Characterisation and sintering of nanophase hydroxyapatite synthesised by a species of *Serratia*

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Abstract. The bacterium *Serratia* sp. NCIMB 40259, which grows as a biofilm on polymeric, glass and metal substrates, produces extracellular crystals of hydroxyapatite (HA) by enzymatic cleavage of β-glycerophosphate in the presence of calcium chloride. Following growth on polyurethane foam, biomineralisation and subsequent sintering, an HA scaffold is formed whose three-dimensional architecture replicates that of the foam and the biofilm. *Serratia* HA was characterised using X-ray diffraction (XRD), Fourier Transform Infra-Red Spectroscopy (FTIR), energy dispersive X-ray analysis (EDX) scanning electron microscopy (SEM), transmission electron microscopy (TEM) and electron diffraction (ED). The nascent, unsintered material consisted mainly of calcium-deficient HA (CDHA) with a Ca/P ratio of 1.61+/− 0.06 and crystal size (TEM) of 50+/− 10nm length. ED of unsintered crystals and crystals sintered at 600° C showed resolvable ring (unsintered) or dot (600º C) patterns ascribed to (0002), (1122) and (0006) planes of crystalline HA. Material sintered at 1200° C consisted of needle-like crystals of length range 54-111nm (XRD) with lattice parameters of a= 9.441 Å and c= 6.875 Å, consistent with HA.

1. Introduction

*Serratia* sp NCIMB 40259 (previously identified as a species of *Citrobacter* [1]) was originally isolated from soil contaminated with heavy metals [2]. It is a non-pathogenic Gram negative bacterium which over-produces an acid phosphatase enzyme located within the cell surface periplasmic space and attached to fimbriae or other surface polymeric material [3]. The enzyme cleaves organic phosphates liberating inorganic phosphate which combines with metal ions to produce metal phosphates that precipitate as extracellular crystals [4]. When the bacteria are supplied with β-glycerophosphate (G-2-P) and calcium chloride, calcium phosphate crystals are produced in the form of hydroxyapatite (HA) [5].

The procedure for manufacture of HA using *Serratia* consists of a three-stages: First the bacteria are grown as a biofilm on a support material in an air-lift fermenter under lactose-limiting conditions, under which the acid phosphatase enzyme activity is up-regulated and the production of fimbriae (hair-like appendages that promote the attachment of the bacteria to the support materials) is induced.
After approximately 6 days the biofilm-coated support materials are transferred to mineralisation medium, which consists of a buffered solution of calcium chloride and G-2-P. The acid phosphatase enzyme remains active under these conditions and HA crystals are produced within the bacterial extracellular polymeric substance (EPS), a structured space. The crystals are then sintered at 1100-1200°C, a process which also destroys the bacteria and any pyrogens. Thermogravimetric analysis showed that all organic material is removed by approximately 500°C. When Serratia biofilm is produced on reticulated polyurethane foam, mineralised and then sintered, all organic materials including the foam are destroyed, yielding a porous calcium phosphate scaffold with interconnected channels that supports the growth of osteoblasts. The biomimetic method is economical and using it HA has even been prepared from phosphates in waste-water. Moreover, since the bacteria can form a biofilm on any shape of support and coat it with a layer of mineral, the method has been considered as a potentially useful non-line-of site method for coating materials of complex architecture with hydroxyapatite.

The aim of this study was to characterise Serratia HA before and after sintering to further evaluate its potential as a bone graft precursor material.

2. Materials and Methods

2.1. Bacterial Growth and Powder preparation.

Serratia sp. NCIMB 40259, used under licence from Isis Innovation, Oxford, UK, was cultured as previously described in an air-lift fermenter on polyurethane reticulated foam cubes (Reticel Ltd, Belgium). After 6 days 25 cubes were transferred to a 25cm long vertical glass column perfused with mineralisation solution containing 25 mM calcium chloride (CaCl₂; Sigma, USA) and 50 mM glycerol-2-phosphate (G-2-P; BDH, UK) in 50mM AMPSO buffer (sodium salt; Sigma, USA), pH 8.6. After 14 days the calcium phosphate-encrusted cubes were withdrawn from the column and air dried at 50°C for 12 hours before being sintered in a furnace for 2 hours at 600°C or 3 hours at 1200°C to produce a solid scaffold. Dried foam cubes were squeezed and sintered scaffolds crushed to obtain the crystal powder for analysis.

