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Growth of calcium phosphate on ion-exchange resins pre-saturated with calcium or hydrogenphosphate ions: an SEM/EDX and XPS study

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Calcium phosphate formed on the surfaces of ion-exchange resins pre-saturated with either Ca^{2+} or HPO_4^{2-} ions has been studied using a combination of scanning electron microscopy (SEM)/energy dispersive X-ray (EDX) analysis and X-ray photoelectron spectroscopy (XPS). Calcium phosphate was formed at a temperature of 36.5 °C via two methods. On Ca^{2+} or HPO_4^{2-} -saturated resins, 1.5 × SBF (simulated body fluid) solution was used while on Ca^{2+} -saturated resins only, a novel biomimetic growth medium using the alkaline phosphatase-catalysed hydrolysis reaction of disodium p-nitrophenylphosphate as a source of inorganic phosphate was employed. SEM micrographs showed that the use of 1.5 × SBF growth medium solution led to extensive coverage of the resins with calcium phosphate. In contrast, calcium phosphate coatings formed via the alkaline phosphatase-catalysed reaction were of a more variable quality whose morphology could be influenced by adding albumin and collagen to the growth medium. Average Ca:P ratios determined by EDX for coatings formed from the 1.5 × SBF growth medium were in the range 1.62–1.74 suggesting that hydroxyapatite had formed. In contrast, Ca:P ratios for the calcium phosphate compounds formed on resins from the alkaline phosphatase reaction were lower at 1.50 suggesting that calcium-deficient hydroxyapatite had formed which was confirmed by inductively coupled plasma (ICP) analysis and X-ray diffraction of isolated amorphous and crystallized powder samples, respectively. Evidence from X-ray photoelectron studies supports a mechanism of formation of the coatings which involves diffusion of the ion out of the interior of the resin to create a high local concentration at the surface thus stimulating precipitation of the coating material on the resin beads.

1. Introduction

Recently, research into substrates designed to stimulate the biomimetic growth of hydroxyapatite on their surface has become increasingly active because of the important contributions such studies can make towards the understanding of biomineralization processes in the body. Previous studies by other researchers have centred around a variety of substrates. Kokubo *et al.* [1, 2] studied the formation of bone-like hydroxyapatite (HAP) on a variety of substrates by soaking the substrate to be coated in a solution of SBF (simulated body fluid) and CaO, SiO_2 beads from which Ca dissolves increasing the ionic activity product of the apatite in the solution leading to the formation of apatite nucleation sites on the immersed substrate. Mathers *et al.* [3] studied the growth of hydroxyapatite on type 1 collagen. They found that crystal nucleation and growth was much faster on collagen samples which had been pre-soaked in acetic acid to form a gel rather than on untreated forms. Dalas and Kallistis [4, 5] phosphorylated Porapak-N, divinylbenzene copolymer and polystyrene polymers in order to induce nucleation and growth of hydroxyapatite on the surface of these materials. Given that biomineralization processes are considered to involve epitaxial or oriented growth of hydroxyapatite, materials allowing the lattice matching of both substrate and hydroxyapatite are sought after in studies of the crystal growth of HAP.

In the present study, ion exchange resins were considered as a novel substrate on which to grow hydroxyapatite given that these resins can be selectively saturated with the ions necessary to form calcium phosphate compounds. Thus, selected cationic and anionic ion-exchange resins pre-saturated with either Ca^{2+} or HPO_4^{2-} ions were used as a means of stimulating calcium phosphate formation at the surface of the resin spheres in 1.5 × SBF. A biomimetic-type growth medium involving the alkaline phosphatase-catalysed hydrolysis of p-nitrophenylphosphate to inorganic phosphate was also considered for use as a novel calcium phosphate growth medium.

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2. Experimental procedures

All chemical salts employed in this study were supplied by *Wako Chemicals Co.* and were used without further purification. Ion-exchange resins used were Dowex and Amberlite brand ion-exchange resins. N_2-purged water obtained by distilling water which had been previously passed through an ion-exchange resin was used in the preparation of all solutions. Pre-saturation of the ion-exchange resins was carried out by stirring a pre-weighed quantity (2–3 g) of the ion-exchange resin in 1 mol l^{-1} $Ca(NO_3)_2$ or $(NH_4)_2HPO_4$ (depending on whether the ion-exchange resin was cationic or anionic) for approximately 24 h followed by filtration and rinsing of the resins with distilled water. The resins were then used as received after this procedure without drying. For the experiments involving calcium phosphate growth on the surfaces of the resin particles, two growth mediums were used. The first growth medium was a 1.5 × SBF (simulated body fluid) solution, which has been used by previous researchers [1, 2] prepared by adding 15 ml each of 2.74 mol l^{-1} NaCl, 0.06 mol l^{-1} KCl, 0.05 mol l^{-1} $CaCl_2$, 0.03 mol l^{-1} $MgCl_2$, 0.0895 mol l^{-1} $NaHCO_3$, 0.02 mol l^{-1} K_2HPO_4 and 0.01 mol l^{-1} Na_2SO_4 to a 200 ml volumetric flask along with 25 ml each of 0.4 mol l^{-1} "TRIS" ($(CH_2OH)_3CNH_2$) and 0.36 mol l^{-1} HCl with the remainder of the volume being made up with distilled water. This gave a solution of ions which was approximately 1.5 times the concentration of SBF (simulated body fluid) which had been used in other studies [1, 2]. The TRIS/HCl acted as a buffering agent which kept the pH within the range 7.25–7.60. Usually 10 ml of this solution was added to around 0.70 g of either Ca^{2+}-saturated or HPO_4^{2-}-saturated resin samples (i.e. "Cl-Dowex" [1-X4, 200–400 mesh, Cl^- form, saturated with HPO_4^{2-}], "H-Dowex" [50W-X8, 200–400 mesh, H^+ form, saturated with Ca^{2+}] and Amberlite IRA-900 [Cl^- form, saturated with HPO_4^{2-}] in plastic flasks. The pH of the solution was subsequently measured before immersion of the plastic flasks for periods ranging from 2–10 days in a covered water bath which was kept at 36.5 °C by thermostatic control. During any given soaking period, the flasks were taken out of the water bath each day, the pH of the old solution measured which was then filtered off to be replaced by a further addition of 10 ml of fresh 1.5 × SBF with subsequent measurement of pH and reimmersion of the flasks in the water bath.

