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PAPER

Multifunctional gallium doped bioactive glasses: a targeted delivery for antineoplastic agents and tissue repair against osteosarcoma

Shirin B Hanaei[1], Raghavan C Murugesan[1], Lucas P Souza[1], Juan I Cadiz-Miranda[1], Lee Jeys[2,3], Ivan B Wall[3] and Richard A Martin[1,*]

[1] College of Engineering and Physical Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, United Kingdom
[2] Oncology Department, The Royal Orthopaedic Hospital, Birmingham B31 2AP, United Kingdom
[3] College of Health and Life Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, United Kingdom
[*] Author to whom any correspondence should be addressed.

E-mail: R.A.Martin@Aston.ac.uk

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Abstract

Osteosarcoma (OS) is the mostly commonly occurring primary bone cancer. Despite comprehensive treatment programs including neoadjuvant chemotherapy and tumour resection, survival rates have not improved significantly since the 1970s. Survival rates are dramatically reduced for patients who suffer a local recurrence. Furthermore, primary bone cancer patients are at increased risk of bone fractures. Consequently, there is an urgent need for alternative treatment options. In this paper we report the development of novel gallium doped bioactive glass that selectively kill bone cancer cells whilst simultaneously stimulating new bone growth. Here we show, using a combination of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide, LIVE/DEAD assays and image analysis, that bioactive glasses containing gallium oxide are highly toxic and reduce both the proliferation and migration of bone cancer cells (Saos-2) in a dose dependant manner. Glasses containing 5 mol% gallium oxide reduced the viability of OS cells by 99% without being cytotoxic to the non-cancerous normal human osteoblasts (NHOst) control cells. Furthermore, Fourier transform infrared and energy-dispersive x-ray spectroscopy results confirmed the formation of an amorphous calcium phosphate/hydroxyapatite like layer on the surface of the bioactive glass particulates, after 7 d incubating in simulated body fluid, indicating the early stages of bone formation. These materials show significant potential for use in bone cancer applications as part of a multimodal treatment.

1. Introduction

Osteosarcoma (OS) is the most common primary bone cancer and affects primarily children and young adults. It is a rare tumour characterised by malignant mesenchymal cells and bone stroma development [1]. Survival rates increased significantly in the late 70s early 80s with the development of chemotherapy. However, the 5 year survival rate has not increased significantly during the last 25–30 years with current rates relatively static around 53%–55% [2]. Current therapeutic approaches for OS are primarily based on neoadjuvant chemotherapy (before surgery), surgery and adjuvant chemotherapy (after surgery) where chemotherapy is based on different combinations of doxorubicin, cisplatin, and methotrexate. Radiotherapy is rarely given for OS due to radio-resistance [2]. Chemotherapy drugs often have unpleasant side effects such as vomiting, hypersensitivity reactions, nausea, neurotoxicity, nephrotoxicity and cardiomyopathy [3, 4]. However, of much greater clinical concern is that drug-resistance has now been reported for key chemotherapy drugs including methotrexate, cisplatin, doxorubicin, and ifosfamide. Patients who respond poorly to chemotherapy have bleak prognosis [5].

Survival rates for patients who have a local recurrence are extremely low. For example, Bacci *et al* reported institutional 5 year survival rates of above 70% for patients without local recurrence compared to just 15.9% for patients who had a local recurrence [6].

Whilst preventing local recurrence is the primary objective a secondary objective must be to repair the bone void caused by the tumour removal. Over 50% of OS long-term survivors have a bone mass deficit [7] and patients have an increased risk of bone fractures [8] particularly in the early stages following diagnosis. Pathological fractures have been reported to reduce survival rates by ∼20% [9]. Usually, preventing local recurrence and regenerating lost bone/preventing fractures are delt with separately. Therefore, developing novel therapeutic platforms with high efficacy against primary OS is crucial. Here we propose an innovative solution using bioactive glasses containing gallium ions to tackle both local recurrence and fracture occurrence simultaneously. The idea is to develop a biomaterial for the treatment of bone cancer based on two important functions: (1) targeted drug delivery to the tumour site (2) introducing a regenerative scaffold to stimulate the growth of new bone.

Bioactive glasses are widely used for bone repair and regeneration applications, they are biocompatible, biodegradable, osteoconductive, osteoinductive and osteogenic [10, 11]. Bioglass® forms a bond with bone tissue and induces osteogenesis reaction by releasing biologically active ions (Ca, P and Si). Whilst there has been a lot of research interest in the development and improvement of bioactive glasses for bone tissue engineering, there has been very few studies on bioactive glasses for bone cancer applications. However, bioactive glasses can be tailored to release a wide range of physiologically important ions including gallium [12].

