiable calcium phosphite clusters were observed. However, the observation of the formation of a thin coating of probable apatite composition on the surfaces of the $Ca(OH)_2$-treated urea/H_3PO_4-modified cotton fibres suggests that thin coatings (of calcium phosphite) also formed along with calcium phosphite clusters during the $Ca(OH)_2$ treatment of urea/H_3PO_3-modified cotton fibres. In the previous study [1] on calcium phosphate growth on urea/H_3PO_3-modified cotton, a thin calcium phosphite film was suggested as one of the possible ways that calcium phosphate growth was nucleated on the fibre surface. The observations from the present study lend support to that hypothesis.

3.5. Soaking of $Ca(OH)_2$-treated urea/H_3PO_4-modified cotton in 1.5 × SBF solution: time dependence experiments

Soaking of the $Ca(OH)_2$-treated urea/H_3PO_4-modified cotton fibres in 1.5 × SBF was found to lead to the deposition of a calcium phosphate layer. Thus the behaviour of the system was very similar to the $Ca(OH)_2$-treated urea/H_3PO_3-modified cotton in this respect. It is believed that the thin coatings of calcium phosphate material on the fibres produced by partial hydrolysis of the cotton PO_4 functionalities by soaking

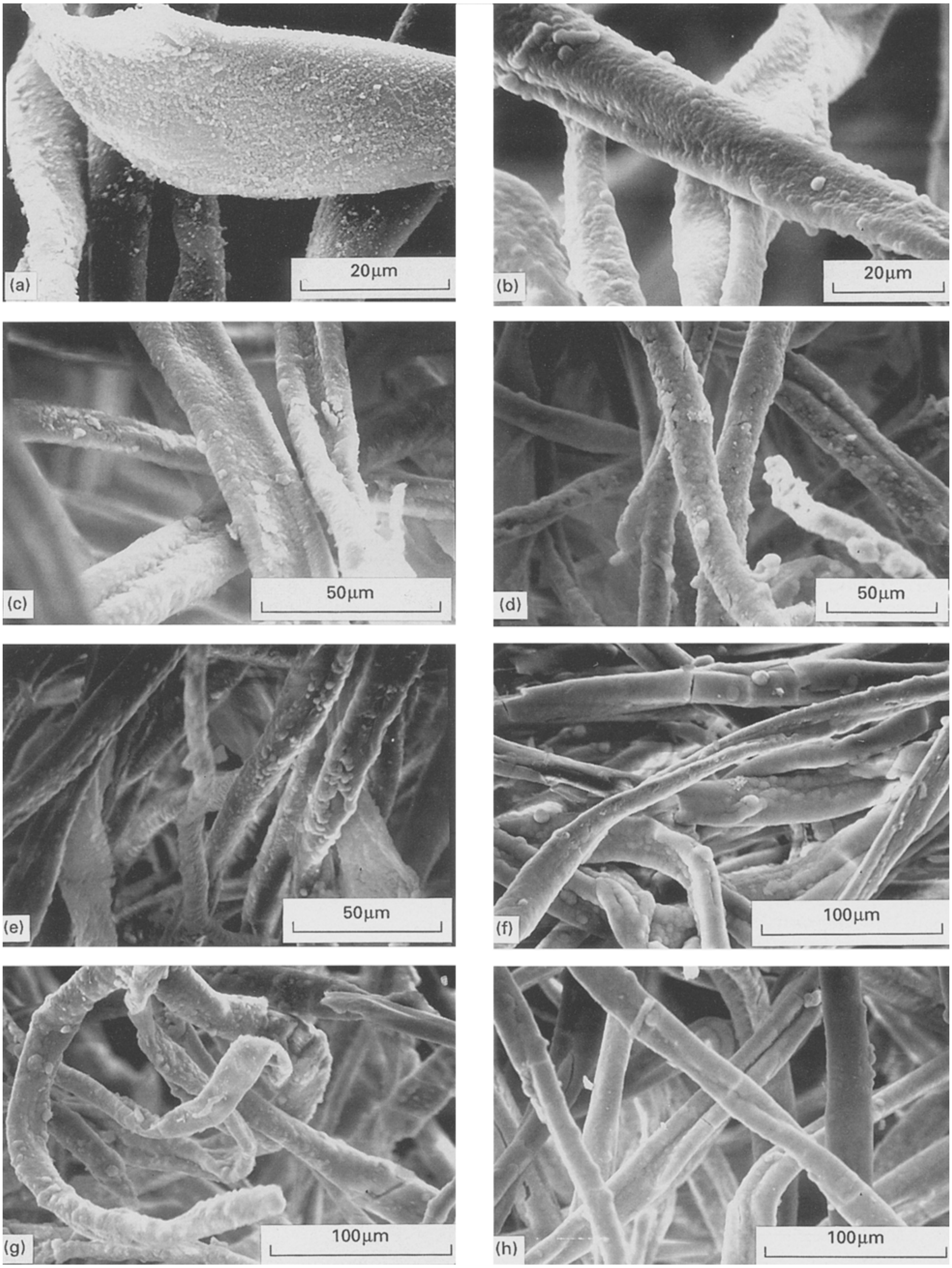

Figure 5 Scanning electron micrographs of platinum-coated samples of (a) urea/H_3PO_4-modified cotton fibres after soaking in saturated $Ca(OH)_2$ (pH ~ 12.4) for approximately one week at room temperature, and $Ca(OH)_2$-treated urea/H_3PO_4-modified cotton fibres after soaking in 1.5 × SBF at 36.5 °C for (b) 1 day, (c) 2 days, (d) 4 days, (e) 6 days, (f) 9 days, (g) 10 days, and (h) 17 days.

in saturated $Ca(OH)_2$ solution act as a nucleation layer upon which the calcium phosphate can grow from 1.5 × SBF solution.

