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Preventing infection of osseointegrated transcutaneous implants: Incorporation of silver into preconditioned fibronectin-functionalized hydroxyapatite coatings suppresses *Staphylococcus aureus* colonization while promoting viable fibroblast growth *in vitro*

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The success of transcutaneous implants depends on the achievement of a soft tissue seal by enabling fibroblasts to win the race for the surface against bacteria. Fibronectin-functionalized hydroxyapatite coatings (HAFn) have been shown to improve dermal tissue ingrowth and attachment. However, during the early postoperative period before a soft tissue seal has formed, bacterial colonization may occur. This study explored the incorporation of silver, a broad spectrum antimicrobial agent, into HAFn coatings with the aim of reducing bacterial colonization. Silver is known to have dose-dependent cytotoxic effects. Therefore, the effects of silver incorporation into HAFn coatings on both *in vitro* human dermal fibroblast viability and *Staphylococcus aureus* colonization were assessed. An electrochemical deposition technique was used to codeposit hydroxyapatite and silver (HAAg) and fibronectin was adsorbed onto this to produce HAAgFn coatings. Surfaces were preconditioned with serum to mimic the *in vivo* environment. Nonpreconditioned HAAg and HAAgFn coatings suppressed bacterial colonization but were cytotoxic. After serum-preconditioning, more than 90% of fibroblasts that grew on all HAAg and HAAgFn coatings were viable. The highest silver content coatings tested (HAAg100 and HAAgFn100) resulted in a greater than 99% reduction in biofilm and planktonic bacterial numbers compared to HA and HAFn controls. Although HAAg100 had greater antibacterial activity than HAAgFn100, the findings of this study indicate that fibroblasts would win the race for the surface against *S aureus* on both HAAg100 and HAAgFn100 after serum-preconditioning. [http://dx.doi.org/10.1116/1.4889977]

I. INTRODUCTION

Conventional socket prostheses for amputees may be complicated by numerous problems including poor fit, repeated fittings, and pressure sores due to uneven pressure distribution.[1,2] Difficulty attaching socket prostheses to short limb stumps, discomfort, and excessive heat and sweating in the socket may also occur.[2] Osseointegrated transcutaneous implants have been developed to overcome these problems by enabling attachment of prostheses directly to bone via a skin-penetrating abutment and consequently resulting in forces being transferred directly to the skeleton.[1,3,4] Direct skeletal attachment of prostheses is associated with increased proprioception and osseoperception, leading to improved range of motion, function, gait, and overall quality of life.[5–8]

Osseointegrated implants have been used successfully in dental practice for over 40 years.[9] However, use in amputees has been complicated by high infection rates, reported to be 18% in a series by Tillander *et al.*[8,10,11] Infection occurs when there is a failure to achieve a tight seal between the soft tissue and the implant, which leads to epithelial downgrowth and marsupialization.[1] The reduced success rate associated with osseointegrated amputation prostheses compared to dental implants is potentially due to the greater soft tissue coverage around limb implants, which increases the interfacial movement.[3] Additionally, oral mucosal fibroblasts exhibit more rapid proliferation, greater extracellular matrix reorganizational ability, as well as increased matrix metalloproteinase expression and growth factor secretion compared to dermal fibroblasts.[12–14]

In order for soft tissue integration to occur and to prevent infection of osseointegrated transcutaneous implants, it is necessary for dermal fibroblasts to win the race for the surface against bacteria. If fibroblasts win the race, a cellular layer covers the surface, and bacteria will be less able to attach. If bacterial cells win the race, infection and ultimately biofilm formation will occur.[15,16] Removal of the implant is

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often the only effective method for eradication of infection once a biofilm has formed. This is due to the increased antibiotic resistance of bacteria within biofilms.[16,17]

A number of strategies have been investigated to increase soft tissue integration. Hydroxyapatite (HA) has been used to coat the skin-implant interface as it increases *in vitro* fibroblast adhesion and *in vivo* soft tissue ingrowth.[1,3,4] Fibronectin is a glycoprotein that contains an arginine–glycine–aspartate (RGD) amino acid sequence that attaches cells via integrins and increases fibroblast adhesion.[18] Fibronectin may be adsorbed onto hydroxyapatite to further enhance the effects of hydroxyapatite on soft tissue attachment.[3,19,20] For example, Pendegrass *et al*. implanted subcutaneous plates into sheep tibia and found that fibronectin-functionalized hydroxyapatite (HAFn) coatings were associated with greater dermal tissue ingrowth and attachment than nonfunctionalized hydroxyapatite coatings.[20]

During the early postoperative period, before the soft tissue seal has formed, it may be useful to incorporate an antimicrobial agent into these coatings in order to prevent bacterial colonization. Silver has been considered an attractive antimicrobial agent because it has a broad spectrum of activity against both gram positive and gram negative bacteria as well as fungi.[21] Silver is known to have dose-dependent cytotoxic effects. However, when used at low concentrations silver has demonstrated antibacterial activity without being cytotoxic.[21–24] Silver may be incorporated into hydroxyapatite coatings using electrochemical deposition. Electrochemical deposition is a non–line-of-sight procedure that takes place at low temperatures and may be used to coat complex structures such as porous titanium.[25] A method of electrochemical deposition developed by Ghani *et al*. has been shown to produce crystalline coatings and a sustained release of silver.[26] This method was used in the present study because electrochemical deposition is able to uniformly coat the inner pores of porous structures.[27] This is important because it is planned that porous titanium will be employed as the underlying substrate to manufacture transcutaneous implants for clinical use as it promotes soft tissue ingrowth.[1,28,29] Other more commonly commercially available methods such as plasma spraying are not able to do this. Although fibroblast viability has been assessed for hydroxyapatite and silver (HAAg) coatings produced by other methods, it has not been previously reported for this method of electrochemical deposition.[30] Additionally, the effect of this coating in combination with fibronectin has not been studied. Our study aimed to assess fibroblast viability and bacterial colonization on fibronectin-functionalized electrochemically deposited hydroxyapatite and silver. It was hypothesized first that these coatings would support growth of viable fibroblasts while preventing bacterial biofilm formation and inhibiting colonization within surrounding planktonic bacterial suspensions. Secondly it was hypothesized that serum-preconditioning of the surfaces (which mimics the *in vivo* environment) would improve the cytocompatibility. The rationale for using preconditioning to improve cytocompatibility is that it is known to remove ionizable metals from biomaterials.[31]

