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Growth of calcium phosphate on surface-modified cotton

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A study of the growth of amorphous calcium phosphate on surface-modified cotton fibres by a combination of scanning electron microscopy/electron diffraction X-ray analysis, micro-FTIR and X-ray photoelectron spectroscopy is reported. Cotton fibres phosphorylated by the urea/phosphorous acid method and then soaked in saturated $Ca(OH)_2$ for approximately one week were found to stimulate the growth of a calcium phosphate coating on their surfaces after soaking in 1.5 × SBF for as little as 1 day. $Ca(OH)_2$ soaking of the fibres is found to produce highly crystalline clusters lodged in the fibres which were confirmed by micro-FTIR to be calcium phosphite monohydrate ($CaHPO_3 \cdot H_2O$). In contrast, phosphorylated fibres not subjected to the $Ca(OH)_2$ treatment did not exhibit calcium phosphate growth upon immersion in 1.5 × SBF solution. Soaking of the $Ca(OH)_2$-treated fibres with time in the 1.5 × SBF solution produced progressively thicker layers of calcium phosphate on the fibres as confirmed by scanning electron microscopy and X-ray photoelectron spectroscopy. In general, calcium phosphate coatings formed over a 1–5 day period soaking in 1.5 × SBF solution appeared to consist of agglomerations of a large number of small spherical particles, while coatings formed after 17 days of soaking were distinctly chunky, thick and non-uniform in appearance. Micro-FTIR indicated that $CaHPO_3 \cdot H_2O$ clusters were still present in cotton samples even after 4 days of soaking, while after 17 days, only the infrared spectrum typical of calcium phosphate was observed. EDX-measured Ca:P ratios of the coatings, although variable, suggested amorphous calcium phosphate. The mechanism of formation of the coating is believed to involve dissolution of the $CaHPO_3.H_2O$ clusters upon introduction of the $Ca(OH)_2$-treated phosphorylated cotton into the 1.5 × SBF solution which elevates the Ca^{2+} ion concentration in the vicinity of the fibres so stimulating calcium phosphate formation. It is postulated that phosphite groups chemically bound to the cotton fibre surface or a calcium phosphite coating on the fibres act as nucleation sites for calcium phosphate growth in 1.5 × SBF solution.

1. Introduction

Conventionally, the lost functions of highly complex tissues such as bone are usually replaced by materials such as alumina that are strong but bioinert and thus not capable of participating in physiological processes. As a consequence, recent biomimetic studies concerned with the stimulation of calcium phosphate growth on suitably designed substrates have become an extremely important part of biomaterials research due to the insights such investigations can give into the natural deposition of bone-like material in the body. The application of such knowledge may also allow the design of coated biomaterials which combine strength, elasticity and bioactivity so that they more closely resemble the function of the tissue they are replacing [1]. Research into the stimulation of calcium hydroxyapatite growth on various substrates has employed a number of techniques including special procedures for raising the ionic activity product of calcium hydroxyapatite in the solution containing the substrate to be coated so stimulating precipitation and the creation of apatite nucleation sites [2], and surface modification (e.g. phosphorylation) giving surface sites which have a crystallographic arrangement similar to that of hydroxyapatite so allowing possible epitaxial growth [3]. In this laboratory, a study of the deposition of calcium phosphate compounds on cationic and anionic (DOWEX and AMBERLITE) ion-exchange resins pre-saturated with Ca^{2+} or HPO_4^{2-} ions respectively was recently reported [4]. In the present paper, this theme was extended by investigating the growth of hydroxyapatite on cotton which becomes an ion-exchange material after phosphorylation. In 1948, it was reported that cotton cellulose phosphorylated by the urea/phosphoric acid method [5] was useful as a cation

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exchange material for Ca^{2+} ion. In the present study, cotton phosphorylated by the urea/phosphorous acid method as reported by Inagaki *et al.* [6] will be used as a substrate for growing calcium phosphates.

2. Experimental

2.1. Solutions and chemicals

All chemicals used were supplied by Wako Pure Chemical Industries Ltd or Katayama Chemicals Ltd and used without further purification. The cotton used in this study was Ciegal Cotton Wipes (100% cotton, 4 ply) supplied by Chiyoda Co 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 × SBF (simulated body fluid) solution, a precise description of which is supplied in an earlier publication [4]. This solution is prepared by dissolving NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, K_2HPO_4 and Na_2SO_4 in distilled water together with "TRIS" ($(CH_2OH)_3CNH_2$) and HCl which acted as a buffering agent keeping the pH of the solution to within a range of 7.10–7.50 during soaking experiments.

2.2. Phosphorylation of cotton

Phosphorylation of cotton samples was carried out following the preparation reported by Inagaki *et al.* [6]. In a typical preparation, two cotton wipes (ca. 3 g) were cut into 20–30 pieces and placed in a round-bottomed flask equipped with a thermometer, mechanical stirrer, condenser and N_2 gas inlet tube. 40 g of urea was subsequently added to the flask and dissolved in 500 ml of dimethyl formamide (DMF). The cotton/urea DMF solution was then heated under N_2 with mechanical stirring up to 130 °C upon which a solution of 27g of phosphorous acid (H_3PO_3) in 100 ml of DMF was added. After addition of the H_3PO_3, the reaction temperature increased to 140–145 °C with the reaction solution becoming opaque and foaming slightly. White fumes were also observed to evolve from the condenser outlet. During the reaction, the cotton did not retain its original ordered weave as exhibited in the cotton wipes but agglomerated to form a tangle of fibres. After 30min reflux, the reaction mixture was stopped, left to cool and subsequently decanted from the cotton fibres which were then rinsed repeatedly in water in order to wash out excess H_3PO_3 and urea. The cotton was filtered and dried in a 40–60 °C oven. Phosphorylation of the cotton fibres is easily recognized by the tendency of the fibres to swell upon addition of water so that a gel-like consistency is obtained. When dried, the fibres shrink in size and become considerably harder and tougher than those of as-received cotton.

