Bacterial biosynthesis of a calcium phosphate bone-substitute material

ANIA C. THACKRAY[1], RACHEL L. SAMMONS[1,*], LYNNE E. MACASKIE[2], PING YONG[2], HARRIET LUGG[1], PETER M. MARQUIS[1]
[1]*Biomaterials Department, School of Dentistry, The University of Birmingham, St. Chad's Queensway, Birmingham, B4 6NN, UK*
E-mail: R.L.Sammons@bham.ac.uk
[2]*School of Biosciences, The University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK*

A species of *Serratia* bacteria produces nano-crystalline hydroxyapatite (HA) crystals by use of a cell-bound phosphatase enzyme, located both periplasmically and within extracellular polymeric materials. The enzyme functions in resting cells by cleaving glycerol-2-phosphate (G-2-P) to liberate free phosphate ions which combine with calcium in solution to produce a cell-bound calcium phosphate material. Bacteria grown as a biofilm on polyurethane reticulated foam cubes were challenged with calcium and G-2-P in a bioreactor to produce a 3-D porous bone-substitute material. The scaffold has 1 mm macropores and 1 μm micropores. XRD showed the crystallites to be 25–28 nm in size, resembling HA before sintering and β-tricalcium phosphate (β-TCP, whitlockite) after. When biofilm was grown on titanium discs and challenged with calcium and G-2-P, a calcium phosphate layer formed on the discs. Biomineralisation is therefore a potential route to production of precursor nanophase HA, which has the potential to improve strength. The scaffold material produced by this method could be used as a bone-filler or as an alternative method for coating implants with a layer of HA.

Introduction

Calcium phosphate bioceramics, including hydroxyapatite (HA), have relatively poor mechanical properties due to the precursor materials used for their production. Chemical production creates processing defects predominantly introduced through hard-particle agglomeration, which lead to weaknesses in the final implant [1]. The strength of such materials varies from 30 to 240 MPa, despite the relatively small grain size of their precursor materials [2].

It is recognised that use of nano-sized grains improves the mechanical properties of bioceramics [11]. However, agglomeration of particles may occur, leading to variation in composition. This causes uneven sintering, leading in turn to defects in the structure of the bioceramic, therefore reducing its strength. To optimise the benefits of nano-size precursors, the particles must be of a uniform shape and size, and a consistent composition.

Production of a material with a grain size of less than 200 nm, but ideally less than 50 nm, and its subsequent manipulation into implants such as bone scaffolds, represents a difficult challenge through conventional processing methods. Previous studies have shown that a method using *Serratia* sp. N14 bacteria (previously classified as *Citrobacter* sp. N14 [3]) to remove heavy metal ions from solution [4] can be modified so that the bacteria accumulate calcium and phosphate [5].

Biomineralisation mediated by microorganisms has potential for nano-particle production by compartmentalisation of crystal growth within or upon the bacterial cells. *Serratia* sp. N14 produces an acid phosphatase enzyme, both periplasmically and within the exopolymeric matrix, that stoichiometrically cleaves glycerol-2-phosphate (G-2-P), to liberate free phosphate ligands from the donor molecule [12]. The activity of the enzyme on non-growing cells, immobilised as a biofilm, remains high, and *Serratia* sp. N14 can remove metal ions from a continuous flow of solution, to produce a high metal load [6]. The calcium phosphate material produced biosynthetically could be used as a precursor material for biomedical hydroxyapatite production.

Aim

The aim of this study was to develop the use of a bioreactor for the production of calcium phosphate crystals from *Serratia* sp. N14 bacteria pre-grown as a

*Author to whom all correspondence should be addressed.

biofilm, and then optimise the conditions for biomineral deposition using the bioreactor to produce a high yield, while still retaining the nano-scale dimensions of the crystals. Further, to investigate the method as a route to production of scaffold structures and as a coating for titanium, the nature of the sintered structure produced was also evaluated.

