Effect of silver content on the structure and antibacterial activity of silver-doped phosphate-based glasses

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Running title: Antibiofilm activity of silver.

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**ABSTRACT**

*Staphylococcus aureus* can cause a range of diseases such as osteomyelitis as well as colonize implanted medical devices. In most instances the organism forms biofilms that are not only resistant to the body’s defence mechanisms but also display decreased susceptibility to antibiotics. In the present study, we have examined the effect of increasing silver content in phosphate-based glasses to prevent the formation of *S. aureus* biofilms. Silver was found to be an effective bactericidal agent against *S. aureus* biofilms and the rate of silver ion release (0.42-1.22 µg.mm$^{-2}$.h$^{-1}$) from phosphate-based glass was found to account for the variation in its bactericidal effect. Analysis of biofilms by confocal microscopy indicated that they consisted of an upper layer of viable bacteria together with a layer (~20µm) of non-viable cells on the glass surface. Our results showed that regardless of the silver contents in these glasses (10, 15 or 20 mol%) the silver exists in its +1 oxidation state which is known to be a highly effective bactericidal agent compared to other oxidation states (+2 or +3). Analysis of the glasses by $^{31}$P NMR and HEXRD showed that it is the structural rearrangement of the phosphate network that is responsible for the variation in silver ion release and associated bactericidal effectiveness. Thus an understanding of the glass structure is important in interpreting the *in vitro* data and also has important clinical implications for the potential use of the phosphate-based glasses in orthopaedic applications to deliver silver ions to combat *S. aureus* biofilm infections.
INTRODUCTION

*Staphylococcus aureus*, a leading cause of nosocomial infections worldwide, is the aetiological agent of a wide range of diseases, from relatively benign skin infections to potentially fatal systemic disorders (41). Many of these diseases, including endocarditis, osteomyelitis, and foreign-body related infections, appear to be caused by biofilm-associated *S. aureus* (14, 18, 27, 38). Biofilms are sessile communities characterized by cells that are attached to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (14). Biofilm formation occurs as a result of a sequence of events: microbial surface attachment, cell proliferation, matrix production and detachment (34). Biofilm-associated bacteria show a decreased susceptibility to antibiotics (10), disinfectants (31) and clearance by host defences (14, 37). Work by Mulligan et al. (29, 30) showed that the inclusion of copper or silver ions in phosphate-based glasses was useful in treating biofilms of *Streptococcus sanguis*. Silver cations exhibit broad antimicrobial action at low concentrations, and they are already being used for the treatment of burn wounds (32) and traumatic injuries (5, 15). Feng et al. (15) studied the antibacterial effect of silver ions on *E. coli* and *S. aureus* and suggested that the antibacterial mechanism was due to DNA not being able to replicate, and proteins becoming inactivated after contact with silver ions.

Phosphate-based glasses are soluble materials that can act as a unique system for the delivery of silver ions in a controlled way (25). The ions are incorporated into the glass structure and are not a separate phase; thus, their rate of release is defined by the overall degradation rate of the glass. Phosphate-based glasses have already been used to deliver
silver ions to help control urinary tract infections in patients needing long-term indwelling catheters (9, 17, 40) and also in wound dressings to prevent infections (9). However in recent work, anomalies have been reported whereby the antimicrobial effect does not follow an expected relationship with silver content (2). This is thought to be due to the speciation and local coordination environment around the silver in the glass and, more generally, the changes in the glass structure. Therefore the aims of this study were; (a) to produce a range of silver-doped phosphate-based glasses (0, 10, 15 and 20 mol% silver), (b) to measure the local coordination environment around the silver and (c) to probe the glass structure and relate this to the results from S. aureus biofilm growth studies. The findings from this study may lead to the potential use of the phosphate-based glasses to deliver silver ions to combat S. aureus biofilm infections.

MATERIALS AND METHODS

Bacterial strain and growth

Staphylococcus aureus NCTC 6571 was routinely propagated on nutrient agar (Oxoid, Basingstoke, UK) at 37°C. Nutrient broth (Oxoid) was used as the medium for the constant depth film fermentor (CDFF) studies.

Preparation of silver-doped phosphate-based glasses

Phosphate-based glasses were produced using NaH$_2$PO$_4$ (BDH), P$_2$O$_5$ (Sigma), and CaCO$_3$ (BDH). For the production of silver-containing phosphate-based glasses, Ag$_2$SO$_4$ (BDH) was also used as shown in Table 1. The amount of chemicals required for particular composition were weighed and placed into a Pt/10%Rh crucible (Johnson Matthey, Royston, UK) when non-silver-containing glasses were produced, while a
vitreous silica crucible (Saint-Gobain Quartz, Tyne & Wear, UK) was used when silver-containing glasses were produced (this was done to avoid silver forming alloys with platinum). The crucible was then placed in a preheated furnace at 1100°C for 1 hour. The molten glass was then poured into graphite moulds, which had been preheated to 370°C. The glass samples were allowed to cool to room temperature, and the resulting glass rods were cut into discs (5 mm diameter and 2 mm thickness) by using a rotary diamond saw (Testbourne Ltd., Basingstoke, UK).

