Preventing Infection of Osseointegrated Transcutaneous Implants: A Preconditioned Hydroxyapatite and Silver Surface that is Cytocompatible and Reduces Staphylococcus Aureus and Pseudomonas Aeruginosa Biofilm Formation

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Introduction: Osseointegrated transcutaneous implants may significantly improve the quality of life for amputees by improving function and proprioception as well as avoiding problems associated with socket prostheses such as repeated fittings, discomfort and pressure sores. If soft tissue cells do not win the ‘race for the surface’ against bacteria, soft tissue integration will not be successful and bacterial biofilm formation will occur. Hydroxyapatite (HA) with adsorbed fibronectin (Fn) has been shown to significantly increase fibroblast adhesion and may increase the soft tissue seal [1]. Silver (Ag) has a broad spectrum of antimicrobial activity and may be incorporated into HA coatings using electrochemical deposition. Electrochemically deposited HA and Ag (HAAg) coatings have been shown to be pure, crystalline and uniform [2]. Ag is associated with dose-dependent cytotoxicity. Preconditioning surfaces is a method that may be used to increase cytocompatibility of surfaces. This study aimed to assess fibroblast viability and bacterial colonisation on electrochemically deposited HAAg with adsorbed Fn surfaces. It was hypothesized that HAAg +/- Fn coatings would have antibacterial activity compared to HA controls and would support viable fibroblast growth after preconditioning.

Methods: Surface preparation: The following surfaces were tested: HA, HA with adsorbed Fn (HAFn), HAAg and HAAg with adsorbed Fn (HAAgFn) and HAAg +/- Fn surfaces that had been preconditioned for 24 hours (P24). 10mm x 3mm titanium alloy discs were immersed in a 0.13M solution of calcium phosphate monobasic (Ca(H2P4)2) containing silver nitrate (100mg/litre). The discs to be coated acted as a cathode. A platinum anode was used. An electrical current of 200mA was applied for 10 minutes. A 20µl droplet containing 500ng Fn was adsorbed onto Fn surfaces. Preconditioning (P24) was carried out by immersing the discs in fetal calf serum for 24 hours.

Surface characterisation: Six areas on three discs per group were analysed (n=3, N=6). Scanning electron microscopy (SEM) was performed to assess the morphology of the coatings. Energy Dispersive X-ray (EDX) analysis was used to determine the atomic percentage of Ag present within the coatings.

Fibroblast viability and metabolism: 5 x 10³ human dermal fibroblasts (1BR3G, ECACC) were seeded onto each disc. Live:dead staining was used to determine the percentage of live cells on each surface (n=3, N=6). Fibroblast metabolism was assessed using the Alamar Blue assay (n=6, N=2). Bacterial colonization: Surfaces were challenged with staphylococcus aureus ATCC 29213 (10^6) and a clinical isolate of pseudomonas aeruginosa F1896 (10^6 and 5 x10^4). Biofilms that formed on the discs after 24 hours were removed by ultrasonication, followed by serial dilutions and plating onto Columbia horse blood agar. Direct colony counts were performed. Three discs per group were used and serial dilutions were plated in triplicate (n=3, N=3). Statistical Analysis: Kruskall Wallis and Mann-Whitney U tests were performed using SPSS 17.0 for Windows (Chicago, US).

Results: Surface characterisation: SEM showed that the electrochemically deposited coatings contained a combination of needle shaped, plate shaped and globular microcrystals. On P24 surfaces crystals appeared to be predominantly aggregated together as globules beneath the preconditioning film (Figure 1). EDX analysis detected the presence of Ag in HAAg surfaces (median atomic percentage 0.66%) and confirmed the absence of Ag in HA coatings. Adsorption of Fn and preconditioning was associated with the appearance of additional elements such as Cl, K, Na, Mg and N (Figure 1). There was a non-significant reduction in Ag content compared on HAAg after preconditioning (p=0.359).

Fibroblast viability and metabolism: Non-preconditioned HAAg surfaces were cytotoxic. However, HAAg P24 surfaces were cytocompatible with more than 90% cell viability and no difference in viability compared to HA and HAFn controls (p=0.182) (Figure 2). There was a reduction in cell metabolism on HAAg surfaces compared to HA (p<0.05). However, preconditioning HAAg surfaces increased the metabolism and there was no difference between HAAg P24 surfaces and HA controls (p=0.374).

Adsorption of Fn onto HAAg did not increase cell viability or metabolism. Bacterial colonization: Staphylococcus aureus biofilm formation was completely suppressed on HAAg and HAAFn. There was some staphylococcus aureus growth on HAAgP24, but this surface still had significant antibacterial activity resulting in a 4.997 x 10^6 reduction in bacteria compared to HA (p=0.002) (Figure 3). Colonization was also reduced on HAAg compared to HA when challenged with 10^6 pseudomonas aeruginosa (p=0.000). However, HAAg P24 did not significantly reduce pseudomonas aeruginosa colonization when challenged with 10^6 bacteria (p=0.878). When challenged with 5 x10^4 pseudomonas aeruginosa HAAgP24 maintained antibacterial activity.
Fn increased staphylococcus aureus colonisation on HA, HA P24 and HAAg P24, but did not increase pseudomonas aeruginosa colonisation.

**Discussion:** Previous release kinetic studies of HAAg at our institution has shown that there is an initial ‘burst’ release of Ag followed by a slower controlled release [2]. This indicates that the release of Ag would be slower from a P24 surface and would explain the improved cytocompatibility. EDX analysis showed that Ag content was not significantly reduced on P24 surfaces. However, it is likely that less of the Ag present would be in a biologically active ionized form after preconditioning due to binding to serum proteins [3]. Further studies to assess the total amount of Ag (rather than the percentage) may be of value. The incorporation of additional elements into P24 surfaces observed may have also contributed to differences in cell viability associated with P24 surfaces. Pseudomonas aeruginosa was less susceptible to the antibacterial activity of HAAg surfaces than staphylococcus aureus. HAAg P24 was only found to have significant anti-pseudomonal activity when a smaller bacterial challenge was used. This may be due to differences in the production of extracellular polymeric substance by these bacteria. As preconditioning in serum mimics what would occur after implantation in vivo, the reversal of cytotoxic effects on HAAg P24 surfaces may represent a delay in cytocompatibility of surfaces of up to 24 hours post-operatively. The in vivo implications of this are currently being investigated. Fn was associated with an increase in staphylococcus aureus colonisation. Staphylococcus aureus has a specific Fn binding site, explaining why this difference was not observed seen with pseudomonas aeruginosa. Fn does not have beneficial effects in terms of cell viability on HAAg in vitro.

**Significance:** The findings of this study indicate that in vitro, fibroblasts win the ‘race for the surface’ on HAAg P24 against staphylococcus aureus and pseudomonas aeruginosa (depending on the size of the bacterial challenge). This study is the first to show that the detrimental effects of HAAg on cell viability are reversed with preconditioning but a significant reduction in biofilm formation remains after preconditioning. This is a step forward towards developing a surface that could prevent infection of osseointegrated transcutaneous implants and other orthopaedic devices.

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Figure 2. Live (green):dead (red) staining images (x10 magnification)

Figure 3. Staphylococcus aureus colony counts

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