II. MATERIALS AND METHODS

A. Surface preparation

Titanium alloy (Ti6Al4V) disks measuring 10×3 mm were used. The following surface coatings were tested: uncoated polished Ti6Al4V (Pol), HA, HAFn, HAAg, and hydroxyapatite with silver and fibronectin (HAAgFn). These surfaces were studied before and after preconditioning by immersion in fetal calf serum (First Link Ltd., UK) for 24 h (P24).

HA was electrochemically deposited onto the surface of the disks. Disks to be HA coated were immersed in a 0.13 M solution of calcium phosphate monobasic [$Ca(H_2P_4)_2$]. The disk to be coated acted as a cathode and a platinum anode was used. An electrical current density of 80 mA/cm^2 was applied for 10 min using a DC Dual Power Supply 6010D. The current was controlled using a FLUKE 867B Graphical Multimeter (Fluke Corporation, USA). Silver nitrate ($AgNO_3$) (100 mg) was added per liter of the $Ca(H_2P_4)_2$ solution to produce an HAAg coating, which was named HAAg100. This method was originally described by Ghani *et al*. and has been shown to produce coatings of 102.2 +/− 4.20 μm thickness with silver incorporated into the HA crystalline structure.[26] Modifications of this coating named HAAg10 and HAAg50 were tested where 10 and 50 mg of $AgNO_3$ was added per liter of the $Ca(H_2P_4)_2$ solution, respectively, and a lower electrical current density of approximately 28 mA/cm^2 was applied for 4 min. Electrochemical deposition of HAAg coatings was carried out in the dark to prevent precipitation of silver. The initial layer of brushite [$Ca(HPO_4)\cdot 2(H_2O)$] that formed (with or without Ag incorporated) was converted to HA by immersion in 0.1 M sodium hydroxide for 72 h.[26,32]

Disks were sterilized with dry heat for 1 h at 160 °C. Human plasma fibronectin (Sigma Aldrich, Dorset, UK) (500 ng) was adsorbed onto surfaces as a 20 μl droplet.

B. Surface characterization

Surface characterization was carried out to determine if there were any differences in the morphology, the composition and the roughness of the coatings as these factors may affect cell attachment and subsequently cytocompatibility. Scanning electron microscopy (SEM) was used to visualize samples and assess the morphology of the coatings (JSM 5500 LV, JEOL, UK). The atomic percentage of silver present within the coatings was determined using energy dispersive X-ray (EDX) analysis (EDAX Genesis V5.216 AMETEK, NJ, USA). Six areas on three disks were analyzed ($n = 3$, $N = 6$). Surface roughness (R_a) was measured using a noncontact surface optical profilometer (Bruker Contour GT).

C. Cell culture

Human dermal fibroblasts (1BR3G, ECACC) were cultured in 225 cm^2 vented flasks (Corning Incorporated, New

York, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, Dorset, UK) containing 4500 mg/l glucose, 1%penicillin/streptomycin (Invitrogen Corporation, Paisley, UK), and 10% fetal calf serum.

D. Fibroblast viability

Cells were seeded onto the disk surface at a density of 5000 cells per disk and cultured for 24 h. The cells were then incubated in a solution containing 2 μM calcein AM and 4 μM ethidium homodimer-1 (Invitrogen Molecular Probes) for 45 min in the dark. A Zeiss fluorescent microscope was used to visualize cells. Three disks per coating were used and six areas per disk were viewed (n = 3, N = 6). The numbers of the live and dead cells were counted, and the percentage of live cells was calculated.

E. Fibroblast metabolism

Cells were seeded onto the disk surface at a density of 15,000 cells per disk. After 24 h, 1 ml of 10% Alamar blue in phenol red free DMEM was added. The cells were incubated at 37 °C with 5% CO_2 for 4 h. Six disks per coating were used and two 100 μl aliquots from each sample were added to a white 96 well plate (FluoroNunc^{TM}) (n = 6, N = 2). Absorbance was measured at 570 nm (reference range, 630 nm) using an Ascent fluoroscan plate reader (Thermo Electron Corporation, MA).

F. Bacterial challenge

Samples in all groups were challenged with 10^6 *Staphylococcus aureus* ATCC 29213. *S aureus* was used for experiments because it is the commonest organism causing infection of osseointegrated transcutaneous implants.[11] Each disk was placed into a 1 ml planktonic suspension of the bacteria in nutrient broth (Oxoid Ltd., Basingstoke, UK) and incubated for 24 h at 37 °C on a shaking tray at 50 rpm.

G. Biofilm direct colony counts

The samples were rinsed in phosphate buffered saline (PBS) (Oxoid) in order to remove poorly adhered bacteria. The attached biofilms that had formed on the surface of the disks were removed by ultrasonication in PBS for 2 min. The resultant bacterial suspension was vortexed for 10 s to separate the bacterial aggregates, and serially diluted in PBS over a 6-log range. Three disks per group were used, and 10 μl of each dilution was plated onto Columbia horse blood agar (Oxoid) in triplicate (n = 3, N = 3). The plates were incubated at 37 °C. After 24 h, the colony forming units (cfu) were counted. The number of cfu per milliliter was calculated.

H. Planktonic direct colony counts

The planktonic bacterial suspensions that disks had been immersed in were vortexed, serially diluted, and plated. Direct colony counts were performed using the same method as for biofilm direct colony counts described in Sec. II G.