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

Phosphorylated cotton was soaked (without stirring) in a saturated solution of 50 ml of $Ca(OH)_2$ (pH ~ 11–12) in 100 ml closed, screw-top plastic bottles for periods of up to 8 days. The $Ca(OH)_2$ solution was renewed every 4 days. After 4 days, the pH had usually decreased to < 9.0. The second soaking of the phosphorylated cotton in $Ca(OH)_2$ produced a smaller decrease in pH from 12.0 to 9.5–10.0. 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.

2.4. Growth of calcium phosphate

In typical calcium phosphate growth experiments, 0.04–0.2 g samples of $Ca(OH)_2$-treated phosphorylated cotton (CPC) were weighed into plastic screwtop flasks to which 10–20 ml of 1.5 × SBF was subsequently added. The pH of the cotton/1.5 × SBF solutions was then measured before immersion of the plastic flasks for periods ranging from 1–17 days in a covered water bath which was kept at 36.5 °C by thermostatic control. When soaking periods exceeded one day, the flasks were taken out of the water bath each day, the pH of the old solution measured, with the old solution being filtered off from the cotton sample and replaced by a further addition of 10–20 ml of fresh 1.5 × SBF. The pH of the fresh 1.5 × SBF/cotton solution was also measured prior to reimmersion of the flasks in the water bath.

Similar soaking experiments were also performed which did not involve the use of $Ca(OH)_2$-treated phosphorylated cotton. In these experiments, untreated phosphorylated cotton as well as phosphorylated cotton saturated with Ca^{2+} ions (by presoaking in 0.01–1.0 mol l^{-1} $Ca(NO_3)_2$ solution for 24 h) were used. At the conclusion of all soaking periods, cotton samples were filtered off, washed with distilled water and dried in vacuo at 40–60 °C before further examination.

2.5. Instrumentation

Scanning electron microscopy (SEM) and EDX analyses were performed using a Hitachi S-530 scanning electron microscope to which a Horiba EMAX-2200 X-ray Microanalyser was attached. EDX analyses (using the ZAF method) were obtained from C-coated samples using CaF_2 and GaP standard spectra included with the EDX software. Micrographs of cotton samples were usually obtained from sputter Pt coated specimens. X-ray photoelectron spectra from 0–600 eV were recorded using AlK_α radiation on a Kratos XSAM 800 X-ray photoelectron spectrometer. Samples were mounted on stubs with double-sided sticky tape together with gold foil which acted as a reference. All Micro-FTIR cotton spectra were recorded on samples encased in a transparent KBr matrix on a JASCO Micro-FTIR Jansen Fourier transform infrared spectrometer. All ^{31}P 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. Spectra were usually obtained after an accumulation of 200–300 transients using a pulse delay of 30 s. When cross-polarized spectra were obtained, a pulse delay of 20 s

was employed. 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. In the case of the phosphorylated cotton, the sample was packed with kaolinite powder (as a compacting aid) in the NMR sample tube.

3. Results and discussion

3.1. Preliminary studies of as-received cotton and untreated phosphorylated cotton soaked in 1.5 × SBF solution

3.1.1. SEM/micro-FTIR/solid state NMR/XPS studies of as-received phosphorylated cotton fibres

Fig. 1a is an SEM micrograph of as-received Ciegal cotton fibres before phosphorylation. The fibres generally exhibited very little foreign matter and were tangled in appearance. EDX analysis revealed no peaks above fluorine which is the limit of detection of the EDX detector used. In addition, X-ray photoelectron spectroscopy showed only C1s and O1s peaks as would be expected from a surface of cotton cellulose fibres. It was confirmed that soaking of the as-received Ciegal cotton in 1.5 × SBF for 14 days did not lead to any growth of Ca–P-containing material on the fibres.

Fig. 1b is an SEM micrograph of cotton phosphorylated by the urea/phosphorous acid method. When cotton is phosphorylated by this method, swollen gel-like fibres are obtained which shrink upon drying to form a very tough material which is difficult to pull apart. Rewetting of the fibres caused the fibres to expand again. Inagaki *et al.* [6] have reported that phosphorylation of cotton fibres leads to the incorporation of phosphite groups into the material. SEM micrographs revealed that the phosphorylated cotton fibres were agglomerated with a higher level of disorder and contortion than was observed in the as-received Ciegal fibres (see Fig. 1b). EDX analysis of the phosphorylated fibres indicated an intense PK_α signal. When being examined at high magnification, it was noticed that the surface of the phosphorylated cotton fibres tended to split under the action of the electron beam. This was believed to be due to fluid within the fibres escaping when subjected to the intense electron beam. A similar phenomenon was not observed with pre-phosphorylated cotton fibres.

Micro-FTIR of the cotton fibres before and after phosphorylation are given in Fig. 2a and 2b. The spectrum of the phosphorylated fibre exhibited a peak at 2360 cm^{-1} which is due to P–H stretching in a phosphite group. It was also obvious that the phosphorylated fibres contained a noticeable amount of aqueous fluid as shown by the broadening of the peak in the O–H stretching region of the spectrum. The introduction of the phosphorus-containing functionalities appears to endow the fibres with gel-like properties. In fact, when cellulose in powdered form is phosphorylated by urea and phosphorous acid, the product is found to be water-soluble [6]. ^{31}P solid state NMR spectra of the phosphorylated fibres (Fig. 3a) feature a sharp peak at 0 ppm and a relatively