The second growth medium was a novel formulation of a biomimetic nature which involved the well-known alkaline-phosphatase-catalysed reaction of p-nitrophenyl phosphate (PNP) to p-nitrophenol and inorganic phosphate. The general solution composition or "standard" solution for this growth medium consisted of around 0.70 g of Ca^{2+}-saturated "H-Dowex" resin, 0.05 mol l^{-1} $Ca(NO_3)_2$ and 0.03 mol l^{-1} disodium p-nitrophenylphosphate using borax buffer as the solvent. The pH of the solution after dissolving the $Ca(NO_3)_2$ and disodium p-nitrophenylphosphate (PNP) in the borax buffer was 9.70. Following equilibration at 36.5 °C of the $Ca(NO_3)_2$ and PNP solution together with the resin 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 syringe into the solution. The solution was then left immersed overnight (12–16 h) in the 36.5 °C water bath followed by measurement of pH and separation of the resin from the solution the next day. To confirm 100% conversion of the PNP to p-nitrophenol, ultraviolet analysis of the diluted filtrate was conducted in the 210–360 nm region where a broad band due to PNP (≈ 310 nm) occurs. Variations to the "standard" solution formulation were made in order to examine the effects on the morphology of the calcium phosphate produced. These were omission of $Ca(NO_3)_2$, using a solution of 0.1% or 1% of albumin (*NBCO Biochemicals* crystallized bovine albumin) and a solution of 0.1% albumin and 0.03 g l^{-1} collagen (*Cellmatrix Type I-A*) in borax buffer as solvent.

Scanning electron micrographs and EDX analyses were obtained using a Hitachi S-530 scanning electron microscope to which a Horiba EMAX-2200 X-ray microanalyser was attached. EDX-measured Ca : P ratios were measured on C-coated samples using CaF_2 and GaP standard spectra provided with the EDX software. Micrographs were usually obtained of platinum-coated specimens. X-ray photoelectron spectra of samples mounted on double-sided sticky tape were recorded on a Kratos XSAM 800 X-ray photoelectron spectrometer using gold foil as a reference. ICP measurements of the Ca:P ratio in various calcium phosphate powder samples prepared in this study were carried out using a Seiko Instrument SPS 7000 A desk-top inductively coupled emission spectrometer. The solvent medium used in such ICP measurements was 1 mol l^{-1} HNO_3. Infrared spectra were recorded on a JASCO FTIR-8000 of KBr pellets of the samples. X-ray diffraction spectra were obtained on an MXP materials analysis and characterization XRD spectrometer using CuK_α radiation.

3. Results and discussion

Table I is a description of the various systems examined by SEM, XPS and ICP in this study. Included in the table are studies conducted on calcium phosphate powders prepared (in the absence of any resins) under the same conditions employed when deposition on resin surfaces is carried out (see later).

In general, calcium phosphate formation was found to occur to a significant extent on the three resins used in this study (namely, Ca^{2+}-saturated H-Dowex, HPO_4^{2-}-saturated Cl-Dowex and Amberlite IRA-900). However, other resins that had been investigated (i.e. Ca^{2+}-saturated H-OH Dowex 11A-8, 50–100 mesh, HPO_4^{2-}-saturated Amberlite IRA-400 and IRA-410 as well as Ca^{2+}-saturated Amberlite IRC-50) were not found to be suitable for the growth of calcium phosphate compound due to either (a) disintegration of the resin particles during resin saturation (Amberlite IRA-400 and Amberlite IRA-410) or (b)

TABLE I Description of samples examined in this study. Samples 1R–3R, 1SR–3SR, 1SBF–5SBF and 1AP–5AP were all studied by both SEM/EDX and XPS. Techniques used on the solid powder samples (1APS–3APS and 1HAPS) are indicated in parentheses

Sample	Description of system and techniques applied
1R	As-received H-Dowex Resin
2R	As-received Cl-Dowex Resin
3R	As-received IRA-900 Amberlite Resin
1SR	Ca^{2+} sat. H-Dowex Resin (before soaking in growth medium)
2SR	HPO_4^{2-} sat. Cl^- Dowex Resin (before soaking in growth medium)
3SR	HPO_4^{2-} sat. IRA-900 Amberlite Resin (before soaking in growth medium)
1SBF	Ca^{2+}-sat. H Dowex Resin, 3 day soak in 1.5 × SBF solution
2SBF	Ca^{2+}-sat. H Dowex Resin, 10 day soak in 1.5 × SBF solution
3SBF	HPO_4^{2-}-sat. Cl^- Dowex Resin, 3 day soak in 1.5 × SBF solution
4SBF	HPO_4^{2-}-sat. Cl^- Dowex Resin, 10 day soak in 1.5 × SBF solution
5SBF	HPO_4^{2-}-sat. IRA-900 Amberlite Resin, 3 day soak in 1.5 × SBF solution
1AP	Ca^{2+}-sat. H Dowex Resin/0.03 mol l^{-1} PNP/0.05 mol l^{-1} $Ca(NO_3)_2$/Alk. Phos.
2AP	Ca^{2+}-sat. H Dowex Resin/0.03 mol l^{-1} PNP/Alk. Phos.
3AP	Ca^{2+}-sat. H Dowex Resin/0.03 mol l^{-1} PNP/0.05 mol l^{-1} $Ca(NO_3)_2$/1%Albumin/Alk. Phos.
4AP	Ca^{2+}-sat. H Dowex Resin/0.03 mol l^{-1} PNP/0.05 mol l^{-1} $Ca(NO_3)_2$/0.1%Albumin/Alk. Phos.
5AP	Ca^{2+}-sat. H Dowex Resin/0.03 mol l^{-1} PNP/0.05 mol l^{-1} $Ca(NO_3)_2$/0.1%Albumin/0.03 g l^{-1} Collagen/Alk. Phos.
1APS	Solid obtained from 0.03 mol l^{-1} PNP/0.05 mol l^{-1} $Ca(NO_3)_2$/Alk. Phos. (no resin used) (SEM/EDX, XPS, IR, XRD, ICP)
2APS	Solid obtained from 0.03 mol l^{-1} PNP/0.05 mol l^{-1} $Ca(NO_3)_2$/0.1% Albumin/Alk. Phos. (no resin used) (SEM/EDX, XPS, IR, ICP)
3APS	Solid obtained from 0.03 mol l^{-1} PNP/0.05 mol l^{-1} $Ca(NO_3)_2$/0.1% Albumin/0.03 g l^{-1} Collagen/Alk. Phos. (no resin used) (SEM/EDX, XPS, IR, ICP)
1HAPS	Commercial Sample of Hydroxyapatite (SEM/EDX, XPS, IR, ICP)

TABLE II Summary of SEM/EDX data for calcium phosphate formation on the ion-exchange resin particles from the 1.5 × SBF solution and the alkaline-phosphatase-catalysed hydrolysis of PNP to p-nitrophenol. Data are included for powders generated from alkaline-phosphatase-catalysed reactions as well as for commercial hydroxyapatite (n/a = not applicable)

Sample	Elements observed in EDX	Ca:P ratio (average) EDX	Ca:P ratio (XPS)	Ca:P ratio (ICP)
1SBF	Ca, P, S	1.63	2.11	n/a
2SBF	Ca, P, S	2.15	1.12	n/a
3SBF	Ca, P, Cl (v. strong)	1.62	1.24	n/a
4SBF	Ca, P, Cl (v. strong)	1.73	1.73	n/a
5SBF	Ca, P, Cl	1.74	1.22	n/a
1AP	Ca, P	1.50	2.11	n/a
2AP	Ca, P	2.09	1.48	n/a
3AP	Ca, P	2.07	1.40	n/a
4AP	Ca, P	1.76	1.56	n/a
5AP	Ca, P	1.48	1.49	n/a
1APS	Ca, P	1.50	1.54	1.40
2APS	Ca, P	1.48	1.19	1.44
3APS	Ca, P	1.55	0.98	1.40
1HAPS	Ca, P	1.70	1.49	1.59

failure to form significant quantities of calcium phosphate at the surface after soaking in the 1.5 × SBF (Amberlite IRC-50 and H–OH Dowex resins).

