

Deposition of substituted apatites with anticolonizing properties onto titanium surfaces using a novel blasting process

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Abstract: A series of doped apatites have been deposited onto titanium (V) substrates using a novel ambient temperature blasting process. The potential of these deposited doped apatites as non-colonizing osteoconductive coatings has been evaluated *in vitro*. XPS, EDX, and gravimetric analysis demonstrated that a high degree of coating incorporation was observed for each material. The modified surfaces were found to produce osteoblast proliferation comparable to, or better than, a hydroxyapatite finish. Promising levels of initial microbial inhibition were observed from the Sr- and Ag-doped surfaces, with the strontium showing prolonged ability to reduce bacteria numbers over a 30-day period. Ion elution profiles

have been characterized and linked to the microbial response and based on the results obtained, mechanisms of kill have been suggested. In this study, the direct contact of coated substrate surfaces with microbes was observed to be a significant contributing factor to the antimicrobial performance and the anticolonizing activity. The silver substituted apatite was observed to out-perform both the SrA and ZnA in terms of biofilm inhibition. © 2010 Wiley Periodicals, Inc. *J Biomed Mater Res Part B: Appl Biomater* 95B: 141–149, 2010.

Key Words: hydroxyapatite, substituted apatites, grit-blast, antimicrobial, anticolonizing

INTRODUCTION

Biomaterial selection for coating hard tissue implants poses a number of challenges. The early osteointegration of an implant material into the host bone is a key parameter in its ultimate long-term success. Attachment can be achieved by positively enhancing the interactions that occur at the implant-bone interface through the deposition of bioactive coatings onto the implant surface. Hydroxyapatite (HA) has been well documented as a proven bioceramic for coating hard tissue implants due to its biocompatible and osteoconductive properties.^{1,2}

Biomaterial-associated infections pose great surgical problems, as revision surgery is not always easy and patients suffer severe discomfort.³ Such infections are common in orthopedic surgery where infection rates range from 0.5–5%, irrespective of the utilization of strict hygienic protocols (aseptic and sterilization procedures) and also the systemic administration of antibiotic prophylactics.^{4–6} These infections are the result of bacterial adhesion and subsequent colonization leading to the inevitable biofilm formation at the implantation site which comprises the stability of the implant.^{7,8} The introduction of bacteria such as *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*) onto implant surfaces before or during surgery have been highlighted as main causes of such infections.⁹ Therefore, there is a need for a bioceramic coating

that will not only have osteointegrative properties but can also effectively resist bacterial colonization.

With regards to this aim doped apatites which can deliver added functionality to the HA bioactive surfaces, through their antimicrobial impact are of major interest. Investigations into the use of ions such as silver (Ag) and zinc (Zn) incorporated into HA have shown positive antimicrobial effects.^{10,11} Strontium ions (Sr), typically present in trace quantities in the mineral phase of bone, have been reported to have an antimicrobial effect *in vitro*.^{12,13} The presence of Zn and Sr has also been shown to increase the activity of osteoblast cells *in vitro*.^{14–16} Several proposed mechanisms have been reported for the antimicrobial mode of action of these metal ions, but results have been mixed and the mechanisms by which these surfaces produce an antibacterial effect has not been fully elucidated. Reported mechanisms include disruption of electron transport chain, inhibition of DNA replication, DNA cleavage, membranous disruption, reactive oxygen formation, and enzyme inhibition.^{11,17–24}

A novel ambient temperature deposition technology called CoBlast™ which is based on a grit blasting process has been developed for the modification of metallic implant surfaces and is described elsewhere.²⁵ In previous studies, the CoBlast technique has been shown to effectively deposit a range of bioceramic materials on titanium with a resultant

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surface modification depth of less than 10 μm .^{25,26} The CoBlast modifications are the result of the mechanical interlocking and tribo-chemical bond formation between the bioceramic material and the underlying metal substrate. The CoBlast HA surface has demonstrated significantly enhanced *in vitro* osteoblastic adhesion and proliferation and has also shown to promote the formation of good quality lamellar bone in an *in vivo* animal study.²⁵

In this study, CoBlast technology was used to deposit various substituted apatites as well as HA onto titanium substrates. A range of *in vitro* tests were undertaken to determine if the dopant materials could produce a modified surface which was simultaneously osteoconductive and anti-colonizing and the results were interpreted in view of detailed surface characterization studies.

MATERIALS AND METHODS

Materials

Titanium (Grade 5, Ti-6Al-4V) coupons was obtained from Lisnabrin Engineering Irl. HPLC grade HCl, deionized water, phosphate buffer solution (PBS), Trypsin EDTA, MTT assay kit, ACS reagent grade dimethyl sulphoxide, Luria Bertoni agar (LB) and acridine orange dye were all purchased from Sigma-Aldrich, UK. Zinc substituted apatite silver substituted apatite and strontium substituted apatite were sourced from Himed Inc. (NY, USA). The substituted apatites were described as being low crystallinity in the unsintered condition. Crystalline hydroxyapatite powder was supplied by S.A.I., France and the abrasive alumina powder was purchased from Comco Inc, USA. MG-63 osteoblast cells were obtained from American Type Culture Collection, Rockville, MD, USA. Minimum Essential Medium (MEM), foetal calf serum, penicillin G sodium, streptomycin, amphotericin B were purchased from PAA Laboratories GmbH, Austria. *S. aureus* (ATCC 1448) was obtained from the laboratory of Prof. Alan Dobson at the Microbiology Department, University College Cork.

