Manufacture and evaluation of bioactive and biodegradable materials and scaffolds for tissue engineering

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For tissue regeneration and tissue engineering applications, a number of bioactive and biodegradable composites, either porous or non-porous, were fabricated. The newly developed materials included tricalcium phosphate reinforced polyhydroxybutyrate and its copolymer, poorly crystallized hydroxyapatite reinforced chitin, and plasma sprayed hydroxyapatite reinforced poly(L-lactic acid). It was shown that these new materials could be successfully produced using the manufacturing techniques adopted. *In vitro* experiments revealed that the incorporation of bioceramic particles in biodegradable polymers rendered the composites bioactive and significantly improved the ability of composites to induce the formation of bone-like apatite on their surfaces. Degradation of composite scaffolds in simulated body fluid was observed and could be due to the simultaneous degradation of polymer matrix and dissolution of bioceramic particles.

Introduction

Just over a decade ago, ''tissue engineering'' started to be used to describe a multidisciplinary endeavor that applies the knowledge of engineering, the life sciences, and the clinical sciences to solve critical medical problems of tissue loss and organ failure [1, 2]. The essence of tissue engineering is the use of living cells, together with either natural or synthetic extracellular components, in the development of implantable parts or devices for the restoration of body functions. One of the key issues in tissue engineering is the development of suitable biodegradable materials (scaffolds) for seeding cells and for the subsequent growth of tissues. Recently, various polymeric scaffolds were produced and evaluated for tissue engineering applications [3].

Over the last two decades, a number of bioactive polymer matrix composites have been developed for tissue replacement [4, 5]. Due to the matrix polymers used, most of these composites are non-biodegradable. In recent years, attention has moved from materials that will remain completely stable in the biological environment to materials that will, in some way, alter their properties or degrade in response to the cellular environment [6, 7]. Biodegradable materials have the advantage of allowing the new tissue, as it grows naturally, to take over their load-bearing or other functions without any of the potential chronic problems associated with the presence of biostable implants. Our experience in developing bioactive and biostable composites for tissue replacement has proven useful for investigating bioactive and biodegradable materials for tissue regeneration. Furthermore, with appropriate modifications, conventional manufacturing techniques can be used to produce bioactive scaffolds for tissue engineering applications. In this paper, our efforts in the production of bioactive and biodegradable materials and scaffolds are presented and results from *in vitro* experiments are reported.

Materials and experimental techniques

Raw materials

Several biodegradable polymers, including polyhydroxybutyrate (PHB) and its copolymer polyhydroxyvalerate (i.e. PHB–PHV; with both PHB and PHB–PHV being supplied by ICI plc, UK), poly(L-lactic acid) (PLLA, provided by Chengdu Institute of Organic Chemistry, Academia Sinica, China) and chitin (supplied by Polysciences Inc., USA), were used in this investigation. The bioactive materials that were incorporated in the polymers were plasma sprayed hydroxyapatite (pcHA) made in-house [8], poorly crystallized hydroxyapatite (pcHA) and tricalcium phosphate (TCP), with both pcHA and TCP being commercially available from Merck, Germany. All raw materials were characterized using various techniques prior to scaffold (or composite) production.

Production of composite materials and scaffolds

Non-porous biodegradable composites were produced using either a standardized thermo-mechanical method

as in the case of TCP/PHB materials or the solution casting method as in the case of pcHA/chitin composites. Structure and composition of composites were assessed subsequently using various techniques including scanning electron microscopy (SEM), thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC).

TCP/PHB composites containing up to 30 vol % (i.e. 52.4 wt %) of TCP were manufactured through a process consisting of compounding, milling, drying and compression molding. Appropriate amounts of TCP and PHB powders were weighed before compounding according to nominal TCP volume percentages in the composites. A Hakke Rheomix 600 internal mixer was used for compounding TCP with PHB. The temperature of the mixer chamber was just above the melting point of PHB and a pair of cam rotors were utilized. The duration of compounding was controlled to be around 20 min, with high rotor speed being used for materials containing high percentages of TCP. The compounded materials were processed into composite powders using a Fritsch cutting mill. The composite powders were then dried overnight in an oven and finally compression molded into plates (110 mm × 110 mm × 2 mm) using a custom made mold and a hot, hydraulic press. Specimens for various tests were made from these thin plates.

