

Investigation of the influence of phosphatase activity of staphylococcus on the dissolution of a synthetic hydroxyapatite biomaterial

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Abstract

Investigation of the influence of phosphatase activity of staphylococcus on the dissolution of a synthetic hydroxyapatite biomaterial

Objective: Hydroxyapatite (HA) is widely used as bone graft and as coating for orthopaedic and dental implants. Staphylococci are accepted as the main pathogens of biomaterial-related infections. The aim of this study was to investigate whether the phosphatase activity of various strains of *S. epidermidis* had an effect on the dissolution of a synthetic hydroxyapatite biomaterial. **Method:** Ceramic discs were prepared by compressing pure hydroxyapatite powder and sintering at 1200°C. The ability of various strains of staphylococci to produce the phosphatase enzyme was evaluated with two different methods. HA discs were incubated with staphylococcal strains of known phosphatase activity and the calcium concentrations of the medium were measured. HA disc surfaces were examined by means of SEM. **Results:** The results of the Ca concentration measurements of the medium in which HA discs were incubated showed that in the presence of four *S. epidermidis* with known phosphatase activity, no dissolution occurred from the HA discs. Scanning electron microscope (SEM) analysis revealed that the strains of *S. epidermidis* tested may adhere to HA discs however no visual signs of dissolution appeared on the surfaces. No correlation was observed between the phosphatase activity of *S. epidermidis* strains and their adherence to HA surface. **Discussion:** Phosphatase activity of the strains of *S. epidermidis* did not have any influence on the dissolution of hydroxyapatite material tested. For future work, it would be beneficial to do similar experiments on HA plasma-sprayed coating samples.

Key Words: Hydroxyapatite, Staphylococcus, phosphatase, dissolution.

Stafilokokların fosfataz aktivitesinin bir sentetik hidroksiapatit biomateriyalin çözünmesine etkisinin incelenmesi

Özet

Amaç: Hidroksiapatit (HA) kemik grefti olarak ve ortopedik ile dental implantlarda yüzey kaplaması şeklinde yaygın olarak kullanılan bir materyaldir. Stafilocoklar biomateriyallere bağlı enfeksiyonlarda esas patojen olarak kabul edilirler. Bu çalışmanın amacı *S. epidermidis*'in farklı suşlarının fosfataz aktivitelerinin, bir sentetik hidroksiapatit biomateriyalin çözünmesinde etkisi olup olmadığını bulmaktır. **Yöntem:** Seramik diskler saf hidroksiapatit tozunun preslenmesi ve 1200°C'de sinterlenmesi ile hazırlandı. Stafilocokların farklı suşlarının fosfataz enzimi üretebilme yetisi iki farklı metotla değerlendirildi. HA diskler fosfataz aktiviteleri bilinen stafilocok suşları ile inkübe edildi ve besiyerindeki kalsiyum konsantrasyonları ölçüldü. HA disk yüzeyleri scanning elektron mikroskobu (SEM) yardımıyla incelendi. **Sonuçlar:** HA disklerin inkübe edildiği besiyerlerindeki Ca konsantrasyonlarının ölçülmesi, *S. epidermidis*'in fosfataz aktivitesi bilinen dört

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farklı suşunun varlığında, HA disklerde çözünme olmadığını gösterdi. SEM ile yapılan analizler, *S. epidermidis*'in incelenen suşlarının HA disklerle tutunabileceğini gösterdi, ancak yüzeylerde gözle fark edilebilir bir çözünmeye rastlanmadı. *S. epidermidis* suşlarının fosfataz aktivitesi ile HA disklerle tutunması arasında bir ilişki gözlenmedi. **Tartışma:** *S. epidermidis* suşlarının fosfataz aktivitesinin, incelenen HA materyalin çözünmesi üzerine bir etkisi bulunmamaktadır. İlerideki çalışmalarda HA plazma sprey kaplı örneklerde benzer deneylerin yapılması faydalı olabilir.

