Hydroxyapatite synthesis on solid surfaces using a biological approach

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Abstract. Many naturally occurring mineralisation processes yield hydroxyapatite (HA) or related salts, but biological routes to calcification have not generally been exploited for production of hydroxyapatite for clinical and industrial applications. Serratia sp. NCIMB 40259 is a non-pathogenic Gram-negative bacterium which is capable of growing as a biofilm on many surfaces and can be used to form HA coatings on a variety of polymeric and metallic materials, including titanium. Here we review previous work and report the results of more recent studies on the influence of titanium compositional and surface properties on Serratia adherence and proliferation and biomineralisation on commercially pure titanium (cp Ti) discs and a Ti mesh. Bacterial adherence was equivalent on cpTi and Ti6Al4V, and biofilms formed on both rough and mirror-polished cpTi surfaces. Embedded alumina particles and alkali treatment did not noticeably alter the precipitation of Serratia HA, nor the structure of the coating in comparison with non-treated substrates. Coatings were retained after sintering at 800°C in argon, although the original curved plate-like crystals changed to nano-scale β-tricalcium phosphate particles. A phosphorous-rich diffusion zone formed at the coating-titanium interface. Bacterial mineralisation may have applications as a method for producing coatings on implants in non load-bearing sites, and non-clinical applications where a high surface area is the major concern.

1. Introduction
Biomineralisation occurs in nature in many forms, some of which are beneficial to humans and some detrimental. In the body, biomineralisation leads to the formation of the skeleton and teeth but pathogenic calcification can also occur in arteries, in skin, on heart valves, as dental calculus, as kidney stones and as encrustations blocking medical devices such as urinary catheters [1, 2]. Although many of these naturally occurring processes yield hydroxyapatite (HA) or related salts, biological routes have not generally been exploited for production of HA for clinical and industrial applications. In this paper we briefly review our previous work on HA formation by Serratia and then describe the results of more recent investigations [3] using this approach to form a HA coating on titanium substrata, starting with biofilms. Finally we briefly discuss an alternative method of producing HA crystals and coatings using Serratia not as a biofilm but as planktonic cells in suspension.
2. Biomineralisation by Serratia: Background

*Serratia* sp. NCIMB 40259 was originally isolated from heavy-metal ion contaminated soil where it survives by removing heavy metal contaminants e.g. cadmium from its surroundings as metal phosphates [4]. Initially identified as a species of *Citrobacter*, it was reclassified as a species of *Serratia* in 2002[5].

The mechanism by which metal phosphates are formed by this species of *Serratia* involves an acid phosphatase enzyme, located in the bacterial cell wall, within the periplasmic space, and on hair-like projections called fimbriae [6]. The enzyme cleaves organic phosphates liberating inorganic phosphate, which combines with metal ions to produce metal phosphates which precipitate within the extracellular polymeric matrix EPM) surrounding the cells. The EPM provides both the nucleation sites for crystal formation and restricts crystal growth [7,8,9].

When *Serratia* are presented in the laboratory with calcium ions from calcium chloride and an organic phosphate (glycerol-2-phosphate), nanoscale crystals of Ca-deficient hydroxyapatite are produced. The Ca/P ratio varies depending on the mineralisation conditions: Mineralisation at pH 8.6 yields calcium deficient HA which may either sinter (at 1200° C) to β-TCP [10] or remain as Ca-deficient HA, depending on the initial Ca/P ratio, the pH and/or the presence of sodium citrate [10]. As in many biological apatites, the absence of the typical HA O-H stretching peak in the FTIR spectrum suggests that the HA crystal lattice is defective [11]. Na⁺ and Cl⁻ ions are present at about 1% in the original crystals and remain as impurities in the final sintered product [12]. A porous scaffold material that supports the growth of osteoblasts in culture was made by culturing the bacteria on polyethylene sponge, allowing biomineral formation and then sintering the mineral-encrusted biofilm to destroy the bacteria and the sponge template leaving only the mineral “skeleton” that replicates the original structure of the biofilm, with a very high surface area [11,12]. By presenting the bacteria with a mixture of CaCl2 and SrCl2 in the biomineralisation solution, co-crystals are formed containing Ca and Sr in the crystal lattice in amounts corresponding to the supplied concentrations [2]. Such materials could be useful as bone-substitute materials including applications in the repair of osteoporotic bone. On the other hand, as a procedure for coating solid surfaces with HA it may be a useful non-line-of-sight method [13], whilst its high surface area makes it potentially advantageous for waste water treatment for removal of phosphates [14] or radionuclides [15,16].

3. Influence of titanium properties on Serratia adherence and proliferation

3.1 Chemical composition

*Serratia* attached equally well to commercially pure (cpTi) and TiAl6V4 alloy [16]. There was no significant difference in proliferation on these after 24h. The bacteria proliferated significantly better on pure vanadium than aluminium (figure 1).
3.2 Influence of grain boundaries and crystal orientation
Using a combination of back-scattered electron imaging to show grain boundaries, and electron back-scattered diffraction to show crystal orientation on polished cpTi, it was found that neither grain boundaries nor grain orientation affected Serratia adherence [16].