I. Statistical analysis

The data were analyzed using SPSS, version 17.0 for Windows (Chicago, US). The Kruskall–Wallis test was performed to compare three or more groups and Mann–Whitney tests were used for pairwise comparisons. The differences were considered statistically significant when $p < 0.05$.

III. RESULTS

A. Surface characterization

SEM of the electrochemically deposited coatings showed the presence of a combination of needle shaped, plate shaped, and globular microcrystals on HA and HAAg surfaces. HAAg100 coatings appeared to have a greater proportion of larger plate shaped crystals and a more disorganized morphology than HAAg50 and HAAg10 coatings. Fibronectin adsorption did not affect the morphology of the coatings. The presence of a protein film was visible on surfaces that had been serum-preconditioned. As it was necessary to dry the samples prior to performing SEM, cracks were visible in the protein film. Few needle and plate shaped crystals were visible on preconditioned surfaces and the crystals appeared to be predominantly aggregated together as globules beneath the film (Fig. 1).

EDX analysis detected the presence of silver in all HAAg coatings and confirmed the absence of silver in the control coatings. HAAg100 coatings had the highest silver content and HAAg10 coatings had the lowest silver content (Table I) ($p < 0.05$). Fibronectin adsorption and serum-preconditioning resulted in the appearance of elements not detected in HA and HAAg coatings such as Cl, K, Mg, and N (Fig. 2). HAAg surfaces that had been preconditioned had a lower silver content than nonpreconditioned HAAg surfaces; however, this reduction was not statistically significant ($p > 0.05$).

Profilometry showed that Pol surfaces were smoother than all HA and HAAg surfaces having significantly lower R_a values ($p < 0.05$). There was no difference in the roughness of HA, HAAg10 and 50 surfaces ($p > 0.05$). However, HAAg100 (with and without fibronectin) was significantly rougher than all other surfaces ($p < 0.05$). Preconditioning of HAAg surfaces resulted in reduced R_a values; however, this reduction was found to be statistically significant only for HAAgFn10 and HAAg50 ($p < 0.05$). The adsorption of fibronectin onto surfaces did not significantly affect R_a values ($p > 0.05$) (Table II).

B. Fibroblast viability

All nonpreconditioned HAAg surfaces were cytotoxic to fibroblasts compared to Pol, HA, and HAFn ($p < 0.05$). HAAg100 and HAAgFn100 exhibited the greatest cytotoxicity and there were no live cells on these surfaces, compared to significantly greater median percentage live cells of 16% on HAAg50 and 55% on HAAg10 ($p < 0.05$). Preconditioning of HAAg surfaces significantly reduced the cytotoxic effects. After 24 h preconditioning, all surfaces

