Structure, dissolution behavior, cytocompatibility, and antibacterial activity of silver-containing calcium phosphate invert glasses

Sungho Lee,1 Takayoshi Nakano,1 Toshihiro Kasuga2
1Division of Materials and Manufacturing Science, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka, 565-0871, Japan
2Division of Advanced Ceramics, Graduate School of Engineering, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya, 466-8555, Japan

Received 15 May 2017; revised 29 June 2017; accepted 1 August 2017
Published online 24 August 2017 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.36173

Abstract: Novel CaO–P2O5–Nb2O5–Ag2O invert glasses with substitution Ag2O for Nb2O5 were successfully prepared using a melt-quenching method. Ag2O in the glasses act as a network modifier oxide, playing the same role as Na2O, which breaks the phosphate chains. Analysis of the ultraviolet-visible absorption spectra of the glasses showed that the glass matrix contained ionic silver species and silver nanoparticles. Approximately 0.05 mM of Nb5+ ions released from the glasses, which would be expected to stimulate osteoblast differentiation. A glass containing 1 mol % Ag2O showed a linear increase in the releasing amount of Ag+ ions with increasing soaking time, whereas glasses containing 3–5 mol % Ag2O showed Ag+ ion concentrations of around 13 μM at day 3, and then maintained similar values until day 7. When the solution was replaced with fresh solution every 2 days, the Ag+ ion dissolution amounts indicated almost constantly 13 μM due to AgCl formation. There were no differences in the numbers of primary osteoblast cells on silver-free and silver-containing glasses after cultivation for 1–7 days. The silver-containing calcium phosphate invert glasses showed cytocompatibility with simultaneous antibacterial activity to Escherichia coli and Staphylococcus aureus. © 2017 The Authors. Journal of Biomedical Materials Research Part A Published by Wiley Periodicals, Inc. J Biomed Mater Res Part A: 105A: 3127–3135, 2017.

How to cite this article: Lee S, Nakano T, Kasuga T. 2017. Structure, dissolution behavior, cytocompatibility, and antibacterial activity of silver-containing calcium phosphate invert glasses. J Biomed Mater Res Part A 2017:105A:3127–3135.

INTRODUCTION

Implant infections are very common and account for a large majority of post-surgical complications. The incidence of biomaterial-centered infections has been reported at 5–10% for dental implants, 5–10% for fixation pins or screws, 2–4% for prosthetic hips, and 3–4% for prosthetic knees.1 These infections are caused by the colonization of bacteria on the biomaterial surfaces.2 Since antimicrobial agents are not effective against bacterial biofilms on material surfaces3; as a result, implant removal often represents the only chance to erode the infection.4 The occurrence of biomaterial-centered infection could potentially be reduced by applying an antibacterial coating on the material surfaces.5 However, the antibacterial activity of the coatings must be maintained for up to 2 to 3 weeks after the implantation to be effective.1

Upon the controlled degradation of the coating, active chemical cues can be released to induce antibacterial activity. Ag+ ions show a wide range antibacterial activities against both gram-positive and gram-negative bacteria, even when applied at low concentrations.6 Consequently, silver is one of the most widely used materials in the development of antibacterial substances; for instance, Ag+ ions are clinically used for treating severe burns or as an active coating for catheters.7,8 Feng et al. suggested the bactericidal mechanism of Ag+ ions, which condense the DNA molecules in bacteria so that they lose their replication abilities; the ions also interact with thiol groups in proteins to inactivate the enzyme activity of bacteria.9 However, Ag+ ions also show some toxicity. The half-maximal (50%) inhibitory concentrations of Ag+ ions were reported to be 2.77 × 10−6 mol/L and 4.25 × 10−6 mol/L for murine osteoblastic (MC3T3-E1) cells and murine fibroblasts (L929), respectively.10 Thus, it is important that the coating material, which carries the Ag+ ions, has excellent chemical durability with controlled degradation behavior.

Such behavior can be achieved with the use of niobium-containing phosphate invert glasses (Nb-PIGs).11,12 Nb-PIGs consist of short phosphate groups such as ortho- and pyrophosphates (Q2 and Q3), which are cross-linked with NbO4 tetrahedra.11,12 Indeed, the addition of niobia was shown to significantly improve the chemical durability of phosphate invert glasses.11,13 Specifically, the total amount of ions released from Nb-PIGs on day 7 decreased from 7.5% to...
2.5% with an increase of the Nb2O5 content from 3 to 10 mol \%. In addition, Nb-PIGs could stimulate the differentiation of MC3T3-E1 cells via the niobate ions dissolved from the glasses.