Anahtar Kelimeler: Hidroksiapatit, Stafilokok, fosfataz, çözünme.

Introduction

Calcium phosphate biomaterials are accepted as osteoconductive materials since they promote bone healing and regeneration. Synthetic calcium phosphate biomaterials include hydroxyapatite (HA), tricalcium phosphate and biphasic calcium phosphate (mixture of HA and β -tricalcium phosphate) (1-3). Pure synthetic HA is very similar to the bone mineral hydroxyapatite (biological apatite) and therefore it is an attractive bone substitute material (4). The clinical applications of HA are mainly in the forms of granules or blocks as bone substitute materials and as coatings deposited on titanium alloy fixtures for orthopaedic and dental implants (5, 6).

Biomaterial-related infections, including those involving biomedical devices in contact with bone are commonly associated with staphylococci. This tendency is related to the ability of the pathogens to adhere to biomaterials and form a stable biofilm. Once a biofilm has been generated, treatment of infection becomes extremely complicated (7). As in compliance with the Gristina's concept of "the race for the surface" (8), one concern about HA is whether it promotes not only bone regeneration and cell growth, but also supports bacterial adherence and proliferation (3). Controversial opinions have been expressed by different authors in terms of the bacterial susceptibility of HA (3, 9-18). Verheyen et al. stated that *S. epidermidis* and *S. aureus* might cause substantial damage to hydroxyapatite coating through bacterial infection (13). Oga et al. demonstrated that *S. epidermidis* more extensively colonised on hydroxyapatite than titanium and stainless

steel (15). On the other hand, some authors have claimed that HA has antibacterial activity (16-18). Therefore, there is still little known about the bacterial adhesion to synthetic HA surfaces and its possible consequences.

Any enzyme which hydrolyses esters of phosphoric acid is called phosphatase. Phosphatases are classified according to their optimum pH activity as acid or alkaline phosphatase. Staphylococci produce both acid and alkaline phosphatases. An acid phosphatase, with an optimum pH of 5.2, is produced by most or all coagulase-negative strains. The enzyme may be secreted into the medium or may be loosely or tightly cell-bound. Phosphatase production may be assayed using different methods. It may be demonstrated by growing the test organisms on a solid medium containing the sodium salt of phenolphthalein diphosphate (19). Phosphatase may be assayed in liquid medium using the artificial substrate *para*-nitrophenyl phosphate; *para*-nitrophenol (yellow at alkaline pH) is released by acid or alkaline phosphatases and can be assayed spectrophotometrically (20).

There is substantial evidence indicating a pathological increase in bone resorption with an increase in stimulation of osteoclastic activity leading to the increase in the synthesis of acid phosphatase enzyme (21, 22). Thus acid phosphatase is accepted to be associated with the bone resorption process; however its role is not yet fully understood (22).

The main objective of this study was to investigate the effect of staphylococci with various phosphatase activities on the dissolution of synthetic HA biomaterial.

Method

Preparation of hydroxyapatite discs

Ceramic discs were prepared from pure hydroxyapatite powder (Captal R HA Sintering Grade Batch No: P 201) supplied by Plasma Biotall Ltd, UK (Figure1). Captal HA was selected for this study because this brand had been previously characterised and shown not to change the pH of water or cell culture medium (23). Ceramic discs were produced using a stainless steel die with an internal diameter of 13 mm. For each disc, 0.3 g HA powder was compressed by 2500 kg pressure exerted by a hydraulic clamp. The discs were sintered at 1200°C in a Carbolite CWF1300 furnace (Carbolite, UK). The fired discs were measured as 10.2 mm in diameter and 1.60 mm in thickness. One face of each disc was then polished using 1 µm diamond abrasive paper and DP Blue lubricant (Struers, Denmark) to obtain a smooth surface on which any defects subsequently caused by bacteria could be seen and to avoid bacteria lodging in surface irregularities. The polished discs were cleaned in a sonic bath with distilled water for 10 min to remove the debris on the surface and then sterilised in a Little Sister II autoclave (Surgical Equipment Supplies, UK).