Sample preparation

Titanium (V) coupons, 15 mm \times 15 mm \times 1 mm, were used in this study as the substrate material. Prior to surface modification, the coupons were ultrasonically cleaned in 1M HCL and then in isopropanol to remove any contaminants. Zinc substituted apatite (ZnA), silver substituted apatite (AgA) and strontium substituted apatite (SrA) were used as-received in powder form for deposition onto the substrates. HA was used to produce a positive control surface. The HA and substituted apatites were deposited onto the titanium coupons using the CoBlast technique, as described in detail elsewhere.^{25,26} All bioceramics were deposited at a pressure of 90 psi, speed of 13 mm/s and a working distance of \sim 20 mm. After the surface treatment step, each sample was air-cleaned using compressed air for 30 s to remove any loose powder from the surface.

Surface characterization

A number of key analytical techniques were used to analyze the CoBlast coatings. X-ray photoelectron spectroscopy

(XPS) was performed using a Kratos Analytical Axis Ultra photoelectron spectrometer with a monochromated aluminium (Al K α) X-ray source. SEM-EDX analysis was carried out using a Jeol JSM 5510 scanning electron microscope (SEM) in conjunction with an INCA X-sight Energy Dispersive X-ray (EDX) spectroscopy detector (Oxford Instruments, Buckinghamshire, UK). The XPS and EDX were used to determine the elemental composition of the coatings. Gravimetric analysis was used to determine the coating mass using an Ohaus DV314C analytical balance. The surface roughness (Ra) was determined using a Talysurf 10 surface profilometer (Taylor Hobson, UK). The concentration of the various ions present in the coatings was determined by dissolving the coating in 1M HCl and analysing the solution using a Thermo IRIS Advantage Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). *In vitro* release studies of the ions were performed. Coated coupons were placed in 20 mL PBS and incubated under physiological conditions (pH7.4, 37°C and agitated at 150 rpm, $n = 3$). The buffer solution was replaced for days 1, 8 and 16 and experiment completed at day 30. The ion release at each time point was determined using ICP-OES.

Cell proliferation and cytotoxicity analysis

Prior to cell culture analysis, each sample set was steam autoclaved at 121°C for 20 min. MG-63 cells were used to assess cell proliferation. Cells were cultured in the MEM media supplemented with 10% fetal calf serum and antibiotic/antimycotic (penicillin G sodium 100 U/mL, streptomycin 100 $\mu\text{g}/\text{mL}$, amphotericin B 0.25 $\mu\text{g}/\text{mL}$) in 75 cm³ tissue culture flasks. Cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C and were subcultured when they reached confluence using 0.25%-Trypsin EDTA to provide adequate numbers of cells for the various *in vitro* culture studies undertaken. MG-63 cells were cultured in direct contact with the sample surfaces for 24 and 72 h. A commercial MTT assay kit was used, employing a modified Mosmann method.²⁷ The MTT assay reagent was prepared as a 5 mg/mL stock solution in PBS, sterilized by Millipore filtration, and stored in the dark. An aliquot of the MTT stock solution (10% of total volume) was added to each well of a six-well plate containing the samples ($n = 4$ for each sample type). After 3-h incubation at 37°C in 5% CO₂, 200 μL of dimethyl sulphoxide was added to dissolve the formazan crystals. The solution was agitated homogeneously for 15 min on a shaker to ensure adequate dissolution. The optical density of the formazan solutions was read by spectrophotometry using an ELISA plate reader (Tecan Sunrise, Tecan Austria) at 570 nm with the background absorbance value measured at 650 nm. The absorbance values recorded were determined to be proportional to the number of cells attached to the membrane surface in each case. All data reported are expressed as mean \pm standard deviation. A statistical one-way analysis of variance (ANOVA) was performed to determine statistically significant differences between the sample types with a value of $p < 0.05$ considered to be statistically significant. The Dunnett's post-

test was applied to compare values between all the sample columns to the control HA sample.

Antimicrobial studies

Antimicrobial testing consisted of two test methods using *S. aureus* as the challenge organism. A modified ASTM standard E2149-01 protocol²⁸ was used to determine the antibacterial effect of the coatings which focuses on the activity of antimicrobial agents immobilized on the surfaces.²⁹ A second test also based on the E2149-01 standard was employed to assess the potential antimicrobial activity of released ions from the sample surfaces.