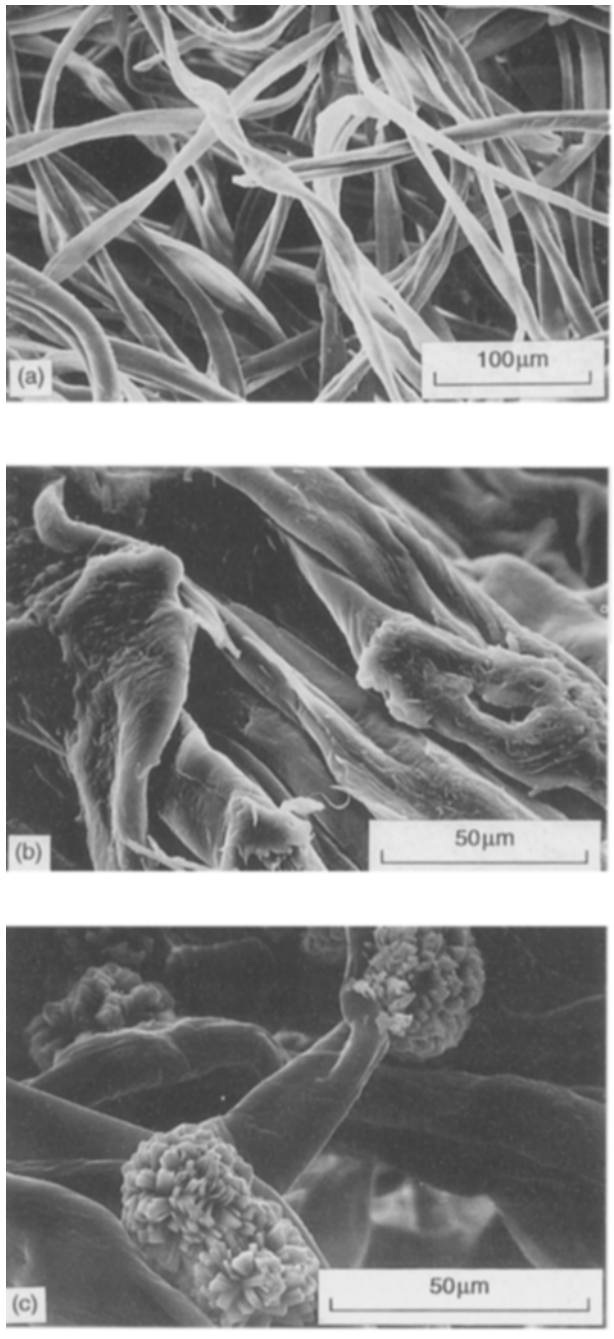

Figure 1 Scanning electron micrographs of (a) as-received cotton, (b) phosphorylated cotton and (c) $Ca(OH)_2$-treated phosphorylated cotton showing calcium phosphite clusters.

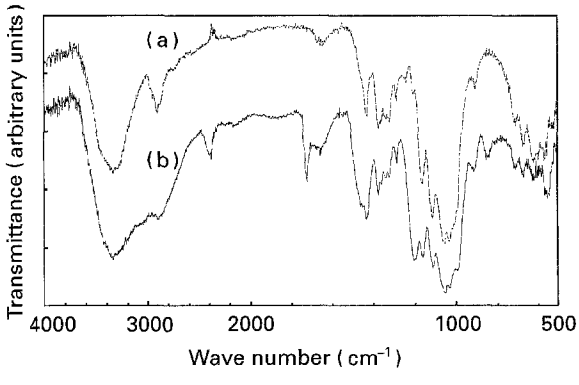

Figure 2 Micro-FTIR spectrum of (a) as-received cotton fibres before phosphorylation, and (b) after phosphorylation.

broader peak at about 7 ppm with associated spinning side-bands at $\pm$ 40 ppm. These peaks are similar in position, respectively, to the major peak positions observed for ^{31}P solid state NMR spectra of calcium hydroxy apatite and calcium phosphite powder samples. The ^{31}P MAS NMR spectrum of phosphite is particularly characteristic giving a spinning sideband pattern arising as a consequence of shielding anisotropy. However, given the sharpness of the peak at 0 ppm and the previous SEM observation on the splitting of the fibres due to fluid escape under the electron beam, it is believed that this peak is due to phosphoric acid contained in the fibres. The 0 ppm peak was at its most intense in a spectrum of fresh cotton sample but re-examination of the cotton after standing with time (mixed with kaolinite packing material) gave a spectrum in which the sharp 0 ppm peak was considerably weaker existing merely as a shoulder on the dominant phosphite signal. This confirms a solution-type species whose peak intensity is thus sensitive to the moisture content of the sample. The storage of the sample mixed with kaolinite powder which is likely to be acting as a desiccant has most likely led to a reduction in this signal intensity. The sharp peak at 0 ppm reappeared when a drop of water was added to moisten the NMR sample so confirming that it was a solution species. The presence of phosphoric acid in the cotton may be explained by the fact that phosphorous acid is known to decompose to phosphoric acid upon heating [7], thus, it is likely that some phosphoric acid may remain in the fibres after washing and drying.

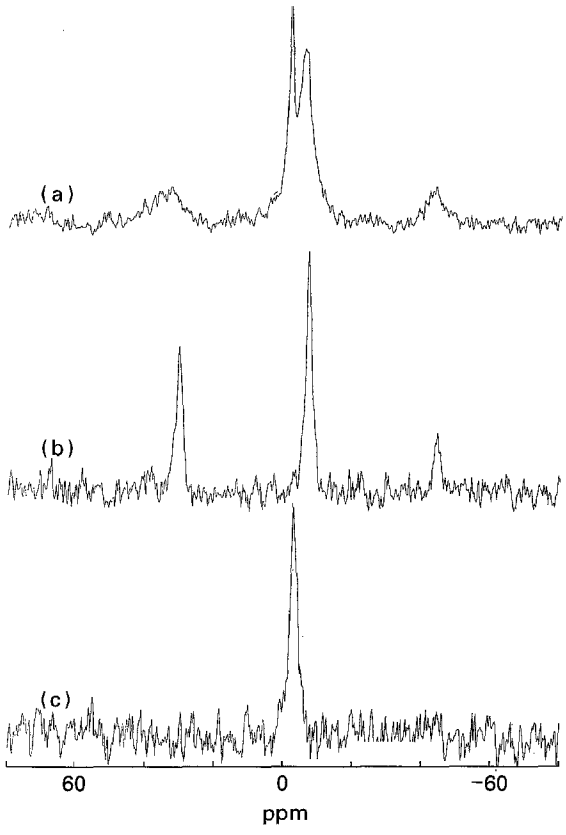

Figure 3 ^{31}P solid state NMR spectra of (a) phosphorylated cotton (without cross-polarization, 292 transients); (b) $Ca(OH)_2$-treated phosphorylated cotton (with cross-polarization, 200 transients); and (c) $Ca(OH)_2$-treated phosphorylated cotton soaked in 1.5 × SBF for 17 days (without cross-polarization, 200 transients).

