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Bacterial adhesion to bisphosphonate coated hydroxyapatite

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Staphylococcus aureus (*S. aureus*) is commonly associated with microbial infection of orthopaedic implants. Such infections often lead to osteomyelitis, which may result in failure of the implant due to localised bone destruction. Bacterial adhesion and subsequent colonisation of the device may occur as a consequence of contamination during surgery, or by seeding from a distant site through the blood circulation. Coating of the hydroxyapatite (HA) ceramic component of artificial hip joints with the bisphosphonates clodronate (C) and pamidronate (P) has been proposed as a means to minimise osteolysis and thereby prevent loosening of the implant. However, the effect of the bisphosphonate coating on bacterial adhesion to the HA materials must be determined before this approach can be implemented. In this study coated HA materials were incubated with the *S. aureus* and the number of adherent bacteria determined using the Modified Vortex Device (MVD) method. The number of bacteria adherent to the P coated HA material was significantly greater than that adherent to uncoated HA (60-fold increase) or to the C coated HA (90-fold increase). Therefore, even though earlier studies suggested that P bound to HA may improve osseointegration, the results presented would suggest that the use of this coating may be limited by the potential increased susceptibility of the coated device to infection.

1. Introduction

Bacterial adhesion has become a major concern for many medical devices, including catheters, middle ear implants and contact lenses [1–4]. It has, therefore, become important to develop materials for implantation which are resistant to bacterial adhesion. Orthopaedic implants are also susceptible to microbial infections and these are frequently due to *Staphylococcus aureus* (*S. aureus*) [5, 6]. Such infections often lead to osteomyelitis, which may result in failure of the implant due to localised bone destruction [7]. Bacteria can adhere to and colonise the material either by contamination during surgery or by seeding from a distant site through the blood circulation [1]. It is therefore important to investigate the extent to which orthopaedic materials resist or promote bacterial adhesion, and to this end a Modified Vortex Device (MVD) test method was used to investigate bacterial adhesion to materials being developed for use on the surface of artificial hips. This method has previously been validated as an alternative to the use of costly animal testing for the assessment of bacterial adhesion to new contact lens materials [4]. The method relies on a whirlpool-type force to remove bacteria adherent to the test material.

Osteolysis with concomitant loosening of the prosthesis is the most common cause of failure in total joint arthroplasty and it is usually initiated by a number of material, device, site and host specific biological and mechanical events [8]. These include fragmentation and wear of the implant materials, release of particles into the tissue to provoke a foreign body reaction, stress shielding due to stiffness mismatch between the implant material and surrounding bone, and activation of cells to produce a variety of cytokines and proteolytic enzymes. It is therefore important to develop successful strategies to minimise osteolysis, as these would reduce the costs to both the patient and healthcare providers associated with revision surgery to replace failed prostheses. Bone remodelling occurs continuously in the skeleton by the co-ordinated actions of osteoblasts, which secrete new matrix, and osteoclasts, which resorb old bone. As osteoblastic and osteoclastic cells are also activated during osteolysis, any agent which reduces the efficiency

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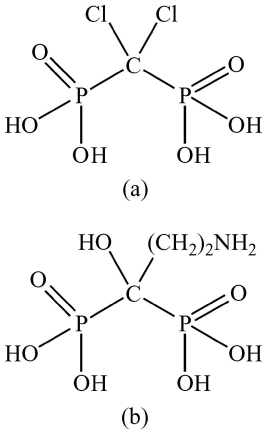

Figure 1 Chemical structures of (a) clodronate, (b) pamidronate.

of bone resorption, will inhibit the osteolysis associated with prosthesis loosening.

The bisphosphonates are a group of stable pyrophosphate analogues characterised by a central carbon atom linking two phosphate groups and two side chains of variable structure (for examples see Fig. 1). These agents are known to be effective inhibitors of osteoclastic activity [9–16] and may therefore have a role to play in reducing bone resorption. The properties and potency of the bisphosphonates in inhibiting osteoclastic activity is determined by the nature of the side chains [17]. Bisphosphonates are used therapeutically in a variety of diseases of enhanced bone resorption, including Paget's disease, hypercalcaemia of malignancy and osteoporosis, so there are data available for the efficiency, safety and pharmacodynamics in humans following both oral and intravenous administration [17].