3.1. Scanning electron microscopy

Scanning electron micrographs of the as-received and Ca^{2+}- and HPO_4^{2-}-saturated ion exchange resins (samples 1R–3R) showed smooth regular spheres with little evidence of any foreign deposited material. In the case of the samples soaked in 1.5 × SBF solution, SEM showed that resins not subject to the pre-saturation treatment did not induce any calcium phosphate growth on their surface when soaked in 1.5 × SBF solution. After the pre-saturation treatment, EDX spectra of the Ca^{2+}- and HPO_4^{2-}-saturated resins showed appreciably intense signals due to the saturating ion.

3.2. Calcium phosphate layers formed from the 1.5 × SBF growth medium solution on ion-exchange resins

Table II summarizes the EDX observations and average Ca:P ratios measured for calcium phosphate formation from 1.5 × SBF solution on the Cl-Dowex, H-Dowex and Amberlite IRA-900 ion-exchange resins (samples 1SBF–5SBF). Fig. 1a–e shows the scanning electron micrographs for the systems summarized in Table II. In general, the resin particles in all systems studied showed extensive coverage by calcium phosphate material. In some systems such as the HPO_4^{2-}-saturated Cl-Dowex resin soaked in 1.5 × SBF for 3 days (3SBF) as well as the HPO_4^{2-}-saturated Amberlite IRA-900 resin (5SBF), thick coatings with apparent peeling off and cracking of the coating were observed (Fig. 1a and c). In the case of the HPO_4^{2-}-saturated Cl-Dowex resin soaked in 1.5 × SBF for 10

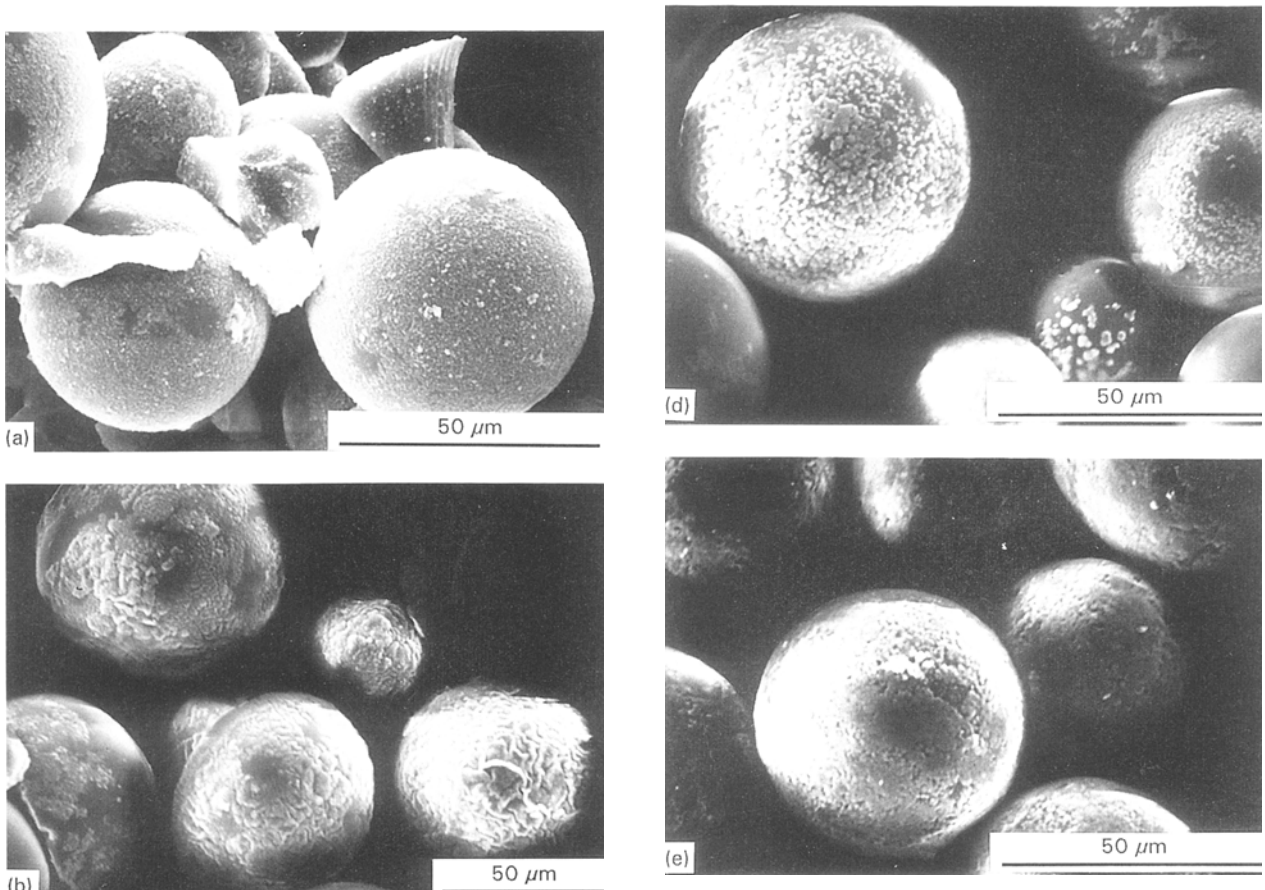

Figure 1 Scanning electron micrographs for calcium phosphate formed on HPO_4^{2-}- and Ca^{2+}-saturated ion exchange resins using the 1.5 × SBF growth medium: (a) sample 3SBF; (b) sample 4SBF; (c) sample 5SBF; (d) sample 1SBF; and (e) sample 2SBF.

