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Relating ion release and pH to in vitro cell viability for gallium-inclusive bioactive glasses

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Abstract A bioactive glass (BG) in which Ga was substituted for Zn was formulated to investigate whether the ionic form of Ga can elicit effects similar to gallium nitrate. The ion release and pH of BG extracts were evaluated, as well as the in vitro cytocompatibility of extracts in contact with mouse fibroblasts and human osteoblasts. After incubation times of 1 year, the glass (*TGa-1*) containing the smaller Ga-addition (8 mol%) released the most sodium (Na) (1420 mg/L), silicon (Si) (221 mg/L), and Ga (1295 mg/L), while the glass (*TGa-2*) containing the larger Ga-addition (16 mol%), exhibited release levels between *TGa-1*, and the 0 mol% Ga (*Control*) glass. The pH of all 3 glass extracts steadily increased over time, with maximums observed after 365 days for *Control* (10.0), *TGa-1* (12.2), and *TGa-2* (9.7). Cell viability analysis suggested that Ga-release produced toxic effects in L-929 fibroblasts, with less than 3 % viability for both *TGa-1* and *TGa-2* extracts after 90, 180, and 365 days; however, no significant decrease in MC-3T3 osteoblast viability was observed for *TGa-1* extracts after any time period, despite the higher ion release and pH values, and a significant decrease to 51 % viability was only observed for *TGa-2* extracts after 365 days. These results suggest that tailoring the release of Ga from BG is not only possible, but also beneficial to the host, thus rendering such glasses useful in bone void-filling applications.

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Introduction

Bioactive glasses (BGs) are materials which have been formulated for applications in dental and orthopedic surgery and can be used to aid in the repair or restoration of mineralized tissues which have sustained injury as a result of either disease or traumatic injury. Upon implantation, partial dissolution of the BG surface occurs, as ion exchange is conducted between the glass surface and the surrounding aqueous medium, ultimately resulting in the formation of a chemical bond between the implanted material and the surrounding tissue [1]. The most well-known BG, Bioglass® 45S5, was developed by Hench et al. in 1969. With a composition of 45 %SiO_2–24.4 %Na_2O–24.4 %CaO–6 %P_2O_5 (wt%), a thin apatite layer forms at the glass-tissue interface upon implantation into a living host, which then facilitates a strong chemical bond between the implant and bone tissue [2]. From these findings, engineers began to design implantable materials with the intent to bond and interact with host tissues, rather than remaining separate and inert upon implantation [3].

In addition to forming strong interfacial bonds between the implant and the host tissue, ionic dissolution products from BGs can elicit other beneficial effects. For instance, it has been observed that the release of silicon and calcium from Bioglass® 45S5 results in a dramatic increase in osteostimulation, which is characterized by an increase in both alkaline phosphatase activity and DNA production in osteoblasts, translating to increased proliferation and differentiation [4]. BGs can be formulated to include different elements which, upon dissolution from the glass network in

their ionic forms, can elicit specific therapeutic effects. One of the most common additional elements to be incorporated in BGs is zinc (Zn), which can increase osteostimulation [5]. The increase in collagen and calcium content that accompanies osteostimulation increases the compressive and flexural strengths of the bone, resulting in increased mechanical properties [6]. Zn is also included in BGs because it exhibits toxicity towards pathogenic bacteria such as *Staphylococcus aureus* (Gram +ve), and *Escherichia coli* (Gram −ve), which cause opportunistic infections at the site of implantation [7].

Although Zn is included in our glass series, the element of most interest in this study is gallium (Ga). Ga has been incrementally substituted for Zn in these glasses (8 and 16 mol%), for several reasons. Although there have been some recent studies conducted on the structural role of Ga in different BG compositions and the effects its inclusion may have on in vitro interactions with simulated body fluid (SBF) [8–10], decades of research have been dedicated to investigating Ga in its pharmaceutical form, gallium nitrate ($Ga(NO_3)_3$). This compound has exhibited the ability to suppress the growth of subcutaneously implanted tumors in mice and rats, while also exhibiting low toxicity compared to other anti-cancer compounds [11]. Gallium nitrate has displayed effectiveness against an array of cancer types, most notably against non-Hodgkin's lymphoma [12–16] and bladder cancer [17–20]. In addition to exhibiting effectiveness in the treatment of different forms of cancer, gallium nitrate has also been shown to combat these diseases without causing myelosuppression (bone marrow suppression) [21], which is a trait that most chemotherapy drugs cannot claim. It has also been shown to decrease levels of calcium in the bloodstream of patients undergoing cancer therapy [22–24] and to reduce the biochemical markers associated with accelerated bone turnover [25].

Although the effects of Ga [3] $^{+}$ have not been studied nearly as thoroughly as the pharmaceutical form ($Ga(NO_3)_3$), the potential ability to harness some of the same therapeutic abilities through release of Ga from a BG network justifies this investigation. Here, a glass with a starting composition of $CaO–Na_2O–ZnO–SiO_2$ was employed. Calcium (Ca^{2+}) and sodium (Na^{+}) act as network modifiers within this glass system and promote glass dissolution and ion exchange, while silicon (Si^{4+}) acts as the network former. Zinc (Zn^{2+}) can act as a network intermediate, assuming either a network-forming or network-modifying role [9]. However, the principal purpose of this study is to evaluate the effect that gallium (Ga^{3+}) has on the structure, long-term solubility, and in vitro cytocompatibility of this glass system. Ga^{3+} is a glass network intermediate [9, 26], which has prompted studies in which Ga^{3+} has been investigated as a substitute for aluminum (Al^{3+}) in glassy materials [27] and is the reason why it has been substituted for Zn^{2+} in prior work conducted by the authors [26]. However, its effect on the long-term solubility of this system has not yet been determined. This study aims to investigate the influence of Ga_2O_3 addition on BG solubility over extended time periods (up to 1 year), and to determine if this influence translates to effects on the in vitro cell viability of L-929 Fibroblasts and MC-3T3-E1 Osteoblasts.

Materials and methods

Glass synthesis

Three glasses were formulated for this study: Two Ga-containing glasses (*TGa-1, TGa-2*) and a Ga-free $CaO–Na_2O–ZnO–SiO_2$ glass *(Control)*. The Ga-containing glasses *(TGa-1, TGa-2)* contain incremental concentrations of Ga_2O_3 at the expense of ZnO (Table 1). The powdered mixes of analytical grade reagents (Fisher Sci., PA, USA) were mixed using silica beads, and then oven dried (100 °C, 1 h) and fired (1500 °C, 1 h) in platinum crucibles and shock quenched into water. The resulting frits were dried, ground using a Gy-Ro Mill (Glen Creston Ltd, South West London, UK) in 10 s intervals at 3400 rpm, and sieved to retrieve glass powders with a maximum particle size of 90 μm.

Preparation of cell culture/ion release extracts

50 g of each glass (*Control, TGa-1, and TGa-2*, where $n = 3$) was sterilized using γ-irradiation at 25kGray (Isotron ltd, Mayo, Ireland) prior to incubation. Ultrapure water obtained using a Milli-Q water purification system (EMD Millipore, MA, USA) was selected as the solvent to prepare extracts. A surface area of 1 m^2 of each sample ($n = 3$) was aseptically immersed in 10 mL of the ultrapure water and agitated for periods of 1, 7, 14, 30, 90, 180, and 365 days at 37 °C. Each of the subsequent extracts were prepared for ion release analysis by adding 1 ml aliquots ($n = 3$) of each extract to 9 ml of ultrapure water, to create a 1:10 dilution. 10 μl aliquots ($n = 3$) of each

Table 1 Glass compositions (mol. fr.)