Growth of the *S. aureus* challenge organism was achieved by the aseptic inoculation of a discrete colony from the surface of LB agar, into 30 mL of fresh LB liquid media and allowed to grow overnight at 37°C in a shaking incubator at 180 rpm, to provide adequate oxygenation for abundant cell growth. Overnight cultures typically achieved a cell density of 10^8 – 10^9 cfu/mL, as determined by standard spread plate counting of serial culture dilutions on Luria Bertani agar. The cultures were subsequently diluted with an appropriate volume of sterile PBS buffer to provide a standardized microbial challenge load of $\sim 1 \times 10^6$ cfu/mL.

Antimicrobial activity of the coatings

The activity of the coatings was performed on coupons following deposition (air-cleaned) and also on coupons following 30-day incubation in PBS buffer (pH 7.4, 37°C, and 150 rpm). Coupons for assessment were sterilized by immersion in 70% ethanol and allowed to dry at a 45° angle in sterile 12-well titre plates in a LAF hood. Once dry, 2 mL aliquots of the microbial suspension containing 1×10^6 cfu/mL were added to each coupon containing well and the plates incubated at 37°C in a shaking incubator at 200 rpm for 1 h.

Antimicrobial activity of the released ion

Coupons for assessment via the leach test were sterilized as previously described and 2 mL of freshly prepared, sterile PBS (pH 7.4) added to each coupon containing well to provide a medium for ion release. Plates were subsequently placed in a shaking incubator (150 rpm) at 37°C for the required length of the trial period, (1, 8, and 16 days, respectively). The test volume of 2 mL was maintained during extended trial periods by making appropriate additions of PBS during the incubation period. At the end of the test period PBS containing leached ions was aseptically removed from each well. In a clean, sterile 12-well plate, 0.5 mL of the microbial challenge (2×10^6 cfu/mL) was mixed with 0.5 mL of respective leachate and the plates incubated at 37°C, shaking at 150 rpm for a period of 1 h.

Quantification of the antimicrobial activity

Totally, 0.1 mL of each test sample (from leach and non leach test) was aseptically transferred to a 1.5 mL microcentrifuge tube containing 0.9 mL of sterile PBS to achieve a 10^{-1} dilution. For each sample coating, tests were conducted in triplicate. Each 1 mL solution was vortexed for

5 s to achieve thorough mixing and 0.1 mL was again transferred to a fresh 0.9 mL of PBS and thoroughly mixed to provide a 10^{-2} dilution. The serial dilution procedure above was repeated to produce 10^{-3} and 10^{-4} dilutions. To enumerate the microbial content of a sample, sterile, solid LB agar plates were divided into four quadrants that is 10^{-1} to 10^{-4} . Subsequently three discrete 10 μ L drops from a single dilution sample were added within the respective quadrant. Once dot plating of dilutions 10^{-1} to 10^{-4} had been conducted the drops were allowed to diffuse into the media by air drying in the flow hood. Dried plates were subsequently incubated overnight (in an inverted position) in a 37°C incubator for a maximum period of 14 h. Colonies present on the plates were enumerated and used to calculate the potential antimicrobial impact of the apatite coating and leached ion by comparison with control samples (HA coating). The results are presented as % kill.

Anticolonization studies

To investigate the potential anticolonization efficacy of the respective sample coatings, a quantitative assessment of biofilm formation was carried out at various points over a 14-day period. The approach used was based upon the previously reported method of Chae and Schraft.³⁰

S. aureus cultivation and coupon sterilization was carried out as previously described. To facilitate bacterial attachment and biofilm initiation, 100 μ L of undiluted *S. aureus* culture was aseptically spotted onto the surface of each sample coupon in discrete plate wells. Plates were then incubated at 37°C for 3 h without shaking. Post incubation, coupons were gently rinsed with 10 mL sterile PBS in the LAF hood to remove planktonic cells, before being placed in plate wells containing 2 mL of sterile LB media, with the coating surface facing upward. After 24 h, and at 48-hour intervals thereafter, coupons were aseptically removed from plates, rinsed with 10 mL of sterile PBS and placed in fresh plate wells again with 2 mL LB media. At time points 1, 7, and 14 days, coupons were aseptically removed from wells and rinsed with 10 mL of sterile PBS to wash off planktonic cells. Attached organisms were then collected by swabbing the coupon surface 100 times with a sterile cotton bud, which was subsequently placed in 2 mL of sterile PBS and vortexed vigorously for 10 s to remove collected cells. Serial dilutions (10^{-1} to 10^{-4}) of the microbial colonies of the test samples were prepared and dot plating procedure performed, as previously described. Enumeration of resultant colony growth associated with the respective coating surfaces, relative to the HA experimental control, were used to determine the % biofilm inhibition of the apatite coatings.