2.2 Powder Characterisation.

2.2.1. Electron Microscopy. Scanning electron microscopy (SEM) was carried out on intact scaffolds using an Environmental Scanning Electron Microscope (ESEM XL30) operated at 15 kV and equipped with Oxford INCA EDX (energy dispersive X-ray) analyser and software. Transmission Electron Microscopy (TEM) was performed on embedded and sectioned powders with a Field Emission Gun (FEI F20 TECNAI) microscope equipped for EDX as above and operated at 200 kV. For electron diffraction (ED) studies the camera length of the TEM was calibrated using evaporated thallous chloride (Agar Scientific, UK).

2.2.2. Fourier Transform Infra-Red Spectroscopy (FTIR). The FTIR spectra of the samples were obtained by diffuse reflectance spectroscopy (4000-500 cm⁻¹) using a MAGNA-IR 860 Spectrometer with a scan rate of 50 mV/s and a resolution of 4 cm.

2.2.3. X-Ray Diffractometry. Phase analysis of the powders was performed on a Philips X’Pert PW3040 X-ray diffractometer (XRD) using CuKα radiation (1.5417 Å) at 40 kV and 30 mA. The scanning range (2θ) was from 20 to 120° at a scan speed of 0.5° min⁻¹ with a step size of 0.05°. Phases were identified by reference to the Joint Committee on Powder Diffraction Standards. The relative crystallinity (Cr) of the HA powder was assessed and determined by comparing the main (211) peak intensity to that of a commercial reference HA (Captal® R Hydroxylapatite: Plasma BioTal® Ltd, UK). The crystallite size of the samples was determined from the XRD data according to the Scherrer equation.
3. Results

3.1 Scanning electron microscopy of sintered scaffold.
Figure 1 shows a sintered scaffold made by growing *Serratia* on foam with 0.5mm pores and sintering at 1200°C. The original polyurethane foam which formed the substrate for biofilm growth has been destroyed leaving the calcium phosphate “skeleton” (figure 1a). Higher magnification images (figure 1 b-d) reveal that the sintered scaffold surface is made up of solid chains of annealed particles presumably replicating the arrangement of bacteria in the original biofilm. The surface is porous with micron and submicron-scale cavities. The individual grain size of the sintered material ranged from 0.5-10µm (figure 1d).

![Figure 1. Sintered Serratia HA scaffold. (a) Complete scaffold, pore size approximately 500µm. (b) Scanning electron micrograph showing an individual “trabeculum” of the scaffold; size bar = 200 µm; (c) Higher magnification view of (b) showing surface structure, size bar = 10µm; (d) Higher magnification showing individual ceramic grains, size bar = 2µm.](image)

3.2 TEM and Electron Diffraction
The size of the unsintered crystals determined by TEM was 50 +/- 10nm length; 4 +/- 1nm width. ED patterns obtained from the unsintered crystals showed resolvable ring patterns ascribed to (0002), (1122) and (0006) planes of crystalline HA. Single crystal patterns characteristic of HA were seen with commercial HA and HA sintered at 600°C (figure 2). ED patterns were not obtained from the material sintered at 1200°C but lattice parameters determined from XRD spectra were consistent with stoichiometric HA, as reported below.
3.3 FTIR
The FTIR spectrum of unsintered HA (figure 3) shows peaks at 1104 and 1043 cm\(^{-1}\) indicating P-O asymmetric stretching in orthophosphate and at 602 and 559 cm\(^{-1}\) indicating P-O-P deformation of the PO\(_4^{3-}\) ion. The broad peak at 3412-3435 cm\(^{-1}\) represents O-H bonding in water. It shows a broad band in the 870 cm\(^{-1}\) region due to the simultaneous presence of P-O-H in HPO\(_4^{2-}\) groups and C-O vibrations in CO\(_3^{2-}\) groups [10]. In the HA sintered at 1200°C the peak 1104-1083 cm\(^{-1}\) is missing. The 958 cm\(^{-1}\) band, characteristic of symmetric stretching of the PO\(_4\) group in HA, is present, as are the peaks at 1043 cm\(^{-1}\) (P-O asymmetric stretching) and 596-572 cm\(^{-1}\) (P-O-P). There is also a small band in the 877 cm\(^{-1}\) region due to the simultaneous presence of P-O-H in HPO\(_4^{2-}\) groups and C-O vibrations from the CO\(_3^{2-}\) groups.