	Control	TGa-1	TGa-2
SiO_2	0.42	0.42	0.42
Ga_2O_3	0.00	0.08	0.16
ZnO	0.40	0.32	0.24
Na_2O	0.10	0.10	0.10
CaO	0.08	0.08	0.08

extract (except for 14 day extracts) were later used for cell viability analysis.

Preparation of glass plates for SBF testing

Analytical grade reagents (Fisher Sci., PA, USA) were batched according to Table I, and mixed using silica beads in order to obtain powdered mixes. These mixes were then oven dried (100 °C, 1 h) and fired (1500 °C, 1 h) in platinum crucibles. Glass castings were then produced by pouring the molten glass into graphite molds. After cooling for 1 h, the glasses were removed from the molds and annealed at their respective glass transition temperatures (T_g). The glass casts were then shaped into plates with approximate dimensions of 15 × 3 × 3 mm. These dimensions were achieved by first cutting with a diamond blade on an Isomet 5000 Linear Precision Saw (1500 rpm, 0.4 mm/min), and then by grinding with 60 μm silicon carbide grinding paper (Buehler, IL, USA) on a Phoenix 4000 grinding machine.

Glass characterization

Particle size analysis (PSA)

Particle size analysis was performed using a Beckman Coulter Multi-Sizer 4 Particle Size Analyzer (BeckmanCoulter, CA, USA). Glass powder samples were suspended in ultrapure water and evaluated in the range of 0.4–20.0 μm, with a run time of 60 s. Relevant volume statistics were calculated and reported for each glass.

Differential thermal analysis (DTA)

The glass transition temperature (T_g) of each glass was measured using an SDT Q600 Simultaneous DSC-TGA (TA Instruments, DE, USA). The samples were heated at a rate of 10 °C/min between 30 and 1300 °C in an air atmosphere with alumina in a matched platinum crucible as a reference.

Simulated body fluid (SBF) study

SBF was produced in accordance with the procedure outlined by Kokubo et al. [28]. The composition of SBF is outlined in Table 2. The reagents were dissolved in order, from reagent 1–9, in 500 ml of ultrapure water using a magnetic stirrer. The solution was maintained at 36.5 °C. 1 M-HCl was titrated to adjust the pH of the SBF to 7.4. Ultrapure water was then used to adjust the volume of the solution up to 1L. Glass plates ($n = 1$) were each immersed in 10 ml of SBF and were subsequently stored for 1, 7, and 14 days in an incubator, at 37 °C.

Table 2 SBF recipe

Order	Reagent	Amount added
1	NaCl	7.996 g
2	$NaHCO_3$	0.35 g
3	KCl	0.224 g
4	$K_2HPO_4{\cdot}3H_2O$	0.228 g
5	$MgCl_2{\cdot}6H_20$	0.305 g
6	1 M-HCl	40 ml
7	$CaCl_2$	0.278 g
8	Na_2SO_4	0.071 g
9	$NH_2C(CH_2OH)_3$	6.057 g

Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDAX)

A Quanta 200F Environmental Scanning Electron Microscope was used to image the samples under a vacuum at a pressure of 0.90 torr. The electron beam was used at an accelerating voltage of 20 kV and a spot size of 4.0. Energy-dispersive x-ray spectroscopy was carried out using an FEI EDAX system equipped with a silicon-drift detector.

Accelerated surface area and porosity (ASAP)

The surface area of each glass was determined using the Accelerated Surface Area and Porosimetry (ASAP) 2010 System Analyzer (Micrometrics Instrument Corp., GA, USA). Approximately 60 mg of each glass was used with a mixture of nitrogen and helium gases, to calculate the specific surface areas through the Brunauer-Emmett-Teller (BET) method.

Ion release profiles

Powder samples were weighed out to contain 1 m^2 of glass per incubation sample ($n = 3$) and were then submerged in ultrapure water for periods of 1, 7, 14, 30, 90, 180, and 365 days. After the incubation times were complete, the aqueous solution from each sample was removed and filtered using Amicon® Ultra-4 Centrifugal Filters (Merck KGaA, Darmstadt, Germany). Dilutions of each extract were then prepared (1:10), and ion release analysis was performed. Solutions were analyzed for Na, Ca, Si, Zn, and Ga content.

The ion release profile of each glass was measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES) on a Perkin-Elmer Optima 8000 (Perkin Elmer, MA, USA). ICP-OES calibration standards for Ca, Si, Na, Zn and Ga ions were prepared from stock solutions on a

gravimetric basis. Three target calibration standards were prepared for each ion, and ultrapure water was used as a control.

pH testing

The pH of each extract solution was measured using an Accumet® Excel XL 15 pH meter (Fisher Scientific, NH, USA). 3 ml aliquots of each sample ($n = 3$) were removed from the 10 ml extract solutions and placed into separate sterile 15-ml centrifuge tubes for pH analysis in order to avoid contamination of extract solutions prior to cell viability analysis.

Cell viability analysis

The established cell lines L-929 (American Type Culture collection CCL 1 fibroblast, NCTC clone 929) and MC-3T3-E1 Osteoblasts (ATCC CRL-2593) were used in this study. L-929 fibroblasts were cultured, as required by ISO10993 part 5 [29], in HyClone® Medium 199/EBSS (Thermo Scientific, MA, USA), which included Earl's balanced salts and L-glutamine, and was supplemented with 10 vol% fetal bovine serum (Thermo Scientific, MA, USA). MC-3T3-E1 osteoblasts were cultured in HyClone® MEM Alpha Modification (1X) media (Thermo Scientific, MA, USA), which included L-glutamine, ribonucleosides, and deoxyribonucleosides, and was supplemented with 10 vol% fetal bovine serum (Thermo Scientific, MA, USA). Cells were maintained on a regular feeding regime in a cell culture incubator at 37 °C, with a 5 % CO_2/95 % air atmosphere. Cells were then seeded into 96-well plates at a density of 10,000 cells per well and incubated for 24 h prior to the addition of extracts. The cytotoxicity of glass extracts was evaluated using the methyl tetrazolium (MTT) assay. 10 µl aliquots of undiluted extract were then added into wells containing cells in culture medium (100 µl) and incubated for an additional 24 h. 10 µl of the MTT assay was then added to each well and incubated for an additional 4 h. After 4 h, the cultures were removed from the incubator, and the resultant formazan crystals were dissolved by first removing all of the aqueous media from each well, and then adding 100 µl of MTT Solubilization Solution (10 % Triton x-100 in Acidic Isopropanol. (0.1 n HCI)). Once the crystals were fully dissolved, the absorbance was measured at a wavelength of 570 nm using a µQuant Plate Reader (Bio-Tek Instruments, Inc., VT, USA). 10 µl aliquots of ultrapure water were used as control additions, and cells were assumed to have metabolic activities of 100 %. Glass extracts in media without cells were also tested and were found not to interfere with the MTT assay.

Statistical analysis

Particle size data for BG particles are presented as means ± standard deviations and represents 3 trials per glass. Ion release, pH, and cell assay data are also presented as means ± standard deviations, and represents data from 3 individual extract replicates per glass, per incubation interval. One-way analysis of variance (ANOVA) was used to compare both ion release, and cytocompatibility of the BG extracts in relation to 1) incubation time, and 2) Ga-content. Comparison of relevant means was performed using the post hoc Bonferroni test. Differences between groups were deemed significant when $p < 0.05$.

Results

Glass characterization

Particle size analysis

PSA was conducted for each glass powder sample, and the results are presented in Fig. 1. Mean particle size was similar for each glass at 11.1, 10.3, and 10.2 µm for *Control*, *TGa-1*, and *TGa-2*, respectively.