**Biofilm production**

A CDFF (University College Cardiff, Cardiff, UK), described previously by Mulligan et al. (30), was used for the production of biofilms. The CDFF which contains a stainless steel turntable can hold up to 15 polytetrafluoroethylene (PTFE) pans; each PTFE pan can hold 5 PTFE plugs. Discs, 5 mm in diameter, were placed on each plug and recessed to a depth of 300 µm. The PTFE pans were then inserted so that they were flush with the turntable. A cylindrical glass vessel and two stainless steel end plates encase the turntable. The top plate contains an air inlet port, to which two 0.2 µm Hepa-vent air filters (Fisher Scientific, Town and Country) were attached. It also contains three media inlet ports. Incoming medium (in this case nutrient broth) drips onto the rotating turntable and is distributed over the PTFE pans by two scraper blades. The scraper blades also serve to maintain the biofilms on the discs at the required depth, equal to the depth of the recess. The bottom plate contains a medium outlet port. The CDFF was sterilized in a hot air oven, using a temperature of 160°C for 1 h. During all experiments, the CDFF was incubated at 37°C. The turntable rotated at a speed of 3 rpm.
Viable counts

At various time intervals, pans were removed aseptically from the CDFF. Each pan was washed with 10 ml of phosphate-buffered saline (PBS; Oxoid). Discs containing biofilms were placed in 1 ml of PBS and vortexed for 1 min to remove the attached biofilms and to disperse them into the suspension. Serial dilutions of the suspensions were carried out in PBS. 25 µl volumes of the suspension and each dilution were spread onto nutrient agar (Oxoid) plates. The plates were then incubated aerobically at 37°C for 48 h. For each type of disc, viable counts (colony forming units; CFUs) were conducted in triplicate.

Scanning electron microscopy (SEM)

Aseptically removed discs were placed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer, to fix the cells, and stored at 4°C overnight. Specimens were then prepared for the scanning electron microscope by first dehydrating in a graded series of alcohols (20%, 50%, 70%, and 90%). The specimens were left in each alcohol for 15 min, before being rinsed three times in 100% alcohol (10 mins each time). Each specimen was then transferred into hexadimethylsilane for 2 mins prior to placing them in a desiccator. Once dry, the specimens were mounted onto aluminium stubs using araldite and sputter-coated with gold/palladium in a Polaron E5000 sputter coater. The specimens were then viewed with a Cambridge 90B SEM operating at 15 kV.

Confocal laser scanning microscopy (CLSM)

A viewing solution was first prepared containing 8ml of PBS together with 2 µl each of components A and B of BacLight™ LIVE/DEAD stain (Invitrogen, UK). The biofilm containing discs were placed into a small cell-culture dish (Bibby Sterilin Ltd, Stone, UK), and covered with the viewing solution and the stains were allowed to develop
in the dark for 10min. The biofilms were then examined via the microscope (Olympus BX51 microscope) which incorporated a Bio-Rad Radiance 2100 laser scanning system and LUMPlanFI 40x water lens. Two-channel (viable ‘Live’/nonviable ‘Dead’) confocal image stacks were collected in 8-bit colour depth at a resolution of 1024×1024 pixels. The z-axis step size was typically 0.6 µm, however this was optimised for each image stack depending upon the total depth of the sample.

**Image analysis**

The initial image analysis and 3D structure construction were performed using the Bio-Rad LaserVox™ image analysis software whilst the structure and distribution of cell vitality (19, 22) was elucidated using ImageJ (v1.33 u, National Institutes of Health, USA). Projection images (plan view) were constructed to return the sum of pixel brightness values through the entire image stack, effectively merging all the individual sections into one greyscale image. The depth-related trends of the viable and nonviable stains through the biofilms were determined by constructing fluorescence profiles. These profiles were created by plotting the total image brightness, for each channel, against depth into the image stack. These data were then normalised against the maximum brightness value within their channel and converted into depth related viability profiles by plotting the normalized viable fluorescence minus the normalised nonviable fluorescence values against depth into the biofilms (19, 20).

**Statistical analysis**

One-way analysis of variance (ANOVA) was used to compare mean viable counts, following arcsinh transformation of data. When a significant difference was detected, a
Tukey test was conducted to find which values were different (GraphPad Software; San Diego, USA.).