Radiofrequency magnetron sputtering (RF-sputtering) could be used to coat implants with Nb-PIGs. For example, an amorphous calcium phosphate (ACP) film was successfully deposited onto titanium using RF-sputtering. The tensile bonding strength between the ACP film and titanium was >60 MPa when the film thickness was 0.5–1 \( \mu \)m. In addition, the ACP film had a smooth surface, which could help to prevent the colonization of bacteria. However, the bonding strength of the ACP film on titanium decreased to 30 MPa after immersing in phosphate-buffered saline for 3 days, indicating the low chemical durability of the ACP film. Thus, given their superior chemical durability compared to ACP, Nb-PIGs could potentially be excellent candidates for the deposition of amorphous films using an RF-sputtering method. In addition, antibacterial activity can be achieved by incorporation of antibacterial inorganic ions to the Nb-PIGs.

We predicted that the addition of Ag2O to Nb-PIGs would result in antibacterial activity, and that the amount of Ag\(^+\) ion dissolution amount would be easily tunable by modulating the glass composition. In this study, new types of CaO-P2O5-Nb2O5-Ag2O invert glasses were successfully developed by substituting Ag2O for Nb2O5, and their structures and dissolution behaviors were evaluated in a Tris buffer solution (TBS). The cytocompatibilities of the glasses were examined using primary osteoblasts isolated from newborn mouse calvariae, and their antibacterial properties were examined using Escherichia coli and Staphylococcus aureus.

**MATERIALS AND METHODS**

**Preparation of the glasses**

Glasses with compositions of 60CaO-30P2O5-(10-x) Nb2O5-xAg2O (mol %, \( x = 0-5 \), denoted by xAg) and 60CaO-30P2O5-5Nb2O5:5Na2O (mol %, denoted by 5Na) were prepared with CaCO3 (99.5%, Kishida Chemical), H3PO4 (85% liquid, Kishida Chemical), Nb2O5 (99.9%, Kishida Chemical), Ag2O (99.0%, Wako Pure Chemical), and Na2CO3 (99.5%, Kishida Chemical). The reagents were mixed with distilled water into a slurry, which was dried under an infrared lamp overnight and stored at 140°C. The mixture was melted in a platinum crucible at 1500°C for 30 min and then quenched by pressing between two stainless steel plates. The composition of xAg was determined by energy dispersive X-ray spectroscopy (EDX, JED-2300, JEOL) and is expressed as the average of three samples.

**Characterization of the glass structure**

The glass transition temperature (\( T_g \)) and crystallization temperature (\( T_c \), defined as the onset of crystallization) of xAg were determined from differential thermal analysis (DTA, heating rate: 5 K/min, Thermoplus TgB120, Rigaku). The glassification degree (\( GD \)), as an indicator of the glass-forming ability, was calculated as follows:

\[
GD = \frac{T_c - T_g}{T_c} \left( \frac{K}{R} \right)
\]

The glass structures were evaluated by Fourier-transform infrared spectroscopy (FT-IR, FT/IR-4100, JASCO) in the region between 500 and 1400 cm\(^{-1}\), using a KBr pellet method. Solid-state \( ^{31}P \) magic-angle spinning nuclear magnetic resonance (MAS-NMR, Varian UNITY Inova 400 plus) spectra were constructed at resonance frequencies of 161.906 MHz with 8-mm rotor spinning at 5 kHz. A single-pulse experiment was performed with a 5-\( \mu \)s pulse width, 60-s recycle delay, and cumulated number of 64. The chemical shift was analyzed in reference to the signal of ammonium dihydrogen phosphate (1.0 ppm). The ultraviolet-visible spectra (UV-Vis, V-550, JASCO) were measured for the silver-doped glasses (1Ag, 3Ag, 5Ag), using the spectrum of non-doped glass (0Ag) as a reference. Polished glasses with a size of 5 \( \times \) 5 \( \times \) 0.3 mm\(^2\) were scanned in the range of 300–600 nm (scan speed: 100 nm/min). Cross-sectional views of 3Ag were observed with a transmission electron microscope (TEM, JEM-2100 F, JEOL) at an acceleration voltage of 200 kV. The sample was prepared by milling with a focused ion beam (FIB, JEM-9320FIB, JEOL).

**Ion dissolution in TBS**

Glass powders were obtained by grinding and sieving (125–250 \( \mu \)m). The dissolution behavior of xAg was evaluated by immersing 15 mg of the glass powders into 15 mL of a 50 mM TBS (pH 7.40, 37°C) for 7 days. The concentrations of Ca\(^{2+}\), phosphate, Nb\(^{5+}\), and Ag\(^+\) ions in TBS were measured with TBS recycle delay, and cumulated number of 64. The chemical shift was analyzed in reference to the signal of ammonium dihydrogen phosphate (1.0 ppm). The ultraviolet-visible spectra (UV-Vis, V-550, JASCO) were measured for the silver-doped glasses (1Ag, 3Ag, 5Ag), using the spectrum of non-doped glass (0Ag) as a reference. Polished glasses with a size of 5 \( \times \) 5 \( \times \) 0.3 mm\(^2\) were scanned in the range of 300–600 nm (scan speed: 100 nm/min). Cross-sectional views of 3Ag were observed with a transmission electron microscope (TEM, JEM-2100 F, JEOL) at an acceleration voltage of 200 kV. The sample was prepared by milling with a focused ion beam (FIB, JEM-9320FIB, JEOL).