DTA to evaluate structural changes in Ga-containing glasses

DTA results are presented in Fig. 2 and show that the glass containing the highest Ga concentration (*TGa-2)* exhibited the highest T_g of 614 °C, along with the highest crystallization temperature (T_c) at 832 °C. The glass with lower

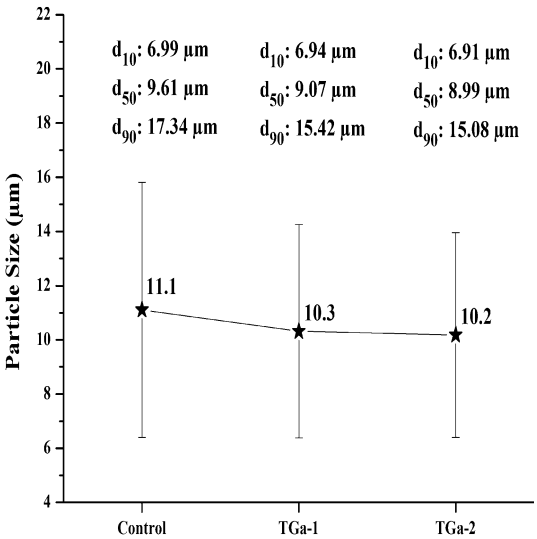

Fig. 1 Particle sizes of Ga-glass series

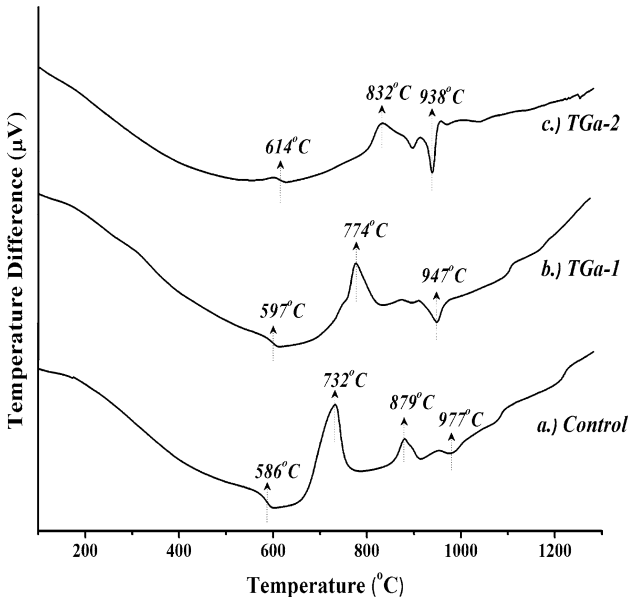

Fig. 2 Differential thermal analysis of Ga-glass series

Ga-content (*TGa-1*) exhibited a slightly lower T_g (597 °C) and T_c (774 °C), and the Ga-free glass (*Control*) exhibited the lowest T_g (586 °C) and T_c (732 °C). *Control* glass also clearly exhibited a second crystallization temperature at 879 °C, along with a melting temperature (T_m) of 977 °C, which was higher than both *TGa-1* (947 °C) and *TGa-2* (938 °C).

SEM to visualize Ca/P depositions on BG surfaces

SEM micrographs of the *Control*, *TGa-1*, and *TGa-2* glasses after 30 days of incubation in SBF at 37 °C, along with the corresponding EDAX spectra for the observed surface depositions, are presented in Fig. 3. Depositions on the surface of the *Control* glass exhibit similar morphology and appear to be apatite-like depositions [30, 31], which was further suggested by the presence of Ca, P, and O in the EDAX spectra. However, there appears to be depositions of two different basic morphologies on the surfaces of both the *TGa-1* and *TGa-2* glasses. EDAX analysis revealed that both the larger, agglomerated depositions and the smaller, dendritic depositions on the glass surfaces contained Ca, P, and O, although quantitative information revealed there were large differences in the Ca:P ratios of the different depositions.

Surface area determination for BGs

Prior to incubating glass powder samples in order to obtain extracts to be used in ICP-OES, pH, and cell viability analysis, the amount of surface area available for each sample was normalized using gas adsorption in combination with the BET method. Powder samples exhibited an average surface area of 2.28, 1.55, and 1.79 m^2/g for the *Control*, *TGa-1*, and *TGa-2* glasses, respectively. Each glass was then weighed out to obtain 1 m^2 of powder per incubation sample. The surface area of each glass, as well as the corresponding amount of glass used in each sample can be seen in Table 3. Glass additions were calculated to be 0.439, 0.645, and 0.559 g for the *Control*, *TGa-1*, and *TGa-2* glasses, respectively.

Elemental analysis of ions released from BGs

The Na-release of each glass over the different time periods is presented in Fig. 4a and displays similar trends for all three sample types. Na-release was observed, but remained relatively constant for the first 30 days of incubation for all glass types, and then an increase in Na-release occurred for all sample types after 90 days. *Control* glass released the least amount of Na over the year-long incubation period, with a release of 22.1 mg/L observed after 30 days, and a maximum release of 179.4 mg/L after 365 days. *TGa-1* glass released a small amount of Na over the shorter incubation periods, releasing 16.6 mg/L after 30 days, but released much more Na after 90, 180, and 365 days than the other two glasses, with a maximum of 1419.7 mg/L after 365 days. *TGa-2* glass released 20.9 mg/L of Na after 30 days and then fell between the other 2 glasses over the longer time periods, releasing a maximum of 565.9 mg/L after 180 days, and exhibiting a very slight decrease to 556.4 mg/L after 365 days.

The Si-release profiles for each glass can be seen in Fig. 4b, which exhibits similar trends for all 3 glass types. *Control* glass released the least Si over each time period, at 11.5 mg/L after 14 days, then decreasing to just 1 mg/L after 30 days. This same trend was observed after long-term incubation, as a maximum of 135.3 mg/L was released after 180 days, which then decreased to 107.1 mg/L after 365 days. Similar trends were seen in the *TGa-1* glass, with short- and long-term maxima of 23.2 and 296.2 mg/L after 14 and 180 days, respectively, and with short- and long-term minima of 9.8 and 221.4 mg/L after 30 and 365 days, respectively. Again, *TGa-2* exhibited the same trends, with short- and long-term maxima of 66 and 193.3 mg/L after 14 and 180 days, respectively, and with short- and long-term minima of 30.2 and 126.6 mg/L after 30 and 365 days, respectively. It is noteworthy that there was no significant difference in Si-release after 365 days between *Control* and *TGa-2* extracts.

Ca-release profiles are presented in Fig. 5a, with all three glasses releasing much less Ca than either Na or Si. Although trends are much less clearly observed for Ca-release, it can be seen that for incubation periods of up to 30 days, *TGa-2* released more than both *Control* and *TGa-*