Glass degradation and ion release

Degradation study

Silver-doped phosphate-based glass rods (5 mm diameter and 2 mm thickness) with different contents of silver ions were placed in plastic containers and filled with 50 ml of deionised water (pH 7±0.5), and placed in an incubator at 37°C. At various time points (6, 24, 48, 120 and 144h) the three disks were taken out of their respective containers, and excess moisture was removed by blotting the samples dry with tissue prior to weighing them. All the disks were placed into a fresh solution of deionised water and placed back into the 37°C incubator. To obtain the rate of weight loss, the initial weight ($M_0$) of each sample was measured as well as the weight at time $t$ ($M_t$) to give a weight loss per unit area thus: weight loss=$\frac{M_0-M_t}{A}$, where $A$ is the surface area ($\text{mm}^2$). The measurements were carried out in triplicate. The data were plotted as weight loss per unit area against time. The slope of this graph gave a dissolution rate value in terms of mg.mm$^{-2}$ h$^{-1}$, which was determined by fitting a straight line of the form $y = mx$ through the origin.

Ion release study

Ion release studies were simultaneously conducted, and the medium was analysed for cation ($\text{Na}^+$ and $\text{Ca}^{2+}$) and anion ($\text{PO}_4^{3-}$, $\text{P}_2\text{O}_7^{4-}$, $\text{P}_3\text{O}_9^{5-}$ and $\text{P}_3\text{O}_{10}^{5-}$) release using ion chromatography (Dionex, UK). Silver ion release was measured using the commercially available silver test kit (Silver Test Kit 1.14831.0001, Merck, UK). The test works on the
principle that in a weakly acidic solution, silver ions react with phenanthroline and eosine to form a red complex, the concentration of which is determined photometrically (at 552 nm). A silver standard solution, 1000 mg/l Ag, provided by the supplier, was used to prepare the calibration curve. In the event of silver concentrations exceeding 5 mg/l, samples were diluted before measurement. For all samples tested, high purity water was used as a reference.

**Structural analysis of the silver-doped phosphate-based glasses**

**31P MAS NMR**

All 31P NMR experiments were performed using a Varian-Chemagnetics CMX 360 MHz Infinity spectrometer equipped with an 8.45 T magnet operating at a frequency of 145.85 MHz. A Varian 4 mm probe was used and the samples were spun at ~12 kHz. A standard one pulse experimental procedure was used. A single pulse of 1.4 µs (corresponding to a tip angle of 30°) and a recycle delay of 450 s were used due to the extremely long T1 relaxation time of ~360 s. The spectra were referenced against a secondary reference of NH4H2PO4 at a shift of +0.9 ppm (relative to 85 % H3PO4).

**High energy X-ray diffraction**

The high energy X-ray diffraction (HEXRD) data were collected on Station 9.1 at the Synchrotron Radiaton Source (SRS) Daresbury Laboratory, UK. The finely powdered samples were enclosed inside a 0.5 mm thick circular metal annulus by kapton windows and mounted onto a flat-plate instrumental set-up. The wavelength was set at λ = 0.5092 Å, and calibrated using the K-edge of a Pd foil; this value was low enough to provide
data to a high value of momentum transfer \( Q_{\text{max}} = 4\pi \sin \theta / \lambda \sim 22 \text{ Å}^{-1} \). The data were corrected using a collection of programs written in-house.

The initial stage of analysis of X-ray diffraction data from an amorphous material involves the removal of background scattering, normalization, correction for absorption and subtraction of the self-scattering term (13). The resultant scattered intensity, \( i(Q) \), can reveal structural information by Fourier transformation to obtain the pair distribution function:

\[
T(r) = T^0(r) + \int_{0}^{\infty} Q i(Q) \sin(Qr) dQ
\]

where \( T^0(r) = 2\pi^2 r \rho_o \) (\( r \) is the atomic separation between atoms and \( \rho_o \) is the macroscopic number density)

**Ag K-edge XANES measurements**

Ag K-edge X-ray absorption spectroscopy (XAS) measurements were made at a temperature of approximately 77 K on Station 16.5 at the SRS. The spectra were recorded in transmission mode using a double crystal Si(220) monochromator and ionisation chambers to detect the incident and transmitted beam intensities, \( I_i \) and \( I_t \) respectively. A silver foil and a third ionisation chamber were placed after the sample’s transmission ionisation chamber to allow an absorption spectrum of the foil to be collected simultaneously for the purpose of calibration of the energy scale. The energy scale was defined by assigning the maximum of the derivative of the Ag foil spectrum to 25521.0 eV.

The data processing comprised conversion of the data to absorption \textit{versus} energy, calibration of the energy scale, removal of the pre-edge absorption by straight-line fitting to \( \log_{10}(I_i/I_0) \) and removal of the post-edge atomic absorption profile by fitting with a
second order polynomial. All the spectra were normalised to have an edge-step of unity. Spectra were also collected from reference materials; AgO (Aldrich), and Ag$_2$SO$_4$ ($\geq$99.99%, Aldrich).

RESULTS

SEM analysis of the attachment of *S. aureus* to silver-doped phosphate-based glasses

SEM analysis of *S. aureus* biofilms on hydroxyapatite (HA), Ag-, Ag10, Ag15 and Ag20 discs showed reduction in *S. aureus* attachment on Ag10, Ag15 and Ag20, with Ag20 being the least, compared to Ag- and HA discs (data not shown).