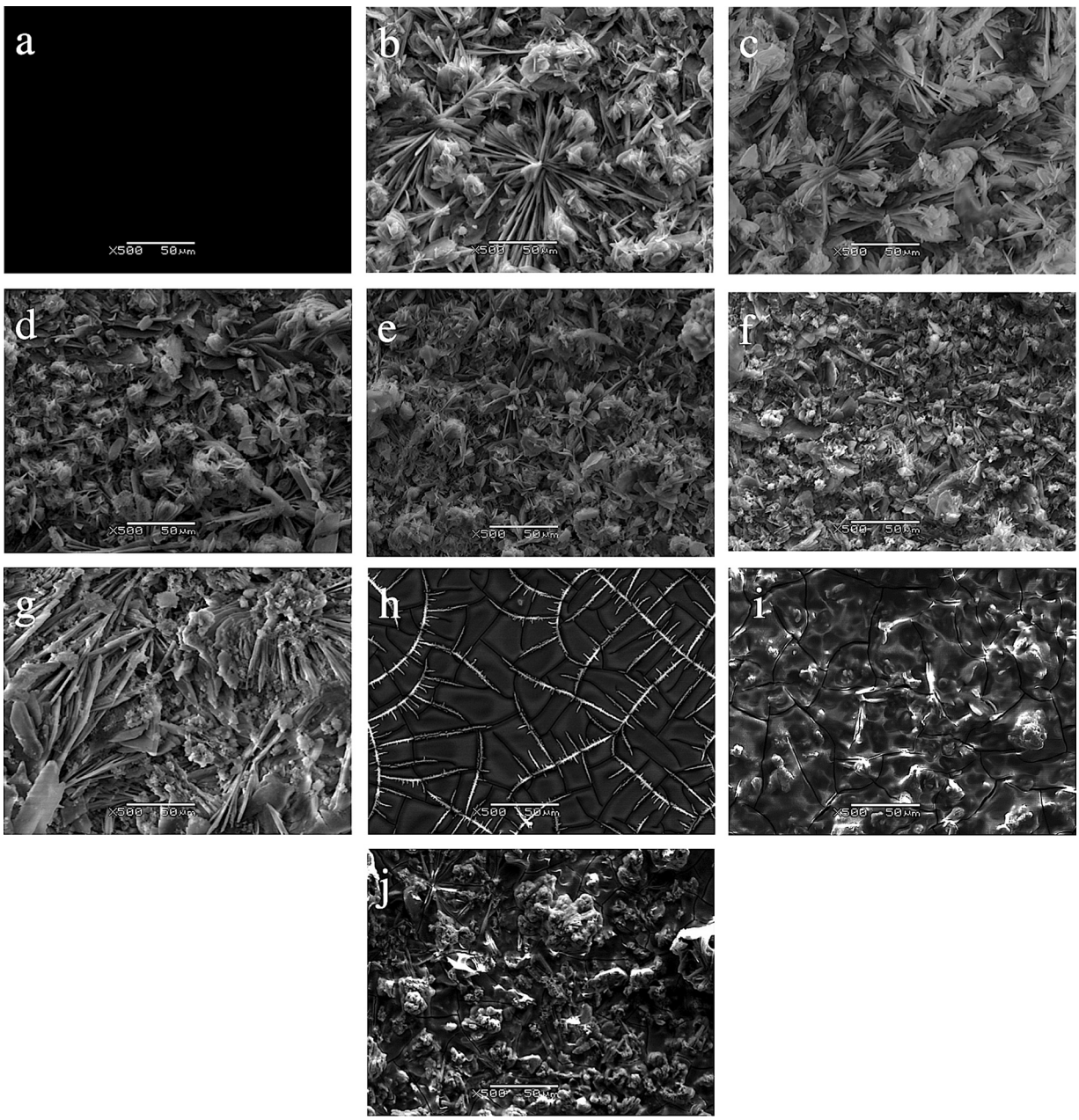

FIG. 1. Scanning electron micrographs showing morphology of coatings (a) Pol, (b) HA, (c) HAFn, (d) HAAg10, (e) HAAg10 Fn, (f) HAAg50, (g) HAAg100, (h) Pol (P24), (i) HA (P24), and (j) HAAg100 (P24). Key: Pol = Uncoated polished Ti6Al4V, HA = electrochemically deposited hydroxyapatite, HAFn = electrochemically deposited hydroxyapatite with adsorbed fibronectin, HAAg = electrochemically codeposited hydroxyapatite and silver, HAAgFn = electrochemically codeposited hydroxyapatite and silver with adsorbed fibronectin, and (P24) = serum-preconditioning for 24 h.

were cytocompatible with more than 90% cell viability, and there was no reduction in viability on HAAg (P24) surfaces compared to Pol, HA, and HAFn controls ($p > 0.05$). Preconditioning did not affect the cytocompatibility of the control surfaces. Adsorption of fibronectin onto surfaces did not improve the cytocompatibility of surfaces (Figs. 3 and 4).

C. Fibroblast metabolism

The greatest cell metabolism was observed on HAFn controls, which resulted in greater metabolism than Pol ($p = 0.008$) but was not significantly different compared to HA ($p = 0.143$). There was significantly less cell metabolism on HAAg surfaces that had not been preconditioned than on Pol, HA, and HAFn surfaces ($p < 0.05$). Decreased cell metabolism was observed on HAAg100 surfaces compared to HAAg50 and HAAg10 surfaces ($p < 0.05$). The cell metabolism on HAAg surfaces increased after preconditioning, compared to HAAg surfaces that had not been preconditioned ($p < 0.05$). There was no difference in cell metabolism between HAAg10, HAAg50, and HAAg100 surfaces after preconditioning ($p > 0.05$) and no difference between these surfaces and Pol and HA controls ($p > 0.05$). However,

TABLE I. Atomic silver percentage for each surface presented as median values and (95% confidence intervals).

Surface	Ag (at. %)
Pol	0.000
HA	0.000
HAFn	0.000
HAAg10	0.205 (0.190–0.230)
HAAgFn10	0.145 (0.090–0.240)
HAAg50	0.280 (0.270–0.330)
HAAgFn50	0.290 (0.230–0.370)
HAAg100	0.665 (0.440–0.820)
HAAgFn100	0.595 (0.440–0.760)
HAAg10 (P24)	0.200 (0.150–0.230)
HAAgFn10 (P24)	0.150 (0.130–0.180)
HAAg50 (P24)	0.270 (0.230–0.390)
HAAgFn50 (P24)	0.290 (0.200–0.400)
HAAg100 (P24)	0.460 (0.310–0.620)
HAAgFn100 (P24)	0.535 (0.440–0.690)

TABLE II. Surface roughness results presented as median values and (95% confidence intervals).

Surface	R_a (μm)
Pol	0.031 (0.028–0.033)
HA	2.952 (2.828–3.451)
HAFn	3.317 (2.802–3.444)
HAAg10	2.804 (2.345–3.504)
HAAgFn10	3.261 (2.602–3.683)
HAAg50	3.022 (2.735–3.678)
HAAgFn50	3.301 (2.411–3.635)
HAAg100	4.774 (4.663–5.327)
HAAgFn100	4.550 (4.302–4.715)
HAAg10 (P24)	2.597 (2.334–3.802)
HAAgFn10 (P24)	2.255 (2.087–3.605)
HAAg50 (P24)	2.333 (2.250–3.028)
HAAgFn50 (P24)	2.540 (1.947–4.059)
HAAg100 (P24)	4.358 (3.296–5.579)
HAAgFn100 (P24)	4.184 (3.477–4.638)

greater cell metabolism was observed on HAFn controls than on HAAg (P24) surfaces ($p < 0.05$). A trend was observed that fibronectin was associated with increased cell metabolism on HAAg surfaces; however, this was only statistically significant when added to HAAg50 ($p = 0.003$), but not when added to HAAg10 and HAAg100 ($p = 0.799$ and 0.977, respectively) (Fig. 5).

D. Biofilm direct colony counts

Greater numbers of bacteria were present within biofilms that formed on HA compared to Pol ($p = 0.008$). There was a further increase in the numbers of bacteria within biofilms formed on HAFn compared to HA ($p = 0.001$). Nonpreconditioned HAAg surfaces reduced bacterial colonization compared to Pol, HA, and HAFn controls ($p < 0.05$). Increasing the silver content increased the degree of antibacterial activity. HAAg100 and HAAgFn100 showed the greatest antibacterial activity, and there was complete suppression of bacterial colonization on these surfaces. After preconditioning, some bacterial colonization occurred on HAAg100 surfaces. However, the numbers of bacteria colonizing HAAg100 (P24) surfaces remained significantly lower than the control surfaces ($p < 0.05$). There was a reduction of 1.997×10^6 cfu/ml on HAAg100 (P24) compared to HA (P24), which is equivalent to a 99.85% reduction in bacterial colonization. There was a reduction of 1.983×10^6 cfu/ml on HAAgFn100 (P24) compared to HAFn (P24) (99.15% reduction). After preconditioning,