3.3 Influence of surface topography on biofilm formation
Biofilm formed on a range of model dental implant surfaces, as well as on Al₂O₃ grit-blasted and mirror polished cpTi shown in figure 2a. There was no influence of surface roughness (ra value) but some indication that surface hydrophobicity might be more important, there being a negative correlation between static water contact angle (figure 2b) and protein after 7 days in culture with T1, T3, T4, T6 (figure 2c). However further work must be done to confirm this.

![Figure 2](image_url)

**Figure 2**: a) cpTi surfaces used for comparison of bacterial proliferation; b) hydrophobicity of the surfaces as measured by water contact angle; c) bacterial load estimated by a protein assay after 7 days in culture in lactose-limiting medium. * indicates statistical significance in t test; n = 3; P = <0.05*; <0.01**.

According to the protein assay and SEM images, biofilm formed on the mirror polished surface (T2) at least as well as on the rougher ones. The bacteria formed densely-packed colonies on T2 and on T6 they formed vertical stacks and palisades in the troughs, with more randomly aligned cells on the ridges (figure 3).
4. Biomineralisation on grit-blasted cpTi

4.1 Structure of the coating before heat-treatment

When biomineralisation occurs, individual cells become coated with an envelope of clusters of needle or plate-shaped crystals. A few uncoated bacterial cells can also be seen amongst the coated ones (figure 4a). A cross-section through the mineralised biofilm shows the porosity of the structure and the thickness of the mineral envelope around each cell (figure 4 b,c). In addition, clumps of cells are surrounded by shells of mineral approximately 0.5µm thick. Selected area diffraction confirmed the presence of HA and the Ca/P ratio (EDX) was 1.57±0.14.

Figure 4: HA coating on grit-blasted cpTi. a: envelopes of needle- or plate-shaped crystals covering cells; a few uncoated cells are also seen. b and c: Cross-section c: Clumps of coated individual cells are surrounded by shells of mineral (arrows).
4.2 Structure of the coating after heat treatment
To burn off the biomass and sinter the crystal coating samples were subjected to the following heating regime: ramp at 5° C/min to 550° C, dwell for 3h, ramp at 5° C/min to 800° C for 10h and then cool at 5° C/min. Figure 5 shows the overall appearance of the coating. Cracks are apparent in the surface. Higher magnification revealed that the coating consisted of spheres of nano-particles with a Ca/P ratio of 1.34±0.04, consistent with β-tricalcium phosphate. A transition layer was formed at the interface with the substrate (figure 5).

Figure 5: Heated HA coating on cpTi. a: overall appearance of the coating (scale bar = 100μm). Higher magnification images in b and c show clusters of nanoparticles and TEM appearance below. d and e: a transition layer at the interface between the coating and the substrate (figure 6 below).

Figure 6: Cross-section of the interface between the HA coating and the cpTi substrate after heating. A line scan shows migration of P from a P-deficient zone into the substrate, confirmed by EDX maps.
5. Biomineralisation on grit blasted titanium treated with sodium hydroxide

The interface between the HA coating and cp Ti which had been grit-blasted with Al₂O₃ and then treated with 5M sodium hydroxide to form a sodium titanate layer was investigated. Focused ion beam milling was used to produce a cross-section 150nm wide. Imaging revealed a layered structure with continuity between the sodium titanate and the HA crystals (figure 7). The sodium titanate layer consisted of a denser lower region and a “fibrous” upper region interdigitating with HA crystals. Embedded Al₂O₃ particles were surrounded by sodium titanate and similarly covered with HA.

6. Biomineralisation of a titanium mesh

*Serratia* are 1-2µm long and possess flagellae that enable them to swim in suspension and theoretically thereby enter cavities and channels that are wider than a few microns. However build up of the biofilm on the outside of a porous structure might prevent bacterial infiltration into the interior. To investigate this, a Ti mesh made from 50 µm diameter wires with a thickness of about 1mm was used as a substrate (figure 8) The meshes were incubated in *Serratia* culture for three days to allow bacterial adhesion and proliferation and then loaded into a bioreactor perfused with mineralisation solution at a flow rate of 1.5 ml/h for nine days. Under these conditions each wire of the mesh was coated with nanoscale crystals, even in the interior. Following heating (550°C for 3 hours & 800 °C for 5 hours in an argon furnace) the coating remained on the surface. EDS mapping confirmed the presence of large crystals of calcium phosphate between the wires and a line scan of the interface showed migration of phosphate from the coating into the Ti in a diffusion zone as with the Ti sustrate described above (figure 8).
Figure 8. Biomineralisation on Ti wire mesh. a and b Appearance of the uncoated mesh and individual wire surface showing Ti crystal structure. c: wires after biomineralisation and heating, showing retention of the calcium phosphate crystals. d: Cross section of the interface between the wire and adjacent HA crystals. An EDX line map from left to right of the image shows migration of P into the titanium substrate.