In the X-ray photoelectron spectrum of the fibres, P2s and P2p (see Table I) as well as N1s peaks could be observed. The N1s peak was believed to be due to a small amount of urea remaining in the fibres from the phosphorylation reaction. The detection of P peaks indicated that there was a significant presence of ion exchangeable phosphite groups at the surfaces of the fibres as opposed to the previously studied spherical ion-exchange resins [4] where most or all of the active ion-exchange groups were contained in the interior of the resin.

TABLE I X-ray photoelectron spectral data from ESCA studies of growth of calcium phosphate on surface-modified cotton

Sample	Ca2p	P2p	C1s	O1s	C (%)	O(%)	Ca (%)	P (%)	Ca:P
C	—	—	285.44	531.95	52.17	44.51	—	—	—
PC	—	133.03	285.46	531.47	45.21	43.77	0	5.65	0
PCCa	347.99	133.98	285.07	532.49	51.16	41.83	2.69	4.32	0.62
PCCa4d	0	133.53	284.59	532.11	61.42	36.18	0	2.40	0
CPC	349.03	133.06	285.51	532.32	55.87	39.72	1.49	1.17	1.27
CPC1d	347.51	133.38	285.42	532.38	58.0	37.06	2.32	2.61	0.88
CPC2d	348.04	133.86	286.39	532.78	49.04	42.32	4.63	4.0	1.16
CPC3d	347.89	133.45	285.94	532.54	45.97	47.84	3.36	2.82	1.19
CPC4d	348.03	134.11	286.32	532.17	36.85	50.61	8.38	4.16	2.01
CPC5d	347.95	133.96	285.97	532.03	37.33	45.65	9.37	7.65	1.22
CPC17d	348.0	133.81	286.0	532.01	44.99	43.99	6.27	4.76	1.32
Apatite[a]	347.66	133.95	284.97	531.45	22.92	46.16	18.49	12.44	1.49
Phosphite	348.03	133.61	284.99	531.94	27.30	48.29	12.88	11.54	1.10

C = Chiyoda Co Ciegahl cotton as received from supplier
PC = Phosphorylated cotton
PCCa = Phosphorylated cotton soaked for 1 day in 0.1 mol l^{-1} $Ca(NO_3)_2$ solution
PCCa4d = PCCa sample soaked for 4 days in 1.5 × SBF (simulated body fluid solution) at 36–37°C
CPC = $Ca(OH)_2$-treated phosphorylated cotton (achieved by soaking in saturated calcium hydroxide for periods of 8–12 days)
CPc*n*d = $Ca(OH)_2$-treated phosphorylated cotton soaked for *n* days in 1.5 × SBF solution at 36–37°C
Apatite = Central Glass Co. calcium hydroxy apatite
Phosphite = Wako calcium phosphite monohydrate.
[a]Using data from reference [4]

3.1.2. Straight soaking of phosphorylated cotton in 1.5 × SBF: SEM/EDX studies

Initially, the phosphorylated cotton as received after reaction was soaked in 1.5 × SBF for 10 days. However, SEM examination revealed that no calcium phosphate compounds had grown on the surface of the fibres. The EDX analysis showed in fact that an uptake of the cationic components of the 1.5 × SBF, namely, Na^+, Mg^{2+}, K^+, and Ca^{2+} with the Ca^{2+} ion being taken up the most by the fibres (see Table II for Ca:P ratio) had occurred. The P signal also remained a dominant feature of the EDX spectrum. Therefore, the phosphorylated fibres were simply behaving as ion-exchange materials in the 1.5 × SBF.

Given that the fibres exhibited a preference for the uptake of Ca^{2+} ion, it was decided to saturate the fibres by stirring in 0.01/0.1 and 1.0 $mol\,l^{-1}$ solutions of $Ca(NO_3)_2$ following the method used in an earlier study of the growth of calcium phosphate on spherical DOWEX and AMBERLITE ion-exchange resins [4]. A maximum saturation level of Ca:P of 0.59–0.64 was observed for the fibres soaked in the 0.1 and 1.0 $mol\,l^{-1}$ $Ca(NO_3)_2$ solutions. X-ray photoelectron spectra of the saturated fibres also showed that Ca and P were present at the surface of the fibres. Despite this, subsequent exposure of the fibres to 1.5 × SBF solution for 4 days failed to produce any calcium phosphate formation on the fibres. SEM showed that Ca:P ratios had decreased from 0.59–0.64 to values in the vicinity of 0.24–0.31 thus suggesting a leaching out of Ca^{2+} ion from the fibres (see Table II). It is likely that these fibres have an ion exchange capacity lower than that of the AMBERLITE and DOWEX resins studied earlier [4] so that the saturated fibres *per se* do not seem capable of stimulating precipitation (by diffusion of Ca^{2+} ion from the fibres) of calcium phosphate from 1.5 × SBF solution.

TABLE II Summary of SEM/EDX data for apatite, calcium phosphorylated cotton fibre studies.[a]

System	Ca:P ratio (± % error)[b]	Comments
PCSBF[c]	0.22	No Ca-P compound formed
PCCa	0.64	No Ca-P compound formed
PCCa4d	0.31	No Ca-P compound formed
CPC	1.06 (5.7)	Calcium phosphite clusters
CPC1d	1.29 (22)	Coating on fibres
CPC2d	1.34 (28)	Coating on fibres
CPC3d	1.21 (11.6)	Coating on fibres
CPC4d	1.37 (12.4)	Coating on fibres
CPC5d	1.51 (7.3)	Coating on fibres
CPC17d	1.60 (24)	Coating on fibres
Apatite[d]	1.70 (1.8)	Powder
Phosphite	0.99 (4.0)	Powder