**Cytocompatibility of the glasses**

Polished glasses with a size of 7 \( \times \) 7 \( \times \) 0.5 mm\(^3\) were prepared for cell culture tests and dry-sterilized at 180°C for 90 min. The cells were cultured in alpha-minimum essential medium (α-MEM, with l-glutamine and phenol red, Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen). The sterilized glass plates were placed into 24-well plates (n = 4), and primary osteoblasts were seeded by adding 1 mL of the culture medium containing cells at a concentration of 4 \( \times \) 10\(^4\) cells/mL. Primary osteoblasts were isolated from newborn mouse calvariae as described in our previous report. In brief, calvariae from newborn C57BL/6 mice were excised under aseptic conditions, placed in ice-cold α-MEM, and then the fibrous tissues around the bone were gently removed. The calvariae were then subjected to a series of collagenase (Wako Pure Chemical)/trypsin (Nacalai Tesque) digestions at 37°C for 15 min each. The first two digests were discarded, and the supernatants of digests 3–5
were neutralized with α-MEM and pooled. The pooled solutions were filtered using a 100-μm mesh. The filtrates were

Table 1. Nominal and Analyzed Compositions (mol %) of xAg and Glass Codes. The Analyzed Compositions are Shown in Parentheses with Standard Deviations

| Glass code | CaO | P2O5 | Nb2O5 | Ag2O
|------------|-----|------|-------|-----|
| 0Ag | 60 | (61.0 ± 1.8) | 30 | (9.0 ± 0.2) | 10 | - |
| 1Ag | 60 | (57.2 ± 2.0) | 30 | (6.5 ± 0.8) | 9 | 1 |
| 2Ag | 60 | (56.9 ± 0.8) | 30 | (5.5 ± 0.5) | 7 | 2 |
| 3Ag | 60 | (56.4 ± 1.1) | 30 | (5.9 ± 0.7) | 6 | 3 |
| 4Ag | 60 | (56.9 ± 0.5) | 30 | (4.0 ± 0.1) | 5 | 4 |
| 5Ag | 60 | (56.8 ± 0.4) | 30 | (3.0 ± 0.1) | 5 | 5 |

were neutralized with α-MEM and pooled. The pooled solutions were filtered using a 100-μm mesh. The filtrates were

Be and the results Tg, Tc, and GD values for xAg, Tg decreased linearly with increasing Ag2O content; the Tg and Tc values decreased from 659°C to 589°C and from 749°C to 669°C, respectively. The GD values did not significantly differ with respect to the Ag2O content, with a value of approximately 0.09 across samples. The Tg, Tc, and GD values of all glasses were 614°C, 673°C, and 0.07, respectively.

Figure 2 shows the results for the Ag2O contents of 4 and 5 mol %, the asymmetric stretching mode of the chain-terminating Qp group (νas(P-O-P), 920 cm⁻¹). The asymmetric vibration mode of the Qp group (νas(P-O-P), 1050 cm⁻¹), the asymmetric stretching vibration of the Qp group (νas(P-O-P), 1000 cm⁻¹), and the symmetric stretching vibrations of O-P-O bridges of the Qp group (νas(P-O-P), 920 cm⁻¹), and the symmetric stretching vibrations of O-P-O bridges of the Qp group (νas(P-O-P), 740 cm⁻¹). The niobate group shows the FT-IR bands corresponding to the asymmetric stretching vibration of the Nb-O-Nb bridges of distorted NbO6 octahedra (νas(Nb-O), 635 cm⁻¹) and the asymmetric

### RESULTS

#### Glass structures

As quenched Ag2O containing glasses were yellowish optically clear and partially crystallized. The crystalline and opaque parts were removed manually by breaking the glasses. Following experiments, optically clear parts were used. The analyzed compositions of xAg are listed in Table 1. The amounts of Ag2O were reasonably close to that of the nominal composition, and the amounts of CaO, P2O5, and Nb2O5 varied by approximately 3.6, 5.6, and 2.5 mol %, respectively. Since the phosphorus Kα (2013 eV) and niobium Lα (2166 eV) peaks overlapped, their compositions may cause mismatch from the nominal ones. The powder X-ray diffraction patterns of xAg showed halo peaks (Supporting Information Fig. S1), which indicated that the materials were amorphous.