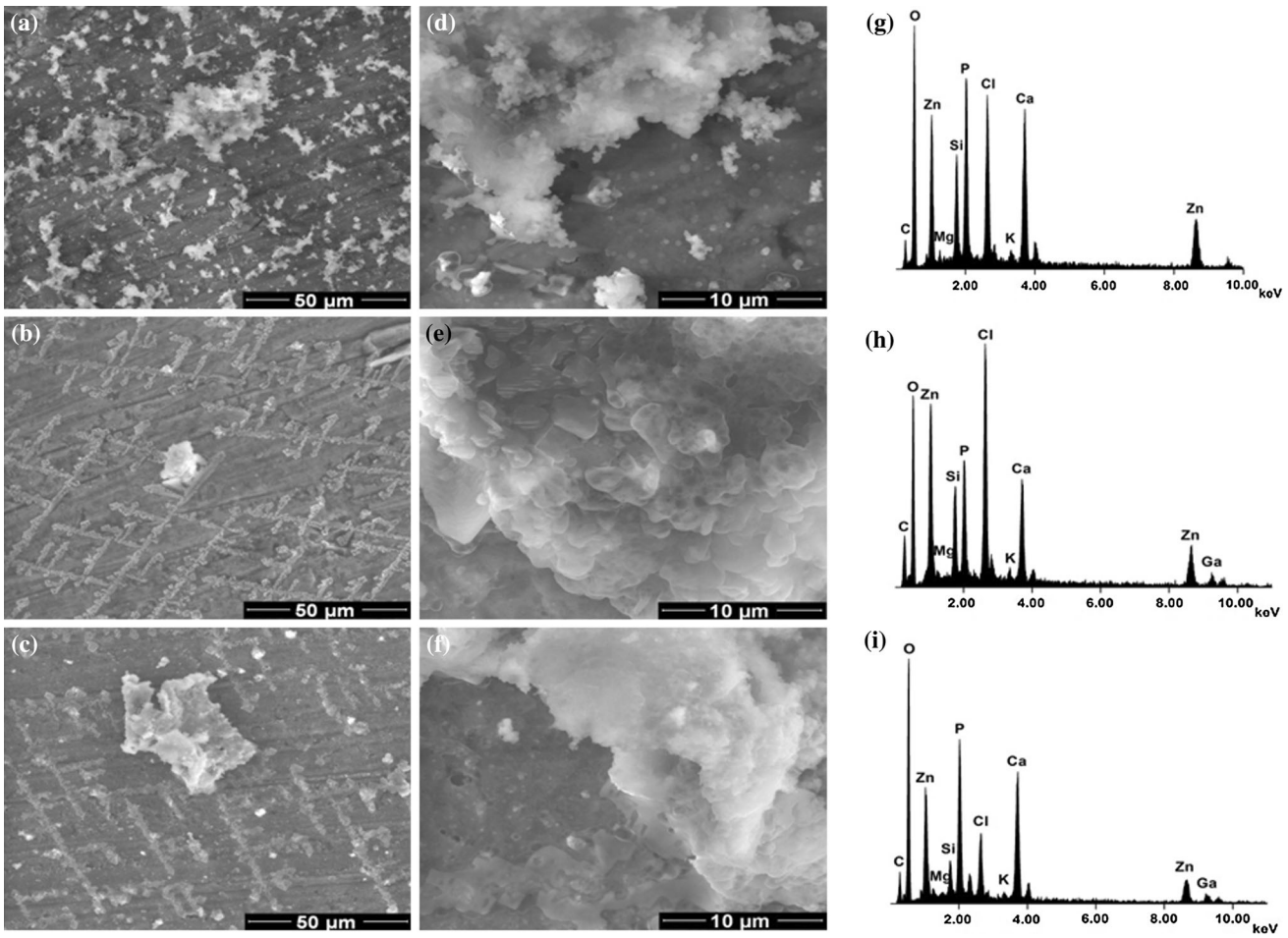

Fig. 3 SEM micrographs and EDAX spectra for depositions on surfaces of **a**, **d**, **g** *control*, **b**, **e**, **h** *TGa-1*, and **c**, **f**, **i** *TGa-2* glass surfaces after 30 days

Table 3 Surface area and weight added per incubation sample for Ga-glass series

	Surface area (m^2/g)	Wt./incubation sample (g)
Control	2.28	0.439
TGa-1	1.55	0.645
TGa-2	1.79	0.559

1, and all of the glasses exhibited similar short-term trends to what was observed in the Si-release profiles, with maxima after 14 days, and then slight decreases after 30 days. There was no significant difference in Ca-release between the three glasses after 90 days, and there were again long-term maxima observed after 180 days for the two Ga-containing glasses, which then decreased after 365 days. All three glasses exhibited decreases between 180–365 days. Also, although *TGa-2* released significantly more Ca (8.3 mg/L) than both the *Control* (1.9 mg/L, $p = 0.012$) and *TGa-1* (2.3 mg/L, $p = 0.016$) after 365 days, the amount of Ca released from each glass was not statistically different from their respective amounts in solution after just 30 days.

Zn-release information is presented in Fig. 5b, displaying low release totals for all 3 glasses. After 30 days, the *Control*, *TGa-1,* and *TGa-2* glasses had each only released 0.3 mg/L of Zn. The amount of Zn released exhibited a significant change after 180 days when compared to 30 day solutions for all 3 glasses, with *Control*, *TGa-1*, and *TGa-2* releasing 4.9, 6.5, and 5.9 mg/L, respectively. As with Si and Ca, a significant decrease in Zn-release was observed for each glass after 365 days.

Ga-release profiles are presented in Fig. 6. As seen in the Si- and Ca-release profiles, maxima in the short-term incubation period occurred after 14 days, with *TGa-1* and *TGa-2* releasing 19.6 mg/L and 123.9 mg/L, respectively. Then, after 30 days, the amount of Ga in solution decreased to 3.4 mg/L and 19.8 mg/L for *TGa-1* and *TGa-2*, respectively. Beyond 30 days, both glasses exhibited substantial increases in Ga-release, and there was not a