[a] New abbreviations used in the Sample column are defined below. For the other abbreviations, see footnotes for Table I.
[b] For Samples PCSBF, PCCa, and PCCa4d, only one EDX measurement was taken while for the remainder of the samples in the table, 3–5 EDX measurements in different areas of the sample were taken and averaged.
[c] PCSBF = phosphorylated cotton soaked for 10 days in 1.5 × SBF (simulated body fluid) solution 36–37°C.
[d] From reference [4]

3.2. $Ca(OH)_2$-treated phosphorylated cotton (CPC)

3.2.1. SEM/EDX studies

After the failure to grow calcium phosphate on the surfaces of the untreated and Ca^{2+}-saturated phosphorylated fibres, it was decided to soak the untreated phosphorylated fibres in saturated $Ca(OH)_2$ in the hope that the highly basic conditions would lead to enhanced uptake of Ca^{2+} ion by deprotonation of OH groups on the phosphite functionality followed by binding of the Ca^{2+} ion. However SEM/EDX examination of the fibres unexpectedly showed highly crystalline clusters which were embedded in or attached to the fibres. Fig. 1c is an SEM of nucleated phosphorylated cotton which illustrates the clusters. EDX spot and area analyses of the clusters usually indicated a Ca:P ratio of 1:1 (see Table II). Although the bulk of the clusters gave Ca:P ratios of this magnitude, other clusters or deposits could be found which were rich in Ca only. These could be residues of the original $Ca(OH)_2$ used or possibly $CaCO_3$.

3.2.2. Micro-FTIR/NMR studies

When micro-FTIR spectra of the embedded clusters were acquired (see Fig. 4a and b), a characteristic spectrum consisting of intense peaks at 3357, 2437, 1664, 1123, 1055, 991, 643, and 581 cm^{-1} was observed. A literature search [8] revealed that the spectrum corresponded to calcium phosphite monohydrate ($CaHPO_3 \cdot H_2O$). This was confirmed by comparing the spectrum with that of commercial (WAKO) $CaHPO_3.H_2O$. In addition, the solid state ^{31}P NMR spectrum of $Ca(OH)_2$-treated phosphorylated cotton (Fig. 3b) corresponded exactly to that for calcium phosphite. Thus it is obvious that the action of saturated $Ca(OH)_2$ solution upon the phosphorylated cotton fibres leads to the partial hydrolysis of the phosphite functionalities in the cotton fibres to free phosphite. Given the static conditions of the soaking experiment, it is likely that high concentrations of phosphite accumulate in regions of the fibre during hydrolysis and consequently crystallise to form the calcium phosphite hydrate compound. The transient peak assigned to residual phosphoric acid which was observed in samples of phosphorylated cotton prior to $Ca(OH)_2$ treatment had completely disappeared.

In the $Ca(OH)_2$-treated cotton fibre system, there was also evidence of coating of fibres with calcium phosphite. X-ray photoelectron studies of $Ca(OH)_2$-treated phosphorylated cotton will be discussed in the following section where spectra of $Ca(OH)_2$-treated samples before and after soaking in 1.5 × SBF from 1–17 days are dealt with.

3.3. Soaking of $Ca(OH)_2$-treated phosphorylated cotton in 1.5 × SBF

3.3.1. SEM/EDX studies

In contrast to the previous attempts at calcium phosphate growth described earlier, the treatment of the phosphorylated fibres in $Ca(OH)_2$ successfully led to the deposition of calcium phosphate on the fibres

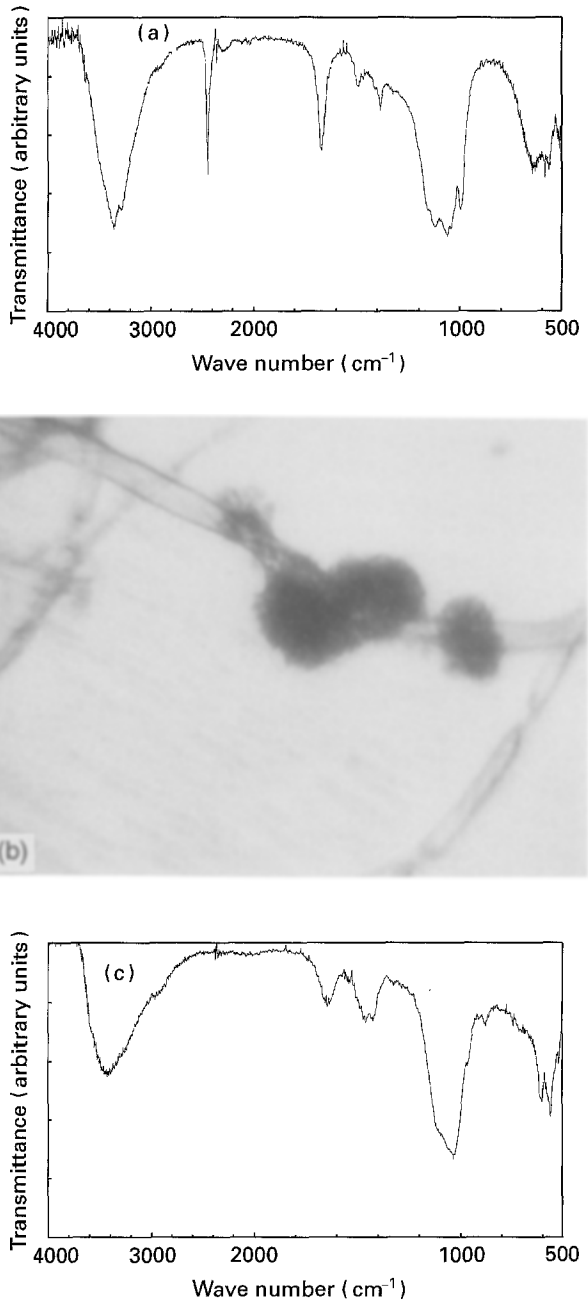

Figure 4 (a) Micro-FTIR spectrum of clusters in $Ca(OH)_2$-treated phosphorylated cotton; (b) micro-FTIR photograph of typical sampled area; and (c) micro-FTIR spectrum of calcium phosphate coating grown on $Ca(OH)_2$-treated phosphorylated fibre after 17 days soaking in 1.5 × SBF solution.

upon immersion in 1.5 × SBF. The growth of calcium phosphate was consequently investigated as a function of soaking time to confirm whether progressive accumulation of calcium phosphate on the cotton fibres would occur.