A 1–17-day soaking experiment in 1.5 × SBF was, therefore, carried out in order to study the deposition of calcium phosphate on the fibres as a function of time (see PO4COT-CAOH*1.5 × SBFnD entry in Table I). The observations and EDX-measured Ca : P ratios from this time dependence study are summarized in Table II.

Fig. 5b–h are scanning electron micrographs of calcium phosphate growth from 1.5 × SBF solution on $Ca(OH)_2$-treated urea/H_3PO_4-modified cotton as a function of soaking time in days. On the first day of soaking in 1.5 × SBF (Fig. 5b), it appeared that the original speckly and bumpy coating deposited on the fibres by $Ca(OH)_2$ treatment was overlaid with a new somewhat thicker coating which also contained bumps and nodules. Judging from the micrographs of the cotton after soaking for 2, 4 and 6 days (Fig. 5c–e), the growth of the calcium phosphate layer appears to proceed by nucleation on the existing coating in the form of circular nodules which then grow in number and size on the surface. For samples soaked for periods of 3–8 days in 1.5 × SBF, calcium phosphate coatings grown on the $Ca(OH)_2$-treated urea/H_3PO_4 fibres were found to be very similar in appearance to each other. However, the coatings formed on fibres soaked for 9 days (Fig. 5f) looked distinctly thicker and rather uniform. Micrographs of the $Ca(OH)_2$-treated urea/H_3PO_4-modified cotton after 10 and 17 days of soaking in 1.5 × SBF (Fig. 5g and h) look similar to the coated fibres after 9 days' soaking. It is apparent that after such a long soaking time, the coatings have thickened to the point that cracks appear in some areas (see Fig. 5h). In general, the calcium phosphate coatings formed on the $Ca(OH)_2$-treated urea/H_3PO_4 fibres are somewhat improved in appearance to those grown on $Ca(OH)_2$-treated urea/H_3PO_3-modified cotton fibres soaked in 1.5 × SBF in which coatings formed initially as a result of the agglomeration of a large number of much smaller particles leading to fibres rather unevenly coated with calcium phosphate after 17 days [1].