Selection of bacteria

In this study strains of *S. epidermidis* tested were given isolate numbers 11, 33, 142, 159, 160, 257, 339, 355, 257. Strains of *Staphylococcus epidermidis* tested were clinical isolates from bone, skin or soft tissue infections obtained from Birmingham University, Queen Elizabeth Hospital NHS Trust Microbiology Department, UK or Nottingham Royal Infirmary Microbiology Department, UK. Tryptone Soya Agar (TSA) (Oxoid, UK) was used as a solid culture media and Tryptone Soya Broth (TSB) (Oxoid, UK) as liquid culture medium.

Determination of phosphatase activity of different strains of *S. epidermidis*

Staphylococci were screened for their ability to produce the phosphatase enzyme using *p*-nitrophenyl phosphate agar (*p*-NPP), as described by Geary and Stevens (19) Phosphatase activity was indicated by a bright yellow pigment around the colonies after 24 hours.

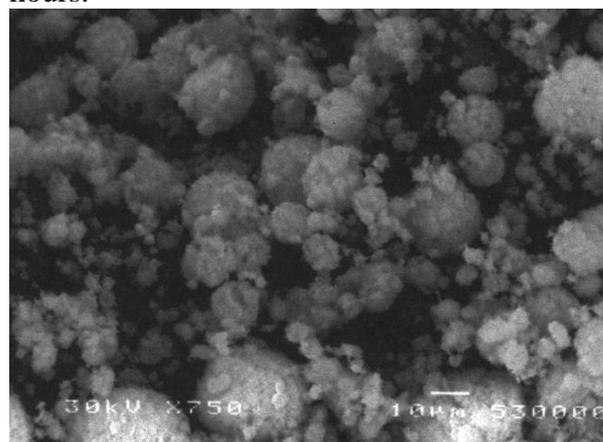


Figure 1. SEM view of Captal HA in powder form, particle size ranging between 5-30 µm (750x).

The phosphatase activity of selected staphylococcal isolates was quantified using the phosphatase test described by Pompei *et al.* (20) which is based on the liberation of *para*-nitrophenol from *para*-nitrophenyl phosphate at pH 7.0. The phosphatase specific activity was expressed as described below:

$$\text{Specific Activity} = \left(\frac{\text{Amount of } p\text{-NPP}}{\text{Reaction time (min)}} \right) \times 1/\text{Amount of Protein}$$
 The protein content of the bacteria was determined using the Coomassie protein assay (Pierce, UK).

Preparation of cultures containing HA discs

An overnight bacterial culture was diluted 1 in 10 with sterile TSB, to provide a suspension containing approximately 1×10^8 cells per ml. In aseptic conditions sterile HA discs were placed in sterile plastic bijou bottles with the polished surface uppermost. For experimental samples 1 ml of diluted bacterial suspension and for control samples 1 ml sterile TSB were pipetted onto each HA

disc. For each group three samples were examined. Bottles were incubated at 37°C for 24 h. HA discs were then removed for testing and the medium was frozen for further examinations. Each HA disc was carefully removed and washed with phosphate buffered saline (Sigma-Aldrich, USA) three times to remove any free bacteria and TSB. Bacteria adhering to HA discs were fixed 2% v/v aqueous paraformaldehyde solution in PBS for 30 min. Subsequently the fixative was removed and the discs were washed 3 times with PBS. The samples were then dehydrated through a series of ethanol solutions of increasing concentration up to 100% and then using hexamethyldisilazane (HMDS, TAAB, UK) the samples were dried for SEM examination.

Scanning electron microscopy (SEM) examination

Samples were gold sputter coated in a Denton Desktop II sputter coater (Microfield Scientific Ltd, UK) and then viewed using a Jeol 5300 LV scanning electron microscope (Jeol Ltd, UK) operating in high vacuum mode at 20 or 30 kV.