For the solution casting of pcHA/chitin composites, anhydrous LiCl was dissolved in N,N-dimethylacetamide (DMAc) by magnetic stirring to make a 5% (w/w) LiCl/DMAc solution to be used as the solvent for chitin. To produce the composites, pcHA particles were first dispersed in the solvent in a beaker before chitin flakes were added. The beaker was then sealed and the solvent containing pcHA particles and chitin flakes was stirred for approximately five days at ambient temperature so as to produce a viscous, milky solution. The resultant composite solution was cast in Petri-dishes. It became chitin gel containing dispersed pcHA particles after DMAc had partially evaporated while water molecules were absorbed into the solution. The chitin gel was prepared in the same way. Subsequently, both chitin and pcHA/chitin composite gel plates were immersed in distilled water for days and then thoroughly washed in order to remove DMAc and LiCl completely. The washed gel plates were gently blotted to visual dryness with filter paper and air-dried under a moderate compressive force to produce membranes. Composite membranes containing 10–60 wt % of pcHA were made. To achieve a homogeneous distribution of pcHA particles in the chitin matrix, manufacturing parameters such as chitin concentration, stirring period, mixing mode and gelation rate were optimized.

Porous biodegradable materials were produced in a solution casting and porosifier extraction procedure. To produce porous psHA/PLLA composites, psHA particles were first dispersed in acetone before PLLA chips were dissolved in the solvent. The composite solution was then mixed with sugar particles of a particular size and subsequently cast into a metal mold, The psHA/sugar/PLLA composite was subjected to extraction of sugar particles in distilled water which was a non-solvent for PLLA but was miscible with acetone. In this way, a highly porous PLLA-matrix scaffold containing 20 wt % of psHA particles could be produced. The manufacture of porous PLLA followed the same procedure without adding psHA particles at the beginning. Porous PLLA and psHA/PLLA samples were cut into rectangular blocks of dimensions of 8 mm × 6 mm × 6 mm and stored in a desiccator until utilization. Porous chitin and chitin composites were produced using the same technique but with a different solvent (i.e. LiCl/DMAc).

In vitro evaluation

An acellular simulated body fluid (SBF) was used for *in vitro* experiments as it had ion concentrations (in milliimoles: Na^+ 142.0, K^+ 5.0, Mg^{2+} 1.5, Ca^{2+} 2.5, Cl^- 147.8, HCO^{3-} 4.2, HPO_4^{2-} 1.0, SO_4^{2-} 0.5) that were nearly the same as those of human blood plasma [9]. SBF was prepared by dissolving reagent-grade chemicals of NaCl, $NaHCO_3$, KCl, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, Na_2SO_4, and $(CH_2OH)_3CNH_2$ in distilled and de-ionized water and buffered to pH 7.4 at 37 °C with HCl. Specimens, porous or non-porous, were immersed in SBF at 37 °C for various periods of time. Changes of the surface structure of immersed specimens were analyzed using various techniques after the specimens had been removed from SBF, washed with distilled water, and dried. Analytical techniques employed included scanning electron microscopy (SEM), thin-film X-ray diffraction (TF-XRD) and Fourier transform infrared spectroscopy (FTIR).

Results and discussion

Both non-porous and porous bioactive and biodegradable composites could be successfully produced for tissue regeneration and tissue engineering applications. SEM examination of non-porous composites showed a uniform distribution of bioceramic particles in the composites (Figs 1 and 2). A homogeneous distribution of bioceramic particles in composites is essential for their mechanical as well as biological performance. TGA results indicated that the difference between the measured mass percentage of bioceramics in composites and the theoretical value (''Rule of Mixtures'' calculations) was negligible and therefore intended compositions for the composites had been achieved.