Figure 2 shows the results for the Ag2O contents of 4 and 5 mol %, the asymmetric stretching mode of the chain-terminating Qp group (νas(P-O-P), 920 cm⁻¹), the symmetric vibration mode of the Qp group (νas(P-O-P), 1050 cm⁻¹), the asymmetric stretching vibration of the Qp group (νas(P-O-P), 1000 cm⁻¹), and the symmetric stretching vibrations of O-P-O bridges of the Qp group (νas(P-O-P), 920 cm⁻¹), and the symmetric stretching vibrations of O-P-O bridges of the Qp group (νas(P-O-P), 740 cm⁻¹). The niobate group shows the FT-IR bands corresponding to the asymmetric stretching vibration of the Nb-O-Nb bridges of distorted NbO6 octahedra (νas(Nb-O), 635 cm⁻¹) and the asymmetric

---

**TABLE I. Nominal and Analyzed Compositions (mol %) of xAg and Glass Codes. The Analyzed Compositions are Shown in Parentheses with Standard Deviations**

| Glass code | CaO | P2O5 | Nb2O5 | Ag2O |
|------------|-----|------|-------|-----|
| 0Ag | 60 | (61.0 ± 1.8) | 30 | (9.0 ± 0.2) | 10 | - |
| 1Ag | 60 | (57.2 ± 2.0) | 30 | (6.5 ± 0.8) | 9 | 1 |
| 2Ag | 60 | (56.9 ± 0.8) | 30 | (5.5 ± 0.5) | 7 | 2 |
| 3Ag | 60 | (56.4 ± 1.1) | 30 | (5.9 ± 0.7) | 6 | 3 |
| 4Ag | 60 | (56.9 ± 0.5) | 30 | (4.0 ± 0.1) | 5 | 4 |
| 5Ag | 60 | (56.8 ± 0.4) | 30 | (3.0 ± 0.1) | 5 | 5 |

---

**FIGURE 1.** Glass transition temperature (Tg), crystallization temperature (Tc), and glassification degree (GD) for xAg as a function of Ag2O content and that of 5Na.
stretching vibration of Nb-O-Nb bridges in the 3D network of regular NbO₆ octahedra ($v_{\text{as}}$(Nb-O), 580 cm⁻¹).²² The bands at 550 cm⁻¹ were assigned to the vibrational coupling of $[v$(Nb-O) (medium Nb-O distances) + (O-P-O)] stretching with deformation modes.¹³

The 3¹P MAS-NMR spectra of $x$Ag are shown in Figure 3(a). The center peaks between 15 and −25 ppm were assigned to the $Q_p^0$ and $Q_p^1$ groups, and the remaining peaks observed on both sides of the center peaks were associated with spinning side bands. The experimental spectra were simulated assuming Gaussian lines for the $Q_p^0$ and $Q_p^1$ groups. With an increase in the Ag₂O content, the content of the $Q_p^0$ group increased from 43% to 46%, whereas that of the $Q_p^1$ group decreased from 57% to 54% [Fig. 3(b)]. The contents of the $Q_p^0$ and $Q_p^1$ groups of 5Na were 48% and 52%, respectively.

Figure 4 shows the UV-Vis absorption spectra of $x$Ag. The absorption peaks were detected at around 430 nm and 320 nm. The wavelength below 310 nm indicates a transmittance of 0%. Figure 5 shows the TEM images of 3Ag; the nanoparticles were dispersed in the glass matrix and their average size was 23.8 ± 1.8 nm.

**Dissolution behaviors of the glasses**

Figure 6 shows the Ca²⁺, phosphate, Nb⁵⁺, and Ag⁺ ion dissolution profiles from $x$Ag into TBS after 1, 3, 5, and 7 days of immersion. The release amounts of Ca²⁺, phosphate, and Nb⁵⁺ ions linearly increased with increasing soaking time. However, the Ag⁺ ion dissolution amounts of 3–5Ag after day 3 were relatively stable, with values of approximately 12–14 μM. The Ag⁺ ion dissolution amounts with TBS replacement are shown in Figure 7(a). The Ag⁺ ion concentration for 3Ag and 5Ag with TBS removed at day 1, 3, 5, and 7 was consistently 13 μM. Figure 7(b–d) shows the SEM image and EDX results of 5Ag after being soaked in TBS for 7 days; the particles composed of Ag and Cl were precipitated on the glass surface. Based on these results, 0Ag, 1Ag, and 3Ag were selected for the cytocompatibility and antibacterial activity tests, since there were no significant differences in the dissolution behaviors between 3Ag and 5Ag.

**Cytocompatibility of the glasses**

Figure 8 shows the cell numbers on 0Ag, 1Ag, and 3Ag. The number of cells increased with increasing culture period, whereas there were no significant differences in cell numbers among the samples in each culture period.

**Antibacterial activity of the glasses**

The antibacterial activities of 0Ag, 1Ag, and 3Ag were examined against *E. coli* and *S. aureus* as shown in Figure 9. The figure represents the number of CFU after being cultivated with the glass powders for 24 h. Controls (0 and 24 h) mean the initial number of CFU and the number of CFU after being cultivated for 24 h without glass powders, respectively. There was no significant difference in the number of CFU between 0Ag and the control (24 h, without
the glass). The numbers of CFU for 1Ag and 3Ag were smaller than those of 0Ag and the control (24 h).