Fig. 6 illustrates the EDX-measured Ca : P ratio for the calcium phosphate coatings as a function of time of soaking 1.5 × SBF solution. The top curve (a) fitted to plotted data designated by open circles refers to Ca : P ratios measured over a wide area of the sample at low magnification (50 ×). The bottom curve (b) fitted to the plotted data represented as filled diamonds is for Ca : P ratios (averaged over seven measurements) obtained at high magnification (8000–30 000 ×) of coated individual fibres. Curve (a) shows that the sample has very Ca : P ratios over a large area which suggests Ca-enrichment due to the presence of calcium carbonate. It is interesting to observe that fluctuations in curve (a) are reflected to some extent in curve (b). For curve (b), however, which should be interpreted as the characteristic Ca : P ratios of the actual coatings deposited on individual fibres, the Ca : P ratios are in general quite scattered but appear to stabilize for thick coatings (formed at soaking times > 9 days) at a value of around 1.60 suggesting amorphous Ca-deficient apatite. This is in fact similar to the Ca : P ratio obtained for calcium phosphate coatings on $Ca(OH)_2$-treated urea/H_3PO_3-modified fibres grown after 17 days soaking in 1.5 × SBF solution [1].

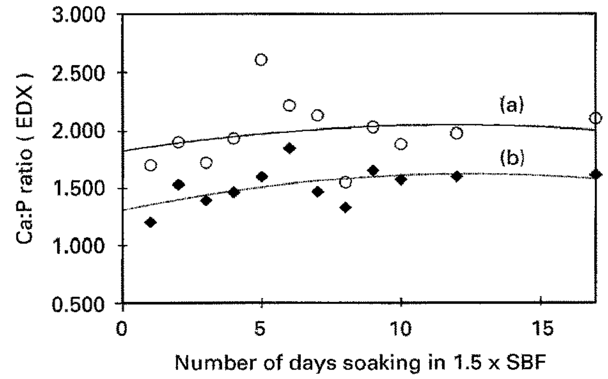

Figure 6 Plots of EDX-measured Ca : P ratios for calcium phosphate coatings grown on $Ca(OH)_2$-treated H_3PO_4-modified cotton fibres soaked in 1.5 × SBF at 36.5 °C as a function of time. Curve (a) [solid line fitting data shown by open circles] is the Ca : P ratios measured over a wide area of the sample at low magnification (50 ×) and curve (b) [dotted line fitting data shown by filled diamonds] is Ca : P ratios measured for coatings on individual fibres at high magnification (8000–30 000 ×). Each data point in curve (b) is averaged over seven individual measurements of Ca : P in seven different areas of the sample.

3.6. Micro-FTIR spectra of calcium phosphate coatings on $Ca(OH)_2$-treated urea/H_3PO_4-modified cotton fibres

Fig. 7a is a micro-FTIR spectrum of the coating material grown on the $Ca(OH)_2$-treated urea/H_3PO_4-modified cotton fibres after 9 days soaking in 1.5 × SBF solution. Fig. 7b is a photograph of the sampled coating on the fibres as sampled by the