Bisphosphonates bind avidly to the bone mineral hydroxyapatite (HA), and are released during skeletal remodelling (17). We propose that a coating of bisphosphonate on the HA ceramic component of artificial hip joints may inhibit osteolysis of the surrounding tissue, reduce the tendency towards loosening, and therefore improve the lifetime of the implant. We have recently reported that osteoblasts cultured on HA materials coated with bisphosphonates show increased synthesis of DNA, protein and collagen compared with cells cultured on uncoated HA [18]. This suggests that bisphosphonate coating may improve osseointegration of HA implants by also increasing osteoblast growth and activity. However, before this approach can be implemented the effect of the bisphosphonate coating on bacterial adhesion onto the HA materials must be determined. In this study HA materials coated with either Clodronate (C) or Pamidronate (P) were incubated with *S. aureus* and the MVD used to determine the number of adherent bacteria associated with the different materials. The structures of these drugs are shown in Fig. 1.

2. Materials and methods

2.1. Preparation of materials

Sodium clodronate (Bonefos, Boehringer Ingelheim, Bracknell, Berkshire, UK), and Pamidronate (Aredia, Ciba, Camberley, Surrey) were prepared as 0.22 M, and 0.038 M solutions in serum-free medium respectively.

To produce pellets of dense HA material (pore size < 3 μm) 55 g of calcium phosphate powder (tribasic 34-4% Ca) was mixed with 10 ml of 4% (w/v) polyvinyl alcohol in distilled water. Using an Instron, 1 g of the mixture was placed in a compaction chamber and a pressure of 750 kg applied to produce 1 cm $\times$ 0.5 cm dense pellets. The samples were then sintered at 1150 °C for 2 h. After sintering, samples were polished using silicone carbide paper, cleaned for 1 h in 70% (v/v) alcohol, and then for a further 1h in distilled water at 37 °C. A number of polished and cleaned samples of the material were then coated with 4 $\times$ 200 μl aliquots of either 3 mg/ml disodium clodronate or 1 mg/ml disodium pamidronate at room temperature. The coated substrates were dried under a controlled, constant heated airflow between each application. The presence of the coating was confirmed using a Joel JSM 6310 Scanning Electron Microscope with an Oxford ISIS Energy dispersive X-ray (EDX) microanalysis attachment using an accelerating voltage of 20 kV.

2.2. Bacterial adherence assay

An overnight culture (18 h) of *S. aureus* was prepared in 100 ml of nutrient broth. The bacteria were washed thrice. By reference to a standard optical density calibration curve the cells were resuspended in 10 ml of sterile phosphate buffered saline (PBS) to a concentration of approximately 10^8 cells/ml. The coated and uncoated samples of HA ceramics were equilibrated with 2 ml of sterile PBS for 1 h, prior to placing the samples into a 24 well plate. 1 ml of the bacterial suspension was added to each well and the plate incubated at 37 °C on a shaker at 120 rpm for 6 h.

On completion of the incubation the coated and uncoated HA ceramics were placed into a fresh 24-well plate and repeatedly washed with 2 ml of sterile PBS to remove any loosely adherent bacteria. Each material was then placed into 5 ml of sterile PBS and vortexed at maximum power for 1minute to remove bacteria which had adhered to the material. Previous work has shown that few bacteria remain attached to surfaces after this vortex process [4]. 200 μl aliquots of the vortex solutions were serially diluted and plated in triplicate onto tryptone soya agar (TSA) plates and incubated at 37 °C for 17 h. The colonies formed from the incubation solutions and vortex solutions were counted.