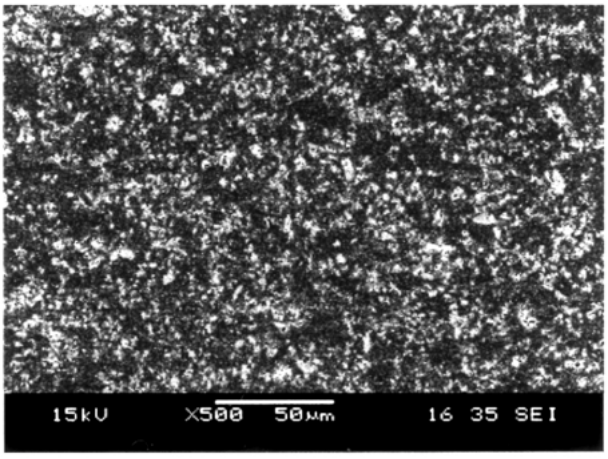


Figure 1 Distribution of TCP particles in the PHB matrix (20 vol % of TCP, polished surface).

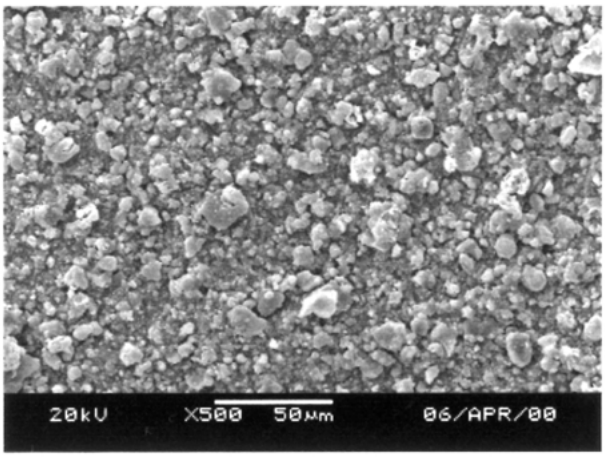


Figure 2 Distribution of pcHA particles in the chitin matrix (50 wt % of pcHA, as-produced membrance surface).

DSC analysis showed that an increase in the TCP content resulted in decreases in both the melting temperature and the crystallinity of PHB. This result suggests that the *in vivo* degradation rate of TCP/PHB composites may be higher than that of unfilled PHB as a lower degree of crystallinity leads to a higher degradation rate of the polymer [10].

For bone tissue engineering, biodegradable scaffolds are required. There are a number of techniques for producing porous polymer structures for the medical use [11]. In this investigation, the solution casting and porosifier extraction technique used was effective for producing highly porous scaffolds. Pore size and pore morphology in porous structures were affected by manufacturing parameters and the porosifier used. Fig. 3 shows the structure of a porous chitin fabricated with a high chitin concentration and large porosifier particles, revealing the average pore size of about 500 µm that is suitable for cell-seeding. Other scaffolds produced exhibited similar structural features.

In the *in vitro* experiments, mineral crystals were observed to grow on TCP/PHB composite specimens (20 vol % TCP) after three days immersion in SBF (Fig. 4a). The crystals grew locally on the specimen surface following the contour of the specimen. The surface of the crystals was very rough. After seven days immersion in SBF, mineral crystals covered the whole surface of the

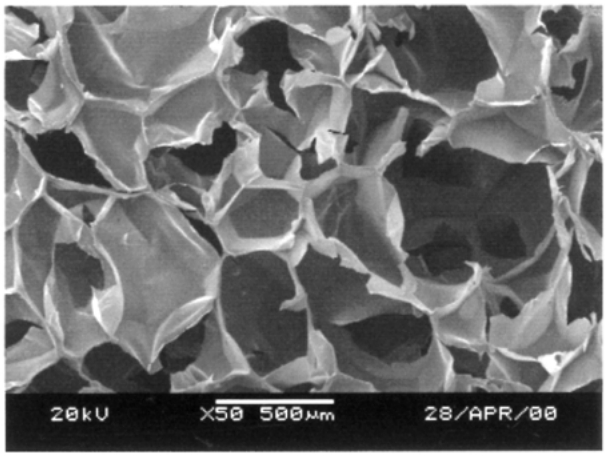
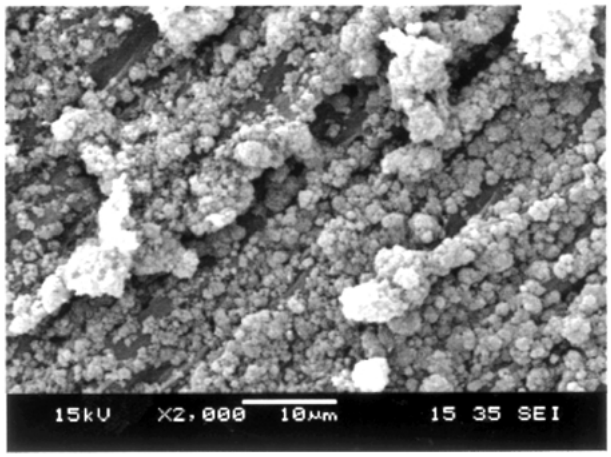