Measurement of calcium release in TSB medium

Calcium released from the HA discs into the culture medium was measured using Sigma Diagnostics Calcium kit (Sigma Diagnostics, USA). The intensity of the colour was measured using a Cecil Series 2 spectrophotometer (UK) at an absorbance of 575 nm and is directly proportional to the calcium concentration in the sample. For each sample three measurements were made. Defrosted samples were centrifuged for 3 min at 13000 r.p.m. using a Camlab 4223 centrifuge to pellet the bacteria and then filtered using Nalgene cellulose acetate membrane syringe filter (Nalge Nunc, USA) with the pore size of 0.45µm to remove remaining bacteria and/or calcium phosphate particles in the supernatant fluid to prevent these interfering with the calcium measurements.

Quantifying bacterial adhesion to calcium phosphate discs

The average number of bacteria on the HA discs were estimated from 3 images taken at random points on each sample at a magnification of 1500x. The digital images were saved using SemAfore software (Jeol Ltd, UK) and adherent bacteria counted in each image and the average number of bacteria per mm² and standard deviation for each disc were calculated.

Results

Results of the phosphatase tests

i. Plate method: Using *p*-NPP agar plates, the phosphatase activity of selected strains of staphylococci was detected by the presence of bright yellow pigmentation into the medium around the inoculation area (Figure 2). Results are shown in Table 1. The degree of yellow staining of colonies grown on agar media was scored as (-) for no stain and as (+) for staining. Seven of the 9 *S. epidermidis* strains were observed to produce phosphatase. No yellow zone was observed around *S. epidermidis* 142 and 355.

ii. Quantitative method in liquid medium:

The results of the phosphatase activity test by quantitative method indicated that among the strains tested, the highest amount of *p*-NP release was detected from strain 33, while the lowest was 159 (Figure 3). Surprisingly, one of the strains that was negative in the plate test showed a higher phosphatase activity in this test than some of the positive strains, whilst strain 159, which showed yellow pigmentation on plates showed very low activity in the biochemical assay.

Influence of staphylococcal bacteria on the dissolution of HA Discs

Four strains of *S. epidermidis*, two with high phosphatase activity (257, 33) one with medium activity (142) and one with very low

activity (159) in the liquid assay were selected to compare their effects on HA dissolution. HA discs were incubated with these strains or controls without bacteria and the calcium concentrations of the medium were measured. The results are shown in Figure 4.

Table 1. Identification of phosphatase activity of staphylococci by *p*-NPP plate test

Staphylococci Species-Strains	Phosphatase Activity
<i>S. epidermidis</i> 257	(+)
<i>S. epidermidis</i> 11	(+)
<i>S. epidermidis</i> 33	(+)
<i>S. epidermidis</i> 160	(+)
<i>S. epidermidis</i> 339	(+)
<i>S. epidermidis</i> 27	(+)
<i>S. epidermidis</i> 159	(+)
<i>S. epidermidis</i> 142	(-)
<i>S. epidermidis</i> 355	(-)

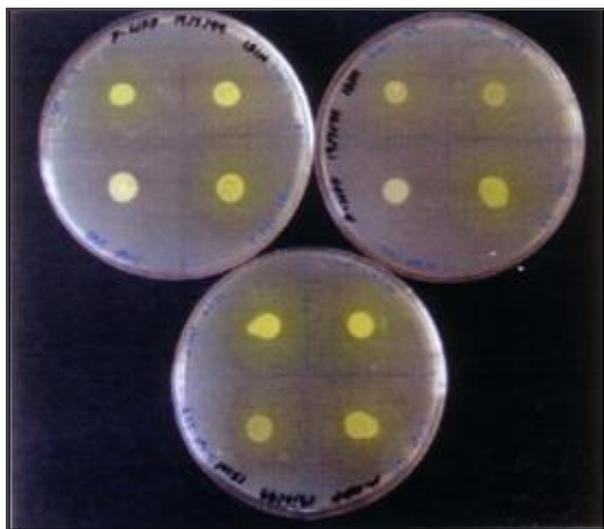


Figure 2. Result of the plate method revealed phosphatase positive strains with bright yellow pigmentation.