2.3. Scanning electron microscopy

Coated and uncoated samples of HA ceramic incubated with *S. aureus* were prepared for SEM following standard procedures. Samples were fixed in 2.5% glutaraldehyde at room temperature for 17 h. The samples were then washed three times with cacodylate buffer and dehydrated through a series of graded ethanol solutions (25, 50, 75, 95, 100%). The samples were subsequently freeze-dried, sputter coated with gold, and observed using Joel JSM 6310 Scanning Electron Microscope.

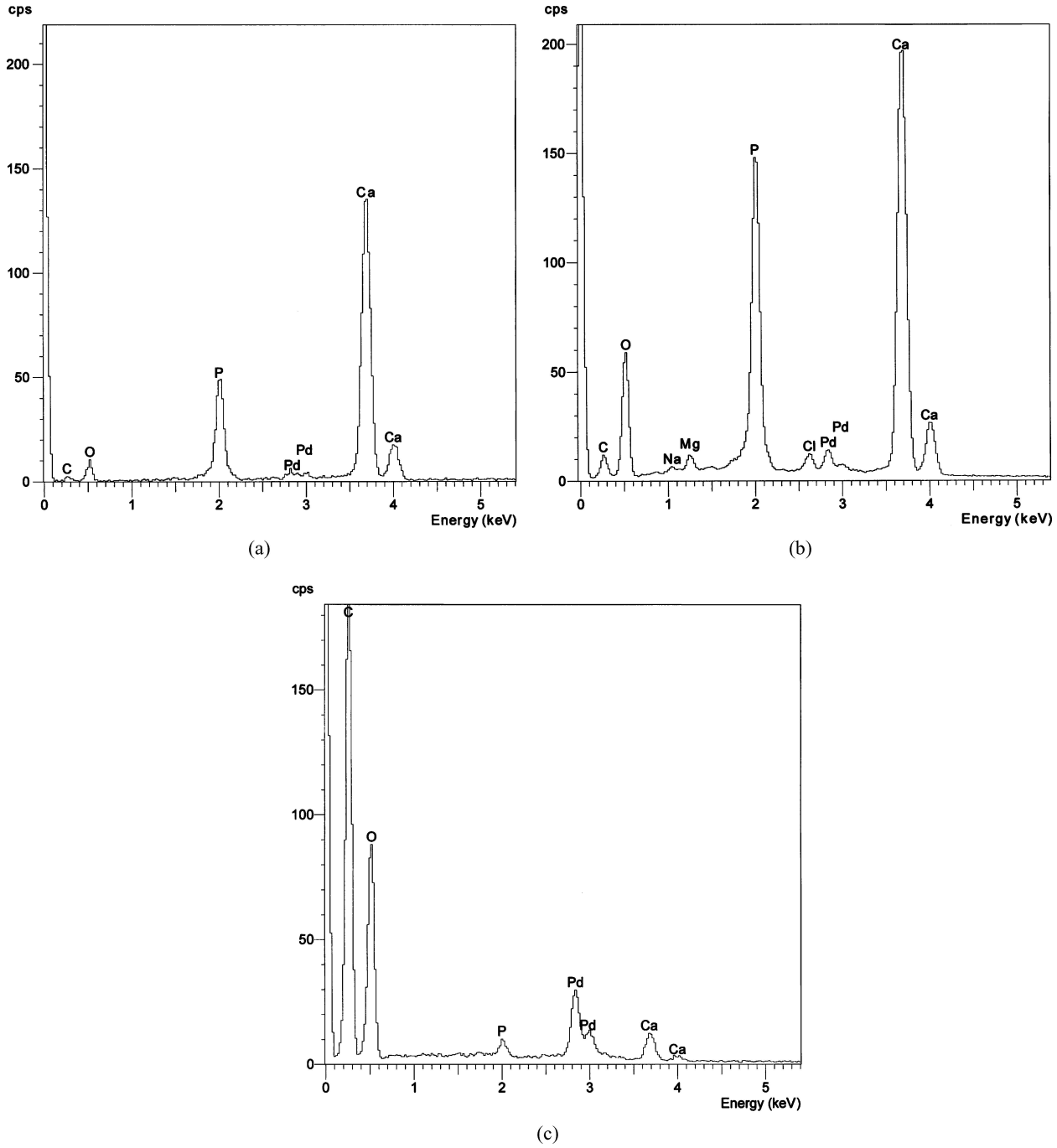

Figure 2 EDX microanalysis spectra for uncoated (a), clodronate coated (b) and pamidronate coated (c) HA disks obtained using a Joel JSM 6310 Scanning Electron Microscope with an Oxford ISIS EDX system at an accelerating voltage of 20 kV.