days (4SBF), distinct layering patterns of the coating material were observed (Fig. 1b). However, coverage did not appear so high as it did for the sample soaked for 3 days (sample 3SBF) because of flaking off of the coating. Debris was usually seen in the vicinity of the resin particles which has been attributed to spalled off coating material. Average Ca: P ratios determined by EDX were found to be in the range of 1.62–1.74 for samples soaked in the 1.5 × SBF solution for periods of less than 10 days, which indicated that the deposited material was close to hydroxyapatite in composition. Indeed, commercial hydroxyapatite powder (sample 1HAPS supplied by Central Glass Co.) examined by EDX gave an average Ca: P ratio of 1.70. For resin samples soaked up to 10 days, Ca: P ratios were found to increase. Ca^{2+}-saturated H-Dowex resin (1SBF, 2SBF) for which the average Ca: P ratio increased from 1.63 to 2.15 showed a larger increase than the HPO_4^{2-}-saturated Cl-Dowex resin (3SBF, 4SBF) which only increased to 1.73 from 1.62. These observations indicate that the Ca: P ratios measured by EDX are influenced by "leaching" of the excess Ca^{2+} from the interior of the resin into the coating due to prolonged soaking in growth media. Thus variable Ca: P ratios deduced from EDX revealed that there were areas of compositional inhomogeneity in the calcium phosphate deposited on the resins. Ca^{2+} ion appears to diffuse more easily than HPO_4^{2-} ion in this regard. This was demonstrated by the visibly increased coating of Ca^{2+}-saturated H-Dowex resin beads with calcium phosphate after soaking periods of 10 days as opposed to 3 days (Fig. 1a and b). In contrast, EDX analyses carried out on a commercial hydroxyapatite powder showed far less variability in Ca: P ratios. Intense Cl EDX signals were observed in addition to Ca and P in the Cl-Dowex and IRA-900 Amberlite resins, although the origin of these signals is likely to emanate from the interior of the resin rather than from the coating itself as X-ray photoelectron spectra of the calcium phosphate coatings show no trace of Cl^- ion (see later).

The variable particle morphology of the deposited coatings makes ascertaining whether or not the growth is epitaxial a difficult exercise. Although there are formations which suggest epitaxial structure, such as apparent layering (as in Fig. 1b) and "flake-like" formation of calcium phosphate on the resin surface (as in Fig. 1c), it is more likely that the coatings have

formed by a precipitation process than epitaxial growth. It is also impossible to elucidate the phases present in the coatings directly by XRD due to insufficient sensitivity caused by the small amount of calcium phosphate present coupled with the fact that the material formed is amorphous in character.

3.3. Calcium phosphate formed on resins from the growth medium employing alkaline phosphatase-catalysed hydrolysis of PNP to p-Nitrophenol and phosphate

3.3.1. "Standard" alkaline phosphatase-catalysed reaction system

Table II (samples 1AP–5AP) summarizes the SEM/EDX data for the calcium phosphate coatings deposited on Ca^{2+}-saturated H-Dowex resins using the novel biomimetic growth medium. Fig. 2a–f shows the scanning electron micrographs obtained from systems involving this reaction. In general, the morphology of the deposited calcium phosphate in the "standard" ($Ca(NO_3)_2$/PNP/alkaline phosphatase) system (1AP) (Fig. 2a) differed significantly from the calcium phosphate formed in the $1.5 \times$ SBF growth medium. Coatings were patchy and uneven with disproportionately large clumps of calcium phosphate material distributed on the resin particles. These clumps of material had probably formed in solution by precipitation of the Ca^{2+} ion from the dissolved $Ca(NO_3)_2$ and had subsequently settled and adhered to the resin. The sparser coating areas bearing a slight resemblance to honeycombs between the regions occupied by the large clumps most likely represent calcium phosphate compounds formed by precipitation of emerging Ca^{2+} from the interior of the resin. The clumps and sparser coating material consist of small spherical particles. If calcium phosphate powder is prepared in the absence of the Ca^{2+}-saturated resins by adding 300 units of alkaline phosphatase to 0.05 mol l^{-1} $Ca(NO_3)_2$ and 0.03 mol l^{-1} PNP at 36.5 °C and the SEM taken (1APS) (Fig. 2b), the powder particles show the same spherical morphology to that observed on the resins. The average EDX-measured Ca:P ratios for the calcium phosphate deposited on the resins in the "standard" system, as well as for the powder generated in the absence of resins, was 1.50. This was lower than Ca:P ratios measured for coatings formed from the $1.5 \times$ SBF growth medium and commercial hydroxyapatite powder (Table II) and suggest that calcium-deficient hydroxyapatite has formed on the resin particles.

3.3.2. The effect of variations to the "standard" alkaline phosphatase-catalysed reaction solution composition on the morphology of the calcium phosphate coatings

Omission of $Ca(NO_3)_2$ Given that the presence of $Ca(NO_3)_2$ in the "standard" solution used in this growth medium led to precipitation and settling of calcium phosphate on the resin particles, it was decided to investigate the effect of omitting the $Ca(NO_3)_2$ altogether from the solution medium on the morphology of the calcium phosphate produced. Another reason leading to the decision to omit $Ca(NO_3)_2$ was the observation that on using one batch of alkaline phosphatase, the presence of $Ca(NO_3)_2$ in the solution medium tended to retard the alkaline-phosphatase-catalysed hydrolysis reaction which led to negligible calcium phosphate formation and significant amounts of unreacted PNP remaining in solution. Omission of $Ca(NO_3)_2$ was found to solve this problem for the particular batch used. When a second, new batch of alkaline phosphatase was used this ceased to be a problem. It is probable that a solution complex may have formed between the PNP ion and Ca^{2+} ion which is probably more resistant to alkaline-phosphatase-catalysed hydrolysis than is the PNP alone. There appear to be no references to such a complex in the chemical literature.

Fig. 2c shows the SEM of calcium phosphate grown from alkaline-phosphatase-catalysed systems in the absence of dissolved $Ca(NO_3)_2$. The morphology of the calcium phosphate is significantly different from that formed in the "standard" system (cf. Fig. 2a). The particles appear more extensively covered with a thicker coating similar to those formed using $1.5 \times$ SBF as a growth medium. The morphology was more in keeping with the growth of calcium phosphate at the surface alone with no contribution from apparent settling on particles of material formed by precipitation in solution. The average Ca:P ratio measured by EDX (≈ 2.09) was found to be very high in this system which reflected the strong leaching out of Ca^{2+} ion in response to the high phosphate concentration growth medium. In this particular system, the morphology of the deposited calcium phosphate appeared similar in texture to the epitaxially grown "flower-like" hydroxyapatite grown on sol–gel-prepared silica as reported by Li *et al.* [2].