3.4. XRD
XRD spectra of nascent unsintered powder and powder sintered at 600°C and 1200°C are shown in figure 4, in comparison with the commercial HA. The unsintered material consisted mainly of calcium-deficient HA (CDHA), with small amounts of sodium chloride (approximately 1%). The Ca/P ratio was 1.61+/- 0.06, determined by EDX. Powder sintered at 600°C and 1200°C was also identified.
as CDHA, with Ca/P ratios of 1.62 and 1.52 respectively and contained small amounts of sodium calcium phosphate in addition to HA. Material sintered at 1200° C consisted of needle-like crystals of length range 54-111nm with lattice parameters of a= 9.441 Å and c= 6.875 Å, consistent with HA [11].

![Figure 4. XRD Analysis of unsintered, nascent HA and powder sintered at 600° C and 1200° C.](image)

**Figure 4.** XRD Analysis of unsintered, nascent HA and powder sintered at 600° C and 1200° C.

### 4. Discussion

The results of this study confirmed and extended preliminary observations that when grown as a biofilm and placed in a solution containing an organic phosphate and source of calcium ions, *Serratia* bacteria produce crystals of HA. FE-SEM of a sintered scaffold revealed the unique, highly porous surface topography of the scaffold, for which the original bacterial biofilm forms the template. A large surface area and porous microstructure is potentially advantageous for cell and biomolecule attachment.

Elemental analysis and TEM showed that the nascent powder consisted of calcium-deficient HA crystals, approximately 50nm in length. Smaller, nanophase crystals (15 – 25nm length) can be obtained if mineralisation is carried out in the presence of sodium citrate and with low levels of G-2-P to retard crystal growth [9]. Nanophase crystals are advantageous because of the potential gains in mechanical strength conferred by nanoscale grain structure in the sintered product if the small size can be retained [11] However, on sintering, growth of the crystals occurred and the grain sizes in the sintered scaffold were micron-scale. It is necessary to further modify the mineralisation and sintering conditions in order to obtain smaller grain sizes.

The sintered crystals consisted of calcium-deficient HA with small amounts of sodium calcium apatite. The presence of sodium ions within the crystal lattice may not be clinically disadvantageous since bone contains approximately 1% sodium [12]. The percentage weight of sodium in the sintered material may vary from approximately 1% [13] to 10% (this study). FTIR spectra showed peaks typical of an apatitic structure [14] but lacked the OH⁻ ion stretching peak which is characteristic of HA. The CO₃²⁻ groups present are most likely to originate from the evaporation of the polyurethane foam support and/or the bacterial cells during sintering. The presence of these carbonate ions could be advantageous in promoting osseointegration [15]. Stoichiometric HA with a typical HA FTIR spectrum may be produced if biomineralisation is carried out at pH9.2, in the presence of citrate [16].
If it is carried out at pH 8.6, the nascent crystals may sinter to HA or β-TCP [7, 16]. Apart from pH, the phase composition of the final product also depends on the Ca/P ratio of the unsintered crystals [17,18], which, in the case of *Serratia* biomineralisation, is governed by the concentration of CaCl₂ and G-2-P in the biomineralisation solution, other ions present (e.g. citrate) and potentially other factors including bacterial/enzyme concentration and the rate of perfusion through the column. Further experiments are necessary to define these parameters. Both HA and β-TCP are useful bone graft biomaterials: HA is used when a permanent bone replacement is required, whereas β-TCP is more soluble and resorbable and is used as a temporary graft material that is gradually replaced by host bone [19].