Effect of increasing silver ion concentration on the viable counts of *S. aureus* in biofilms for 48 h

Initial viable count experiments were conducted on Ag10, Ag15 and Ag20 (Table 1). Both Ag- and HA discs were used as controls (Figure 1). Each point represents the log$_{10}$ of the mean number of viable count of three biofilms from one representative CDFF run. Error bars represent standard deviations. It should be noted that at least three runs for each experiment were performed to confirm the results found. The data were not pooled because slight differences in the inoculation produced differences in the absolute CFU numbers obtained. However, the relative differences found were very repeatable.

(a) Ag10

The Ag10 glasses showed no significant difference between the log$_{10}$ of the mean number of viable cells (6.08±0.11) compared to both Ag- (6.11±0.13) ($p=0.77$) and HA discs (6.19±0.12) ($p=0.11$) at 6h (Figure 1). However, at 24h, the Ag10 discs displayed statistically significant ($p \leq 0.001$) difference between the log$_{10}$ of the mean number of viable cells.
viable cells (4.65±0.17) compared to both the controls, Ag- (6.32±0.32) and HA discs (6.37±0.11). The log$_{10}$ of the mean number of viable cells at 48h on Ag10 discs (6.42±0.08) started to recover from the previous low at 24h, but was still less than both the controls, Ag- (8.21±0.06) ($p=0.0001$) and HA (7.96±0.33) ($p=0.001$). There was approximately a 1.2 log$_{10}$ reduction in CFUs for the Ag10 glasses compared to controls that were maintained for the first 48h.

(b) Ag15

Similar to the Ag10 glasses, the Ag15 glasses also showed no significant difference between the log$_{10}$ of the mean number of viable cells (6.02±0.20) compared to both the Ag- discs (6.12±0.13) ($p=0.51$) and HA discs (6.19±0.13) ($p=0.28$) at 6h (Figure 1). By 24 h, the Ag15 discs (4.03±0.11) displayed a statistically significant ($p \leq 0.0003$) difference in the log$_{10}$ of the mean number of viable cells compared to both the controls, Ag- (6.33±0.32) and HA discs (6.37±0.11). Similar to the Ag10 glasses, the log$_{10}$ of the mean number of viable cells at 48h on Ag15 discs (7.14±0.13) started to recover from the previous low at 24h but was still less than both the controls, Ag- (8.21±0.06) ($p=0.0002$) and HA discs (7.96±0.33) ($p=0.002$). There was an approximately 1.5 log$_{10}$ reduction in CFUs maintained for the first 48h by Ag15 glasses compared to the controls.

(c) Ag20

Only the Ag20 glasses showed a statistically significant difference in the log$_{10}$ of the mean number of viable cells (5.44±0.24) compared to both the Ag- (6.11±0.13) ($p=0.015$) and HA discs (6.19±0.13)($p=0.01$) at 6h (Figure 1). As with the other glasses, the log$_{10}$ of the mean number of viable cells at 24h on the Ag20 discs (5.10±0.04) started
to recover from the previous low at 6h, but showed a statistically significant difference to both the controls, Ag- (6.33±0.32) \( (p=0.003) \) and HA discs (6.37±0.11) \( (p=0.0001) \). After 48 h, the Ag20 discs (7.26±0.21), displayed a significant difference in CFUs compared to the controls, the Ag- (8.21±0.06) \( (p=0.002) \) and HA discs (7.96±0.33) \( (p=0.036) \). There was an approximately 1 log\(_{10}\) reduction in CFUs that was maintained for the first 48 h by the Ag20 glasses compared to the controls.

Due to the early onset of a bactericidal effect (from 6h of biofilm growth) by the Ag20 glasses, it was chosen along with Ag15 glasses (which displayed maximum bactericidal effect at 24 h of biofilm growth compared to other glasses) for the second set of CDFF studies with time points up to 144h.

**Effect of Ag15 glasses on viable counts of *S. aureus* biofilms up to 144h**

In the second set of experiments, the Ag15 glasses showed no significant difference in the log\(_{10}\) of the mean number of viable cells (5.79±0.08) compared to the Ag- discs (5.97±0.11) \( (p=0.084) \), but a statistically significant difference from HA discs (6.36±0.19) \( (p=0.009) \) at 6h (Figure 2). The difference in CFUs became more apparent at 24h as the Ag15 (4.56±0.2) displayed a statistically significant \( (p \leq 0.002) \) difference in the log\(_{10}\) of the mean number of viable cells compared to both the controls, the Ag- (7.29±0.09) and HA discs (7.02±0.57). After 48 h, the log\(_{10}\) of the mean number of viable cells on the Ag15 (6.80±0.49) started to recover from the previous low at 24h, but was still less than both the controls, the Ag- (7.85±0.15) \( (p=0.024) \) and HA discs (7.9±0.15) \( (p=0.021) \). However, at time points > 48 h, the Ag15 glasses showed a sharp increase in the CFUs compared to the controls and the log\(_{10}\) CFU for all samples reached
values similar to those for the controls by 120h and remained similar, at approximately 8.3 $\log_{10}$ CFU, until 144 h (Figure 2).