Figure 3 Porous chitin produced using the solution casting and porsifier extraction technique.



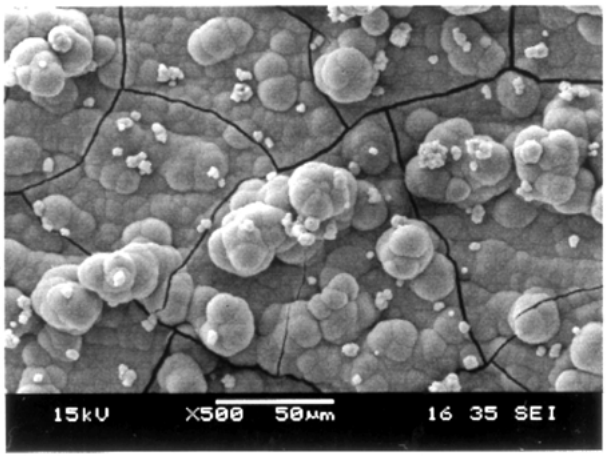


Figure 4 Formation of bone-like apatie on non-porous TCP/PHB composite (20 vol % of TCP) after immersion in SBF. (a) Three days immersion; (b) 14 days immersion.

specimen, but the mineral layer formed was very thin and the original contour of the specimen could still be seen. After 14 days immersion in SBF, a thick and dense mineral layer was formed on the specimen surface (Fig. 4b). Fig. 5 shows TF-XRD patterns of the 20 vo1% TCP/PHB composite before and after immersion in SBF. After seven days immersion, peak heights of TCP and PHB obviously decreased but no apatite peaks were noted. The broad apatite peaks were observed after 14 days immersion in SBF and original TCP and PHB peaks were suppressed. The intensity of apatite peaks increased with an increase in immersion time. FTIR spectra of apatite formed *in vitro* were similar to that of synthetic HA. The characteristic absorption bands of phosphate appearing at 565, 604 and 962 cm^{-1} were observed for TCP/PHB specimens after their immersion in SBF (Fig. 6). The spectra of apatite formed *in vitro* also had a strong absorption band at 873 cm^{-1} which corresponded to the vibration mode of carbonate. Other carbonate peaks at 1415 and 1454 cm^{-1} were observed as well. Moreover, while hydroxyl stretch was observed at 3570 cm^{-1} in the spectrum of commercial HA, there was no evident peak at the same wave number for the apatite formed *in vitro*, indicating the incorporation of carbonate in the structure of apatite and the carbonate substitution for hydroxyl in the apatite. The intensity of phosphate and carbonate peaks increased with the increase in immersion time, indicating the growth of apatite *in vitro*. These results