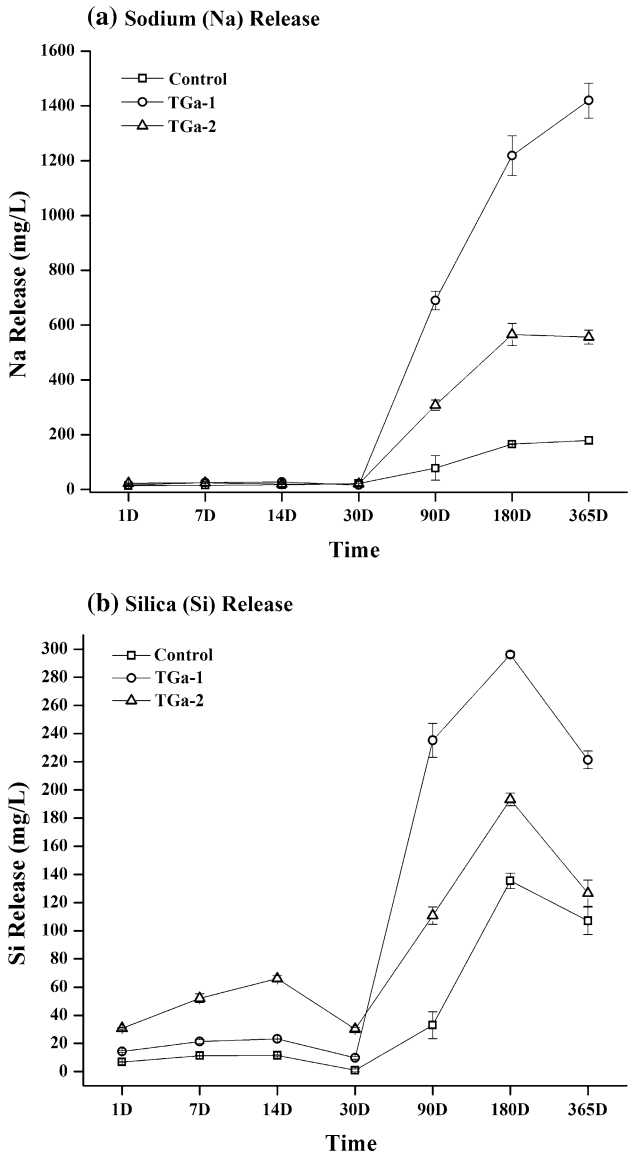

Fig. 4 **a** Na- and **b** Si-release profiles for Ga-glass series

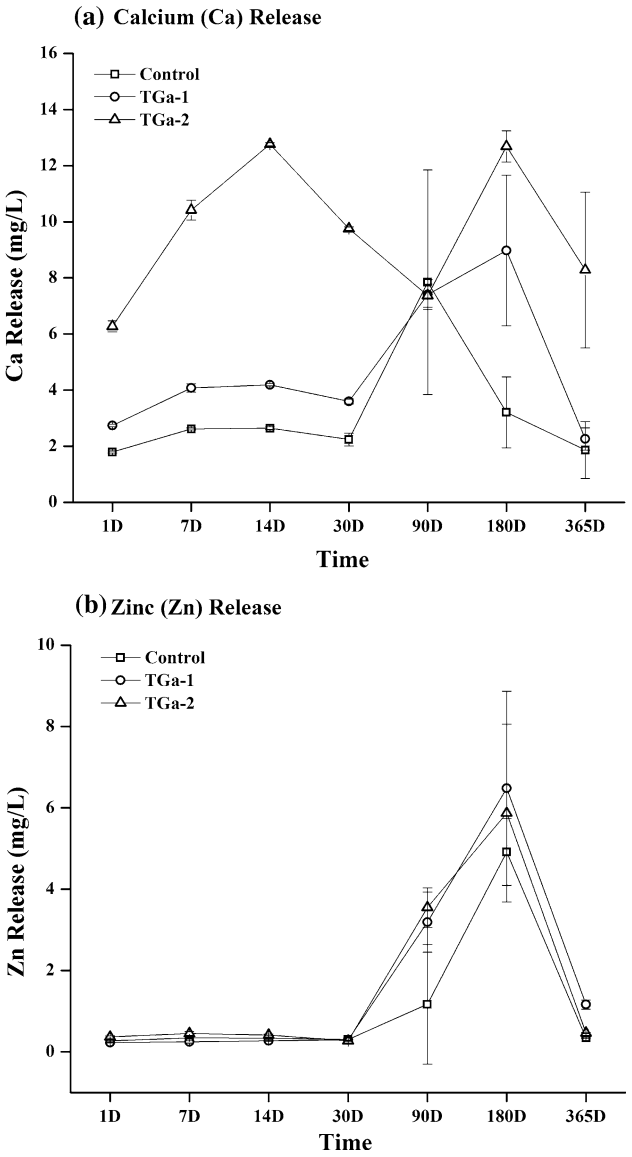

Fig. 5 **a** Ca- and **b** Zn-release profiles for Ga-glass series

significant difference observed between the two until the final 365-day period, when *TGa-1* had released 1295.3 mg/L, and *TGa-2* had released 1006.3 mg/L.

pH of BG extracts

pH data for all 3 glasses are presented in Fig. 7 and shows pH fluctuations which are very similar to the observed Si- and Na-release profiles. As with Si- and Na-releases, all 3 glasses exhibited very similar pH levels after 30 days, with *Control*, *TGa-1*, and *TGa-2* expressing pH's of 7.2, 7.4, and 7.4, respectively. Also in line with the trend observed in the Si-release profiles, *Control* and *TGa-2* expressed similar pH levels after 365 days of 10.0 and 9.7, respectively, while *TGa-1* possessed a higher pH of 12.2.

Cell viability analysis

L-929 fibroblast viability

Cell viability analysis was conducted using the extracts which were obtained from incubating glass powder samples in ultrapure water. Figure 8a presents L-929 fibroblast viability data for extracts obtained after 1-, 7-, and 30-day incubation periods and shows that viability was not affected by any of the 3 glasses after 1 and 7 days but was nearly equally reduced by all 3 glasses after 30 days, with *Control*, *TGa-1*, and *TGa-2* glasses expressing average viabilities of 68, 77, and 79 %, respectively. Figure 8b presents the results of cell viability analysis after 90-, 180-, and 365-day incubation periods, and shows that the

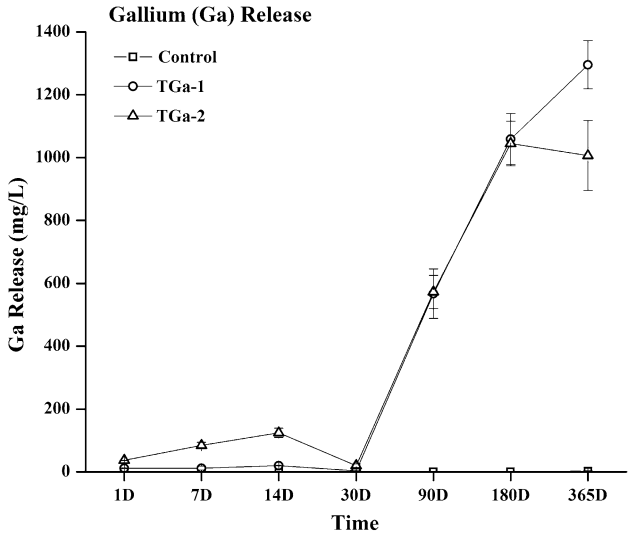

Fig. 6 Ga-release profiles for Ga-glass series

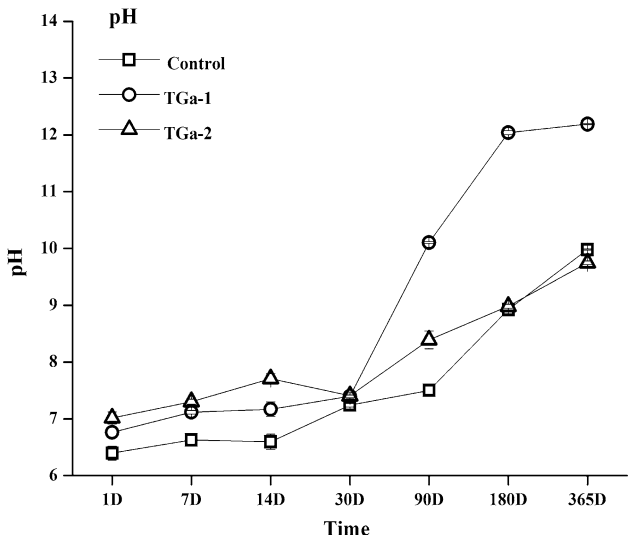

Fig. 7 pH of extracts from Ga-glass series

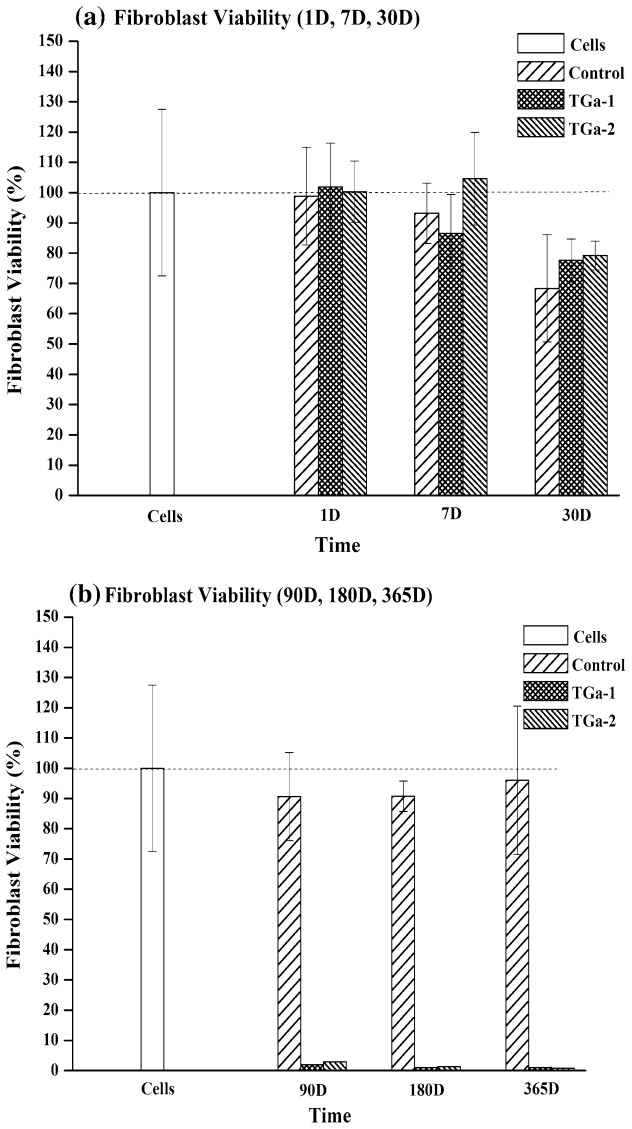

Fig. 8 L929 fibroblast viability for extracts of Ga-glass series obtained after **a** 1, 7, and 30 days, and **b** 90, 180, and 365 days