Experimental methods

Serratia sp. N14 [13] bacteria were grown in minimal medium under continuous carbon (lactose) limited culture at 30 °C in an airlift fermentor as described previously [7, 8]. The fermentor contained 1 cm^3 polyurethane reticulated foam cubes (20 pores $inch^{-1}$, Recticel Ltd, Belgium) threaded onto cotton strings and washed with distilled water. The cubes were left for 6 days in the fermentor to allow biofilm development on the reticulated surfaces [8], then transferred to the bioreactor for further experiments.

Twenty-five biofilm-coated cubes were arranged in a 25 cm long glass column through which calcium chloride ($CaCl_2$) and G-2-P in solution were pumped upward at a rate of 1 ml h^{-1} (Fig. 1). Use of a bioreactor allows careful regulation of the bacterial environment enabling control of pH, temperature and solution concentrations. Over approximately 14 days in an incubator at 30 °C, the biofilm-coated cubes became progressively encrusted with a white crystalline material, producing $\sim 180\,mg \pm 2.8$ (mean $\pm$ SD) per cube. A control column containing heat-killed bacteria under identical conditions gave no white deposit, showing that the mineral formation was due to enzymatic activity.

The residue-covered cubes were withdrawn from the column and air-dried, before being sintered in a Carbolite 1300 HA furnace (2 h; 1200 °C). The crystals were characterised by Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD) and Energy Dispersive X-ray Analysis (EDX). A sample of the crystals was subjected to Thermogravimetric Analysis (TGA) whereby the decreasing weight of the sample was recorded as the temperature was increased from room temperature up to 1200 °C. Identical characterisation tests were carried out on samples of commercially available HA (Captal R Hydroxylapatite: Plasma BioTal® Ltd, UK).

Titanium discs (Titanium International Ltd, UK) of 14.0 mm diameter × 1.0 mm thickness were grit-blasted and washed with distilled water, then introduced into flasks containing bacteria grown in batch culture [9]. The cultures were incubated at 30 °C for 6 days to allow a biofilm to grow on the discs. They were then transferred to the challenge solution, containing pH-buffered AMPSO (Sigma, UK), $CaCl_2$ (Sigma, UK) and G-2-P (BDH, UK) for a further 7 days. The thickness of the coating at 20 random points at the edge of the titanium discs was measured by SEM using SemAfore image analysis software.

Results

Calcium and phosphate were taken up by the *Serratia* biofilm in the bioreactor, to produce a 3-D calcium phosphate scaffold material. It was found that the highest rate of crystal production occurred when the bioreactor was perfused with 50 mM AMPSO, buffered to pH 8.6, 25 mM $CaCl_2$ and 50 mM G-2-P.

The cubes were dried at 50 °C for 12 h, then squeezed to obtain the crystals. The SEM showed needle-shaped clusters of crystals, 2–3 μm in length. XRD identified the crystals as HA by comparing the spectrum with that of commercial HA and the XRD standard spectrum (Fig. 2). From the spectra, the crystals were calculated to be 25–28 nm in size [9]. After sintering, XRD showed the crystals to be consistent with β-tricalcium phosphate (β-TCP) (Fig. 3).

The FTIR analysis carried out on a sample of dried powdered crystals before sintering, as well as on a sample of commercially available HA showed the positions of adsorption peaks at wavelengths of 1087, 1046 and 962 cm^{-1}, indicating the presence of P–O–H bonds, identical to those present in commercial HA. The EDX showed that the average calcium to phosphate ratio in the samples was 1.60, compared to 1.70 for the