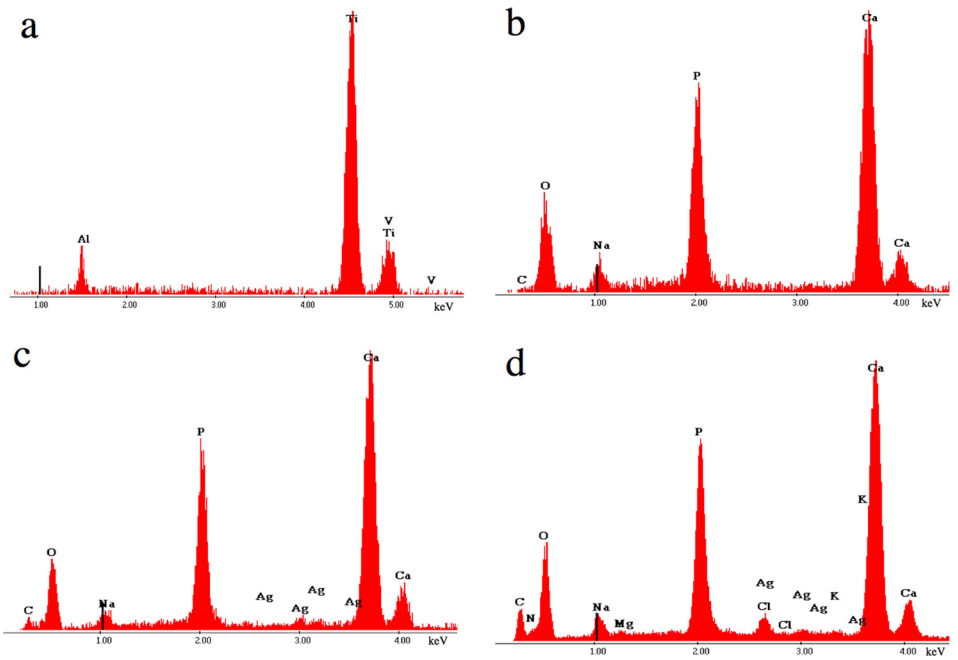

FIG. 2. Energy dispersive x ray analysis spectra for (a) Pol, (b) HA, (c) HAAg100, and (d) HAAgFn100 (P24).

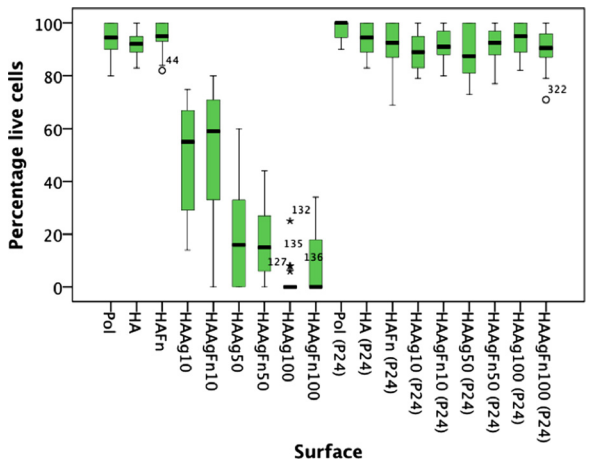

FIG. 3. Box-plot showing the percentage of live fibroblasts on each surface.

HAAg10 and HAAg50 lost their antibacterial activity compared to Pol, but fewer bacteria colonized these surfaces than HA ($p = 0.04$ and 0.02, respectively). It was noted that reduced numbers of bacteria colonized Pol, HA, and HAFn control surfaces after they were preconditioned ($p = 0.001$, 0.088, and 0.000, respectively). In contrast increased numbers of bacteria colonized HAAg and HAAgFn surfaces after they were preconditioned ($p < 0.05$). HAAg100 was the only surface that maintained an antibacterial effect after preconditioning compared to Pol (P24) surfaces ($p = 0.008$). Fibronectin resulted in an increase in bacterial colonization on HAAg100 (P24) but did not affect the bacterial colonization on other HAAg (P24) surfaces (Fig. 6).

E. Planktonic direct colony counts

HAAg100 and HAAgFn100 had the greatest antibacterial activity and were the only surfaces to reduce bacterial colonization compared to all three control surfaces before and after serum-preconditioning ($p < 0.05$). HAAg100 (P24) was associated with a 2.493×10^7 cfu/ml reduction in planktonic colonization compared to HA (P24) (99.72% reduction). HAAgFn100 (P24) reduced planktonic colonization by 1.994×10^7 cfu/ml compared to HAFn (P24) (99.68% reduction). Fibronectin increased bacterial colonization when adsorbed onto HAAg50 (P24) ($p = 0.000$). This effect was not observed on other surfaces (Fig. 7).

IV. DISCUSSION

The key results of this study show that electrochemically deposited HAAg100 and HAAgFn100 suppress bacterial