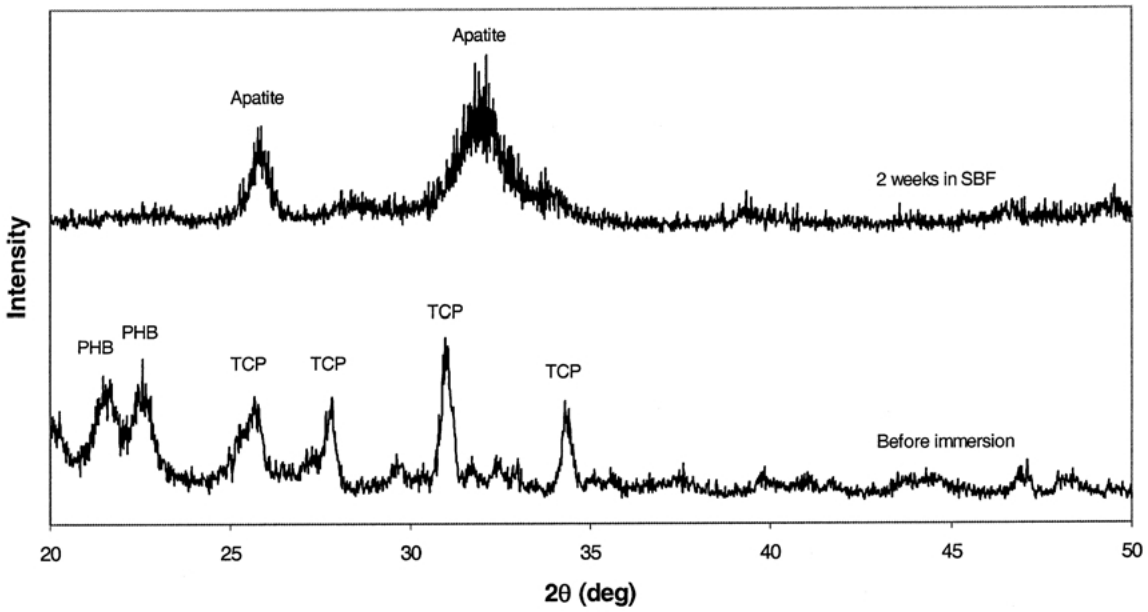


Figure 5 TF-XRD patterns of 20 vol % TCP/PHB composite.

suggest that the apatite formed on the surface of composites in SBF was carbonated apatite, which is similar in composition and structure to bone apatite and was also found on bioactive glasses [12], plasma sprayed HA coatings [13] and alkaline treated titanium [14] after they were immersed in SBF. TCP/PHB composites exhibited high *in vitro* bioactivity through the formation of a bone-like apatite layer on their surfaces. The bioactivity of these composites can be tailored by varying the TCP content in the composites.

For the pcHA/chitin composites, the nucleation of mineral appeared as an island-like substance on the composite surface after 28 days immersion in SBF (Fig. 7a) and a uniform mineral layer formed after 72 days immersion in SBF (Fig. 7b). No evident dissolution of the composite surface was observed before the nucleation of mineral took place. However, for chitin membranes, no mineralization was observed on their surfaces during the immersion time in SBF and the membranes became fragile and would disintegrate upon a light pressure being exerted. It was found through SEM examination that there were many degradation sites on the surface of chitin membranes after prolonged immersion in SBF (Fig. 8). Both TF-XRD patterns and FTIR spectra of the mineral formed *in vitro* were similar to those shown in Figs 5 and 6, confirming that the mineral was bone-like apatite. The incorporation of