3. Results

Fig. 2 shows the EDX spectra for the uncoated and coated HA disks. The presence of chlorine in Fig. 2(b) and the enhanced carbon peak in Fig. 2(c) confirm the presence of clodronate and pamidronate respectively on the surface of the coated HA disks.

Fig. 3 shows the bacterial colony forming units (CFU) on the surface of the 3 different hydroxyapatite material discs, uncoated HA, HA coated with C (HAC) and HA coated with P (HAP). The figure shows that after 17 h of incubation on the TSA plates there were greater quantities of bacteria present in the vortex solution for HAP compared with the HA and HAC samples. There were 5 experiments which contained triplicate samples, and these were termed "Run 1–5". For the HAP samples all 5 runs showed a significant increase ($p < 0.05$; by ANOVA followed by Dunnett's test) in the number of bacteria which had attached to the surface compared with the HA samples. The mean CFU/cm^2 for HAP was 61125 compared with 961 for HA and 702 for HAC an increase of almost 60 and 90 fold, respectively.

Examination by SEM of the HA samples incubated with *S. aureus* for 6 h clearly shows the presence of bacterial colonies on the surface of the disks (Fig. 4).

4. Discussion

Bacterial adhesion is a major problem in the medical device field. Many devices which can be put at risk

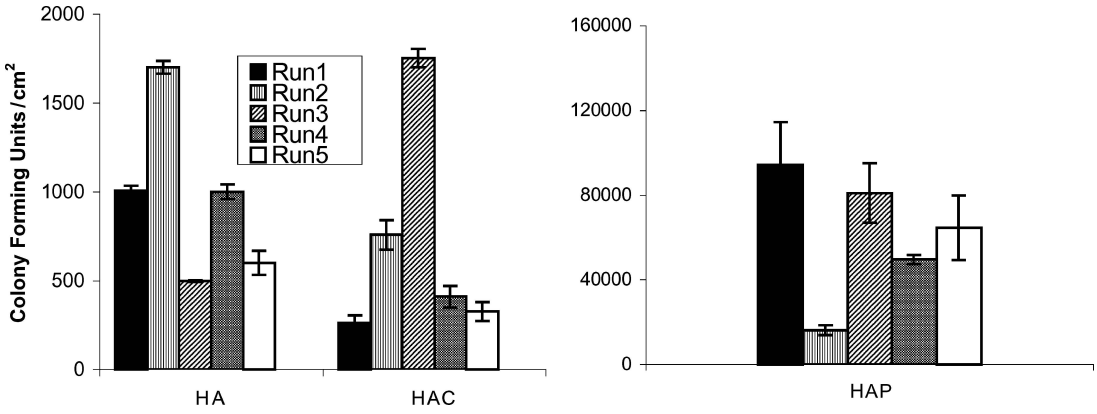

Figure 3 Bacterial Colony forming units on the surface of uncoated hydroxyapatite (HA), and HA coated with either clodronate (HAC) or pamidronate (HAP) for 6 h (note different scales on the *y*-axes). Results are mean ± SEM, of 5 experiments Run 1–5, which contained triplicate samples, $^{*}P < 0.05$ compared with uncoated HA, by ANOVA followed by Dunnett's test.