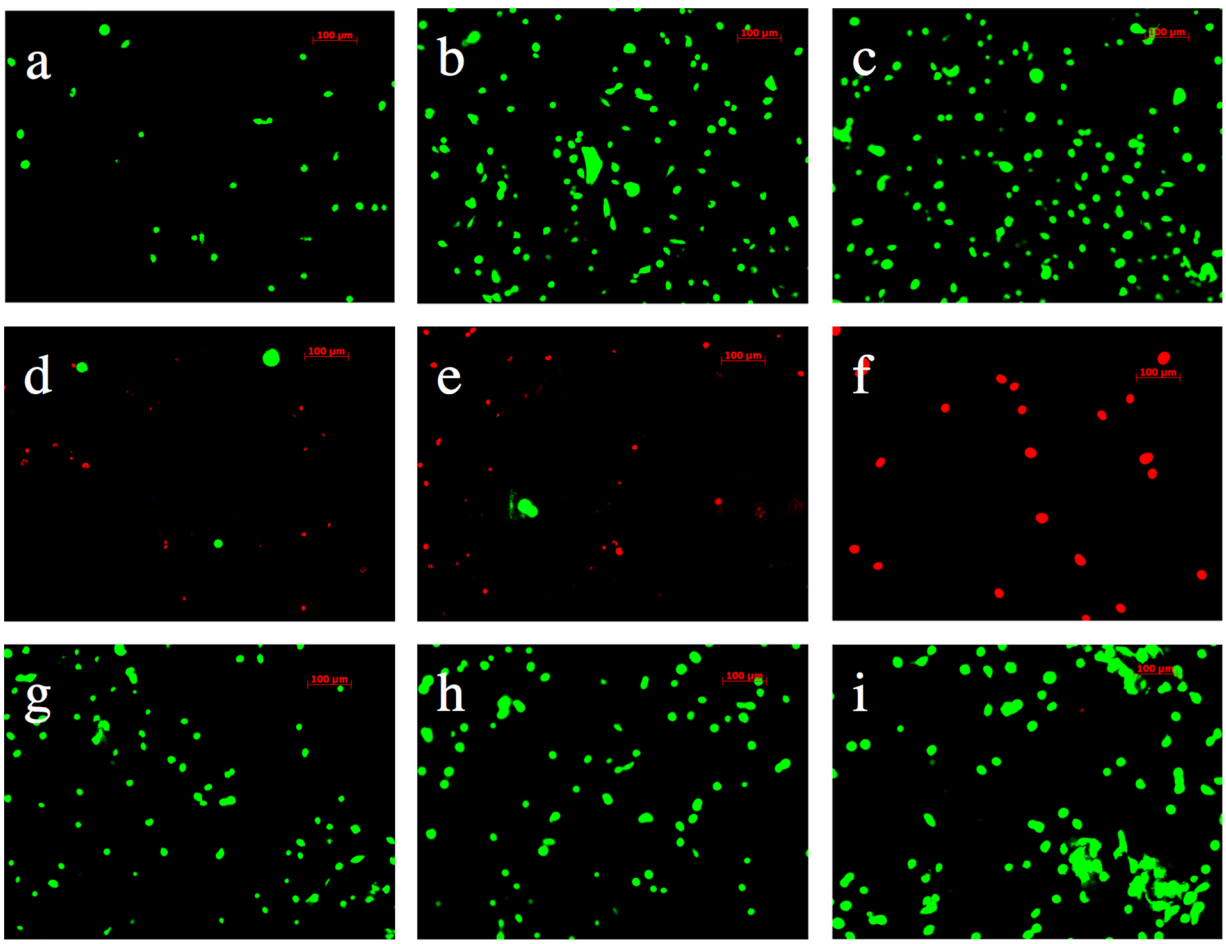

FIG. 4. Live (green):dead (red) staining images of fibroblasts stained with calcein AM and ethidium homodimer-1 (a) Pol, (b) HA, (c) HAFn, (d) HAAg10, (e) HAAg50, (f) HAAg100, (g) HAAg10 P24, (h) HAAg50 (P24), and (i) HAAg100 (P24).

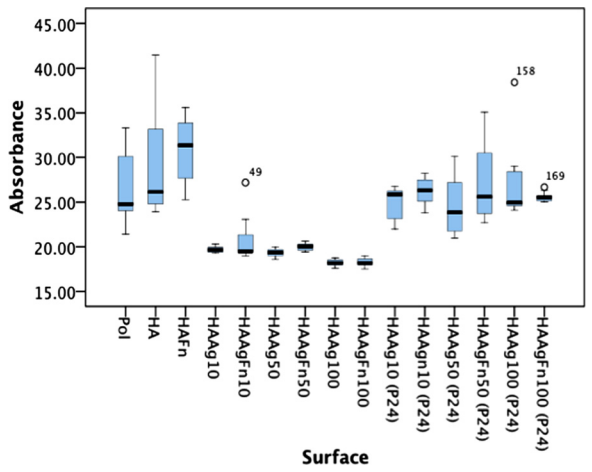

FIG. 5. Box-plot showing Alamar Blue absorbance levels for each surface tested.

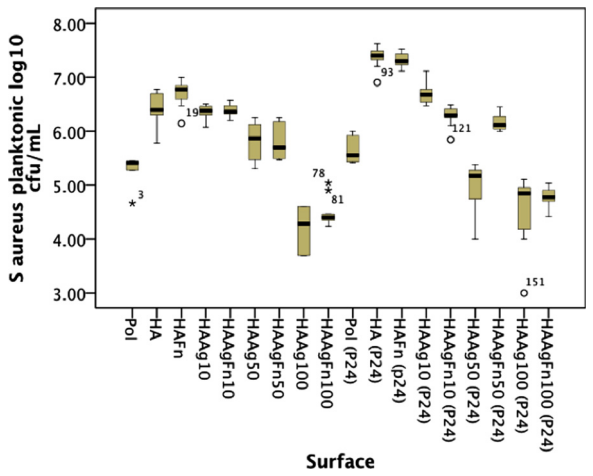

FIG. 7. Box-plot showing the number of colony forming units per milliliter within planktonic suspensions presented as $\log_{10}$ transformed numbers.

colonization before and after preconditioning in fetal calf serum. After preconditioning, all silver containing surfaces were cytocompatible and supported the growth of fibroblasts. Cell metabolism also increased after preconditioning, which may have been due to increased cell proliferation.