Control glass extracts only slightly reduced viability (90, 90, and 96 %, respectively), compared to the extract-free control cells over all 3 time periods, while both *TGa-1* and *TGa-2* exhibited significant decreases in viability. *TGa-1* extracts obtained after 90, 180, and 365 days decreased viability to 2, 1, and 1 %, respectively, while the *TGa-2* extracts produced similar viability levels of 3, 1, and 1 %, respectively.

MC-3T3-E1 osteoblast viability

MC-3T3-E1 osteoblast viability data for extracts obtained from all 3 glasses after 1, 7, and 30 days are displayed in Fig. 9a and show that osteoblast viability exhibits some small changes over these time periods. However, statistical analysis proved that none of these fluctuations were significant at a 95 % confidence level with respect to incubation time or Ga-content. Extracts removed after 1 day of incubation resulted in a slight decrease for *Control* (89 % viability), while increases of nearly equivalent magnitudes were observed for *TGa-1* and *TGa-2* as they expressed viabilities of 117 and 120 %, respectively. However, after 7 days, *Control* and *TGa-1* extracts expressed nearly identical viabilities of 114 and 115 %, respectively, while *TGa-2* extracts caused a decrease to 86 % viability. The trend once again changed after 30 days, as extracts of *TGa-1* and *TGa-2* produced viabilities of 121 and 132 %, respectively, while *Control* extracts resulted in an average viability of 93 %.

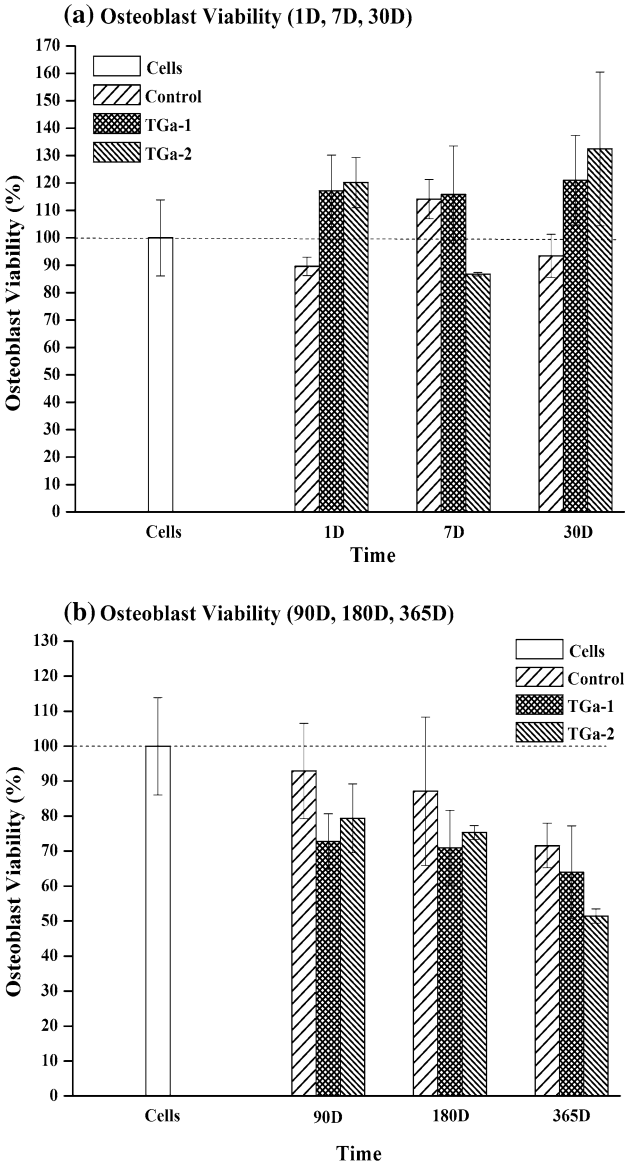

Fig. 9 MC-3T3-E1 osteoblast viability for extracts of Ga-glass series obtained after **a** 1, 7, and 30 days, and **b** 90, 180, and 365 days

Figure 9b presents MC-3T3-E1 viability data for extracts obtained from the 3 glasses after 90, 180, and 365 days and exhibits more consistent trends than were observed over the shorter time periods. In this data, a general decrease in viability with respect to time was observed for all 3 extract types. After 365 days, the presence of *Control* and *TGa-1* extracts exhibited insignificant decreases to 71 and 64 % viability, respectively, while *TGa-2* extracts exhibited a significant decrease to 51 % ($p = 0.01$) viability. The only significant viability decreases within datasets for the same extract types, were observed for *TGa-2* extracts between 1 and 365 days ($p = 0.047$) and between 30 and 365 days ($p = 0.009$).

Discussion

Glass characterization

In a study published in 1980 by Gross et al., it was observed that the additions of multi-valent cations such as aluminum (Al) and titanium (Ti) to glass compositions based on 45S5 Bioglass® resulted in the inhibition of rapid bonding to bone tissue in animal models, due to decreased solubility of the implants [32]. However, if the intention of an implant is to deliver a payload of therapeutic ionic dissolution products over an extended period of time, then perhaps there is a tailorable limit of multi-valent cations which can be added to a batch composition to allow for both bone bonding, and long-term ionic dissolution. A core goal of this study is to demonstrate an ability to control the stability of the glass structure and its relationship with glass dissolution, while also exhibiting the ability of the Ga-containing glasses to release potentially therapeutic ions, including Si, Ca, Zn, and Ga. We also aim to demonstrate that these glasses will allow calcium phosphate to deposit on their surfaces, in order to demonstrate the possibility of using these glasses as implant materials and potentially harness some of the anti-cancerous and bone-promoting properties that the Ga ion may possess.

DTA was conducted in order to determine if the incorporation of Ga into the glass network would influence T_g. Increasing Ga-content caused an increase in T_g and T_c, suggesting that the addition of Ga into these glasses creates a more stable structure with a reduced tendency towards crystallization, implying that Ga is acting primarily as a glass network former. This concurs with structural characterization work previously conducted on this glass series [26], which utilized ^{29}Si-MAS-NMR to show that increased Ga-content resulted in a larger presence of higher-ordered Q-species, suggesting that increased Ga-content results in a more connected glass structure, comprised a higher fraction of bridging oxygens (BO) between Si ions.