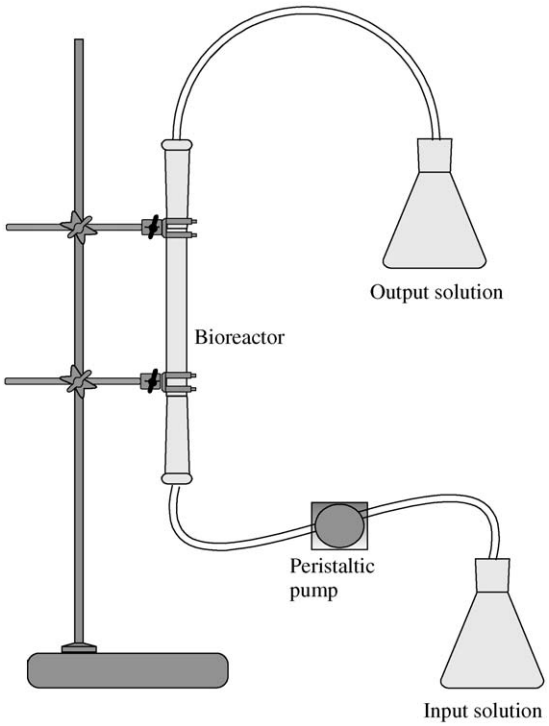

Figure 1 Bioreactor apparatus.

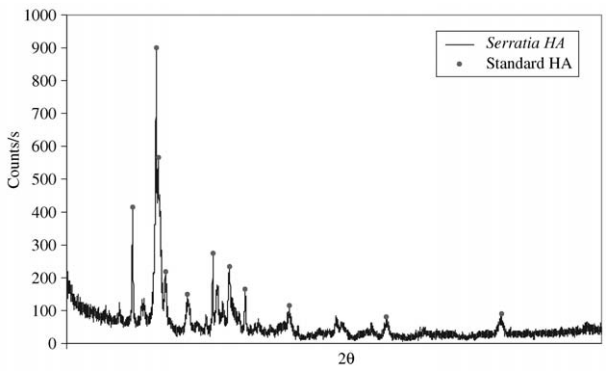

Figure 2 XRD of material before sintering.

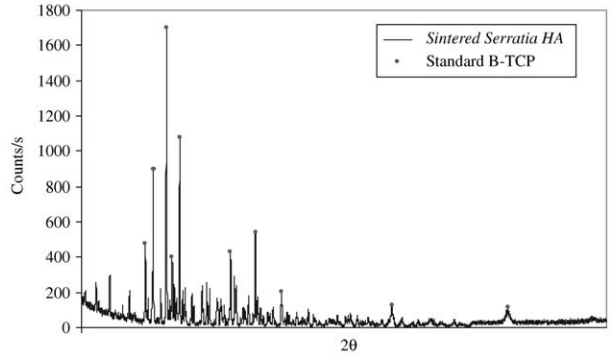

Figure 3 XRD of material after sintering.

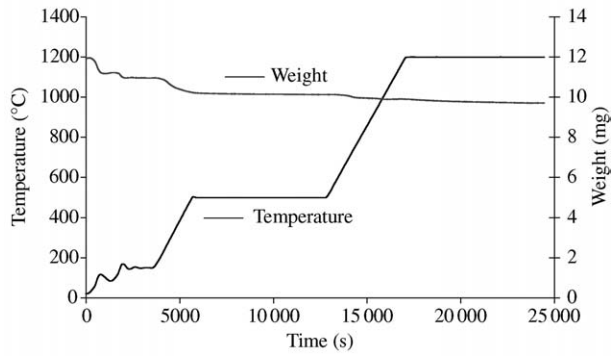

Figure 4 Thermogravimetric analysis.

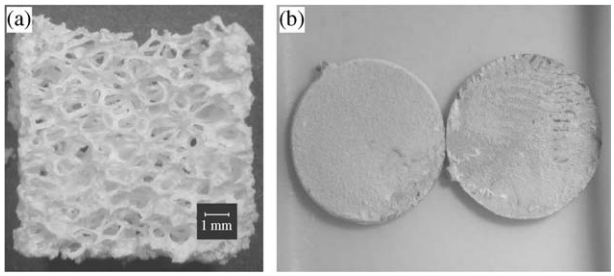

Figure 5 (a) Sintered scaffold structure and (b) coated titanium discs.

commercial sample, which is close to the expected ratio of 1.67. TGA (Fig. 4) revealed weight loss events at specific temperatures. There was 8.19 wt % initial weight loss up to 200 °C, a decrease of 6.14 wt % at approximately 500 °C and another small decrease of 2.86 wt % at approximately 1000 °C.