Previous studies using inductively coupled plasma mass spectrometry (ICP MS) have shown that silver is released from HAAg produced using this method of electrochemical deposition. Furthermore, the release slows down to a sustained controlled release.[26] As a result, it is hypothesized that after preconditioning surfaces, the release would be slower, hence accounting for the improved cytocompatibility. Further elution kinetic studies measuring the amount of silver released into culture media would be valuable to investigate this. The observation of reduction of planktonic bacterial numbers in the present study also indicates that silver is released from HAAg surfaces into the surrounding fluid and consequently silver levels within the coating would be expected to be reduced. However, EDX analysis results showed that the reductions in the atomic percentage of silver

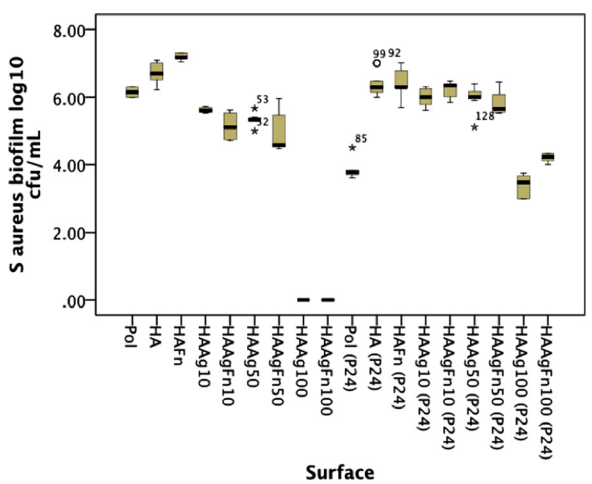

FIG. 6. Box-plot showing the number of colony forming units per milliliter within biofilms presented as $\log_{10}$ transformed numbers.

observed after preconditioning were not significant. This finding may be due to loss of other elements in addition to silver. It could also be due to the number of silver ions initially released being a very small proportion of the total number of silver ions. Further studies to determine the overall amount of silver within the coatings using atomic absorption spectroscopy or ICP MS (rather than a percentage as measured by EDX) may be of value. Extraction of silver into nitric acid could be used to quantify the amount of silver within the coating after preconditioning. It is likely that silver ions interact with proteins adjacent to the surface making them less toxic but the number of ions involved in this process would be very difficult to measure. It has already been shown by Sandrucci *et al*. that preconditioning removes silver ions from silver-containing surfaces, which indicate that the overall silver levels (but not necessarily the percentage) within the substrate would be reduced.[31] This is an explanation for the improved cytocompatibility associated with preconditioning.

An alternative explanation for the lack of significant reductions in percentage silver on P24 surfaces that should be considered is that silver content reduction may not be the only reason for the increased cytocompatibility associated with preconditioning. As the percentage of silver was not reduced on P24 surfaces, it is possible that the effect of P24 could be associated with other factors such as the deposition of proteins. One observation that supports this theory is that the percentage of live cells on HAAg P24 surfaces was equal to that of controls that did not contain silver. Furthermore, there were no differences in the cell viability between HAAg10, 50, and 100 (P24) surfaces. Furno *et al*. concluded that silver ions were able to penetrate protein conditioning films and maintain their bactericidal activity.[33] Similarly, in our study, a substantial amount of the silver may have resided in the preconditioning film resulting in the reductions in silver content detected by EDX being insignificant. The finding of improved viability on preconditioned surfaces may be partly due to less of the silver being in a biologically active ionized form due to binding to serum proteins such as

albumin.[34] However, it should be noted that the study by Furno *et al*. suggested that not all silver bound to proteins is inactivated as their conditioned substrates did not exhibit any loss of antibacterial activity.[33] Indeed, in our study although some reduction of antibacterial activity was seen on HAAg100 after preconditioning, bacterial growth was still significantly less compared to controls. Another finding was that after preconditioning, additional elements such as Cl, K, N and Mg were incorporated into the coatings. Other studies have concluded that immersion in simulated body fluids affects the composition and consequently may influence the biological behavior of HA coatings.[32,35] It has also been noted by other studies that preconditioning may affect the crystallinity of ceramics and result in different apatite phases, which may contribute toward the increased cytocompatibility.[36]

The increased time period and higher current density used for electrochemical deposition may have contributed to the increased roughness of HAAg100 compared to HAAg50 and HAAg10. Higher current densities are associated with more vigorous hydrogen evolution and the overall process is less efficient as a larger proportion of brushite crystals are unable to survive the turbulent environment of a high current density and are thus lost.[37] Redepenning *et al*. reported that the crystals that do survive this environment would be expected to adhere better, resulting in coatings with an overall greater integrity.[37] The same current density and time was used to produce HA coatings, which were not as rough as HAAg100, indicating that the silver content affects the topography of these surfaces. Previous x-ray diffraction analysis of HAAg showed peaks that were sharper than those observed for HA, suggesting that the Ag^+ ion incorporation may increase the crystallinity.[26] The reduction in R_a observed on preconditioned surfaces was not statistically significant for all surfaces. Nevertheless, it was evident from the scanning electron micrographs that the crystal morphology was markedly different after preconditioning. This may be due to the fact that when a hydroxyapatite surface is immersed into a simulated body fluid, resorption of hydroxyapatite and the deposition of an apatite layer on the surface as described by Kokubo *et al*. may occur.[38,39] Fibroblasts are known to be sensitive to surface topography and previous studies have shown that reductions in roughness even on a nanometric scale are associated with improved fibroblast attachment, proliferation, or viability.[40–45] However, many factors such as wettability and surface chemistry influence fibroblast responses and may change the way fibroblasts to respond to surfaces. This may enable cells to grow on rougher surfaces despite the fact that they usually respond better to smoother surfaces.[42,46,47]

HAAg50 and 10 did not perform as well as HAAg100 in terms of antibacterial activity. The only potential advantage of lower silver content coatings is that before preconditioning they were less cytotoxic than HAAg100. This raises the possibility that they may be able to promote earlier attachment of viable fibroblasts but this is not conclusive. After preconditioning, there was no difference in HAAg50 and 10 groups in terms of cytotoxicity compared to HAAg100. It is possible that with a smaller bacterial challenge HAAg10 and HAAg50 may have been able to demonstrate a greater antibacterial effect. The administration of a bacterial challenge of 10^6 *S. aureus* cfu for a 10 mm × 3 mm disk (surface area of 251 mm^2) could be considered an excessively large number of bacteria, which should rarely be encountered clinically in the early postoperative period before a soft tissue seal forms. It was encouraging though to ascertain that HAAg100 was able to withstand such a large bacterial challenge.