SBF studies are a preliminary in vitro method used to simulate the immersion of a sample into physiological fluid, in order to evaluate whether or not the multi-step cascade of events will be triggered, and result in the deposition of calcium phosphate on the sample surface [28]. The SEM images and EDAX spectra show that although depositions containing Ca, P, and O did not deposit with as much uniformity across Ga-containing glass surfaces as they did across the *Control* glass, they did deposit. In addition, evaluation of quantitative information obtained through EDAX revealed that *TGa-1* was the only glass upon which depositions with Ca:P ratios similar to stoichiometric hydroxyapatite (HA) were seen. Depositions

on the surface of the *Control* glass possessed a Ca:P ratio of 1.10 (based on wt% detected), while the smaller, dendritic depositions present on the surface of *TGa-1* possessed a Ca:P ratio of 1.74, which is much closer to the Ca:P ratio of 1.67 possessed by stoichiometric HA. The dendritic depositions seen on the surface of *TGa-2* possessed a Ca:P ratio of 1.00. In addition, the larger, brighter depositions seen on the surfaces of both *TGa-1* and *TGa-2* possessed respective Ca:P ratios of 1.29 and 1.08. This information suggests that the increased presence of Ga from 8 to 16 mol% reduced solubility and caused irregular depositions to form on the glass surfaces and that based on the Ca:P ratios of surface depositions, *TGa-1* is a stronger candidate than *TGa-2* to allow stoichiometric HA to deposit on its surface and function as a bioactive scaffold.

A vital characteristic of a BG is the ability of the surface to react with an aqueous environment through ion exchange. In order to study in vitro ion release, ICP-OES was employed and revealed several unexpected trends. A previous study conducted by Ahmed et al. demonstrated that as the ratio of glass network former (P_2O_5) to glass network modifier (Na_2O) in glass fibers increased, T_g increased, while solubility in distilled water decreased [33]. Prior studies such as these, along with our current DTA results, lead us to hypothesize that increased glass network stability would lead to lower ionic dissolution upon submersion in aqueous solution. However, for each of the 5 elements analyzed, the lowest release totals were actually observed from the Ga-free *Control* glass, assuming that precipitation of ions out of solution did not occur at different rates between samples. Although the Na- and Zn-releases were nearly equal between *Control* and *TGa-2* after 30 days, significantly more Si was released from the 16 mol% Ga-containing *TGa-2* than *Control*, with *TGa-1* releasing a quantity between the two. These results directly contradict our predictions, as it was expected that increased Ga-content would correlate with increased stability, but these ion release results show that as Ga-content increases, more of the primary glass network-forming constituent (Si), is released, suggesting that increased Ga-content translates to faster dissolution of the glass over a period of 30 days. However, the long-term trials (90, 180, 365 days) show that the 8 mol% Ga-containing *TGa-1* glass exhibits greater release of each constituent (other than Ca), than *TGa-2*. This trend leads us to suspect that a threshold exists, pertaining to the amount of Ga that can be included in such a BG formulation before ion release begins to be inhibited, and Ga^{3+} begins to act less like a modifying glass network intermediate, and more like the intermediate Zn^{2+} which it was substituted for, over long-time periods. In 1996, Hill published "An alternate view of the degradation of bioglass," in which he proposed that the mechanism of bioglass degradation presented by Dr. Larry Hench was not particularly predictive of the reactivity of BGs as a function of their composition [34] Hill theorized that silicate glasses can be considered as inorganic polymers of oxygen crosslinked by Si atoms, and that the properties of these glasses may be predicted using the crosslink density of the glass network. Using these principles, he suggested that decreased crosslink density would result in lowered T_g, thus resulting in increased reactivity and solubility, and that the transition from a less reactive three-dimensional network to a more reactive linear polymer occurs at a network connectivity (NC) of 2. However, he also suggested that glasses with a NC just above 2 could still remain highly reactive, and that small changes in composition can greatly affect reactivity and biological properties. Prior characterization work on this glass series predicted the NC values of each glass assuming Ga acted as both a network former and modifier [26], and utilizing compositional data obtained from XPS analysis, suggested that the respective connectivities of *Control*, *TGa-1*, and *TGa-2* were 1.51, 2.16, and 2.82 if Ga acted purely as a former, and 1.51, 1.51, and 1.71 if Ga acted purely as a modifier. Considering the prior ^{29}Si MAS-NMR data, as well as the currently presented DTA data, it is suggested that Ga performed primarily in a glass network-forming role in this series, which according to the NC calculations, would indicate that *TGa-1* possesses a NC slightly above 2, while *TGa-2* possesses a NC much closer to 3. Although this aids in the explanation of the larger long-term ion release from *TGa-1* compared to *TGa-2*, it does not explain their increased reactivity in comparison to *Control* or the increased solubility of *TGa-2* compared to *TGa-1* over the first 30 days. Hill also theorized that if alumina is included in a bioactive silicate glass, the Al^{3+} ion will take on a tetrahedral coordination surrounded by four oxygens, and in the absence of phosphorus, its charge deficiency will be compensated in the first instance by an Na^+ ion, and in the second instance by half of a Ca^{2+} ion [34]. If we assume that Ga^{3+} will perform similarly to Al^{3+} in this series of glass structures, then we can assume that GaO_4^- tetrahedra will exist, with Na^+ ions acting as the primary charge compensators, and Ca^{2+} in a secondary compensation role. The ICP-OES results in this study show that despite the increase in T_g observed with increased Ga-content, the largest amounts of Na^+, Si^{4+}, and Ga^{3+} are released over the course of 365 days from *TGa-1*, followed by *TGa-2*, and then *Control*. This finding suggests that although the introduction of Ga^{3+} increases NC, the GaO_4^- tetrahedra require a charge compensator, and as Na^+ ions from the glass surface interact with H_3O^+ ions in solution, the primary charge compensating ions are removed from the glass, exposing charged network-forming tetrahedra, which may explain the observed increase in solubility. Also, since all 3 glasses were batched to contain the same amounts of

Na_2O and CaO (10 and 8 mol%, respectively), and *TGa-1* contains 8 mol% Ga_2O_3, while *TGa-2* contains 16 mol%, it is suggested that a greater fraction of the total number of GaO_4^- tetrahedra present are charge compensated by monovalent Na^+ ions in *TGa-1* than in *TGa-2*, which is why the increased Na^+ release from *TGa-1* in comparison to the other two glasses translates to more disruption of the network, and larger release of Si^{4+} and Ga^{3+}. This may also be supported by Ca^{2+} release measurements. These measurements showed that *TGa-2* released more Ca^{2+} for all time periods (with the exception of 90 days), than both *TGa-1* and *Control*, which may suggest that this ionic species was performing more frequently in a charge compensating role in GaO_4^- tetrahedra in *TGa-2*, resulting in increased Ca^{2+} release compared to *TGa-1*, despite their similar Ga^{3+} release totals. Additionally, inhomogeneity of the glass particles is one possible explanation for the increased solubility of *TGa-2* compared to *TGa-1* over the first 30 days of incubation. It may be possible that GaO_4^- tetrahedra with Na^+ ions as charge compensators arranged more readily near the surface of *TGa-2* particles than *TGa-1*, and tetrahedra with Ca^{2+} or Zn^{2+} ions as charge compensators were more prevalent in the bulk, resulting in increased degradation initially, and slower degradation over longer time periods, as the less mobile divalent cations [34] came into contact with solution. This suggestion will also be further investigated by the authors using EDAX line scans across cross sections of the different glasses to determine if there are compositional inhomogeneities present between the bulk and surfaces of the particles. These results are most encouraging, as it suggests that we may be able to manipulate a Ga-containing BG composition to tailor the charge compensation occurring within the glass network and ultimately control the rate of dissolution over long-time periods.