Use of albumin and albumin/collagen solution. The decision to add albumin to the "standard" reaction solution was in order to complex the solution calcium and so avoid the problem described earlier of the less than 100% conversion of PNP to p-nitrophenol and phosphate caused by the possible complexation of PNP with Ca^{2+} ion. Initially, a 1% albumin solution was used which allowed 100% conversion of PNP to p-nitrophenol in the presence of $Ca(NO_3)_2$. However, as shown in Fig. 2d and Table II, the morphology of the deposited calcium phosphate completely changed from that of the "standard" system. Particles were found to be completely covered and stuck together with a thick calcium phosphate coating. The average EDX-measured Ca:P ratio was found to be 2.07 (Table II). which could also be attributed to "leaching" out of excess Ca^{2+} ion from the resin into the coating brought about by the propensity of Ca^{2+} ion to complex with albumin. It was interesting to note from the SEM of this system that in areas of the SEM sample, material was observed which gave only Ca signals upon EDX analysis. This strongly suggested that such

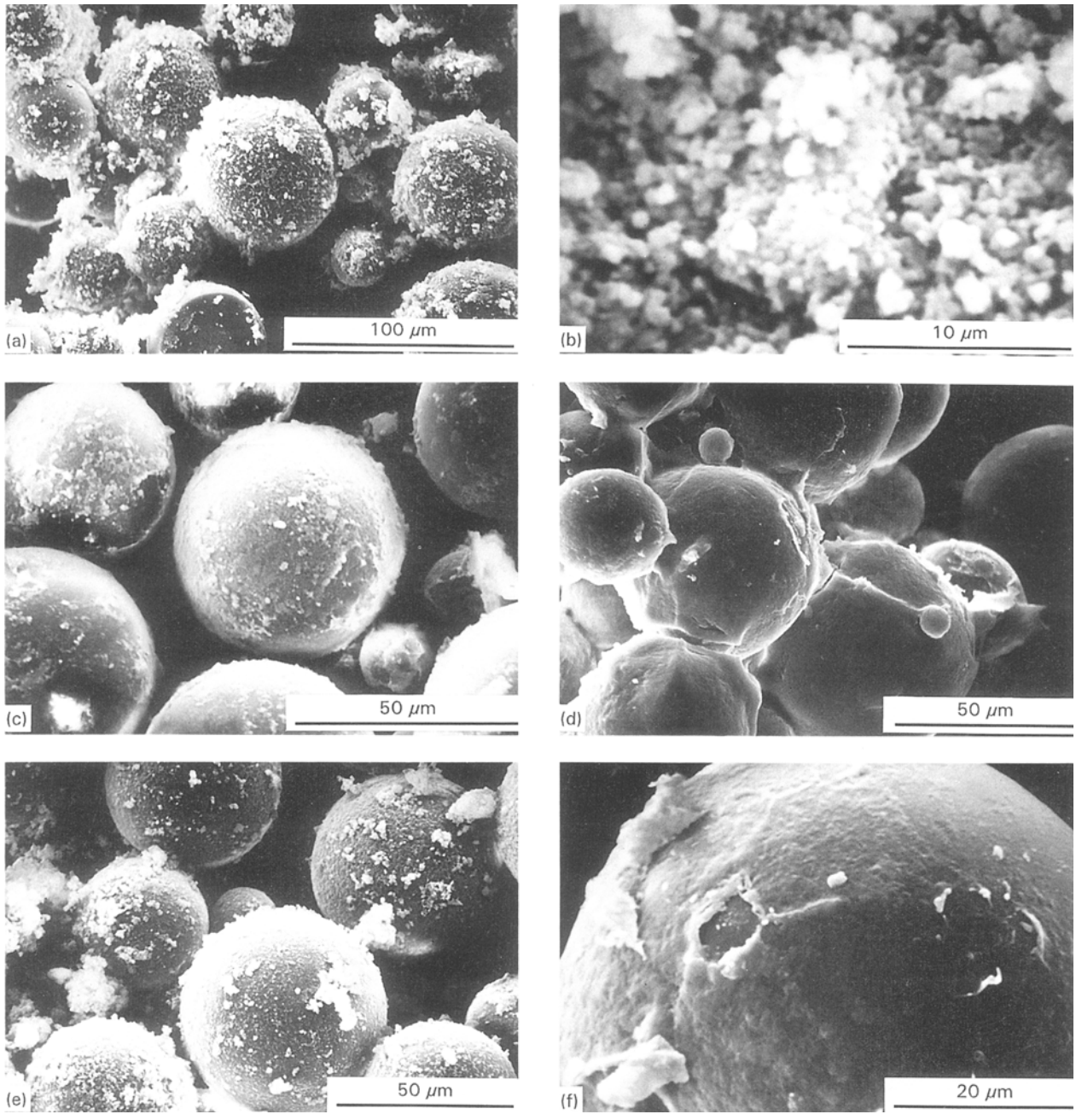

Figure 2 Scanning electron micrographs for calcium phosphate formed on Ca^{2+}-saturated H-Dowex ion exchange resins from the alkaline-phosphatase-catalysed reaction of PNP to p-nitrophenol and phosphate: (a) sample 1AP; (b) sample 1APS; (c) sample 2AP; (d) sample 3AP; (e) sample 4AP; and (f) sample 5AP.

material consisted of Ca^{-} albumin complex. This result is not surprising as previous researchers have reported that albumin adsorbs readily to hydroxyapatite surfaces [6]. Moreover, in the present study, IR analysis of a sample of calcium phosphate solid generated by the alkaline phosphatase reaction in albumin in the absence of the resin (sample 2APS) showed albumin-associated peaks.

When a lower concentration (0.1%) albumin solution was used in the reaction (sample 4AP), the morphology of the deposited calcium phosphate was almost identical to that produced in the "standard" reaction formulation (compare Fig. 2a with Fig. 2e). The average EDX-measured Ca:P ratio, however was found to be 1.76 (see Table II) and may also reflect the "leaching" of excess Ca^{2+} ion into the coating. In contrast, a solid sample generated in 0.1% albumin solution by the alkaline-phosphatase-catalysed hydrolysis reaction (sample 2APS, Table II) in the absence of resin gave a lower average EDX-measured Ca:P ratio of 1.48 which serves to highlight how ion leaching from the resin can distort Ca:P ratio values for calcium phosphate deposited on resins.

Given that hydroxyapatite and collagen are major constituents of natural bone, it was of interest to carry out an experiment involving the growth of calcium phosphate on resin particles by the alkaline-phosphatase-catalysed PNP reaction in a solution containing 0.1% albumin and 0.03 $g\,l^{-1}$ collagen (sample 5AP, Table II). In this case, the morphology of the deposited calcium phosphate changed markedly from the usual patchy, non-continuous coating to a thin

sheet-like coating that enveloped the particles (Fig. 2f). The average EDX-measured Ca:P ratio was 1.48 (Table II) and thus very similar to that measured for coating material prepared in the absence of albumin and collagen. It is possible that the collagen has influenced the calcium phosphate morphology in a similar fashion to that of albumin by adsorbing to the calcium phosphate layer although the solution appears to have caused less leaching out of excess Ca^{2+} ion into the coating than when albumin is used alone.

3.4. X-ray photoelectron spectroscopy

Previous workers have made extensive use of X-ray photoelectron spectroscopy of hydroxyapatite for the characterization of powders and measurement of Ca:P ratios [7], determination of fluoride uptake by hydroxyapatite [8], and the study of surface modifications of hydroxyapatite in aqueous media [9]. In the present study, XPS was used to obtain chemical information on the nature of the deposited material on the resin particles as well as an alternative method for measuring Ca:P ratios from surface atomic concentrations of Ca and P. It was believed that XPS-measured ratios would not be subject to leaching effects as were EDX-measured Ca:P ratios.