After firing the crystal-encrusted foam, a 3-D macroporous structure was produced (Fig. 5(a)) which, as can be seen at high magnification (Fig. 6), contains microporosity in the range of 1–2 μm.

When the layer was produced on titanium discs, the thickness of the coating (biofilm plus HA) at the edge of the titanium discs was of an even depth of 20–30 μm (Fig. 5(b)). The optimal solution that produced this thickness coating contained 50 mM AMPSO (pH 8.6), 5 mM $CaCl_2$ and 25 mM G-2-P.

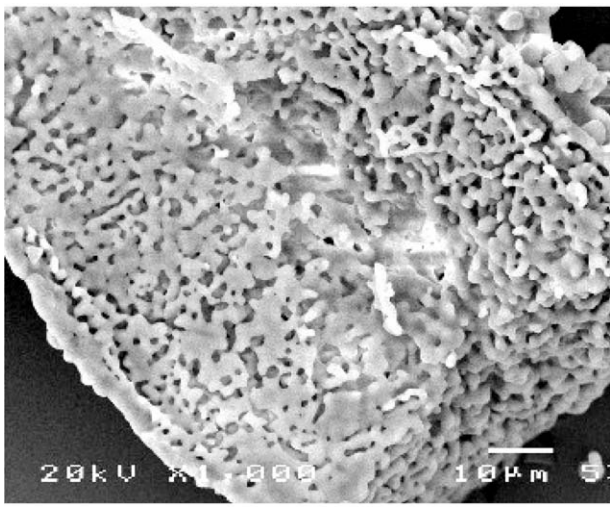

Figure 6 Sintered scaffold material. (× 1000; 20 kV).

Discussion

The phosphatase enzyme located periplasmically and within the extracellular polymeric matrix of the *Serratia* sp. N14 stoichiometrically cleaves G-2-P in solution, liberating inorganic phosphate ions. These ions interact with Ca^{2+} and calcium phosphate synthesis occurs [5, 9]. FTIR and XRD showed the crystals to have an HA structure, but a slightly lower Ca : P ratio than that of commercial HA was found by EDX. This may indicate small levels of ion substitutions, suggesting the presence of calcium deficient HA or the presence of a small amount of brushite.

A decrease of 8.19 wt % below 200 °C was consistent with sample surface water loss and the decrease of 6.14 wt % at approximately 500 °C was attributed to the decomposition of the organic component of the sample. The small loss of 2.86 wt % at approximately 1000 °C is consistent with the associated conversion of HA into β-TCP as confirmed by XRD [10]. The mass of the samples decreased as the density of the sample decreased with the phase change. The sintering analysis gave an indication of the temperature at which the organic components of the scaffold burned off. This is required for removal of the biomass component and optimisation of the sintering schedule to produce a material with optimum porosity and density.

The method of challenging biofilm-coated foam cubes in a bioreactor permitted the formation of a porous 3-D scaffold material. After sintering, the lattice structure had macropores of 1000 μm and micropores of 1 μm diameter, and appeared to resemble β-TCP (whitlockite). The sintered grain size varied from 1–10 μm suggesting that, despite the nanophase dimensions of the material, some explosive grain growth phase had occurred. In order to retain the nanoscale dimensions of the crystals, it is necessary to further optimise the sintering and processing conditions.

A significant feature of the scaffold structure is the uniformly high level of microporosity at the submicron level. This was generated by volatilisation of biomass components, leaving the surrounding crystals, which subsequently densified on sintering. Microporosity should be conducive to bone cell adhesion while macroporosity will facilitate bone cell ingrowth and vascularisation [14].