This study confirmed that *Serratia* biomineralisation is a potentially useful alternative method for obtaining crystals of HA which could be used as precursors for compaction into granules or solids for use as a bone-filler. *Serratia* HA (or β-TCP) scaffolds fulfil many of the requirements of an ideal bone graft material in having a porous microstructure for bone cell attachment and interconnected channels for ingress of blood vessels. The unique surface microstructure of the scaffold, which results from the biofilm architecture, may have advantages for cell and biomolecule attachment, and this will be explored in further studies. The study highlighted the need for further experimentation to define the influence of different factors in controlling the final phase composition of the sintered products. The mechanical properties of the material are also currently inadequate for implantation, with a maximum load at failure of a sintered scaffold of only approximately 12 N [13]. Further optimisation of the sintering conditions is necessary to improve the density and fracture toughness.

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**References:**

[1] Pattanapipitpaisal P, Mabbett AN, Finlay JA, Beswick AJ, Paterson-Beedle M, Essa A, Wright J, Tolley M, Badar U, Ahmed NA, Macaskie LE. 2002 *Environ. Technol.* 23 731-46.
[2] Macaskie LE and Dean ACR 1984 *J. Gen. Microbiol.* 130 53-62
[3] Bonthrone KM, Quarmby J, Hewitt C, Allan, VJM, Paterson-Beedle, M, Kennedy JF and Macaskie, LE 2000 *Environ. Technol.* 21 123-34
[4] Macaskie LE, Bonthrone KM, Yong P and Goddard DT 2000 *Microbiol.* 146 1855-67
[5] Sammons RL, Marquis PM, Macaskie LE, Yong P, Basner C. 2004 *Phosphorus in Environmental Technology* ed E Valsami-Jones (London IWA publishing) chapter 6 pp 582-609
[6] Allan VJM, Callow ME, Macaskie LE and Paterson-Beedle M. 2002 *Microbiol.* 148: 277-28.
[7] Thackray A, Sammons R, Macaskie L, Yong P, Lugg H and Marquis P 2004 *J. Mater. Sci: Mater. Med* 15 403-06
[8] Yong P, Macaskie LE, Sammons RL, Marquis PM 2004 *Biotechnol Lett.* 26 1723-30
[9] Macaskie LE, Yong P, Paterson-Beadle M, Thackray AC, Marquis PM Sammons RL, Nott, KP, Hall LD 2005 *J. Biotechnol* 118 187-200
[10] Tas AC and Aldinger F J 2005 *J. Mater Sci: Mater Med* 16 167–174
[11] Murray MGS, Wang, J. Ponton, CB and Marquis PM 1995 *J.Mater. Sci. 30* 3061-74
[12] Elliot JC 1994 *Structure and chemistry of the apatites and other calcium orthophosphates. studies in inorganic chemistry 18* (Amsterdam, Elsevier)
[13] Thackray, AC 2005 PhD Thesis: Bacterial biosynthesis of a bone substitute material (University of Birmingham, UK)
[14] LeGeros RZ 1991 *Calcium phosphates in oral biology and medicine. Monographs in Oral Science*, vol. 15 ed H Myers (Basel Karger)
[15] Porter A, Patel N, Brooks R, Best S, Rushton N, Bonfield W 2005 *J. Mater Sci: Mater Med* 16 899-907.
[16] Lugg H 2005 PhD Thesis: *A Study of extracellular calcium phosphate biomineralisation and
intracellular inclusion body formation by Serratia sp. N14 (University of Birmingham, UK)

[17] Raynaud S, Champion E and Bernache-Assolant 1999 J.Inorg. Phosph. Res. Bull. 10 214-19

[18] Paleevskis E, Dindune A, Kanepe Z, Krastins J, Janackovic D, Mihailscu IN 2006 Latv. J. Phys. Tech. Sci 4 63-70.

[19] Perry CR 1999 Clin Orthop. Rel. Res. 360 71-86.