The result of this experiment demonstrated that in the presence of *S. epidermidis*, more calcium was detected in the medium when HA discs were present. However, in these experiments additional controls, with bacteria only,

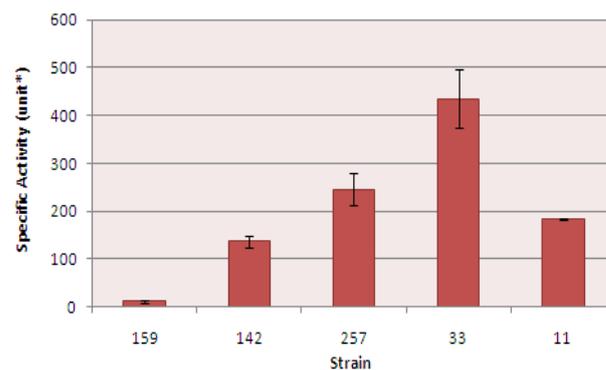


Figure 3. Phosphatase specific activities of five strains of *S. epidermidis*. Columns show phosphatase specific activities (*unit: nmol/min/mg protein x 10⁻⁷ ± standard deviation)

showed that the bacteria themselves elevated the calcium concentration in the medium. This contribution, together with that of the HA disc alone, could account for the higher calcium measurements in the samples with both HA and bacteria, indicating that no, or little, dissolution had occurred. There was no apparent correlation between the phosphatase activity of the strain and calcium dissolution. The HA discs surfaces were examined by SEM but no visual signs of dissolution were detected (Figure 5,6,7). Grain boundaries appeared to be intact and no damage to the integrity of the surface caused by bacteria was seen.

Quantifying bacterial adhesion to calcium phosphate discs

The numbers of bacteria adherent to the surface after 24h were calculated and shown in Figure 8. The results showed that more bacteria of strain 257 may adhere to the HA discs than the other strains. This was followed by strain 33 with almost half the number of adherent bacteria detected with strain 257. Amongst the 4 strains tested, phosphatase activity of *S. epidermidis* 257 was found to be moderate while for strain 33 it was the highest.

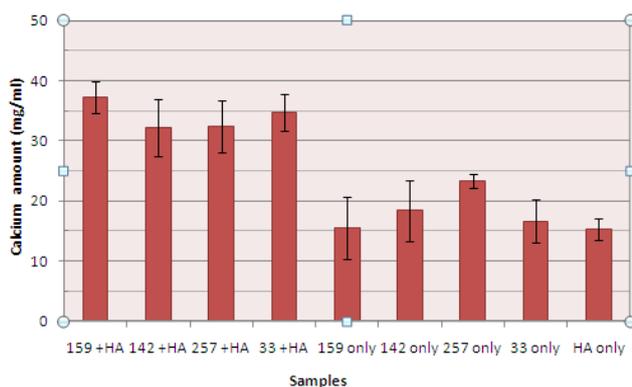


Figure 4. Calcium released from HA discs in the presence of *S. epidermidis* strains with different phosphatase activities.

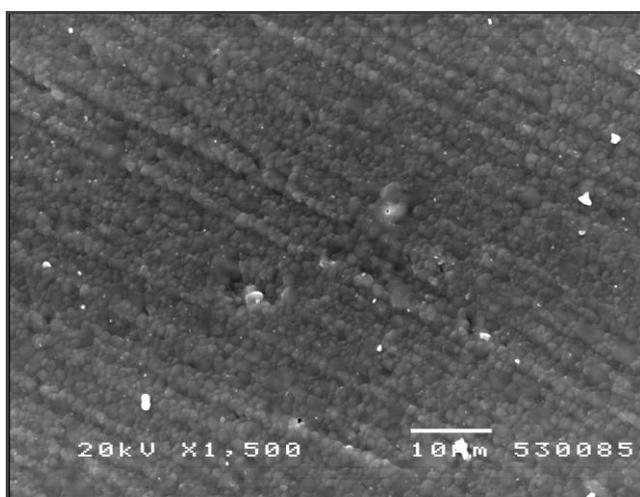


Figure 5. SEM view of the HA discs before incubation with *S. epidermidis* (1500x)