7. Use of planktonic *Serratia* to promote calcium phosphate precipitation

Hydroxyapatite crystals may develop on Ti, which has been treated with 5M NaOH to produce a sodium titanate surface, when it is placed in simulated body fluid containing calcium and phosphate ions, by an ion exchange mechanism [17]. The ability of planktonic (free swimming) *Serratia* bacteria, harvested from the biofermenter outflow, to promote crystal formation on alkali-treated Ti in biomineralisation solution was tested and the results shown in figure 9. Detonated nanodiamonds (DND) were added to some samples as these have previously been shown to promote HA nucleation under these conditions [18]. However DND appeared to inhibit crystal formation or growth to some extent, giving a lower yield of precipitated crystals. All the titanium discs were coated with crystals although the clusters of crystals formed in the presence of nanodiamonds appeared more consolidated. Further work is necessary to elucidate whether biomineralisation by *Serratia* bacteria in suspension involves the action of the acid phosphatase as in the initial stages following biofilm formation or whether crystal nucleation is simply promoted by components of the bacterial cell wall or extracellular matrix.
Figure 9. Biomineralisation promoted in mineralisation medium by planktonic *Serratia* in suspension and on alkali-treated Ti discs placed in the same container. a: A lower yield of crystals was obtained as a precipitate in the presence of DND; b, c: back-scattered electron images of the precipitate on the titanium discs revealed a denser particulate structure with DND (c).

8. Conclusion
Coating titanium discs with HA using bacterial biomineralisation may be superior to other methods when surface area is essential but physical strength is not so important, such as implants in non-load bearing sites and as catalyst carriers, or for water purification. The coating structure is not determined by the topography of the substrate, but the structure of the biofilm, yielding an extremely high surface area with nano-, micro- and macro-scale roughness. This unique structure and the nano-scale calcium-deficient HA may be beneficial *in vivo* to facilitate tissue mineralisation and bone ingrowth whilst its high surface area may find applications for drug release.

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10. References
[1] Ciftiçioğlu N, McKay D S 2010 *Pediatr Res*. 67: 490
[2] Sammons R, Wang, A, Thackray A, Yong P, Kuboki Y, Ametani A and Macaskie L 2010 *Nano Biomedicine* 270
[3] Wang A 2011, PhD thesis, University of Birmingham, UK
[4] Macaskie LE, Dean A C R 1984 *J Gen Microbiol* 130 53
[5] Pattanapipitpaisal P et al 2002 *Environ Technol* 23 731
[6] Bonthrone K M, Quarmby J, Hewitt C J, Allan V J M, Paterson-Beedle M, Kennedy J F and Macaskie L E 2000 *Environ Technol* 21 123
[7] Jeong B C, Hawes C, Bonthrone K M and Macaskie L E 1997 *Microbiol* 143 2497
[8] Finlay J A, Allan V J M, Conner A, Callow M E, Basnakova G, Macaskie L E 1999 *Biotechnol Bioengineering* 63 87
[9] Macaskie L E, Bonthrone K M, Yong P, Goddard D T 2000 *Microbiol-UK* 146 1855
[10] Thackray A C, Sammons R L, Macaskie L E, Yong P, Lugg H, Marquis P M. 2004 *J Mater Sci Mater Med* 15 403
[11] Medina Ledo H, Thackray A C, Jones I P, Marquis P M, Macaskie LE, Sammons R L. 2008 *J Mater Sci Mater Med* **19** 3419

[12] Sammons R L, Thackray A C, Medina-Ledo H, Marquis P M, Jones I P, Yong P, Macaskie LE 2007 *J Phys Conf Ser* **93**, 012048

[13] Macaskie L E, Yong P, Paterson-Beedle M, Thackray A C, Marquis P M, Sammons R L, Nott K P, Hall L D 2005 *J Biotechnol* **118** 187

[14] Yong P, Macaskie LE, Sammons RL, Marquis PM, 2004 Biotechnol Lett 26: 1723-1730

[15] Paterson-Beedle M, Macaskie LE, Lee C H, Hriljac J A, Jee K Y, Kim W H  2006 *Hydrometallurgy* **83** 141

[16] Handley-Sidhu S, Renshaw J C, Yong P, Kerley R, Macaskie L E 2011 *Biotechnol Letts* **33** 79.

[17] Takadama H, Kim H-M, Kokubo T, Nakamura T 2001 *J. Biomed Mater Res* **55** 185

[18] Pramatarova L, Radeva E, Pcheva E, Hikov T, Krasteva N, Dimitrova R, Mitev D, MontgomeryP, Sammons R and Altankov G 2011 The advantages of polymer composites with detonation nanodiamond particles for medical applications *On Biomimetics* ch. 14, ed. L Pramatarova (Croatia; InTech Publications) pp 297-320