By growing a biofilm layer on titanium, then challenging it with $CaCl_2$ and G-2-P, a calcium phosphate layer was formed on the metal. This technique could suggest a potential route to coating implants with a

nanophase layer of HA. The thickness can be controlled to a certain extent by extending or reducing the length of time the biofilm-coated discs are in contact with the challenge solutions. A longer contact time should produce a thicker layer of calcium phosphate on the disc.

Conclusion

It has been demonstrated that the biomineralisation method of using bacteria can produce a uniformly porous precursor bone scaffold material with nanoscale crystal dimensions. Following further optimisation of the sintering conditions, the density of the material and therefore its strength and fracture toughness [1] for use in the body should be improved. The calcium phosphate scaffold material produced by this method could be used as a bone-filler in its existing form, or the powder produced can be processed into granules or a specified solid shape for filling voids. The method of using bacteria to form a calcium phosphate coating on titanium has potential for orthopaedic and dental applications as a metal implant coating. Also, an even thickness coating of hydroxyapatite can be produced on all areas of metals, unlike plasma-spraying, with which there is difficulty in coating areas not in the line-of-sight.

Acknowledgments

This project was supported by the BBSRC (Grant No. 6/E11940). Thanks to Ms C. Hardy for assistance with XRD.

References

1. W. D. KINGERY, H. K. BOWEN and D. R. UHLMAN, in ''Introduction to Ceramics'', 2nd edn (John Wiley & Sons, Inc., USA, 1976).
2. B. D. RATNER, A. S. HOFFMAN, F. J. SCHOEN and J. E. LEMONS, (eds) in ''Biomaterials Science. An Introduction to Materials in Medicine'' (Academic Press, USA, 1996).
3. P. PATTANAPIPITPAISAL, A. N. MABBETT, J. A. FINLAY, A. J. BESWICK, M. PATERSON-BEEDLE, A. ESSA, J. WRIGHT, M. R. TOLLEY, U. BADAR, N. A. AHMED, J. L. HOBMAN, N. L. BROWN and L. E. MACASKIE, *Environ. Technol.* **7** (2002) 731.
4. L. E. MACASKIE, R. M. EMPSON, A. K. CHEETHAM, C. P. GREY and A. J. SKARNULIS, *Science* **257** (1992) 782.
5. R. L. SAMMONS, P. M. MARQUIS, L. E. MACASKIE, P. YONG and C. BASNER, in ''Phosphorus in Environmental Technology'', edited by E. Valsami-Jones (IWA, 2003) (in press).
6. L. E. MACASKIE, *J. Chem. Technol. Biotechnol.* **49** (1990) 357.
7. J. A. FINLAY, V. J. M. ALLAN, A. CONNER, M. E. CALLOW, G. BASNAKOVA and L. E. MACASKIE, *Biotechnol. Bioeng.* **63** (1999) 87.
8. V. J. M. ALLAN, M. E. CALLOW, L. E. MACASKIE and M. PATERSON-BEEDLE, *Microbiology* **148** (2002) 277.
9. P. YONG, R. L. SAMMONS, P. M. MARQUIS, H. LUGG and L. E. MACASKIE, in Proceedings of the International Conference on Biohydrometallurgy, Athens, September 2003 (Elsevier, 2003) (in press).
10. J. C. ELLIOTT, in ''Structure and Chemistry of the Apatites and Other Calcium Orthophosphates. Studies in Organic Chemistry – 18'' (Elsevier, The Netherlands, 1994).
11. M. G. S. MURRAY, J. WANG, C. B. PONTON and P. M. MARQUIS, *J. Mater. Sci.* **30** (1995) 3061.
12. B. C. JEONG and L. E. MACASKIE, *Enzyme Microb. Technol.* **24** (1999) 218.
13. L. E. MACASKIE and A. C. R. DEAN, *J. Gen. Microbiol.* **130** (1984) 53.
14. E. WHITE and E. C. SHORS, *Dent. Clin. North. Am.* **30** (1986) 49.

Received 4 October
and accepted 10 October 2003