3.4.1. General observation

Table III summarizes the peak positions, atom concentrations and Ca:P ratios obtained from the XPS study. Fig. 3 shows the X-ray photoelectron spectrum of the as-received Cl-Dowex resin (sample 2R). The most intense peaks in the spectrum are Cls and Ols which are due to the resin itself. In general, C at % values for Dowex and Amberlite resins before deposition of calcium phosphate (as-received and Ca^{2+}- and HPO_4^{2-}-saturated resins, samples 1R to 3R) used in this study varied from 69–74.4% while O at % values were in the range 19–25.4% (Table III). Peaks associated with the ion-exchange functionalities in the resin (e.g. the quaternized N [plus exchangeable Cl^- ion] and sulfonate group in the Cl and H-Dowex resins, respectively) were either very weak or did not appear at all indicating that relatively few ion-exchange sites existed at the surfaces of the resins and that the bulk of these sites resided in the interior of the resin particles. It was noted that spectra of samples of Ca^{2+}-and HPO_4^{2-}-saturated Dowex and Amberlite resins before exposure to any calcium phosphate growth medium (samples 1SR–3SR in Table III) did not usually exhibit any peak due to the saturating ion. This agrees with the previous observation from X-ray photoelectron spectron of the as-received resins and confirms that the bulk of the saturating ion resides within the resin beads. Thus the mechanism of formation of the calcium phosphate coatings on the Ca^{2+}- and HPO_4^{2-}-saturated resins must principally involve a diffusion of the ion from the interior of the resin, so creating a sufficiently high local concentration of ions at the surface to facilitate precipitation of calcium phosphate from the growth medium. Evidence for diffusion and leaching to the surface was obtained from the X-ray photoelectron spectrum which showed only Ca-associated peaks of modest intensity for a sample of Ca^{2+}-saturated H-Dowex resin sample exposed to a solution containing 0.03 mol l^{-1} PNP, 0.05 mol l^{-1} $Ca(NO_3)_2$) and only 2.5 units of alkaline phosphatase (which had consequently not led to the deposition of any calcium phosphate at the surface).

Fig. 4 is the X-ray photoelectron spectrum of the HPO_4^{2-}-saturated Cl-Dowex resin after being soaked for 3 days in 1.5 × SBF solution (sample 3SBF). Peaks due to Ca2s, Ca2P, P2s and P2p can be observed in the spectrum due to the calcium phosphate coating. The Cls peak is significantly reduced in intensity due to the coating of the resin by calcium phosphate material and undergoes a shift from 284.0 eV (for the uncoated resin) to ≈ 285 eV, which is the characteristic Cls binding energy of adventitious carbon from adsorbed hydrocarbons which are present on any XPS sample. At the same time, the Ols peak increases

TABLE III Summary of XPS data of systems described in Table I. Binding energies are given in eV

Sample	Ca2p	P2p	Cls	Ols	C(%)	O(%)	Ca(%)	P(%)
1R	—	—	284.0	532.0	69.20	25.34	0	0
2R	—	—	284.0	531.18	69.02	19.14	0	0
3R	—		283.6	531.02	73.02	16.40	0	0
1SR	—	—	284.0	531.93	68.28	24.37	0	0
2SR		132.50	284.0	531.50	70.32	21.83	0	0.78
3SR	—	—	284.1	531.53	74.36	22.16	0	0
1SBF	347.98	133.99	284.44	531.94	58.29	29.04	6.56	3.13
2SBF	347.97	133.96	284.97	531.98	44.71	36.4	9.13	8.14
3SBF	347.93	133.96	285.0	531.52	34.40	42.13	13.01	10.46
4SBF	347.94	133.98	284.87	531.66	50.09	33.13	10.63	6.15
5SBF	347.97	133.97	284.90	531.97	47.94	35.14	6.87	1.22
1AP	347.60	133.97	284.55	531.93	57.62	30.21	6.35	3.01
2AP	347.55	133.97	284.9	531.47	31.91	43.97	14.4	9.72
3AP	346.43	132.63	284.36	530.83	53.91	31.53	5.62	4.01
4AP	347.43	133.44	284.93	531.42	37.55	38.20	12.12	7.78
5AP	346.94	133.0	284.39	530.94	39.49	35.86	9.97	6.70
1APS	347.99	133.98	285.17	532.05	22.24	51.66	15.84	10.26
2APS	347.53	133.48	284.49	531.49	48.44	34.27	8.16	6.86
3APS	347.43	133.46	284.46	531.50	53.46	31.50	5.45	5.54
1HAPS	347.66	133.95	284.97	531.45	22.92	46.16	18.49	12.44

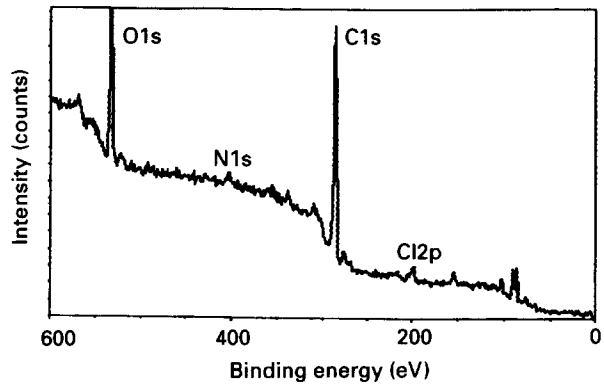

Figure 3 X-ray photoelectron spectrum of as-received Cl-Dowex resin (sample 2R).

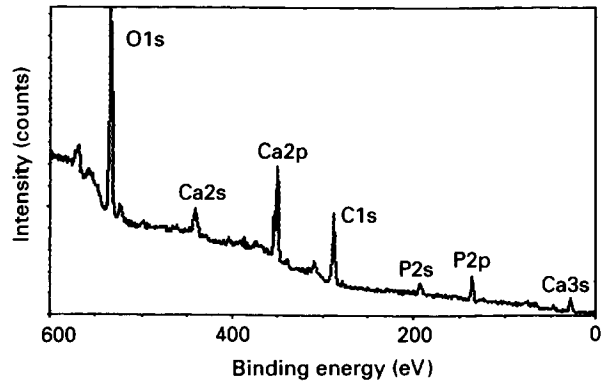

Figure 4 X-ray photoelectron spectrum of HPO_4^{2-}-saturated Cl-Dowex resin soaked in 1.5 × SBF for 3 days (sample 3SBF).