Fluorescent images of the biofilm on the coupons were taken using an acridine orange stain. Coupons following incubation were subjected to drying and heat fixing of biofilm in an oven at 105°C for 3 min. Two milliliter of the 20 μ g/mL acridine orange stain was added to plate wells containing the heat fixed coupons, and incubated at room temperature in the dark for 5 min to allow nucleic acid and stain interactions sufficient time to occur. Coupons were

TABLE I. Elemental Composition of the Coatings Via XPS (% atm)

Coating	C	Ca	O	P	Ti	Ion
HA	17.1	15.5	53.9	10.0	0.6	N/A
AgA	11.3	15.6	57.7	13.9	0	Ag – 0.9
SrA	8.4	14.6	53.5	18.2	0	Sr – 1.9
ZnA	10.0	15.2	56.3	13.5	0	Zn – 3.6

subsequently removed from the solution and rinsed with sterile PBS, air dried, and stored in the dark at room.

RESULTS

Surface characterization

XPS and EDX analysis of all the treated samples revealed surfaces rich in Ca, P and O, as shown in Tables I and II. The modified apatites presented with a low dopant ion levels of less than 5%, with the silver producing the lowest level of dopant ion incorporation and Zn producing the highest levels. Adventitious carbon was also present on all coatings analyzed using XPS, as is common on apatite coatings. The XPS analysis was largely devoid of titanium signals, while low Ti levels were detected in the EDX analysis. This suggests that the low levels of Ti detected by EDX are due to the high sampling depth (10 μm) of this method and not due to incomplete coverage of the surface by the apatite. This would suggest that the coating applied is less than 10 μm in thickness as reported elsewhere.²⁶ On the basis of the low levels of Ti in the EDX results, the Sr doped materials appears to have a significantly higher depth of surface treatment than the other materials.

This is supported by gravimetric analysis which revealed a coating mass of between 0.32 and 1.6 mg/cm² per coupon, with the Sr doped apatite coating exhibiting the greatest coating mass (Figure 1). The concentration of the dopant ions present in each coating was determined by dissolving the complete coating in acid and then determining the ionic concentrations using ICP analysis (Figure 2). SrA and ZnA were determined to contain the greatest amount of the respective dopant present in each coating. This appears to correlate with the surface roughness values detected, with *R_a* values of 1.7, 2.2, 2.9, and 3.6 μm for HA, AgA, SrA, and ZnA, respectively.

ICP was used to determine the concentration of ions released into PBS buffer from each coating. As seen from Figure 3, low levels of ions were released over the 30-day period with ZnA producing the lowest level of dopant ion release. The % ion released was determined using the

TABLE II. Elemental Composition of Coated Samples Via SEM-EDX (% atm)

Coating	Ca	O	P	Ti	Ion
HA	9.2	65.8	6.7	14.7	N/A
AgA	10.6	69	8.8	10.1	Ag – 0.6
SrA	15.6	68.4	13.0	1.6	Sr – 1.1
ZnA	12.7	68	11.0	4.5	Zn – 1.9

TABLE III. Surface Roughness Analysis (*R_a*, *n* = 3)

Coating	Surface Roughness (μm)
Uncoated Ti	0.4 ± 0.1
HA	1.7 ± 0.3
AgA	2.2 ± 0.3
SrA	2.9 ± 0.3
ZnA	3.6 ± 0.3

initial concentration of ions in the coating before elution (Figure 2) and the amount released in the elution samples over 30 days (Figure 3). It was found that over 90% of the Sr and Zn still remained in the coatings at 30 days (Figure 4), whereas 90% of the Ag was released. These findings found to link directly to the antimicrobial properties of the treated surfaces.

Cell proliferation

The extent of osteoblast cell proliferation on the doped apatite substrates is presented in Figure 5. After 24 h there was a significant increase in proliferative rates on the SrA surface (*p* < 0.01) compared with the control HA surface. At 72 h there was also a significant increase in cell proliferation on both the SrA and ZnA surfaces (*p* < 0.01) in comparison with the control HA surface, indicating that the surfaces are at least as osteoconductive as the well characterized HA surface. No evidence of cytotoxicity was observed using MG63 cells on any of the samples evaluated.

Antimicrobial studies

The antimicrobial properties of the samples were determined for freshly prepared samples and for samples that were allowed to elute ions over 30 days. As can be seen from Figure 6, both AgA and SrA produce promising antimicrobial coatings with 57% and 49% kill respectively when freshly prepared. On the contrary, ZnA demonstrated low antimicrobial performance (26% kill) against the Gram positive bacterium. After 30 days of incubation the antimicrobial surface activity of AgA had decreased to 9% bacteria cell reduction while the SrA activity remained unchanged (Figure 6).

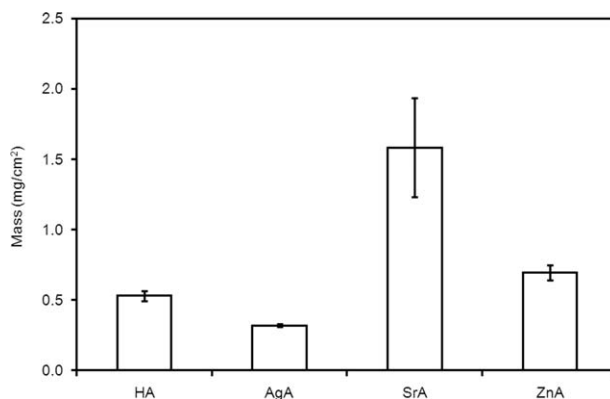


FIGURE 1. Coating mass of the various modifications (gravimetric analysis, *n* = 3).