Fig. 5a is an SEM micrograph of $Ca(OH)_2$-treated phosphorylated cotton fibres soaked in 1.5 × SBF for 1 day. In general, the growth of calcium phosphate from 1.5 × SBF begins immediately after one day of soaking. However, it was evident that large areas of uncoated fibres were still present as well as fibres showing calcium phosphate coatings in the preliminary stages of formation. In addition, calcium phosphite clumps could still be observed in the fibres but not as commonly as in the sample of $Ca(OH)_2$-treated phosphorylated cotton prior to exposure to 1.5 × SBF. This suggests that the clusters have partially dissolved upon introduction of the cotton into the 1.5 × SBF solution. A combination of EDX area and spot analyses on the 1-day soaked sample gave variable Ca:P ratios ranging from 1.0 to 1.73. EDX analyses of the thin nascent calcium phosphate coatings on the fibres are complicated by probable contributions to the P peak intensity of phosphite groups bound to the underlying cotton fibres which the electron beam may also be sampling. For the fibre illustrated in Fig. 5a, spot analyses carried out in the coated and uncoated regions of the fibre yielded Ca:P ratios of 1.11 and 0.68, respectively. The latter Ca:P ratio (0.68) for the uncoated region of the fibre is only slightly higher in magnitude than the maximum value of Ca:P ratios observed for phosphorylated fibres pre-saturated with 0.1 mol l^{-1} $Ca(NO_3)_2$. It was noted that the calcium phosphate was usually deposited in the form of small spheres.

After 2 days of soaking in 1.5 × SBF, most of the cotton fibres appeared to be covered with a coating consisting of spherical calcium phosphate particles which was growing in thickness (see Fig. 5b). For coated areas of fibres, an average Ca:P ratio of 1.34 ± 28% (see Table I). In several of the individual EDX analyses, Ca:P ratios around 1.5 were obtained which is similar to values assigned to amorphous calcium phosphate by previous workers [4, 9, 10]. By the third day of soaking, the fibres were extensively covered with calcium phosphate material. On some fibres, large clumps of calcium phosphate material starting to grow on pre-existing coatings were in evidence (see Fig. 5c). Individual EDX analyses in different areas of the fibres were still variable giving values of Ca:P ratios from 1.06 to 1.43. SEM micrographs of fibre samples soaked in 1.5 × SBF for 4 to 5 days were similar with the calcium phosphate coatings looking thicker in appearance. Some fibres were becoming caked in calcium phosphate coatings (see Fig. 5d). EDX measurements of Ca:P ratios were also becoming more consistent which is probably due to the thickening coatings of calcium phosphate which may give Ca:P ratios which are less influenced by contributions from phosphite groups chemically bound to underlying fibres. For the 5-day soaked sample, the average Ca:P ratio was 1.5, similar to that for amorphous calcium phosphate.

The cotton samples soaked in 1.5 × SBF solutions for up to 17 days gave very different coatings (see Fig. 5e). In this case, the fibres had become mostly caked with a lumpy, non-uniform coating of calcium phosphate. This is evidence that the coating appears to grow continually over this long soaking period. An average Ca:P ratio of 1.60 with a large percentage error of 24% was obtained. This was because one of the measurements was particularly Ca-rich (Ca:P = 2.22 for a coated fibre). This unusual phenomenon of calcium enrichment in the coating was also observed in studies of calcium phosphate growth on Ca^{2+}-ion presaturated ion exchange resins after long soaking periods [4]. In those studies, it was suggested that Ca^{2+} ion leaching into the coating from the resin could be responsible. Given that