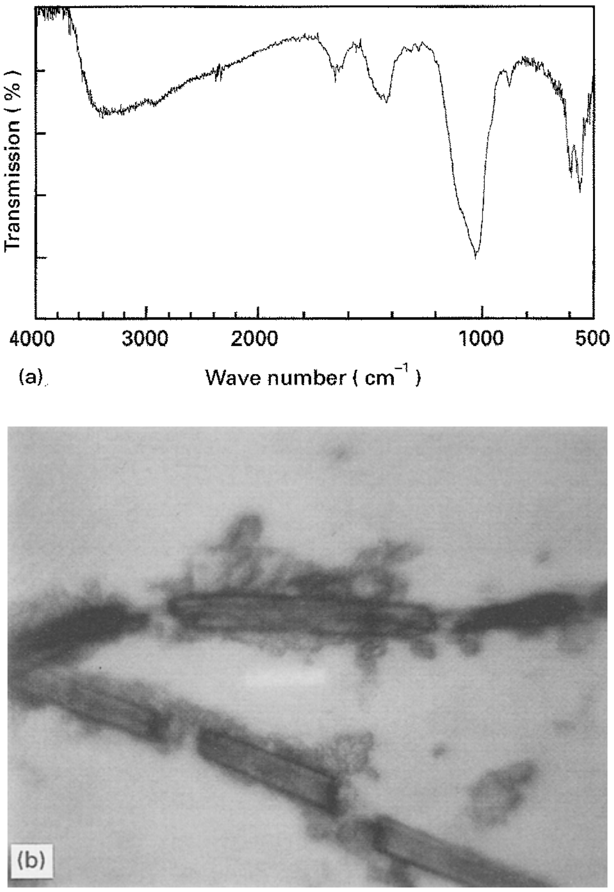

Figure 7 Micro-FTIR studies of the coating material deposited on the $Ca(OH)_2$-treated urea/H_3PO_4-modified fibres after 9 days soaking in 1.5 × SBF solution at 36.5 °C: (a) micro-FTIR spectrum of coating material; and (b) photograph of actual sampled area in micro-FTIR showing the thick coating associated with the fibres.

micro-FTIR instrument. Characteristic phosphate-associated vibrational bands at 1031, 601 and 563 cm^{-1} are observed. The spectrum is almost identical to spectrum observed for coatings grown on $Ca(OH)_2$-treated urea/H_3PO_3-modified cotton soaked in 1.5 × SBF as reported earlier [1]. It is evident that carbonate is present to a noticeable extent in the samples as demonstrated by the broad peak at 1424.6 cm^{-1}. This agrees with the EDX data from the coating samples mentioned earlier. The absence of a sharp peak at 3500–3600 cm^{-1} due to apatite hydroxy groups suggests that the coating is amorphous in nature. There also appears to be water in the coating as evidenced by the broad (indicating hydrogen bonding) ν(OH) stretching peak at 3388 cm^{-1} and the peak at 1654 cm^{-1} due to the bending mode of water.

3.7. X-ray photoelectron studies of calcium phosphate coatings on $Ca(OH)_2$-treated urea/H_3PO_4-modified cotton fibres

Table III is a summary of the XPS data collected for urea/H_3PO_4-modified cotton (PO4COT), $Ca(OH)_2$-treated urea/H_3PO_4 cotton (PO4COT-CASAT), and calcium phosphate coated (after immersion in 1.5 × SBF solution) $Ca(OH)_2$-treated urea/H_3PO_4 cotton (PO4COT-CAOH*1.5 × SBF*n*D, $n = 1$–10, 12, 17). In general, Ca : P ratios calculated from the atomic conc. (%) values were very scattered, ranging from 1.07 to 3.04. Given that XPS samples over a larger area than SEM/EDX, it is likely that the nature of the sample, i.e. fibres with random orientations and incompletely coated with material, is the cause of the scattered data obtained. As calcium phosphate coatings become thicker on the fibres, it is likely that handling of the samples can cause spalling of material from the fibres which will also influence the amount of Ca or P measured on the fibres by XPS. A similar phenomenon was observed in XPS analysis of the urea/H_3PO_3-modified cotton fibres [1].

In general, the $Ca(OH)_2$-treated urea/H_3PO_4-modified cotton sample (before soaking in 1.5 × SBF) gave a very high Ca : P ratio of 3.04 (see Table III) suggesting a surface heavily enriched in Ca as previously implied from SEM/EDX measurements. Soaking in 1.5 × SBF subsequently leads to a reduction in Ca : P to various values that fluctuated between 1.07 and 2.03. It appeared that for most samples, the surfaces of coatings were always heavily enriched in Ca.

Fig. 8a is a narrow scan over the C1s region for $Ca(OH)_2$-treated urea/H_3PO_4 fibres soaked for 17 days in 1.5 × SBF solution. Three sub-bands occurring at binding energies corrected for charging at 285.05 eV, 286.52 eV and 288.87 eV could be fitted to the experimental profile. The sub-band at 288.87 eV at lowest intensity has been assigned to carbonate present in the calcium phosphate coating. In general, all the C1s narrow scan profiles acquired in the XPS study showed evidence of a carbonate peak which exhibited (fitted) binding energy values from 288.0–289.1 eV. This confirms that carbonate is a persistent component of the calcium phosphate coating on the cotton fibres since it was observed in samples ranging from the $Ca(OH)_2$-treated urea/ H_3PO_4 fibres (before immersion in 1.5 × SBF) up to the sample soaked for 17 days in 1.5 × SBF as shown in Fig. 8a. In addition, a weak COO (carbonyl-assigned) component band could also be fitted to the experimental C1s narrow scan profile obtained for the urea/H_3PO_4-modified cotton sample (PO4COT) before $Ca(OH)_2$ treatment. This has been assigned to residual urea remaining in the sample. Indeed, a weak N1s peak at around 400 ppm was also detected in the PO4COT XPS spectrum which confirms this assignment.