Another key finding in the ICP-OES results was that both of the Ga-containing glasses successfully released Ga from the glass network into solution, over all of the time periods, ranging from 11.3 mg/L (*TGa-1*) and 36.4 mg/L (*TGa-2*) after 1 day, to 1295.3 mg/L (*TGa-1*) and 1006.4 mg/L (*TGa-2*) after 365 days. This was an important observation, as the main purpose of including Ga in this glass series is to facilitate its release from the network to allow for interaction with cells within the local environment. With regard to the decrease in release at 365 days after release maxima at 180 days observed several times in these studies, one explanation could be that since these glass powder samples were only incubated in 10 ml of ultrapure water without exchange for such long-time periods, perhaps saturation limits were exceeded and ions which had been released from the glass structure precipitated back onto the particle surfaces. Decreases were also observed in the release profiles after 14 days of incubation, and minima were obtained at 30 days before increasing again, indicating a prior period where precipitation of ions out of solution and onto the particle surfaces occurred. Similar behavior has been observed before by Oliveira et al. [35]., when a series of SiO_2–P_2O_5–CaO–MgO BGs exhibited the ability to develop dual silica-calcium phosphate layers when incubated in SBF. This group also demonstrated that glasses richer in MgO develop a thicker silica gel layer on their surface, and that this can play a decisive role in layer detachment through the gel. The decrease observed in the presented release profiles after 14 days, the increase until 180 days, and then a second decrease to 365 days suggests that ions precipitated out of solution to form gel layers on the particle surfaces between 14–30 days of incubation, portions of these gel layers then dissolved or detached between 30–90 days, and further degradation of the glasses occurred until between 180–365 days, when ions again precipitated out of solution onto the particle surfaces, thickening the silica gel layers. To further investigate these claims, future work will be conducted to observe cross sections of the glass particles after different incubation periods using SEM and EDAX to determine if surface layers are present, to determine the compositions of these layers, and compare the EDAX results to ICP-OES information. In addition to ICP-OES, pH studies were conducted in order to aid in the understanding of this observed trend; however, the consistent increase in Na-release for all 3 glasses and the large Ga-release totals of the two Ga-containing glasses may have made it difficult to observe the effects of the relatively small decreases in Si, Ca, and Zn content in solution.

It is important to monitor pH in addition to ion release, as deviations from physiological pH levels can have detrimental effects on host tissue. The pH results after 30 days displayed expected trends, as relatively low levels of each element were detected in the 3 glasses during ICP-OES studies, which we expected would lead to similar pH levels for all 3 extracts. Additionally, Si^{4+}, Ca^{2+}, and Ga^{3+} releases were highest for *TGa-2* over the initial 30 days of incubation, while Na^+ and Zn^{2+} were nearly identical for all 3 glasses, which suggested a higher degradation rate, which would translate to a higher pH. The pH results after 365 days mostly produced the predicted tendencies, as it was expected that *TGa-1* extracts would possess the highest pH levels from 90–365 days, due to the elevated release levels observed during ICP-OES analysis. However, it was also predicted that *TGa-2* extracts would possess a higher pH than *Control* extracts after 365 days, since the ICP-OES results indicated that *TGa-2* released significantly more Na^+ than *Control*, and since it released a large amount of Ga^{3+}, while *Control* released none. However, pH testing actually revealed that after 365 days, *TGa-2* extracts exhibited a pH of 9.7, while the average pH of

Control extracts was 10.0. This result suggests that Na-release from *TGa-2* (which was significantly lower than *TGa-1*) had less influence on pH than in *TGa-1* extracts, and Ga-release had no effect on pH, which resulted in the slight acidity introduced by Si-release exhibiting more influence on the pH of extract solutions. Overall, all three glasses used in this study exceeded expectations, as prior work conducted by Cerruti et al. [36]. demonstrated that upon submersion in DI water, Bioglass® 45S5 powder raises the solution pH to ~10.5 after only 2 days, while only one of the glasses in the current study (*TGa-1*) exceeds a pH of 10 and that did not occur until after 180 days of submersion. While these results are encouraging, due to the length of time required to elicit large pH increases from glass samples shaking at 37 °C, they do not directly translate to clinical relevancy as the solutions were not changed at any point throughout incubation, which does not accurately replicate physiological conditions.

Cell viability evaluation

Due to the similarity in effects produced by all 3 glasses on fibroblast viability over the first 30 days, and the difference in effects produced by the non-Ga-containing *Control* glass extracts and the extracts of the two Ga-containing glasses *TGa-1* and *TGa-2* after 90, 180, and 365 days, it seems plausible that Ga is the element responsible for the dramatic decrease in viability. This suggestion is supported by the ICP-OES data. Na-release profiles show similar release levels for all 3 glasses up to 30 days and then an increase in Na-release from *TGa-1* and *TGa-2*. However, *TGa-1* released significantly more Na than *TGa-2* after 90, 180, and 365 days, which would suggest that unless Na-release from both glasses had surpassed toxic levels for L-929 fibroblasts after 90 days, then extracts from *TGa-2* should exhibit higher viability levels than those from *TGa-1* over these time periods, which they do not. A similar trend was observed in the Si-release profiles; however, Si-release levels for the *Control* glass were very similar to *TGa-2* after 365 days, so if Si was the main element contributing to change in L-929 fibroblast viability, then extracts from these two glasses should produce similar viabilities after 365 days, which they do not. Since the only element contained in *TGa-1* and *TGa-2* that is not contained in the *Control* glass is Ga, and the ICP-OES results did not show a dramatic increase in Ga-release from the Ga-containing glasses until after 90 days, we can conclude that the main culprit in decreasing L-929 fibroblast viability is the presence of elevated levels of Ga in solution. This conclusion is similar to that of a study by Schedle et al. [37]., which found that ^{3}H-thymidine incorporation by L-929 fibroblasts was completely inhibited in the presence of at least 0.1 mmol/L Ga^{3+}; however, fibroblasts in the current study were able to sustain higher concentrations of Ga^{3+} before exhibiting cytotoxic effects.

Unlike the results obtained from the L-929 fibroblast studies, MC-3T3-E1 viability analysis does not directly correlate with any of the ion release profiles and instead appears to be more efficiently explained using the results obtained from pH testing. It has been previously shown that osteoblasts are sensitive to changes in extracellular pH, as these changes may alter the potential across the cell membrane, resulting in the inhibition of ion exchange systems and the subsequent build-up of intracellular H^{+}, which can lead to protein denaturation [38, 39]. Kaysinger et al. studied the effects of pH levels of 7.0–7.8 on human osteoblasts and determined that osteoblasts exhibited cellular activity at all pHs, although plateaus in the data suggested that their preferred pH was 7.2 [40]. This finding helps to interpret the MC-3T3-E1 viability data obtained in the current study, as the viabilities produced by the *TGa-1* and *TGa-2* extracts do not display an observable decrease until after 90 days, when their respective pH values were 10.1 and 8.4, which are both well above 7.2. In addition, the extracts of all 3 glasses exhibit their lowest viabilities of all the incubation times after 365 days, which is when each extract type expressed its highest pH. As with the pH results, the cell viability data for MC-3T3-E1 osteoblasts exceeded expectations, as these assays were conducted in a static environment rather than a dynamic environment, which they would be exposed to in a host, and despite this disadvantage, there was only one significant decrease observed over all of the time periods for the 3 glasses (*TGa-2* 365 day extracts, compared to 1 and 30 day extracts).

Conclusion

This study characterized the ion release profiles and pH of extracts of a Ga-containing BG series for periods of up to 365 days and related that information to cell viability information using L-929 fibroblasts and MC-3T3-E1 osteoblasts. Ion release studies were conducted and produced unexpected results, as both of the Ga-containing glasses released significantly more Na and Si than the *Control* glass after at least 90 days, in addition to releasing large amounts of Ga, suggesting that Ga was acting in more of an intermediate role. Increased pH was also observed for extracts from these glasses when compared to extracts from the *Control*. Cell viability data suggested that the large amounts of Ga released by *TGa-1* and *TGa-2* after at least 90 days induced toxic effects on the L-929 fibroblasts. However, these data also suggested that these extracts did not negatively affect the viability of MC-3T3-E1 osteoblasts, leading to the conclusion that Ga-containing BGs have potential for bone void-filling applications.

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