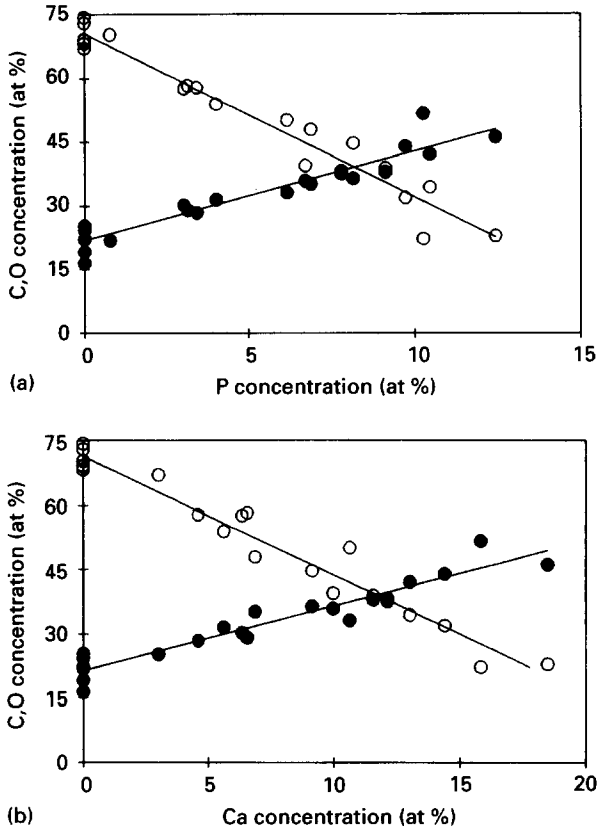

Figure 5 Correlation plots of Cls (○) and Ols (●) surface atomic concentrations with (a) Ca2p and (b) P2p surface atomic concentrations from X-ray photoelectron spectra of uncoated resins and resins coated with calcium phosphate (accumulated data from all X-ray photoelectron spectra recorded in study).

in intensity overall due to the O contained in the phosphate component of the coating.

It was noted that when coverage is high, weak resin-associated surface features such as S 2s and S 2p (for the H-Dowex resin) disappear from the spectra. If the C and O concentration values (at %) are plotted against P and Ca concentration values (at %), linear graphs can be obtained (Fig. 5a and b). This proves conclusively that a phosphate-type compound has deposited only on the resin particles. In addition, the binding energy positions of the Ca 2p (347.6–348.0 eV) and P2p peaks (≈ 134.0 eV) are generally in the range expected for calcium phosphate compounds [10].

3.4.2. Spectra of resin samples soaked in the 1.5 × SBF calcium phosphate growth medium

Specific observations The resin showing the best coverage by calcium phosphate grown from 1.5 × SBF was the Cl-Dowex resin (samples 3SBF, 4SBF). When 10 day soaking periods were used in the case of the Cl-Dowex and H-Dowex resins, the primary aim had been to ascertain whether thicker coatings and/or better coverage of the resin particles could be achieved over the longer soaking time. In general, 10-day soaking did lead to an increase in calcium phosphate coverage of the Ca^{2+}-saturated H-Dowex resin although this was not found to be so for the HPO_4^{2-}-saturated Cl-Dowex resin. The difference could reflect the higher degree of leaching of Ca^{2+} ion from the resin relative to HPO_4^{2-} ion. When the Cls peak (≈ 285.0 eV) of calcium-phosphate-coated (1SBF–5SBF) samples was examined closely, a weak shoulder at the higher binding energy end of the peak (≈ 290 eV) could usually be detected. This was assigned to carbonate in the calcium phosphate coating. In the Ca^{2+}-saturated H-Dowex resin system, the carbonate component increased in intensity for the 10-day soaking time sample relative to that soaked for 3 days. Given that the average EDX-measured Ca:P ratio also increased from 1.63 to 2.15 (Table II), the excess Ca^{2+} ion detected could well be in the form of carbonate-containing hydroxyapatite which would give an increased Ca:P value relative to hydroxyapatite.

3.4.3. Spectra of resin samples soaked in the alkaline phosphatase-catalysed reaction growth medium

Specific observations In resin samples coated with calcium phosphate deposited from the alkaline phosphatase reaction, the experiment in which $Ca(NO_3)_2$ was omitted from the "standard" solution formulation gave the highest calcium phosphate coverage, higher in fact than that observed for the calcium-phosphate-coated Cl-Dowex resin samples (Table IV). In the case of sample 3AP (Table I and Table III), albumin coating of the deposited calcium phosphate was believed to be influencing the detection of Ca and P by XPS

since the SEM/EDX of sample 3AP showed a thick calcium phosphate coating and intense Ca and P signals while only modest Ca and P peak intensities could be observed in the XPS. The presence of albumin in this sample was confirmed by the observation of a Nls peak and a shouldering peak of noticeable intensity due to carbonate at the high binding energy end of the Cls peak. Carbonate component was also observed in the Cls peak of samples of calcium phosphate generated from the alkaline phosphatase method even when albumin was not employed in the growth medium showing that carbonate-containing hydroxyapatite is among the calcium phosphate phases formed when this growth medium is used.

3.4.4. Ca:P ratios measured by XPS for the calcium phosphate-coated resin samples and calcium phosphate powders

Ca:P ratios measured for samples 1SBF–5SBF, 1AP–5AP, 1APS–3APS and 1HAPS have been summarized in Table II. The SBF samples, with the exception of 4SBF, did not show very good agreement with the EDX-measured Ca:P ratios. Usually, the Ca:P values were too low implying a surface enriched in P. The Ca:P ratios determined for calcium phosphate coatings generated by the alkaline phosphatase method proved more interesting. For samples 2AP and 3AP for which EDX-measured Ca:P ratios showed evidence of "leaching" or excess Ca, the XPS-derived values gave Ca:P values close to that measured by EDX for the isolated calcium phosphate powder (sample 3APS, 1.50) generated by the alkaline phosphatase reaction. This would suggest that the use of XPS has bypassed the problems experienced with "leaching" contributing to EDX-measured Ca:P ratios In fact, the XPS-measured Ca:P ratio sample 3APS agreed well with the average EDX-measured Ca:P ratio (Table II). However, it was noted that the XPS-measured Ca:P ratio for sample 1AP (calcium phosphate deposited from the "standard" alkaline phosphatase solution) was 2.11 compared to 1.50 for the EDX. This sample differs from samples 2AP and 3AP by overall coverage of resin particles. It will be recalled that the typical morphology observed in SEM micrographs of calcium phosphate formed from the "standard"alkaline phosphatase solution covered the resin particles in a patchy and non-uniform manner thus leaving areas of bare resin surface. This was reflected in the XPS spectrum of sample 1AP which featured weak resin-surface-associated S peaks whereas spectra of samples 2AP and 3AP did not. Thus leached out resin-associated Ca^{2+} is also being sampled so giving the higher Ca:P ratio.

3.5. Inductively coupled plasma, infrared and X-ray diffraction studies of powders generated by the alkaline phosphatase method

As mentioned previously, direct examination of the calcium phosphate deposited on the resin particles by such techniques as XRD and IR proved to be very difficult because of the amorphous character and paucity of analysable material as well as the awkwardness of sampling. An attempt was made to analyse coating material directly by crushing coated resin beads flat into a matrix of transparent KBr followed by microscopic IR analysis of the perimeters of the flattened resin. However, this procedure only produced very weak and noisy spectra of the coating material which offered limited information. In theory, the Ca:P ratios of calcium phosphate deposited on the ion exchange resins could be probed using ICP by dissolution of the coating in 1 $mol\,l^{-1}$ HNO_3 but if this were to be done, additional Ca^{2+} or HPO_4^{2-} still entrained in the resins would also be extracted so distorting any measured Ca:P ratios.