**DISCUSSION**

With an increase in the Ag2O content in xAg, the Tg and Tc values decreased. This behavior is similar to that observed in our previously developed CaO-P2O5-Nb2O5-Na2O invert glasses; that is, the Tg and Tc values decreased with increasing Na2O content.11 The 31P MAS-NMR spectra of xAg showed similar results to our previous reported phosphate invert glasses11,12; the glasses consist of Q0p and Q1p groups without Qp2 group. Thus, xAg consist of Q0p and Q1p, similar to the phosphate invert glasses. The 31P MAS-NMR results showed that the Q0p content in xAg increased with increasing Ag2O content. FT-IR band intensities corresponding to Q0p (1000 and 1050 cm−1) increased with increasing Ag2O content. This supports the 31P MAS-NMR results. Whereas, the band intensities of chain-terminating Q1p group (1105 cm−1) increased with increasing Ag2O content. The Q0p group in the phosphate invert glass was crosslinked by niobate to form O-P-O-Nb-O bond.11,12 Thus, the band intensity of chain-terminating Q1p group may be influenced by Q0p terminal in O-P-O-Nb-O bond. Ahmed et al. reported that the phosphate glass network structure was not changed by substituting Ag2O for Na2O.23 Christie et al. simulated Ag2O/Na2O-containing metaphosphate glasses; the Ag+ or Na+ ions in the glasses were bond to approximately the same number of phosphate chains.24 Ag2O in the phosphate invert glasses would act as a modifier oxide, similar to Na2O. Consequently, the FT-IR spectra of 5Ag and 5Na showed no significant differences. Q0p group in 5Ag indicated slightly smaller amount than that of 5Na. This may indicate 5Ag contains a smaller amount of network modifier than that of 5Na. That is, Ag+ ions in 5Ag showed smaller amount than Na+ ions in 5Na, since silver nanoparticles were formed in 5Ag glass matrix. The Q0p group increased slightly with increasing Ag2O content in xAg (x = 0–5), due to increase of network modifier in xAg (that is, increasing Ag+ ions in the glass network). The field strength (F) is defined as follows:

\[
F = \frac{Z}{a^2}
\]

where Z is the ionic charge and a is the interatomic distance between cation and oxygen. When the Zs of Ag and Na are 1, the bonding length (that is, the interatomic distance, a) for Ag-O and Na-O in metaphosphate glasses are approximately 2.29 Å and 2.36 Å, respectively. Consequently, the F values of Ag and Na were estimated to be 0.19 and 0.18 valance/Å2, respectively. 5Ag contains a larger amount of Q0p compared with 5Na, since it contains a smaller amount of network modifier as discussed above. The GD of 5Ag (0.09) was larger than that of 5Na (0.07), since the field strength of Ag is larger than that of Na, and the amount of Q1p in 5Ag is slightly larger than that of 5Na. The GDs of xAg were found to be approximately 0.09, which is close to values of the previously reported metaphosphate invert glasses containing TiO2 (0.08) or Nb2O5 (0.09).11,12 In addition, xAg showed comparably good glass-forming ability, even though Ag2O was substituted for Nb2O5.

The UV-Vis absorption peaks around 430 nm corresponded to surface plasmon resonance (SPR) of the electrons in the conduction bands of silver, indicating the formation of silver colloids.26,27 The nanoparticles in the 3Ag glass matrix would be silver nanoparticles (Fig. 5). The silver particles radius γ can be calculated as follows:

\[
\gamma = \frac{1}{2} \left( \frac{4\pi}{3} \right)^{1/3} \frac{a}{\sqrt{F}}
\]

FIGURE 4. UV-Vis absorption spectra for xAg.

FIGURE 5. TEM images of 3Ag with (a) low and (b) high magnification views.
\[ \gamma = \frac{A v_F}{2 \pi c (\Delta \lambda / \lambda_p^2)} \]  

where \( A \) is the line-broadening constant (1.2), \( v_F \) is the Fermi velocity of the free electrons \((1.4 \times 10^6 \text{ m/s})\) for silver, \( c \) is the velocity of light \((3.0 \times 10^8 \text{ m/s})\), \( \lambda_p \) is the SPR wavelength, and \( \Delta \lambda \) is the full width at half maximum of the SPR peak. The calculated radius of 1Ag, 3Ag, and 5Ag was 4.8, 6.7, and 5.1 nm, respectively (that is, diameter of 9.6, 13.4, and 10.2 nm, respectively). The average silver nanoparticle size in 3Ag measured from the TEM images.
was 23.8 ± 1.8 nm. The difference between the calculated and measured diameters of the silver nanoparticles may be underestimated, since the broadening of the plasmon absorption bands can be attributed to not only the particle size but also to the size distribution and interactions between the particles.20 There was no significant difference in the silver SPR peak width and calculated particle size among the samples; this may indicate that the distribution of silver particle size was similar. The absorption peak detected around 320 nm is attributed to the presence of ionic silver species associated with aggregates (Ag\(^{0}\)).27,29 which may coordinate with the phosphate glass network structure. The absorption under 310 nm was constant (that is, transmittance 0%) due to the threshold absorption of the phosphate glass network matrix.21,27