Discussion

Phosphatases are involved in bone mineralisation although their roles are complex and not entirely understood. It is thought that alkaline phosphatase can provide inorganic calcium and phosphate from organic sources. In addition, acid phosphatases are involved in bone destruction being one of the characteristic markers of osteoclasts. Phosphatases have also been shown to be active in bacterial mineralisation. Jeong *et al.* (24) showed that when uranium was present in the medium, uranium phosphate was produced by a *Citrobacter* species due to the action of phosphatase enzyme. Thackray *et al.* (25) has demonstrated that when calcium and

phosphate are present in the medium extracellular calcium phosphate is produced by *Serratia* bacteria by a similar mechanism.

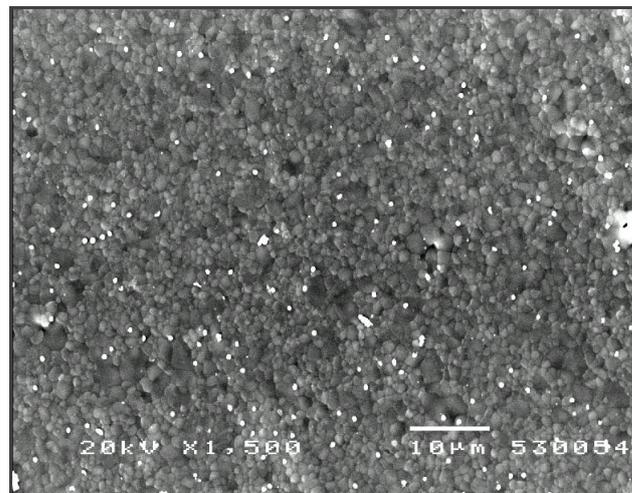


Figure 6. SEM view of HA disc after incubation with *S. epidermidis* 142 (1500x)

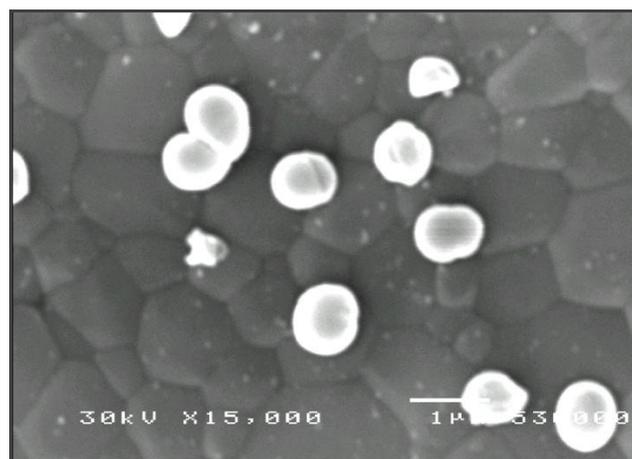


Figure 7. A close-up view of the HA disc with the adhered bacteria on the surface (15000x)

It is possible that a reverse reaction may also take place, causing liberation of Ca^{2+} and PO_4^{3-} ions from calcium phosphate. In the present study whether phosphatase positive or negative strains of *S. epidermidis* could differentially liberate Ca^{2+} and PO_4^{3-} ions from calcium phosphates, causing dissolution was investigated. Two methods were selected to detect the phosphatase activities of the bacteria in the present study: A qualitative *p*-NPP plate method, developed by Geary and Stevens (19), which was based on hydrolysis of di-sodium-*p*-nitrophenyl phosphate in *p*-

nitro phenyl phosphate agar by bacterial phosphatase. Phosphatase activity was recognised by a bright yellow pigment which diffused into the medium around the bacterial inoculation spot.