**Effect of Ag20 glasses on viable counts of *S. aureus* biofilms up to 144h**

The Ag20 glasses showed the greatest difference in the $\log_{10}$ of the mean number of viable cells (5.33±0.20) compared to both the Ag- (6.31±0.11) ($p=0.002$) and HA discs (6.23±0.16) ($p=0.004$) at 6h (Figure 3). As in the first set of CDFF runs, the $\log_{10}$ of the mean number of viable cells at 24 on the Ag20 (5.66±0.07) discs started to recover from the previous low at 6h, but showed a significant difference to both the controls, the Ag- (7.±0.18) ($p=0.0003$) and HA discs (7.23±0.03) ($p=0.0001$). This effect continued at 48h with the Ag20 discs (7.57±0.16), displaying a significant difference to both the controls, the Ag- (8.27±0.03) ($p=0.002$) and HA discs (8.42±0.05) ($p=0.0009$). Compared to the Ag15 glasses, at 120h, the $\log_{10}$ CFUs on the Ag20 glasses stayed at a reduced level (8.05±0.24) compared to the Ag- discs (8.51±0.19) ($p=0.03$), but did not exhibit any statistically significant difference from HA discs (8.59±0.40) ($p=0.096$). More importantly, at longer time points, the $\log_{10}$ CFUs stayed at a reduced level, approximately 0.6 $\log_{10}$ CFU reductions, compared to both the controls ($p \leq 0.005$) even until 144 h (Figure 3).

**Identification of dead bacterial layers using CLSM**

The use of water immersion lenses and a liquid viewing medium (PBS) in the present study enabled the observation of biofilms in their natural hydrated state (Figure 4). Viability mapping, as described by Hope et al. (20), which encompasses viability changes in the z axis was performed (Figure 5). As seen in normal viewing of BacLight™ LIVE/DEAD stained images, in the present study, the viable cells fluoresce green and the
nonviable cells fluoresce red (Figure 4). The biofilms were submerged in the stains (at a relatively high concentration) for at least 15 min before the CLSM scan. The molecular weights of the BacLight™ LIVE/DEAD stain components are similar (component A= 550–750 Da (proprietary information) and component B = 668.4 Da), and both have a net positive charge. It is therefore unlikely that there is any significant difference in their diffusion characteristics into biofilms. The viability distributions in the biofilms were observed in this study using CLSM image analysis. Regions of biofilms composed of viable bacteria with a layer of non-viable bacteria at the interface with the antimicrobial-releasing materials were analysed further (Figure 5).

Viability mapping

Depth-related viability profiles (Figure 5) through the 2 day-old biofilms returned positive values (i.e. increasing) in the upper ~20 µm of the confocal image stack. This indicated that the proportion of viable fluorescence, compared to nonviable fluorescence, increased with depth (i.e. the vertical distance into the biofilm from its highest point) in this region. Between a depth of ~20 and ~40µm, the viability profile values decreased, suggesting that the proportion of viable fluorescence decreased. Moreover, at these depths the viability profiles values fluctuated from low to high. This may be because the confocal laser/fluorophore emissions becoming absorbed by the biofilm, causing a corresponding reduction in the brightness of the optical sections.

Degradation and ion release of silver-doped phosphate-based glasses

The degradation rates obtained, by applying a line of best fit through the weight loss per unit area of each glasses against time (data not shown), for the Ag10, Ag15 and Ag20
glasses were 1.22, 0.41 and 0.42 µg.mm$^{-2}$.h$^{-1}$ respectively (Figure 6). Both the Ag15 and Ag20 glasses showed no perceptible differences in their degradation rate profiles (Figure 6). However, the profile of the Ag10 glasses did exhibit an increased degradation rate compared to Ag15 and Ag20 glasses. The result showed that rate of silver ion release is correlated to rate of degradation with statistically significant ($p \leq 0.005$) difference between Ag10 compared to Ag15 and Ag20 but no significant difference ($p \geq 0.692$) between Ag15 and Ag20 glasses (Figure 6). Rate of release of other cations such as Na$^+$ displayed statistically significant ($p \leq 0.016$) difference between Ag10 compared to Ag15 and Ag20 but no statistical difference ($p \geq 0.666$) between Ag15 and Ag20 glasses (Figure 6). Similarly, with Ca$^{2+}$ ion, statistically significant ($p \leq 0.0001$) difference was observed only between Ag10 compared to Ag15 and Ag20 but not between Ag15 and Ag20 glasses ($p \geq 0.167$). Among the anions (PO$_4^{3-}$, P$_2$O$_7^{4-}$, P$_3$O$_9^{3-}$ and P$_3$O$_{10}^{5-}$), P$_3$O$_9^{3-}$ was the anion released to the greatest extent and it was also found to correlate strongly with rate of degradation of the glasses (Figure 6). As in the case of cations, the rate of P$_3$O$_9^{3-}$ ion release showed statistically significant ($p \leq 0.016$) difference between Ag10 compared to Ag15 and Ag20 but no significant difference ($p \geq 0.666$) between Ag15 and Ag20 glasses.