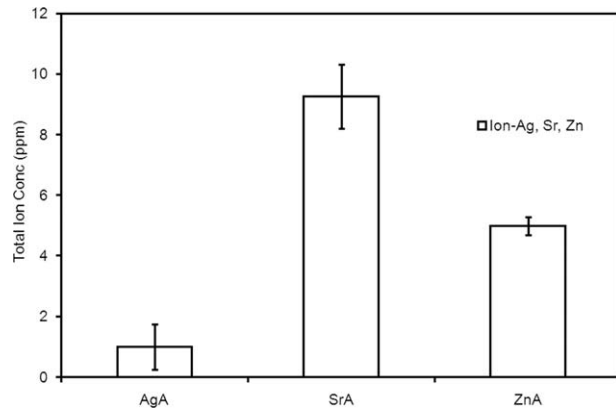


FIGURE 2. Concentration of dopant ion present in the coatings (ICP-OES analysis, $n = 3$).

As well as the antimicrobial properties of the coupons, the antimicrobial impact of the ions eluted from the coatings was also investigated by collecting the leachate and conducting a separate bacterial challenge and the results are displayed in Figure 7. In general, low antibacterial activity was observed for the leachate when compared with results obtained from the direct surface test. It was found that $<26\%$ kill was obtained from the eluted ions irrespective of the surface under investigation.

Anticolonizing performance

The ability of the surfaces to inhibit biofilm growth was also assessed over a 14-day period and the results are presented in Figure 8. All coatings produced a similar result at day 1 (32–42% biofilm inhibition). However, the Ag apatite coating out-performed the other two surfaces at days 7 and 14. Fluorescent microscopy of the biofilm formation of the HA-coated control and the AgA sample at day 7 can be seen in Figure 9. These images highlight the orange stained particles which represent the metabolically active bacterial cells on the surface. As can be seen, the AgA sample revealed reduced microbial colonization compared with the HA coated control, as evidenced through dramatically

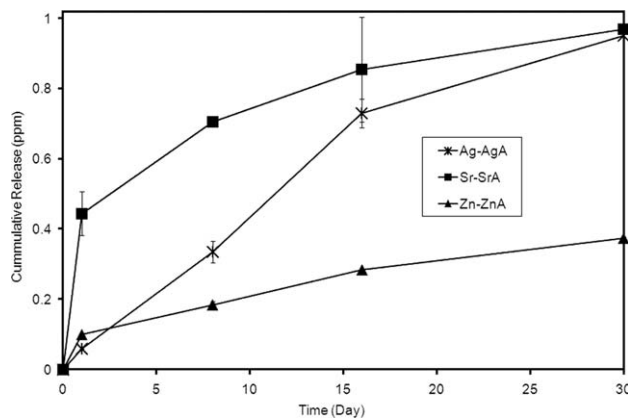


FIGURE 3. Cumulative ion release from the coatings over 30 days determined using ICP-OES (37°C , 150 rpm, $n = 3$).

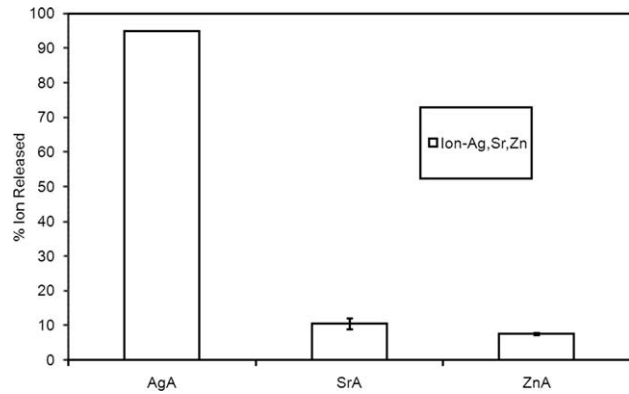


FIGURE 4. % Ion released from the coatings over 30 days (37°C , 150 rpm, $n = 3$).

reduced levels of viable bacterial cells on the Ag-doped surface.