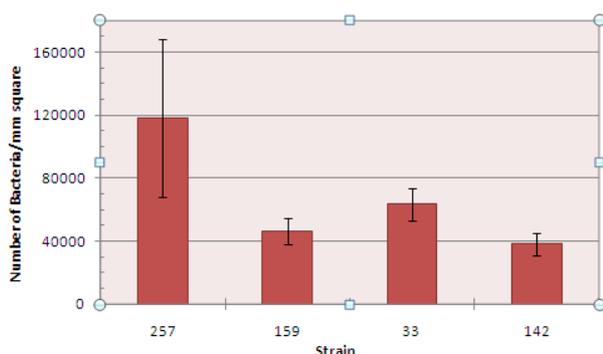


Figure 8. Adherence of phosphatase positive and negative strains of *S. epidermidis* to HA disc surface after 24h. Phosphatase activities of strains: 257 (moderate), 159 (low), 33 (high) and 142 (moderate).

Seven of the nine strains of the *S. epidermidis* were found to be phosphatase positive. Quantitative determination of phosphatase activity was carried out using a liquid method described by Pompei *et al.* (20). Phosphatase activity was estimated by liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate and enzymatic activity was expressed as the amount of *p*-NP produced per minute per microgram of protein. Five strains of *S. epidermidis*, 33, 257, 11, 142 and 159, were tested. The phosphatase activity of the strains was found to decline with the following order: 33>257>11>142>159. Comparison of these two methods showed that the plate method was relatively rapid leading to quick assessment of phosphatase activity of the bacteria tested. The difficulty with this technique was the similarity of the colours of the agar and the pigmentation area. They were both yellow in colour and this caused some difficulty in discerning the borders of the bright yellow pigmentation diffused into the agar medium. The liquid method gave more accurate results permitting comparison of the different strains of *S. epidermidis* however it was more time consuming and less economical. An interesting finding when the results of the two tests were compared was

that *S. epidermidis* strain 142 was found to be phosphatase negative and strain 159 found to be phosphatase positive by the plate method, while strain 142 was found to have greater enzymatic activity than strain 159 using the liquid method. The diversity in this result might be related to the way of liberating *p*-NP phosphate from the bacterial cells. In the plate method the enzyme is secreted naturally and diffuses into the medium whilst in the liquid method toluene is used to lyse the cells. Strain 142 could be less permeable to the enzyme. The reaction could also be time or pH dependant. SEM examinations of the HA discs when incubated with 4 different strains of *S. epidermidis* with different phosphatase activity revealed that all strains of staphylococci adhered and grew on the HA disc surfaces; however, no considerable relation was observed between the phosphatase activity of *S. epidermidis* strains and the number of bacteria adhered on the HA surfaces. When the calcium concentrations of the medium in which the HA discs were incubated were measured, it was observed that in the presence of *S. epidermidis*, more calcium was detected in the medium. However, in these experiments additional controls, with bacteria only, showed that the bacteria themselves elevated the calcium concentration in the medium. This contribution, together with that of the HA disc alone, could account for the higher calcium measurements in the samples with both HA and bacteria, indicating that no, or little, dissolution had occurred. Furthermore there appeared to be no appreciable difference between the amounts of calcium release caused by the high and low phosphatase strains. These results suggest that phosphatase did not have any influence on the dissolution of the Captal HA fired discs; however, the sample sizes were too small for further rigorous statistical evaluation. In a further investigation performed by the present authors (26) the effects of *S. epidermidis* strains on the dissolution of various powder form HA and TCP materials were examined. Their results indicated that *S. epidermidis* caused varying degree of dissolution of the

calcium phosphate materials, but this was more dependent on the material. No appreciable difference on Ca release was observed between the strains with high or low phosphatase activities.

In the present study only fired disc form of HA biomaterial was investigated. This material is used commercially for producing hydroxyapatite coatings. For future work, it would be beneficial to do further experiments on plasma-sprayed coating samples of the same material to determine whether plasma spraying process would alter their dissolution behaviour under bacterial exposure.

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