The other peaks (285.05, 286.52 eV) fitted to the experimental C1s narrow scan profile illustrated in Fig. 8a were interpreted with caution since these may originate from carbon species other than those associated with the sample (e.g. sticky tape hydrocarbons plus adventitious carbon). The bulk of the C1s peak intensity is, however, most likely to be due to the C contained in the cotton cellulose since the atomic conc. (%) values of C1s decreased linearly with increase in Ca atomic conc. (%) (see Fig. 8b) and

TABLE III XPS data from the urea/H_3PO_4-modified cotton samples

Sample	Ca2p[a]	P2p[a]	C1s[a]	O1s[a]	C (%)	O (%)	Ca (%)	P (%)	Ca:P
PO4COT	–	133.51	284.66	531.74	57.75	33.65	–	2.50	0
PO4COT-CASAT	347.57	133.49	285.58	532.12	39.08	48.27	8.38	2.76	3.04
# 1D[b]	347.73	133.53	284.64	532.03	62.61	31.23	3.18	2.97	1.07
# 2D	347.58	133.01	284.07	531.49	56.40	36.40	4.62	2.59	1.78
# 3D	347.98	134.09	284.92	532.11	51.02	38.88	6.77	3.33	2.03
# 4D	348.96	133.98	285.29	532.55	46.28	39.80	8.75	5.17	1.69
# 5D	348.41	133.46	285.93	532.35	40.46	43.05	10.23	6.26	1.63
# 6D	348.30	133.94	285.93	532.06	40.29	44.69	9.58	5.45	1.76
# 7D	348.33	134.04	285.48	532.39	46.86	39.16	8.00	5.98	1.34
# 8D	348.07	133.20	285.14	532.10	46.17	41.14	7.51	5.18	1.45
# 9D	347.28	133.13	285.16	531.83	53.69	36.86	6.31	3.14	2.01
# 10D	347.23	133.17	284.05	531.61	50.32	37.15	7.46	5.07	1.47
# 12D	348.22	133.56	285.50	532.45	51.88	36.72	7.60	3.80	2.00
# 17D	348.18	134.02	285.42	532.40	43.54	41.75	8.30	6.41	1.29

[a] Binding energy in eV corrected with respect to a gold foil reference binding energy.

[b] # = an abbreviation for "PO4COT-CAOH*1.5 × SBF".