DISCUSSION

Irrespective of which coatings are applied on hard tissue implants to enhance the osteointegration process, the host tissue cells (osteoblasts) and bacterial micro-organisms compete for the implants surface in a process coined as “race to the surface”.³¹ Bacteria capable of attaching to the implant surface, synthesize a matrix consisting largely of extracellular polysaccharides that encompasses the cells to form a biofilm which enables the bacteria to escape the effects of antibiotic therapy and host clearance. The pathogen usually grows in coherent micro-colonies in the adherent biofilm, which is often so extensive that the underlying bone at the implant surface is infected and the new bone growth is compromised. A colonized implant may rapidly result in chronic infection and the only successful treatment is removal of the infected implant.⁷ Inhibiting bacterial adhesion is regarded as the critical step in preventing implant associated infections thereby, promoting tissue interactions with the implant.³² This study strongly supports the potential future use of substituted apatites as

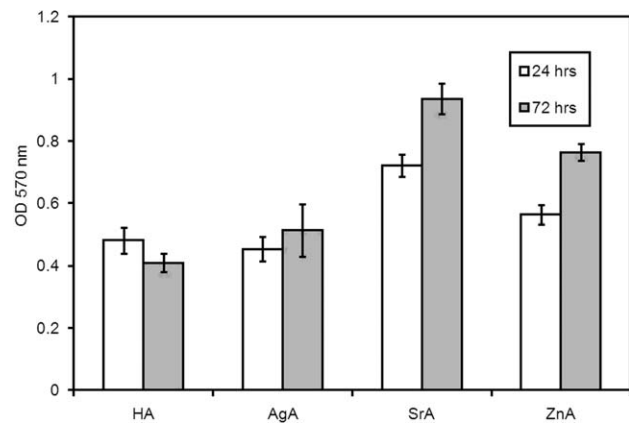


FIGURE 5. Optical density measurements for MG-63 cell proliferation after 24 and 72 h incubation with samples ($n = 4$).

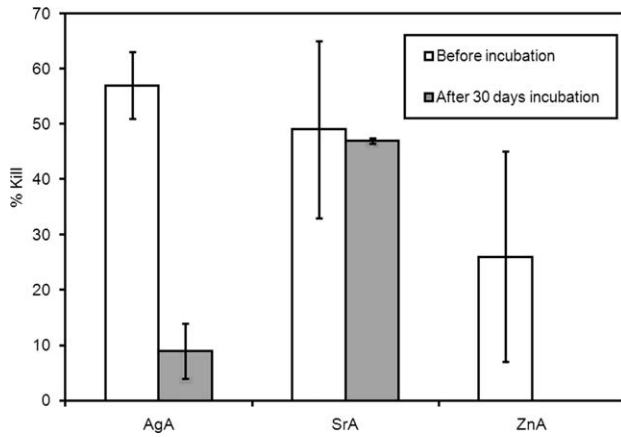


FIGURE 6. Antimicrobial activity as % kill of the coatings using *S. aureus* as the bacterial challenge organism before and after elution at 30 days ($n = 3$).

orthopedic and dental coatings which could combine excellent osteo-conductive properties with the key function of infection prevention.

The CoBlast technique is based on the convergent flow of an abrasive and a dopant stream onto the implant surface which can effectively impregnate the metal with the dopant material.²⁵ The surface chemistry results in their totality are all in good agreement that CoBlast technology is effective in depositing bioceramics onto metal substrates and this is consistent with previous studies.^{25,26} Modifications of less than 10 μm resulted in this study. The combination of ICP measurements, gravimetric analysis and low Ti readings in EDX suggests that the highest coating deposition rates were obtained for the Sr and Zn doped materials, with lower levels of deposition being recorded for the HA and Ag doped ceramics. Surface roughness is also a key parameter which governs cell attachment which leads to the subsequent cell proliferation stage. It has been reported that CoBlast HA significantly increased the surface roughness of blank Ti which also demonstrated increased osteoblastic activity compared to a uncoated Ti surface.²⁵ In this study, it was

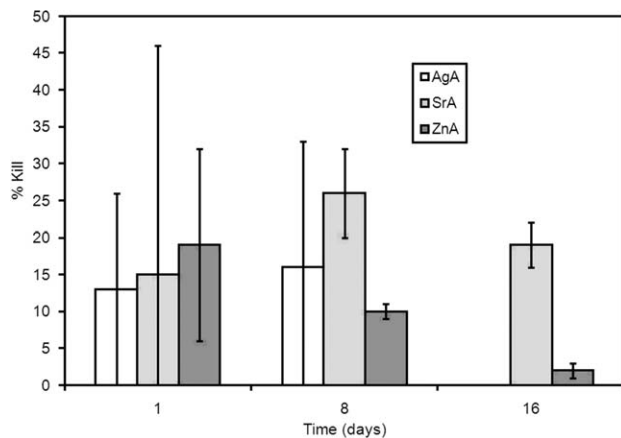


FIGURE 7. Antimicrobial activity as % kill of the released ion from the various coatings using *S. aureus* as the bacterium challenge ($n = 3$).