As a consequence, it was necessary to generate isolated calcium phosphate powder samples under approximately the same conditions as employed when a resin was present and examine such powders by IR and XRD as an indirect means of determining more and more detailed chemical identification of the deposited material. The synthesis of isolated powders could only be carried out with ease in the "standard" alkaline phosphatase growth medium system since this process involves a combination of solution precipitation and deposition of material on the surfaces of resins. Thus by simply omitting the resin from the "standard" solution, an isolated powder sample could be easily generated. Three powder samples (samples 1APS–3APS) were generated using the alkaline-phosphatase-catalysed reaction system under the different conditions employed for generating calcium phosphate on the corresponding resin systems (see samples 1AP, 4AP and 5AP in Table I). In addition, a powder sample of commercial hydroxyapatite was examined for comparison. Generation of similar isolated powder samples from the 1.5 × SBF growth medium solution was not carried out. Previous workers who have used 1.5 × SBF have found that calcium phosphate material formed from immersion of substrates in this growth medium is hydroxyapatite.

3.5.1. ICP Analysis

The Ca:P ratios derived from ICP analyses for powder samples 1APS–3APS and 1HAPS dissolved in nitric acid are summarized in Table II. The HAP powder examined gave a slightly lower Ca:P ratio (1.59) than expected. However, samples 1APS–3APS all exhibited significantly lower Ca:P ratios than the commercial hydroxyapatite. These values agreed to within ± 10% of average EDX-measured and XPS-measured values of Ca:P ratios with the exception of the XPS-derived ratios measured for samples 2APS and 3APS whose flaky and chunky form may have have contributed to the very low Ca:P ratios observed (Table III). The similarity of Ca:P ratios (1.40–1.44) for samples 1APS–3APS indicated that calcium phosphate compounds of similar composition were being produced in the alkaline phosphatase reaction despite the different additives employed.

In general, for processes involving the precipitation of calcium phosphate compounds, HAP is the favoured phase because it has the lowest solubility product of the possible phases that may form [11]. Previous workers [12] have noted that calcium phosphate powders prepared from decomposition of a calcium chelate complex in the pH range 5.0–8.0 gave Ca:P ratios of 1.43 as determined by ICP. In the present study, pH conditions were similar in the "standard" alkaline-phosphatase-catalysed reaction mixture used for growing calcium phosphate although the initial pH was about 9.70 before addition of the alkaline phosphatase but dropped to 7.50–8.00 after completion of the reaction and separation of the resin (or powder) from the solution. It was also evident from XPS studies of the isolated powders confirmed that carbonate is incorporated in the calcium phosphate. This observation can also be extended to calcium phosphate deposited on the resins.

3.5.2. Infrared spectroscopy and X-ray diffraction studies

XRD spectra of the calcium phosphate solid generated from the "standard" alkaline-phosphatase-catalysed reaction system (sample 1APS) reveal that it is amorphous. The IR spectrum of the solid (Fig. 6a) bears some similarity to that of commercial hydroxyapatite but with slight peak broadening in evidence. After heating the amorphous solid to 700 °C for 1 h, a weak peak was able to be observed at $\approx 3570\,cm^{-1}$ which could be assigned to ν(OH) of HAP ($Ca_{10}(PO_4)_6(OH)_2$). However, an XRD spectrum of the crystallized solid showed that a mixture of TCP (tricalcium phosphate, $Ca_3(PO_4)_2$) and HAP had been produced. It is believed that the growth medium using the alkaline-phosphatase-catalysed reaction of PNP to phosphate produces an amorphous calcium-deficient hydroxyapatite on the resin particles. The observation of TCP in the XRD spectrum can be attributed to conversion of amorphous HAP to TCP by dehydration as a result of the 1 h 700 °C heat treatment.

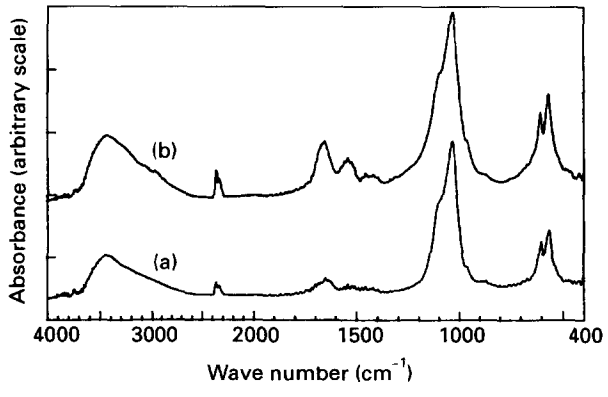

Figure 6 Fourier transform infrared spectra of solids produced in the absence of ion exchange resins from alkaline-phosphatase-catalysed hydrolysis reaction for (a) the "standard" reaction solution (sample 1APS) and (b) the reaction carried out in 0.1% albumin solution (sample 2APS)

Infrared spectra of samples of calcium phosphate sample 2APS were found to exhibit a somewhat broadened calcium phosphate spectrum with additional peaks at 1651, 1541, and 1456 cm^{-1} (Fig. 4b). When an IR spectrum of albumin alone was examined, the same peaks were observed which confirmed that albumin was present in the calcium phosphate powders produced in the 0.1% albumin medium. The infrared spectrum of sample 3APS also featured the albumin-associated peaks. It was difficult to discern any features that might be assigned to collagen although the calcium phosphate-associated peaks appeared broader than those observed in the infrared spectrum of sample 2APS. It is evident that albumin has adsorbed on the surfaces of the calcium phosphate samples prepared by alkaline-phosphatase-catalysed reaction medium, a fact borne out by the X-ray photoelectron spectroscopic studies. Albumin is known to coat HAP surfaces rapidly [6] and is a factor which retards the formation of HAP in blood serum.

4. Conclusions

It has been shown that calcium phosphate formation can be stimulated on ion-exchange resins presaturated with Ca^{2+} or HPO_4^{2-} ions by soaking in a conventionally used 1.5 × SBF growth medium or a novel biomimetic-type growth medium involving the alkaline-phosphatase-catalysed reaction of PNP to inorganic phosphate and p-nitrophenol. The mechanism of formation involves diffusion of ions from the interior of the resin to the surface thus creating a high local concentration which stimulates deposition of calcium phosphate compounds. In both cases amorphous calcium phosphate is believed to deposit on the resin particles. When the 1.5 × SBF growth medium is used, material with a composition close to that for hydroxyapatite is deposited. In contrast, calcium phosphate produced from the alkaline-phosphatase-catalysed hydrolysis reaction of PNP regardless of additives such as albumin or collagen produces calcium-deficient hydroxyapatite.

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