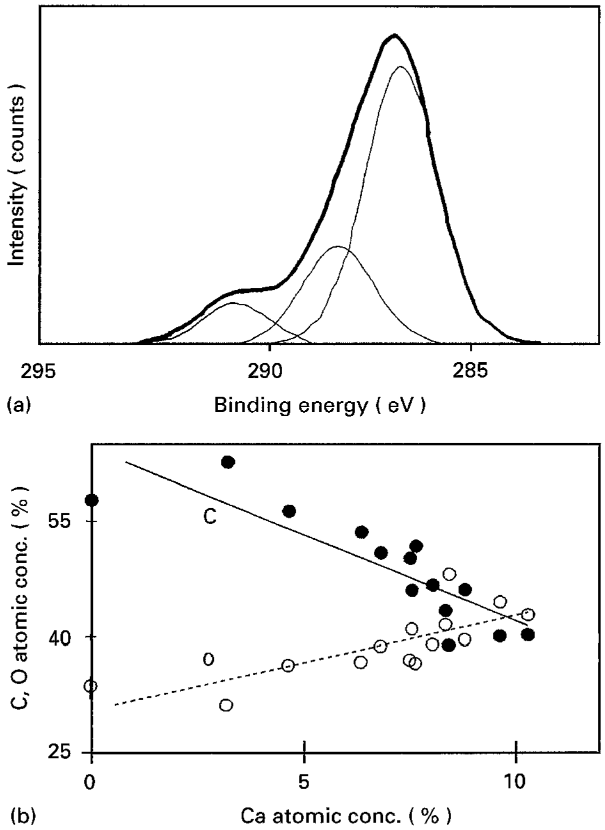

Figure 8 (a) Narrow scan XPS C1s profile obtained for the $Ca(OH)_2$-treated urea/H_3PO_4 fibres soaked for 17 days in 1.5 × SBF solution at 36.5 °C. (b) Plots of C and O atomic conc. (%) values versus Ca atomic concentration (%) values for all phosphorylated cotton samples studied by XPS.

P atomic conc. (%) values (as derived from the wide scans) which indicates coating of the fibres with calcium phosphate material. The O1s atomic conc. (%) values, on the other hand, increased linearly with Ca and P atomic conc. (%) values due to increased O presence at the surface brought about by the deposition of phosphate (PO_4) on the cotton fibres.

3.8. Phosphorylated cotton systems: general comment

It seems from studies reported in the earlier investigation of urea/H_3PO_3-modified cotton fibres as well as the present investigation that the introduction of phosphorous-containing functionalities, be they $P(H)O_3$ or PO_4, do not *per se* induce calcium phosphate growth on the cotton fibres when they are subsequently soaked in a calcium phosphate growth medium. Calcium phosphate growth only becomes possible after $Ca(OH)_2$ treatment. Although the $Ca(OH)_2$ treatment is retroactive in the sense that it partially reverses the earlier phosphorylation via hydrolysis, the precipitation of the hydrolysis products with the Ca^{2+} ensures that these products exist in intimate contact with the cotton where they function as a stimulus or a nucleation layer for the growth of coatings of calcium phosphate on the fibres (and not as loose precipitate held in the fibres) when the sample is soaked in a calcium phosphate growth medium. Thus $Ca(OH)_2$ treatment provides the favourable local conditions leading to calcium phosphate growth on a substrate that would otherwise not easily nucleate the growth of such material. Such a technique or one based on it may be useful to consider when designing materials that mimic biomineralization processes where favourable local conditions are crucial for their occurrence.

4. Conclusions

Further soaking experiments involving the urea/H_3PO_3-modified cotton system have reaffirmed the importance of the $Ca(OH)_2$ treatment of the fibres for the stimulation of growth of calcium phosphate on the cotton fibres.

Cotton fibres phosphorylated using an alternative urea/H_3PO_4 method have also been reported. Comparisons with the urea/H_3PO_3-modified cotton fibres indicate that the urea/H_3PO_4-modified fibres exhibit similar properties. $Ca(OH)_2$ treatment of the urea/H_3PO_4-modified fibres leads to the formation of thin coatings consisting possibly of Ca-deficient apatite. Soaking of the $Ca(OH)_2$ treated urea/H_3PO_4-modified cotton fibres in 1.5 × SBF at 36.5 °C leads to growth of calcium phosphate on the fibres which becomes thick after a prolonged period (e.g. 9 days) of soaking in 1.5 × SBF. Nucleation is believed to occur on the thin coatings formed by $Ca(OH)_2$ treatment. Micro-FTIR of the coatings grown in 1.5 × SBF on $Ca(OH)_2$-treated urea/H_3PO_4 fibres confirm that they consist of calcium phosphate and calcium carbonate. In general, the overall quality and uniformity of the coatings appears superior to those formed on $Ca(OH)_2$-treated urea/H_3PO_3 fibres as reported earlier.

The observation of thin calcium phosphate-containing coatings (which act as a nucleation layer for further calcium phosphate growth) on the urea/H_3PO_4-modified cotton fibres after $Ca(OH)_2$ treatment lend support to the hypothesis from an earlier study that the mechanism of coating growth on $Ca(OH)_2$-treated urea/H_3PO_3-modified cotton fibres soaked in 1.5 × SBF solution is possibly nucleated on a thin layer of calcium phosphite on the fibres.

With reference to the cotton systems, the technique of phosphorylation and partial hydrolysis of introduced phosphorous-containing 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 the favourable local conditions leading to the nucleation and growth of calcium phosphate.

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