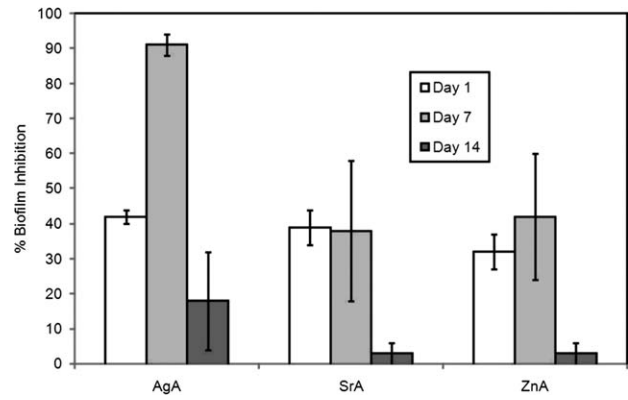


FIGURE 8. Anti-colonising activity as % biofilm inhibition of the coatings using *S. aureus* as the bacterium challenge ($n = 3$).

found that the deposition of the substituted apatites significantly increased the surface roughness of the surface compared to the HA control with ZnA and SrA creating the greatest effect. This would suggest that the thicker and better coating coverage of both ZnA and SrA (EDX, XPS, and

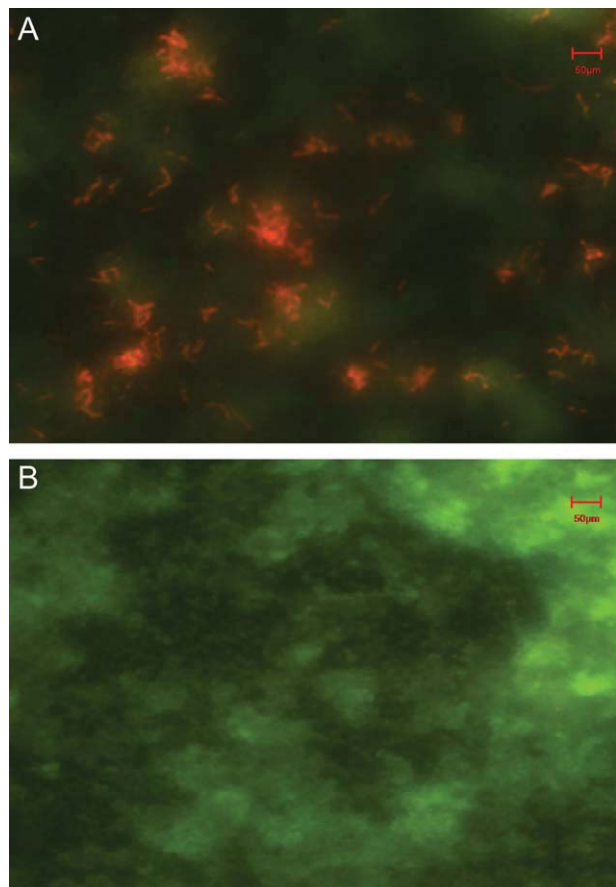


FIGURE 9. Fluorescent microscopy of biofilm formation at day 7. $\times 400$ magnification. Orange fluorescence indicates cell attachments. A: HA coated control coupon. B: AgA-coated titanium coupon. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

gravimetric results) lead to increased surface roughness as more of the dopant was deposited.

The surfaces examined in this study were all shown to be biocompatible with no evidence of cytotoxicity relative to MG-63 osteoblast cells. The proliferation results (Figure 5) appear to agree with the findings of Capuccini et al. who found that in comparison to a HA control surface there was an increase in osteoblast activity on strontium-substituted hydroxyapatite coatings on titanium substrate.¹⁴ It has been suggested that Sr may affect cell replication by two possible mechanisms; through the triggering of mitogenic signaling pathways or the release of autocrine growth factors.¹⁵ Ito et al. have shown that the incorporation of zinc into implant materials may increase bone formation.¹⁶ The proliferation results presented herein suggest that the surfaces are osteoconductive with no evidence of toxicity to osteoblast cells. Low levels of Ag ions are known to be clinically safe, with systematic accumulation of 4–6 g required to produce cytotoxic effects in humans.^{33,34} Exposure thresholds of 8.75 mmol/kg/day of strontium produces toxic effects whereas no such data exists for Zn.³⁵ The ion elution data produced herein reveal low levels of ion release, <1 ppm, over the duration of the study, which would correlate with the lack of toxicity towards mammalian osteoblast cells observed *in vitro*.

Despite the absence of toxicity observed during the osteoblast proliferation study, the surfaces were found to have a significant toxic effect on bacterial cells which were in contact with the surface. While the ions eluted from the surface were found to have a low level of kill (Figure 3), the direct action of the modified surfaces on the bacteria produced measurable decreases in bacteria numbers for both the silver and strontium doped bioceramics (Figure 6). Zn was found to have minimal antimicrobial properties initially and after 30 days in solution had no measureable antibiotic effect against *S. aureus*.

In the case of the silver coatings, the antibacterial effect was found to diminish with time and the coatings had minimal antibacterial properties at day 30. The ion elution studies indicate that the low level of Ag ions (4%) remaining in the surface after 30 days elution is responsible for the reduced kill at this time point. Conversely, the high levels of ions (90%) still present in the corresponding Sr-doped surface after 30 days would explain the retention of the antimicrobial properties of that surface over the 30 days of the trial.