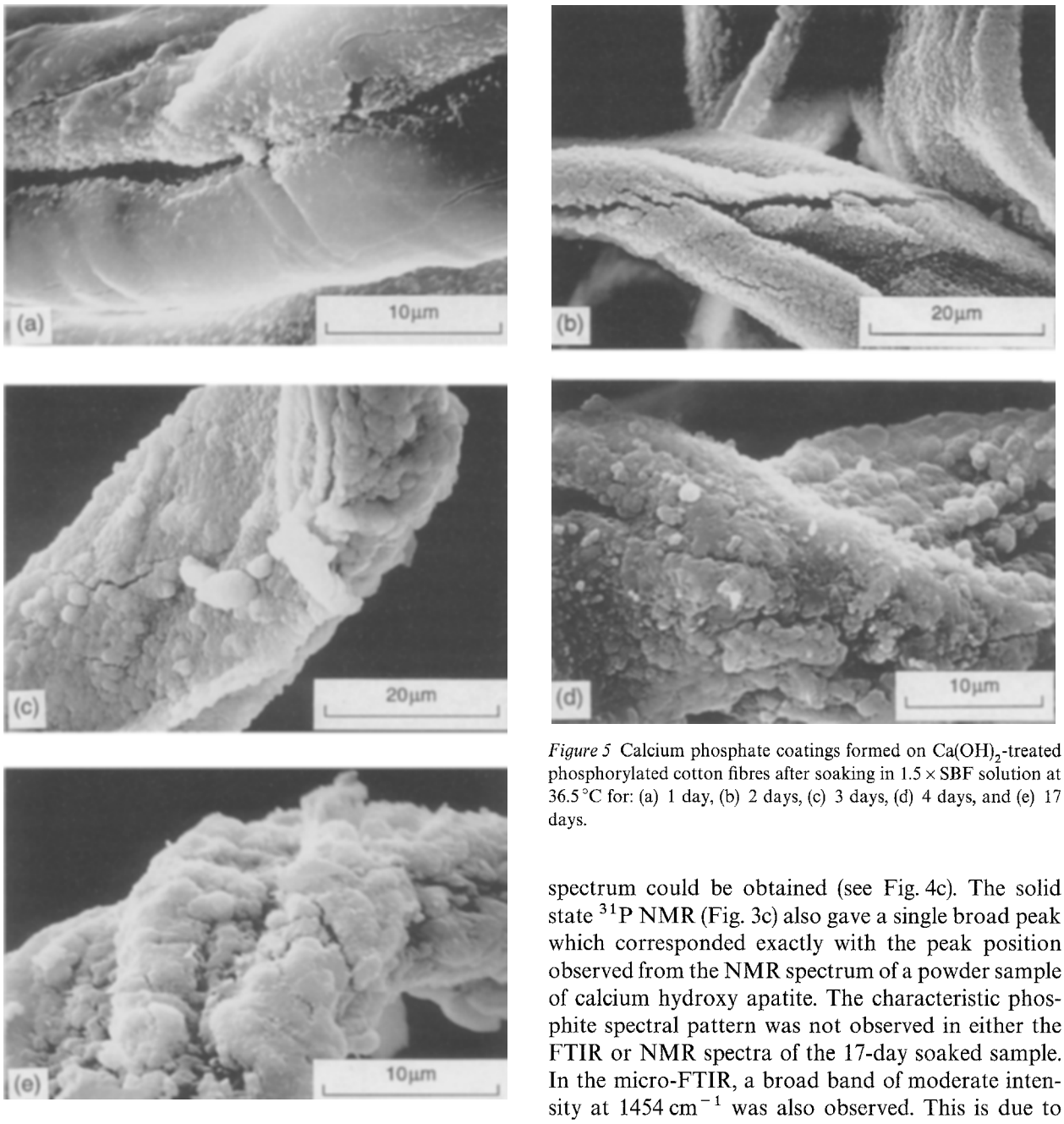

Figure 5 Calcium phosphate coatings formed on $Ca(OH)_2$-treated phosphorylated cotton fibres after soaking in 1.5 × SBF solution at 36.5 °C for: (a) 1 day, (b) 2 days, (c) 3 days, (d) 4 days, and (e) 17 days.

the phosphorylated fibres in the present study also possess ion exchange properties, it is possible that the fibres also leach Ca^{2+} ion into the calcium phosphate coating over time.

3.3.2. Micro-FTIR/NMR studies

Micro-FTIR and solid state ^{31}P NMR studies of the 1.5 × SBF-soaked cotton were carried out in order to confirm that calcium phosphate was being deposited. However, characterization of the calcium phosphate coating in early stages of growth (after 1–4 days of soakings in 1.5 × SBF) was difficult given the thinness of the coatings produced. In fact, calcium phosphite clusters were still observed in some samples by micro-FTIR after 4 days of soaking in 1.5 × SBF. It was only after 17 days of soaking $Ca(OH)_2$-treated phosphorylated cotton in 1.5 × SBF that an infrared spectrum similar to that of a typical calcium phosphate spectrum could be obtained (see Fig. 4c). The solid state ^{31}P NMR (Fig. 3c) also gave a single broad peak which corresponded exactly with the peak position observed from the NMR spectrum of a powder sample of calcium hydroxy apatite. The characteristic phosphite spectral pattern was not observed in either the FTIR or NMR spectra of the 17-day soaked sample. In the micro-FTIR, a broad band of moderate intensity at 1454 cm^{-1} was also observed. This is due to carbonate ion which implies that the amorphous calcium phosphate phase formed contains carbonate in its structure.

3.3.3. X-ray photoelectron spectroscopy

$Ca(OH)_2$-treated phosphorylated cotton. X-ray photoelectron spectra of $Ca(OH)_2$-treated phosphorylated cotton exhibited very weak Ca and P peaks (see entry "CPC" in Table I). These were weaker in fact than Ca and P-associated peaks observed in the X-ray photoelectron spectrum of phosphorylated cotton soaked for 1 day in 0.1 mol l^{-1} $Ca(NO_3)_2$ solution (see entry "PCCa" in Table I). The weak intensity observed is consistent with most of the Ca and P in the sample being present as calcium phosphite clusters randomly lodged within the cotton fibre sample and thus not directly accessible to this surface analysis technique. The Ca : P ratio obtained from a surface element table calculated from spectra was found to be 1.27. In contrast, the EDX-derived Ca : P ratio for a large area of

the sample (magnification 50× corresponding to a scale size of 1 mm) was found to be 1.63. The excess of Ca over P is an important difference when comparisons are made with Ca:P ratios (Both XPS- and EDX-derived) for the phosphorylated cotton sample which was only saturated with 0.1 mol l^{-1} $Ca(NO_3)_2$ (sample PCCa, Tables I and II). In these cases, Ca:P ratios were found to be only 0.62–0.64. Moreover, if phosphorylated cotton is not soaked in $Ca(OH)_2$ for the period of time needed to allow formation of the calcium phosphite clusters, calcium phosphate coatings are not observed on the surfaces of fibres after soaking in 1.5 × SBF.

$Ca(OH)_2$-treated phosphorylated cotton soaked in 1.5 × SBF. The X-ray photoelectron spectroscopic results for $Ca(OH)_2$-treated phosphorylated cotton soaked as a function of time in 1.5 × SBF are summarized under the entries of CPC*n*d (n = 1–5, 17) in Table I. A typical X-ray photoelectron spectrum of calcium phosphate growth on the cotton fibres (after 5 days soaking in 1.5 × SBF) is given in Fig. 6. It was obvious from surface analyses that calcium phosphate coverage on the $Ca(OH)_2$-treated phosphorylated cotton showed an overall increase with time of soaking in the 1.5 × SBF solution. The Ca2p, P2p and O1s binding energy positions did not change dramatically with coverage. Binding energy positions for Ca2p and P2p were in the range expected for calcium phosphate and calcium phosphite species (Table I). There was a small shift of the Cls binding energy from 285.5 to 286.0 eV as calcium phosphate coverage increased on the fibre surfaces. This may be due to small contributions from carbonates in the calcium phosphate. This was supported by micro-FTIR spectra of the $Ca(OH)_2$-treated phosphorylated cotton sample soaked for 17 days in 1.5 × SBF which revealed a carbonate-associated peak at 1454 cm^{-1} (Fig. 4c).