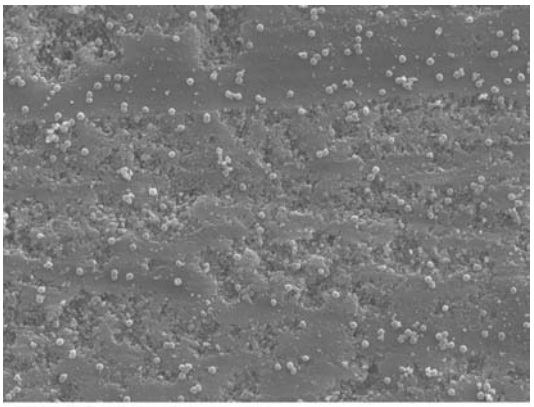

Figure 4 Scanning electron microscopy photographs of *S. aureus.* fixed with glutaraldehyde on uncoated hydroxyapatite, ×2500 magnification.

by recurrent infections, such as middle ear implants, catheters, and contact lenses are routinely tested for susceptibility to bacterial adhesion and colonisation [1–4]. However, in the orthopaedic industry this type of testing is often overlooked even though prosthesis-associated infections represent a major clinical problem [5, 6]. *S. aureus* is directly implicated in the localised bone destruction associated with infected orthopaedic implants and bacterial arthritis [7]. It was therefore considered important to study bacterial adhesion onto the uncoated and bisphosphonate coated HA materials.

An adapted MVD method was used to determine bacterial adhesion, as this procedure has been used successfully to quantify adhesion to contact lens materials [4]. We used this method to quantify the number of adherent bacteria on the coated and uncoated HA materials. The results showed that compared with HA and HAC, there is a dramatic 60–90 fold increase in the number of bacteria which adhered to the HAP material .

The adhesion of *S. aureus* to some surfaces has been shown to be due to its ability to bind specific host matrix proteins [6, 7]. The adhesins that mediate the binding to host proteins, termed MSCRAMMs, are 'microbial surface components which recognise adhesive matrix molecules'. These MSCRAMM components could mediate adhesion on prostheses by binding to host proteins that cover the implant surface *in vivo* [5, 2, 19]. Delmi and co-workers [5] investigated the influence of fibronectin on *in vitro* and *in vivo* *S. aureus* adhesion to stainless steel (steel), pure titanium (Ti) and titanium alloy (Ti-Al-Nb) alloy, metals used commonly for orthopaedic biomaterials. Results from this study showed that all three metallic surfaces that were coated *in vitro* with fibronectin promoted greater adhesion of *S. aureus* than the untreated metals. It has been found that fibronectin binds to *S. aureus* via the amino-terminal domain of its structure [2] and that this interaction is biologically significant in the pathogenesis of *S. aureus* infections. This suggests that the adhesion of such large numbers of *S. aureus* to P may be due to its structure. Fig. 1 shows that P, (3-amino-1-hydroxypropylidene)bisphosphonate contains an amino group, whereas C does not. The cationic amino group on P may attract bacteria by either direct electrostatic interaction or through a direct surface protein interaction. It is possible that P provides an amino acid mimic on the surface of the HA which interacts with the MSCRAMM component. It thus recognises the simulated host protein on the surface of the material, and mediates increased bacterial adhesion to the HAP surface. At present, there is no evidence for a difference in MSCRAMM binding to the two coated surfaces.

This forms part of an ongoing study of the potential use of bisphosphonates as coatings for orthopaedic prostheses. It is the first indication that even though HAP may improve osseointegration [18], it may not be a suitable coating for implants as it promotes adhesion of the bacteria *S. aureus* to the surface of the material. A prosthesis coated with *P* would therefore be prone to infection. The dependence of the effect of P on bacterial adhesion on the coating concentration is at present not known, and it may be possible to find a coating concentration which improves osseointegration but minimises bacterial adhesion. At present, C would be a better coating for implants, as there were very few bacterial colonies adhering to the surface, and, in fact, C appears to slightly inhibit bacterial growth. However, it should be noted that, with orthopaedic implants, the complex in vivo response and the clinical risk of infection can not easily be predicted by a simple *in vitro* adhesion test.

This study demonstrates the importance of determining the effect of modifying the biomaterials for medical devices on their susceptibility to bacterial infection.

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