Due to the importance of silver release in this study, the actual amount of silver ion released at each time point is highlighted in figure 7. As expected, no silver was detected from the Ag- glasses throughout the silver release study. At 6h, there were no significant difference ($p \geq 0.066$) in silver ion release among Ag10, Ag15 and Ag20 glasses (Figure 7) which continued up to 48h between Ag10 and Ag20 glasses ($p \geq 0.078$). Ag20 released higher amounts of silver at 24 and 48h compared to Ag15, but there were no significant
difference in the silver ion release at 120 and 144h between Ag15 and Ag20 glasses (p≥0.09). However, from 48h onwards the Ag10 glasses released the highest amount of silver ions compared to both Ag15 and Ag20 glasses (Figure 7).

Structural analysis of the silver-doped phosphate-based glasses

Examining the results in Figure 1 for the bactericidal effectiveness of the silver-doped phosphate-based glasses on S. aureus biofilms after 48 h, excellent correlation with the silver release curve shown in Figure 6 was found: above 10 mol% silver, there was a reduction in both the bactericidal activity and silver ion release. The reduction in silver ion release also correlated with a flattening out of the rate of degradation curve in Figure 6, where the expected (on the basis of the relative solubilities of sodium and silver salts) reduction in dissolution rate with increasing substitution of silver ions for sodium ions did not continue above 15 mol%. In order to understand the variation of properties with silver content, the structure of the glass was examined using, $^{31}$P MAS NMR, high-energy XRD and Ag K-edge XANES.

The structure of phosphate glasses is known to consist of PO$_4^{3-}$ tetrahedra connected together by between 1 and 3 bridging oxygen atoms (BOs) to form a network (8). The connectivity of this phosphate network is commonly described by Q$^n$ notation, where $n$ refers to the number of BOs in the PO$_4^{3-}$ group. Thus a Q$^3$ PO$_4^{3-}$ unit has 3 BOs to other PO$_4^{3-}$ units and one non-bridging oxygen (NBO), whereas a Q$^0$ PO$_4^{3-}$ unit has 4 NBOs and is unconnected to other PO$_4^{3-}$ groups. This connectivity is affected by the glass composition. Vitreous P$_2$O$_5$ has a structure composed entirely of Q$^3$ units; whereas
addition of metal oxides to phosphate glasses reduces this connectivity and introduces \( Q^1 \) and \( Q^2 \) groups into the structure.

In the \(^{31}\text{P} \) MAS NMR spectra for the silver-doped phosphate-based glasses, shown in Figure 8, the single most prominent peak observed occurs at a chemical shift of -27 ppm and is assigned to \( Q^2 \) groups (26). Two weaker resonances are also observed at -6 and -37 ppm; the latter manifests itself as a broad tail on the low chemical shift side of the main peak. They are assigned to the presence of phosphorus in \( Q^1 \) and \( Q^3 \) environments, respectively. The presence of \( Q^1 \) and \( Q^3 \) environments in samples containing \( \geq 10 \text{ mol\%} \) silver is indicative of disproportionation of \( Q^2 \) units according to the equation, \( 2Q^2 \rightarrow Q^1 + Q^3 \). In other words, \( Q^2 \) groups, which are the structural units that make up phosphate chains and rings, are converting to \( Q^1 \) units, which represent \( \text{P}_2\text{O}_7 \) dimers and chain-terminating phosphate groups, and \( Q^3 \) groups, which represent cross-linking between the phosphate chains. Examining Figure 8, it can be seen that the intensity of the \( Q^1 \) and \( Q^3 \) features increases with silver content, suggesting a structural change in the glass that occurs as a function of silver content. The \(^{31}\text{P} \) NMR results therefore show that as the silver content of these glasses increases, there is a structural change from phosphate rings and polymeric chains to shorter, more cross-linked chains.

The HEXRD pair-distribution functions shown is Figure 9 give information on the average P-O bonding in the glasses. The peak centred at \( \sim 1.55 \) Å in these functions is composed of two components: a shorter distance of \( \sim 1.49 \) Å due to P-NBO bonds and a longer distance of \( \sim 1.60 \) Å P-BO bonding (8). It can be seen from Figure 9 that the shape of the P-O peak in the samples studied here changes as the silver content increases (10 → 20 mol%). This change reflects a change in the distribution of BOs and NBOs between
phosphorus atoms as a function of silver content, consistent with $Q^2$ groups

disproportionating into $Q^1$ and $Q^3$ groups. In agreement with the $^{31}$P NMR data, this result
suggests a change in the connectivity of the phosphate network with higher silver
loadings.