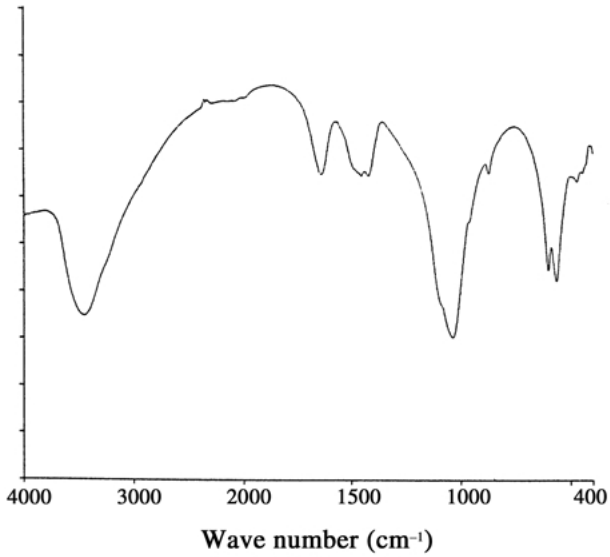
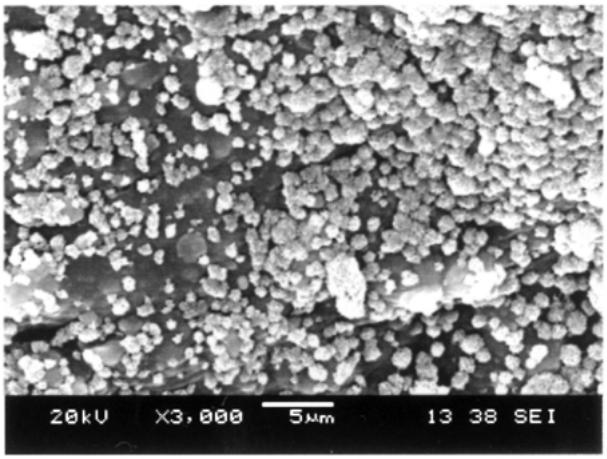
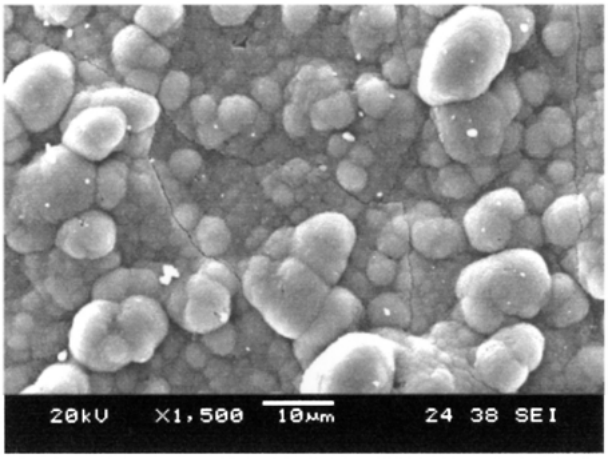


Figure 6 FTIR spectrum of 20 vol% TCP/PHB composite after its immersion in SBF.



(a)



(b)

Figure 7 Formation of bone-like apatite on non-porous pcHA/chitin composite (20 wt % of pcHA) after immersion in SBF. (a) 28 days immersion; (b) 72 days immersion.

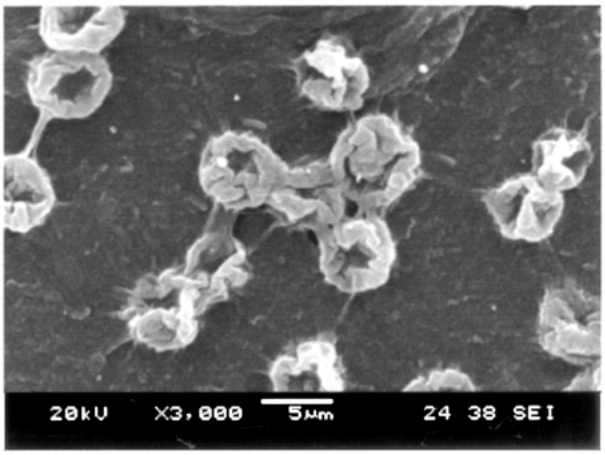
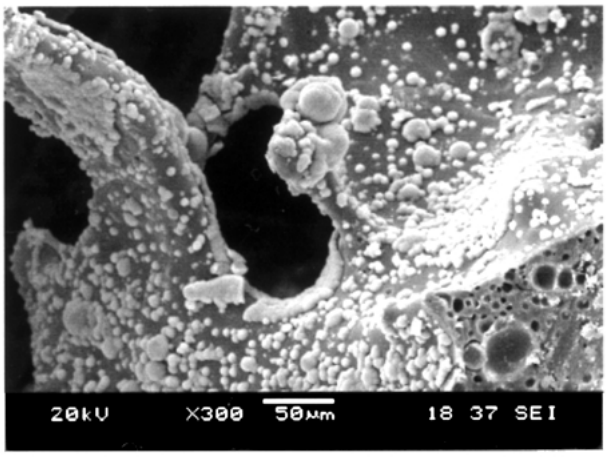