3.3.4. Mechanism of formation of coating

It is obvious that pre-soaking of the phosphorylated fibres in saturated $Ca(OH)_2$ is essential for the growth of calcium phosphate on the surface when the fibres are soaked in 1.5 × SBF. A further observation is that if the $Ca(OH)_2$ soaking is not carried out to the stage at which clusters of calcium phosphite are observed

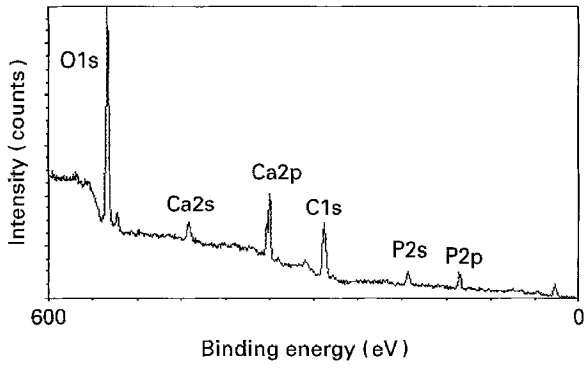

Figure 6 X-ray photoelectron spectrum of calcium phosphate grown on $Ca(OH)_2$-treated phosphorylated cotton fibres after 5 days soaking in 1.5 × SBF.

lodged in the fibres, then growth of the calcium phosphate coating after soaking in 1.5 × SBF is not observed. In such a case, only loss of the Ca^{2+} ions held by phosphite groups bound to the fibre into the solution is observed. EDX analyses of an area at low magnification corresponding to a scale bar reading of 1 mm on the micrograph of the sample reveal that in samples of $Ca(OH)_2$-treated phosphorylated cotton, Ca:P ratios are always > 1, i.e. there is an excess in Ca over P. It has also been observed that the calcium phosphite clusters in $Ca(OH)_2$-treated phosphorylated cotton eventually dissolve out of the sample as the calcium phosphate coating grows over the soaking time period. Further experiments to confirm this general dissolution were conducted by soaking $Ca(OH)_2$-treated phosphorylated cotton samples in solutions containing (i) TRIS ($(CH_2OH)_3CNH_2$)/HCl and (ii) 1.5 × SBF to which no K_2HPO_4 had been added. Both cotton samples showed no evidence of any calcium phosphate coating nor could any calcium phosphite clusters be detected, which confirms that they had completely dissolved after soaking in these solutions. Thus it appears that part of the mechanism of deposition of calcium phosphate involves dissolution of the calcium phosphite leading to an elevation of the Ca^{2+} ion level which in turn must trigger the formation of the calcium phosphate. Calcium phosphite is relatively more soluble than hydroxyapatite thus the clusters sacrificially dissolve leading to the deposition of the less soluble calcium phosphate.

An important question that needs to be asked is how the calcium phosphate is being nucleated on the surface of the fibres. Although, the phosphorylated fibres appear to have ion exchange properties (with a preference for Ca^{2+} ion), it is obvious that this material behaves very differently from the spherical DOWEX sulfonate ion exchange resins studied earlier [4] which when presaturated with Ca^{2+} ion and soaked in 1.5 × SBF or a biomimetic-type medium exhibited calcium phosphate formation upon their surfaces. Thus, Ca^{2+} saturation alone of the fibres is not enough to trigger calcium phosphate formation on the fibres. It is only after a source of solid dissolvable calcium (in the form of calcium phosphite clusters) is formed at a high pH (where the cluster material is insoluble) and then introduced into a solution medium of lower pH (where the cluster material is relatively more soluble) that calcium phosphate formation can occur. One possible mechanism may involve initial dissolution of the calcium phosphite clusters and reprecipitation on the fibres so giving thin coatings of calcium phosphite which may act as nucleation sites. The evidence supporting this mechanism is given by EDX-measured Ca:P ratios of the nascent calcium phosphate coatings which although scattered are frequently close to 1.0 suggesting a calcium phosphite base. An alternative mechanism could involve the phosphite sites bound to the cotton fibre surface which are capable of binding Ca^{2+} ion and thus acting as nucleation sites for the calcium phosphate coating in a process involving uptake of the Ca^{2+} ions emanating from the locally dissolving calcium phosphite clusters and subsequent formation of calcium

phosphite nuclei, which then function as nuclei for calcium phosphate formation. Yet another possibility is that phosphoric acid previously held inside the phosphorylated fibres has diffused out to form calcium phosphate nucleation centres on the surface of the cotton. This mechanism, however, is not favoured because of (1) the absence of NMR evidence suggesting the presence of calcium phosphate, and (2) the unreliability of infrared spectral data because of the overlapping of spectral peaks (especially ν(PO)) due to phosphate and phosphite species.

In natural mineralization [11], materials must be transported to a calcification region in the body where local conditions must be such that precipitation takes place. The collagen in natural bone tissue is known to have a strong affinity for binding phosphate ions which would create a high concentration of phosphate at specific sites [12]. Thus, in the present system, the cotton is performing a similar function to collagen, i.e. phosphorus-based centre bound either to the cotton or part of a salt film for nucleation appears to be of importance. The calcium phosphite clusters provide favourable local conditions for triggering the formation of calcium phosphate. Given that the understanding of the mechanism of deposition is still not complete, this line of research will be continued with varying conditions in order that better insights might be obtained into the nucleation mechanism, which in turn may make a useful contribution to the existing knowledge on the natural processes of biomineralization.

4. Conclusions

Calcium phosphate growth on cotton fibres phosphorylated with urea/phosphorous acid, soaked in $Ca(OH)_2$ and then in $1.5 \times$ SBF solution has been achieved for the first time. Coatings composed of aggregates of spherical calcium phosphate particles gradually become extremely thick and non-uniform over time effectively caking the fibres with material. Tentatively suggested mechanisms for calcium phosphate formation involve either dissolution of calcium phosphite clusters (produced by the $Ca(OH)_2$ soaking) which serves to elevate the Ca^{2+} ion concentration in the local vicinity of the fibres leading either to Ca^{2+} ion uptake by fibre-bound phosphite groups or a thin calcium phosphite coating that provides nucleation sites for the calcium phosphate coating to grow on.

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