Interestingly, the effect of preconditioning was different on controls and HAAg surfaces. Less bacterial colonization was observed on Pol disks after they were preconditioned. Similar findings have been previously reported by other studies where adhesion of bacteria was significantly inhibited on surfaces treated with serum or proteins.[48–52] Hydrophilic proteins in serum such as albumin are believed to be responsible for this phenomenon as bacteria adhere better to hydrophobic surfaces.[51–53] It has also been suggested that preconditioning surfaces forms a barrier to bacterial adhesion.[48] Conversely, Antoci *et al*. found an abundance of live adherent bacteria on titanium rods coated with serum.[54] The reason for these conflicting findings is unclear, but differences in the initial surface characteristics of the underlying substrate may be relevant. Further research on the wettability of these surfaces may be of value. Reductions in bacterial colonization were also seen on HA and HAFn but were less marked than those seen on Pol. Indeed, large numbers of bacteria in the range of more than 10^6 colonies remained present on HA and HAFn surfaces after preconditioning, meaning this reduction is unlikely to be clinically useful. The reduction in bacterial colonization on Pol is also not likely to be exploited clinically as Pol would be unsuitable as a surface for osseointegrated transcutaneous implants due to inadequate soft tissue attachment *in vivo*.[1] Preconditioning HAAg surfaces had the opposite effect to preconditioning controls, and there was an increase in bacterial colonization. If silver from preconditioned HAAg coatings had become bound to proteins and some silver had been inactivated as discussed earlier, the postulated increase in wettability would be expected to have been counteracted.

The adsorption of fibronectin onto HAAg100 (P24) surfaces increased bacterial colonization to the extent that HAAgFn100 (P24) was not statistically significantly antibacterial compared to Pol. However, it was still antibacterial compared to HA and HAFn, which is important because HA surfaces are used clinically (whereas an uncoated Pol surface would not be used clinically). In addition to the possibility of some inactivation of silver associated with fibronectin, *S. aureus* contains fibronectin binding sites.[55] The results of this study indicate that silver does not counteract the effect of these binding sites. This negative effect of fibronectin on antibacterial activity could be less marked with other bacterial species that do not contain fibronectin binding sites. However, as *S aureus* is the most common bacterial species causing infection of transcutaneous implants it may be

considered that this is the most important bacterial species for the coating to have antibacterial activity against. Additionally, this study showed that adsorption of fibronectin onto HAAg did not counteract the detrimental effects of silver on fibroblast metabolism or viability. In the absence of silver, fibronectin increased metabolism but not viability (as viability was already more than 90% on Pol and HA controls). The RGD sequence of fibronectin that is responsible for cell binding may be less likely to increase bacterial colonization and would ultimately be more commercially acceptable.[56] However, the RGD sequence would not be expected to have an advantage over fibronectin on cell viability and metabolism in combination with HAAg.

Fetal calf serum was chosen over simulated body fluids for preconditioning as it contains serum proteins such as albumin and globulins and so was more representative of the *in vivo* environment than simulated body fluids. Nonetheless, it could be argued that a limitation of this study is that plasma would have been more representative as it contains clotting factors such as fibrinogen in addition to serum proteins. This may be relevant as *S. aureus* contains fibrinogen-binding proteins. The finding that preconditioning is necessary before HAAg surfaces become cytocompatible, suggests that in clinical practice HAAg may be cytotoxic initially; and a short time period (up to 24 h) may be required for cells to attach to these surfaces. The clinical implications of this delay are not known. In clinical practice, preconditioning prior to implantation with autologous serum in order to promote earlier soft tissue attachment would be possible but logistically less convenient. An assessment of preconditioning for shorter time periods would reveal whether cytocompatibility is achieved at an earlier stage than 24 h. *In vivo* experiments will be carried out in the near future and will address some of the limitations of this *in vitro* study.

It would be useful to assess the effect of HAAg and HAAgFn surfaces on other cell types and other bacterial species to widen the potential clinical applicability of this coating. In contrast to fibroblasts, osteoblasts are known to respond better to rougher surfaces. Osteoblasts have been reported to respond well to surfaces with similar R_a microroughness levels to the surfaces investigated in this study.[45,57,58] It is well known that HA increases osteoblast adhesion. Studies have also shown that the addition of fibronectin improves osteoblast responses further.[59–61] If osteoblasts were found to respond well to HAAg/HAAgFn, this technique could be useful for the intraosseous portion of osseointegrated transcutaneous implants as well as more widely for other orthopedic implants such as arthroplasty prostheses. This would be advantageous because hip and knee arthroplasties are associated with an infection rate of approximately 1%, which is associated with significant morbidity and substantial economic costs.[62] Finally, further modification of the topography of these coatings could be achieved using alterations of the current density, silver content of the electrolyte solution and the time period used for electrochemical deposition.

V. CONCLUSIONS

This study is the first to show that electrochemically deposited HAAg surfaces lose their cytotoxic effects when preconditioned and support viable fibroblast growth. More than 90% of fibroblasts cultured on all preconditioned surfaces were viable. Before preconditioning complete suppression of *S. aureus* biofilm formation was observed on HAAg100 and HAAgFn100. After serum preconditioning more than 99% of the antibacterial activity of HAAg100 and HAAgFn100 compared to HA and HAFn controls was maintained. This indicates that fibroblasts win the race for the surface against *S. aureus* on HAAg100 and HAAgFn100 after serum-preconditioning. This is a step forward toward developing a surface that could prevent infection of osseointegrated transcutaneous implants and other orthopedic devices.

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