It may be difficult to evaluate dissolution behavior of 1Ag using glass plates, due to their high chemical durability. Thereby, glass powders were used for the present test to easily estimate their dissolution behavior. The Ca\(^{2+}\) and phosphate ion dissolution amounts were increased with increasing Ag\(_{2}\)O content, that is, with decreasing Nb\(_{2}\)O\(_{5}\) content, in 3Ag; niobia significantly improves the chemical durability of the phosphate glasses.11,13 The Nb\(^{5+}\) ion dissolution amount of 1–5Ag at day 7 was approximately 0.05 mM; this is expected to enhance the differentiation of osteoblasts.14,30 The Ag\(^{+}\) ion concentrations of 3–5Ag after day 3 were stable with a value of 13 \(\mu\)M [Fig. 6(d)]. The Ag\(^{+}\) ion dissolution amount of 3Ag and 5Ag with TBS replacement was also 13 \(\mu\)M for every soaking period, and the EDX spectrum showed the peaks of Ag and Cl [Fig. 7(c)]. Saravanapavan et al. reported silver-containing sol-gel glass foams, and Ag\(^{+}\) ion dissolution behaviors in simulated body fluids (SBF) was maintained in equilibrium with silver chloride (AgCl) precipitation.31 Silver-doped bioactive glass also showed AgCl precipitation after being soaked in SBF.32 Thus, the Ag\(^{+}\) ion dissolution amount of 3–5Ag increased until 13 \(\mu\)M and then kept constant due to AgCl precipitation. However, the Ag\(^{+}\) ion dissolution amount of 1Ag was lower than 13 \(\mu\)M after being soaked for 7 days; this may show no AgCl precipitation. Bellantone et al. reported that Ag\(_{2}\)O-doped bioactive glasses showed broad-spectrum bactericidal activity against \textit{E. coli}, \textit{Pseudomonas aeruginosa}, and \textit{S. aureus} with an Ag\(^{+}\) ion concentration of 1–10 \(\mu\)M.33 The total Ag\(^{+}\) dissolution amount for 1Ag until day 7 was 11 \(\mu\)M in the TBS exchange condition, which may be sufficient to show antibacterial activity. 3–5Ag had an Ag\(^{+}\) ion dissolution amount of 13 \(\mu\)M with AgCl precipitation after day 3, which could also show antibacterial activity. However, dissolved Ag\(^{+}\) ions can be toxic to cells,10 and precipitated AgCl on the surface plays a substantial role in cytotoxicity.32 Hence, it is necessary for Ag\(_{2}\)O-containing biomaterials to confirm their cytocompatibility.

The glass plates were used for the cytocompatibility test, since the application for \(x\)Ag will be expected to be coating materials on the metal. There were no significant differences in the cell numbers among the 0Ag, 1Ag, and

![FIGURE 8](https://example.com/figure8.png) Cell numbers after 1–7 days on \(x\)Ag. Error bars represent standard deviations.

![FIGURE 9](https://example.com/figure9.png) Colony forming units (CFU) of (a) \textit{E. coli} and (b) \textit{S. aureus} after cultivation with \(x\)Ag for 24 h. "0 h control" indicates the seeded number of \textit{E. coli} and \textit{S. aureus}, and error bars represent standard deviations.
3Ag surfaces after cultivation for 1, 3, 5, and 7 days (Fig. 8). Thus, 1Ag and 3Ag showed cytocompatibility, and the dissolved Ag\(^+\) ions from 1Ag and 3Ag showed no cytotoxicity. In our previous work, Nb-PIG containing 3 and 7 mol % of Na\(_2\)O improved alkaline phosphatase (ALP) activity.\(^\text{14}\) The dissolution behavior of xAg are comparable with those of Nb-PIGs,\(^\text{11}\) since xAg and Nb-PIGs are comprised of almost similar glass structure. Accordingly, xAg were expected to enhance differentiation of osteoblast cells by dissolved Nb\(^{5+}\) ions from the glasses.