It is interesting to note that the leachates were not found to perform as well as when the modified surfaces were in contact with the microbes. In part, this could be attributed to the fact that the concentration of the ions released within the test time periods examined (1, 8, 16 days) was low relative to the MIC of the challenge organism, *S. aureus*. The MIC for Ag and Zn has been reported to be 0.5 mg–10 mg/L and 200–500 ppb, respectively; however, no such data exists for Sr.^{36,37} Indeed, Jung et al. demonstrated that a Ag ion concentration of 0.2 ppm in solution is necessary to effect log reductions in a *S. aureus* test culture.³⁸ However, the ion release rates indicated in Figure 6

of this study, reveal that such a concentration would not accumulate in the coupon leachate until ~15 days of incubation.

On the basis of comparisons of the antimicrobial activity of the coating and the leachate, the data presented herein suggests that the direct contact of microbes with the coating may be a significant contributing factor for the antibacterial activity of the samples, as the antimicrobial effect was significantly amplified by the presence of the substrate surfaces in the nonleaching test. It has been reported that a dual antimicrobial mechanism exists for substituted apatites, where the antimicrobial action was not only attributed to the low release of ions but also from the generation of the free radicals from the apatite surface.^{39,40} However, the mechanisms of kill may not be the same for each of the materials evaluated herein and possible antimicrobial mechanisms are discussed below for each of the surfaces.

From the results obtained, the release of Ag⁺ ions from the AgA coating would appear to contribute to the antimicrobial response, which is in agreement with the literature.¹⁰ However, the poor performance of the leachate in comparison to the potency of the direct contact with the surface suggests other mechanisms of antimicrobial action may also be responsible for the activity of silver containing surfaces. The authors are aware that high local concentrations of Ag⁺ ions at the surface of the coupon may be responsible for the higher potency observed during the non-leach studies compared with the leachate studies.

The mechanistic action of Sr²⁺ is less well understood than that of Ag. Previous studies have shown antibacterial effects of Sr ions released from glass cements and also Sr containing hydroxyapatites. Little is currently known with respect to microbial resistance to Sr; as only a limited number of published studies on the *in vitro* or *in vivo* antibacterial applications of the ion are available.^{12,13} However, our results suggest a significant potential for SrA coatings to be further investigated/developed in antimicrobial applications given the sustained antibacterial impact observed in this study, when compared with AgA coatings. Moreover, the beneficial effect of Sr ions on osteoblast cells creates the possibility to produce a surface which inhibits microbial colonization while simultaneously promoting osteo-integration, thereby providing an optimal surface for orthopaedic and dental implant fixation. Poor antibacterial effects of Zn compounds against *S. aureus* have been previously reported¹⁰ and in the current study, ZnA coatings were not found to have significant antimicrobial activity.

The % biofilm inhibition attributed to the various coatings assessed over a 14-day period illustrated the anticolonization potential of the surfaces. The anticolonizing results revealed that the AgA coating had the greatest impact in preventing microbial attachment compared to the other coatings. Both the SrA and ZnA coatings produced similar anticolonizing performance although the impact was significantly less effective than AgA after day 1. Subsequent qualitative analyses by fluorescence microscopy indicated that the AgA coating effectively prevented *S. aureus* attachment, as opposed to inhibiting the proliferation of attached

cells, Figure 9. These observations correlate well with the earlier nonleach test results, suggesting the importance of microbe-coating surface interactions in the observed anti-bacterial impact.

It is generally accepted that the surface topography of the implant is one of the most important parameters that influence cellular reactions. Roughened implants demonstrate significant osteo-conductive properties due to enhanced osteoblast adhesion.⁴¹ However, increased surface roughness significantly influences the susceptibility of implants to bacterial adherence.⁴² However, in this study, the surface composition played more of an important role in cellular adhesion and the subsequent proliferation compared to the surface topography which was clearly evident in the MTT assay results. In addition, the less rougher surface of the control, the HA sample, was observed to be more susceptible to colonization in comparison to the rougher substituted apatite samples as seen in the % kill and % biofilm data. This further supports the importance of direct surface-bacteria interaction which appears to control the colonization process through effects exerted by the dopant ion. This interaction appears to dominate the colonization process irrespective of any surface topography or ion elution effects.

CONCLUSIONS

A series of substituted apatites (AgA, SrA, ZnA) were effectively deposited onto biomedical grade titanium metal. The study presented demonstrates that the surface treatments investigated offer the dual benefits of osteoconductive properties essential for bone integration with the added potential of microbial colonization inhibition without cytotoxic effects. The direct contact of the microbes with the coating may be a significant contributing factor to the anticolonizing activity of the samples, as it was shown that the antimicrobial effect was significantly amplified by the presence of the substrate surfaces. AgA was observed to out-perform both the SrA and ZnA in terms of biofilm inhibition. Further optimization of the coating performance can be achieved through varying the loading of the dopant ion, tailoring the elution profile and controlling the surface morphology. These novel coatings possess new opportunities in orthopedic and dental applications for impeding infection while enhancing osteointegration, thereby increasing the success rate and improved patient outcomes for hard tissue implants.

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