The Ag K-edge XANES measurements yield information on the oxidation state of silver
and its local structural environment. The XANES spectra from the three silver-doped
phosphate glasses studied here were identical, demonstrating that the oxidation state and
local environment of silver is the same in each. For this reason only the spectrum from
the Ag10 glass is shown in Figure 10. The Ag K-edge XANES spectra from the reference
compounds are also shown in Figure 10. The position of the X-ray absorption edge in
each spectrum contains information on the oxidation state of the silver present. The edge
position of AgO, which contains a mixture of Ag$^{\text{I}}$ and Ag$^{\text{III}}$ ions (28), appears at the
highest energy since it requires more energy to remove electrons from the higher valence
ions. The absorption edges of Ag$^{\text{I}}$ compounds appear at lower energy. The edge positions
of the silver-doped phosphate glasses all overlay the edge position of Ag$_2$SO$_4$. Given that
Ag$_2$SO$_4$ is a Ag$^{\text{I}}$ compound, this result suggests that the silver in the glasses is present as
Ag$^{\text{I}}$. The similarity in the shape of the XANES spectra from the phosphate glasses and
that from Ag$_2$SO$_4$ suggests that the structural environment of silver in the glasses is
similar to that in the sulphate. Since Ag$_2$SO$_4$ contains silver ions surrounded by a
distorted octahedron of oxygen atoms (16), it follows that the silver ions in the
phosphate-based glasses reside in a very similar environment.
DISCUSSION

Previous work suggests that silver-doped phosphate-based glasses, with a fixed phosphate content of 50 mol % and a fixed calcium oxide content of 30 mol %, are capable of broad-spectrum bactericidal activity against planktonic bacteria including *S. aureus* (2). However, in a biofilm environment, microbes exhibit reduced susceptibility to antimicrobial agents. Silver has shown to be bactericidal against *Streptococcus sanguis* biofilms when phosphate-based glasses were used as a means of delivering the ions in a controlled manner (30). The results of the present study show that the release of an optimal amount of silver ions from the silver-doped phosphate-based glasses that can cause significant reduction of *S. aureus* biofilm growth occurs in 24h. From this point onwards the silver ions released from the glasses did not prevent the re-emergence of viable bacteria from the biofilms (Figure 1). Moreover the CLSM analysis confirmed the production of a dead bacterial layer at the interface between the biofilm and the silver-releasing phosphate-based glasses (Figure 5b).

Chaw et al. (11) reported that low concentrations of silver ions are unsuitable for the treatment of biofilm infections. Although higher silver concentrations have increased effectiveness against sessile cells (3, 30), they nevertheless face the challenge of maintaining their ionic form in applications containing large amount of halides and other ions (e.g. Cl⁻, HCO₃⁻ and CO₃⁻) and proteins (24, 35) due to the production of the insoluble silver salts, which results in silver ion inactivity. As silver ions are highly reactive and bind strongly to the electron donor groups containing oxygen or nitrogen (36) in the extracellular matrix (EM), we suggest that they must be able to bind to molecules such as proteins and polysaccharides within the EM. Therefore it is plausible
that the formation of a dead bacterial layer (Figure 5b) at the interface with the silver releasing phosphate-based glasses resulted in re-emergence of viable bacteria after 24h growth of *S. aureus* in the present study. Other factors, such as the diffusion limitation of silver ions from the phosphate-based glasses or the switching on/off of quorum sensing signals that triggered the efflux pump, which protected *S. aureus* from the toxic silver ions need to be addressed.

Viability mapping can be used to examine the penetration of the bactericidal effect of antimicrobial compounds into biofilms. Whilst it is obviously useful to make direct measurements of the penetration of the antimicrobial compound itself (usually by microelectrodes) into the biofilm, it is the penetration of the antimicrobial effect that is of greater importance with regard to the remediation of the most recalcitrant microbial biofilms (22). The fidelity of BacLight™ LIVE/DEAD stain does not allow one to categorically state that an individual cell which has taken up the nonviable stain (propidium iodide) is unable to reproduce in culture (4). However it is sufficient to allow us to visualize gradients in the spatial distribution of these stains and interpret these motifs as indicators of gradients in cell vitality. In a recent study Beyenal et al. (7) used an optical microsensor to probe biofilms of *S. aureus* which were labelled with a yellow fluorescent protein. The microsensor measured fluorescence in the biofilms directly and reported depth-related profiles similar to the bell-curves obtained by CLSM (21). This suggested that metabolic activity (vitality) increases with depth in the outer layers of a biofilm before decreasing in the deeper regions.