The antibacterial activity of the glasses was tested against E. coli and S. aureus, using shake method. This method was used as a fundamental investigation tool to monitor the effect of the glass compositions. The pH of Nb-PIGs in α-MEM after 24 h showed no significant differences between Nb-PIG containing 0, 3, and 7 mol % of Na\(_2\)O.\(^\text{14}\) When phosphate invert glasses containing TiO\(_2\), which acts a similar role as Nb\(_2\)O\(_5\) in the phosphate invert glasses,\(^\text{12}\) were soaked in TBS, they showed almost no pH change after 7 days, due to their high chemical durability.\(^\text{24}\) Consequently, 0Ag, 1Ag, and 3Ag in 1/500 NB media can show no significant pH change during the antibacterial test; the pH of media is considered not to be dominant source of antibacterial activity for xAg. The E. coli and S. aureus CFU of 1Ag and 3Ag after 24-h culture showed almost the same levels as the initial values. Consequently, 1Ag and 3Ag showed bacteriostatic activity. However, there were almost no bacteria left after culture with 3Ag containing a larger amount of glass powder (5 mg/mL), which indicated bactericidal activity. The Ag\(^+\) ion dissolution amounts of 1Ag and 3Ag after 1 day were 2.05 and 8.90 \(\mu\)M, respectively; the amount can indicate antibacterial activity.\(^\text{23}\) Consequently, dissolved Ag\(^+\) ions are dominant source for exhibiting antibacterial activity. Therefore, substituting even a small amount of Ag\(_2\)O, such as 1–3 mol %, can confer a calcium phosphate invert glass with antibacterial activity without cytotoxicity.

CONCLUSIONS

In this work, Ag\(_2\)O-containing calcium phosphate invert glasses were successfully developed, and their structure, dissolution behavior, cytocompatibility, and antibacterial activity were evaluated. Ag\(_2\)O in phosphate invert glasses acted as a modifier of oxides, playing a similar role to Na\(_2\)O. The silver in xAg existed as ionic silver species, coordinated with the phosphate network structure, and silver nanoparticles dispersed in the glass matrix. The Ag\(^+\) ion dissolution amount of 1Ag was 11 \(\mu\)M, and that of 3–5Ag was 13 \(\mu\)M after day 3 due to AgCl precipitation. Ag\(_2\)O-containing phosphate invert glasses showed cytocompatibility and antibacterial activity against E. coli and S. aureus.