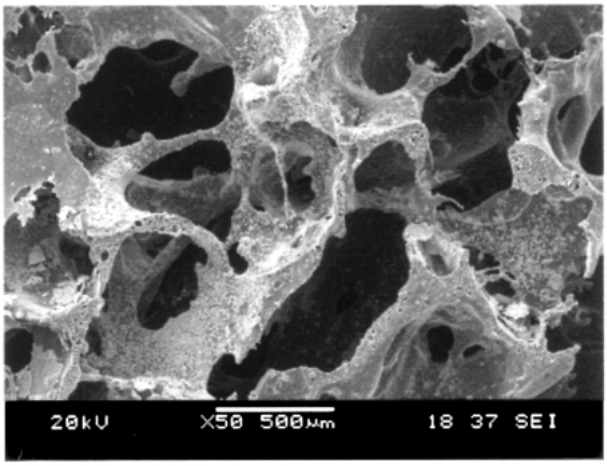
Figure 8 Degradation sites on a chitin membrance after its immersion in SBF for 72 days.

pcHA particles has rendered the pcHA/chitin composites bioactive and significantly improved the ability of composites to induce the formation of bone-like apatite on their surfaces.

Results from *in vitro* experiments also showed evidently that psHA particles enhanced the formation of bone-like apatite on the surface of psHA/PLLA composite scaffolds when they were immersed in SBF. Fig. 9 shows the formation of bone-like apatite on composite scaffolds. After seven days immersion, a large number of ''islands'' of bone-like apatite were formed on the pore surface of composite scaffolds (Fig. 9a), indicating high bioactivity of the composite. It was also observed that within the same immersion period, some struts of composite scaffolds degraded (Fig. 9b), possibly due to the simultaneous degradation of PLLA and dissolution of psHA particles in SBF. In contrast, almost no nuclei of bone-like apatite were found on the pore surface of PLLA scaffolds during their immersion in SBF (Fig. 10). The introduction of bioactivity into biodegradable scaffolds by incorporating particulate bioceramics may enhance cell-seeding and hence the subsequent tissue growth in tissue-engineered products.



(a)



(b)

Figure 9 Porous psHA/PLLA composite (20 wt % of psHA) after immersion in SBF. (a) Formation of bone-like apatite; (b) degradation of struts in the scaffold.

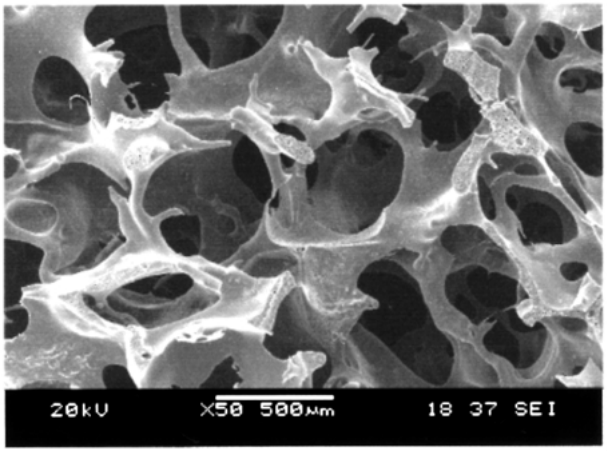


Figure 10 Non-formation of bone-like apatite on porous PLLA after its immersion in SBF.

Conclusions

For tissue regeneration and tissue engineering applications, a number of bioactive and biodegradable composites, either porous or non-porous, were successfully produced using manufacturing techniques adopted. These new materials included tricalcium phosphate reinforced polyhydroxybutyrate and its copolymer, poorly crystallized hydroxyapatite reinforced chitin, and plasma sprayed hydroxyapatite reinforced poly(L-lactic acid). *In vitro* experinments showed that the incorporation of bioceramic particles in biodegrable polymers rendered the composites bioactive and significantly improved the ability of composites to induce the formation of bone-like apatite on their surfaces. Degradation of composite scaffolds in simulated body fluid could be attributed to the simultaneous degradation of the polymer matrix and dissolution of bioceramic particles. The introduction of bioactivity into biodegradable scaffolds may enhance cell-seeding and hence the subsequent tissue growth in the scaffolds.

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