Although high concentrations of free silver ions are needed for bactericidal action against biofilms, it is very important not to sacrifice any cyto/biocompatibility aspects of the
material while maintaining an effective antimicrobial effect. The amount of silver released from silver-doped glasses investigated in this study is well below the levels that are cytotoxic for human cells (33). The report suggested that the minimum bactericidal concentration of silver is 0.1 ppm, and the cytotoxic concentration is 1.6 ppm for human cells (33). The actual amounts quantified from the profiles observed in Figure 7, were respectively 0.083, 0.055 and 0.064 ppm.h\(^{-1}\) for the Ag10, Ag15 and Ag20 glasses. All within the limits specified above. However, it must be noted that it was unclear if the levels of 0.1 ppm and 1.6 ppm stated by Saravanapavan et al. (33) were total values, or whether they were rates in hours or days etc.

The structural analysis using \(^{31}\text{P}\) NMR revealed that \(Q^2\) species are the dominant structural unit in the glasses investigated. This agrees with the predicted model for metaphosphate glasses (i.e. 50 mol% \(\text{P}_2\text{O}_5\)) where the network should be based exclusively on \(Q^2\) tetrahedra, forming chains and/or rings (1, 8). Recently it was found that the phosphate network was unaltered by exchanging sodium with silver for up to one quarter of the initial sodium content (Ahmed et al. unpublished results). This also correlated well with the XRD studies where the crystalline phase identified after annealing the glass at glass crystallisation temperature, \(T_c\), was a cyclic \(Q^2\) species (namely, \(\text{P}_3\text{O}_9\)).

As can be observed from the dissolution profiles in Figure 6, for silver-doping levels above 10 mol%, the rate of release of silver ions decreases and the overall degradation of the glass stabilises. This change can be correlated with a structural change that can be observed in the \(^{31}\text{P}\) NMR and HEXRD results. This change is related to a rearrangement of the phosphate network from \(Q^2\) chains and rings to shorter, more branched chains as
indicated by the presence of increasing amounts of $Q^1$ and $Q^2$ species with increasing silver content. The Ag K-edge XANES spectra from the Ag10, Ag15 and Ag20 glasses are all identical confirming that there is no change in the silver oxidation state or local environment as a function of silver content. Given this, we can conclude that it is the structural rearrangement of the phosphate network that is responsible for the variation in silver ion release and associated bactericidal effectiveness. The literature shows that silver in its +1 oxidation state is highly effective against planktonic bacteria (6, 15, 23). The Ag K-edge XANES spectra from the glasses studied here confirm that the silver is present as $\text{Ag}^1$ in all three compositions.

Apart from the current applications, such as coating of a catheter with silver ions to avoid bloodstream infections (12, 39), a strategy of using the silver ions' bactericidal effect on biofilms in combination with other antimicrobial ions, such as copper zinc or gallium, can be explored for the future testing of antimicrobial effectiveness. This synergistic approach may work well with the silver ions destabilizing the biofilm matrix with other antimicrobial ions and subsequently killing the bacteria.

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REFERENCES


FIGURE LEGENDS

Figure 1. Log_{10} CFU/mm^2 of *S.aureus* in biofilms formed on HA, Ag-, Ag10, Ag15 and Ag20 discs.

Figure 2. Log_{10} CFU/mm^2 of *S. aureus* in biofilms formed on HA, Ag-, and Ag15 discs.

Figure 3. Log_{10} CFU/mm^2 of *S. aureus* in biofilms formed on HA, Ag-, and Ag20 discs.

Figure 4. CLSM images after 48h of *S. aureus* biofilms on (a) Ag- (b) Ag10 (c) Ag15 and (d)Ag20 discs. Viable (green) and non-viable (red) bacteria.

Figure 5. A viability profile through 2 day old biofilms grown on Ag20 discs (a). The y-axis is the normalised viable minus nonviable fluorescence values of 3 separate viability profiles. These data were further normalised to a range of 0 to 1. A cross-sectional view of the biofilms part used for viability profiling (b).

Figure 6. Relationship between cation and anion release rates, and rate of degradation of silver-doped phosphate glasses as a function of silver content.

Figure 7. Cumulative silver ion release vs. time for Ag-, Ag10, Ag15 and Ag20 glass compositions investigated.

Figure 8. ^31^P MAS NMR spectra of the Ag-, Ag10, Ag15 and Ag20 glasses (a). A highlight of the prominent peak (b)

Figure 9. HEXRD pair-distribution functions of phosphate-based glasses Ag10 (solid line), Ag15 (dashed line) and Ag20 (dotted line) showing the peak due to P-O bonding.

Figure 10. Ag K-edge XANES spectra: Ag10 glass (solid line) Ag_2SO_4 (dashed line) and AgO (dotted line).
Table 1. Composition of phosphate-based glasses used in this study

<table>
<thead>
<tr>
<th>Glass code</th>
<th>Glass code used in the text</th>
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<tr>
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<td>30</td>
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Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.

(a)

(b)

Live-Dead (normalised fluorescence) vs. Depth (µm)
Figure 6.
Figure 7.
Figure 8.
Figure 9.
Figure 10.