REFERENCES

1. Busscher HJ, van der Mei HC, Subbiahdoss G, Jutte PC, van den Dungen JJAM, Zaat SAJ, Schulz MJ, Grainger DW. Biomaterial-associated infection: Locating the finish line in the race for the surface. Sci Transl Med 2012;4:153rv10.
2. Grisztina A. Biomaterial-centered infection: Microbial adhesion versus tissue integration. Science 1987;237:1588–1595.
3. Campoccia D, Montanaro L, Speciale P, Arciola CR. Antibiotic-loaded biomaterials and the risks for the spread of antibiotic resistance following their prophylactic and therapeutic clinical use. Biomaterials 2010;31:6363–6377.
4. Campoccia D, Montanaro L, Arciola CR. A review of the clinical implications of anti-infective biomaterials and infection-resistant surfaces. Biomaterials 2013;34:8018–8029.
5. Campoccia D, Montanaro L, Arciola CR. A review of the biomaterials technologies for infection-resistant surfaces. Biomaterials 2013;34:8533–8554.
6. Landsdown ABG. Silver I: Its antibacterial properties and mechanism of action. J Wound Care 2002;11:125–130.
7. Schierholz JM, Lucas LJ, Rump A, Pulverer G. Efficacy of silver-coated medical devices. J Hosp Infect 1998;40:257–262.
8. Percival SL, Bowler PG, Russell D. Bacterial resistance to silver in wound care. J Hosp Infect 2005;60:7–13.
9. Feng QL, Wu J, Chen GO, Cui FZ, Kim JO. A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus. J Biomed Mater Res 2000;52:662–668.
10. Yamamoto A, Homma R, Sumita M. Cytotoxicity evaluation of 43 metal salts using murine fibroblasts and osteoblastic cells. J Biomed Mater Res 1998;39:331–340.
11. Maeda H, Lee S, Miyajima T, Obata A, Ueda K, Narushima T, Kasuga T. Structure and physicochemical properties of CaO–P\(_2\)O\(_5\)–Nb\(_2\)O\(_5\)–Na\(_2\)O glasses. J Non-Cryst Solids 2016;432:60–64. Part A.
12. Lee S, Maeda H, Obata A, Ueda K, Narushima T, Kasuga T. Structures and dissolution behaviors of CaO–P\(_2\)O\(_5\)–TiO\(_2\)–Nb\(_2\)O\(_5\) (Ca/\(P\) > 1) invert glasses. J Non-Cryst Solids 2015;426:35–42.
13. Mazali I, Barbosa L, Alves O. Preparation and characterization of new niobophosphate glasses in the Li\(_2\)O–Nb\(_2\)O\(_5\)–CaO–P\(_2\)O\(_5\) system. J Mater Sci 2004;39:1907–1995.
14. Obata A, Takahashi Y, Miyajima T, Ueda K, Narushima T, Kasuga T. Effects of niobium ions released from calcium phosphate invert glasses containing Nb\(_2\)O\(_5\) on osteoblast-like cell functions. ACS Appl Mater Interfaces 2012;4:5684–5690.
15. Narushima T, Ueda K, Goto T, Masumoto H, Katsube T, Kawamura H, Ouchi C, Iuchi Y. Preparation of calcium phosphate films by radiofrequency magnetron sputtering. Mater Trans 2005;46:2245–2252.
16. Ueda K, Narushima T, Goto T, Katsube T, Nakagawa H, Kawamura H, Taira M. Evaluation of calcium phosphate coating films on titanium fabricated using RF magnetron sputtering. Mater Trans 2007;48:307–312.
17. Ueda K, Narushima T, Goto T, Taira M, Katsube T. Fabrication of calcium phosphate films for coating on titanium substrates heated up to 773 K by RF magnetron sputtering and their evaluations. Biomater 2007;2:5160.
18. Matsuogaki I, Isobe Y, Saku T, Nakano T. Quantitative regulation of bone-mimetic, oriented collagen/apatite matrix structure depends on the degree of osteoblast alignment on oriented collagen substrates. J Biomed Mater Res A 2015;103:489–499.
19. Lee S, Uehara H, Maçon ALB, Maeda H, Obata A, Ueda K, Narushima T, Kasuga T. Preparation of antibacterial Zn\(_2\)O–CaO–P\(_2\)O\(_5\)–Nb\(_2\)O\(_5\) invert glasses. Mater Trans 2016;57:2072–2076.
20. Ouchetto M, Elouadi B, Parke S. Study of lanthanide zinc phosphate invert glasses: Beyond the network connectivity. Phys Chem Glasses 1991;32:22–28.
21. Ahmed AA, Ali AA, Mahmoud DAR, EI-Fiqi AM. Study on the preparation and properties of silver-doped phosphate antibacterial glasses (Part I). Solid State Sci 2011;13:981–992.
22. Dausaze M, Kaminos E, Fargin E, Rodriguez V. Structural rearrangements and second-order optical response in the space charge layer of thermally poled sodium niobium borophosphate glasses. J Phys Chem C 2007;111:14560–14566.
23. Ahmed I, Abou Neel EA, Valappil SP, Nazhat SN, Pickup DM, Carta D, Carroll DL, Newport RJ, Smith ME, Knowles JC. The structure and properties of silver-doped phosphate-based glasses. J Mater Sci 2007;42:9827–9835.
24. Christie JK, Ainsworth A, Leeuw NH. Investigating structural features which control the dissolution of bioactive phosphate glasses: Beyond the network connectivity. J Non-Cryst Solids 2016;432:Part A:31–34.
25. Varshneya AK. Fundamentals of Inorganic Glasses. New York: Academic press; 1994.
26. Filippo E, Serra A, Manno D. Polyvinyl alcohol capped silver nanoparticles as localized surface plasmon resonance-based hydrogen peroxide sensor. Sensors Actuators B: Chem 2009;138:625–630.
27. Lu JQ, Bravo-Suarez JJ, Takahashi A, Haruta M, Oyama ST. In situ UV-vis studies of the effect of particle size on the epoxidation of ethylene and propylene on supported silver catalysts with molecular oxygen. J Catal 2005;232:85–95.
28. Yang X, Li W, Li Z, Wei Y, Huang W. Depth profiles of Ag nanoparticles in silicate glass. Appl Phys A 2008;90:465–467.
29. Jiménez JA, Lysenko S, Liu H. Photoluminescence via plasmon resonance energy transfer in silver nanocomposite glasses. J Appl Phys 2008;104:054313.
30. Tamai M, Isama K, Nakaoka R, Tsuchiya T. Synthesis of a novel b-tricalcium phosphate/hydroxyapatite biphasic calcium phosphate containing niobium ions and evaluation of its osteogenic properties. J Artificial Organs 2007;10:22–28.
31. Saravanapavan P, Gough JE, Jones JR, Hench LL. Antimicrobial macroporous gel-glasses: Dissolution and cytotoxicity. Key Eng Mater 2004;254:1087–1090.
32. Vernè E, Nunzio SD, Bosetti M, Appendino P, Vitale Brovarone C, Maina G, Cannas M. Surface characterization of silver-doped bioactive glass. Biomaterials 2005;26:5111–5119.
33. Bellantone M, Williams HD, Hench LL. Broad-spectrum bactericidal activity of Ag₂O-doped bioactive glass. Antimicrob Agents Chemother 2002;46:1940–1945.
34. Morikawa H, Lee S, Kasuga T, Brauer DS. Effects of magnesium for calcium substitution in P₂O₅–CaO–TiO₂ glasses. J Non-Cryst Solids 2013;380:53–59.