Gallium (Ga) compounds play an important role in the management and treatment of a variety of cancers [13, 14]. Ga is the most widely used metal for the treatment of cancer after platinum. The antitumour properties of Ga were confirmed in 1971 by Hart and Adamson [15]. Studies conducted on calf thymus DNA have demonstrated that Ga can bond to DNA and disrupt helix DNA [16]. In addition, Ga can induce apoptosis by triggering condensation of chromatin [17]. It also prevents DNA replication by forming complexes with TfR and blocks iron uptake, which results in cellular iron deprivation. It interferes with iron-dependent growth pathways in tumour cells by acting as an iron mimetic [18]. Many studies have shown the high potential of Ga based compounds for cancer therapy, for example, $Ga(NO_3)_3$ demonstrated the highest efficacy for tumour suppression with the lowest toxicity. $Ga(NO_3)_3$ has been intravenously administered with a dosage up to 300 mg Kg^{-1} using protracted venous infusion. However, side effects such as granulocytopenia grade 3 and 4 despite the use of growth factors, renal function alteration grade 3 or 4, hypocalcemia grade 3 or 4, thrombocytopenia and temporary blindness have been reported in some of the tested patients [19].

Bioactive glasses can be used to introduce localised drug delivery which increases the bioavailability of Ga compared to the intravenous infusion [20]. In this context, Ga doped bioactive glasses have been reported as a potential scaffold for dual action of the cancer therapy and bone tissue engineering [12, 20, 21]. Despite extensive research on different types of bioactive glass/ceramics for bone tissue engineering, there is limited research on targeted and controlled release of anti-cancer reagents properties for bone cancer. Preliminary studies by Rahimnejad Yazdi *et al* and Souza *et al* have reported bioactive glasses doped with 3 mol% Ga_2O_3 can reduce osteosarcoma (Saos-2) cell activity by ∼50% after 3 d [20, 21]. However, given how rapidly cancer cells can multiply, a significantly higher rate of kill is needed if these glasses are going to be a viable to treatment option. Souza *et al* have reported that glasses with up to 3 mol% Ga are biocompatible with no sign of local toxicity *in vivo* [21], thus providing additional confidence that the maximum gallium concentrations have not yet been achieved. In this study we have developed novel gallium doped sodium calcium phosphor-silicate based bioactive glasses to treat OS. The glasses used in the present study contain significantly higher gallium concentrations, which has been achieved by extending the glass forming regions by employing rapid quenching techniques. The cytotoxic and anti-tumour effect of these Ga doped bioactive glasses were tested against human derived osteosarcoma cells (Saos-2) using 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) and LIVE/DEAD assays with normal human osteoblasts (NHOst) used as the non-cancerous control group.

2. Material and methods

2.1. Glass fabrication

Ga doped bioactive glasses were synthesised using the melt-quenched method. Precursors including H_6NO_4P, Na_2CO_3, $CaCO_3$, SiO_2, and Ga_2O_3 were weighed to give the appropriate molar compositions $(Ga_2O_3)_x(SiO_2)_{46.1-3.06x}(CaO)_{26.9}(Na_2O)_{24.4}(P_2O_5)_{2.6}$. As shown the silica content was reduced by a factor of three times the molar addition of Ga_2O_3. This was to prevent the glasses from becoming less soluble upon the addition of Ga_2O_3. A total of six bioactive glasses were fabricated with increasing Ga_2O_3 concentrations corresponding to 0, 1, 2, 3, 4 and 5 mol% Ga_2O_3. The composition of the 0% Ga glass, which was used as the control, corresponds to 45S5.

Precursors were thoroughly mixed, placed in a 90% platinum-10% rhodium crucible (GLC alloys Ltd Middlesex, UK) and then heated using an electric furnace. The precursors were heated from room temperature at a rate of 10 °C min^{-1} to 1450 °C and held at this temperature for 90 min. The molten liquid was poured into graphite moulds at room temperature. The 5 mol% of Ga doped bioactive glass was fabricated via fritting method where the molten liquid was quenched directly into distilled water, the sample was then quickly removed and dried. Each glass composition was ground with planetary ball mill (PM100, Retsch) and sieved to obtain a particle size distribution between 40 and 63 μm. Glass powders were used for characterization analysis and cell studies. Each glass composition was kept in a desiccator to prevent absorption of atmospheric moisture.

2.2. Bioactive glass characterisation

2.2.1. Elemental compositional analysis

Elemental analysis of the gallium doped bioactive glasses was undertaken using Energy-dispersive x-ray spectroscopy (EDS) using a JEOL JCM-6000 Plus Neoscope Scanning Electron Microscope equipped with EDS detection laser. EDS measurements were undertaken using an excitation energy of 15 KeV. Bioactive glasses were measured initially to determine their compositions directly after manufacture. A second measurement was also undertaken on the glasses after they had been exposed to simulated body fluid (SBF) to determine the change in surface composition and potential bioactivity as described in section 2.2.5.

2.2.2. Ion release

Inductively coupled plasma optical emission spectrometry ICP-OES measurements were undertaken to quantify the concentration of ionic dissolution products released from the glasses. Stock solutions were prepared at concentrations of 10 and 20 mg ml^{-1} in distilled water using a shaker incubator (200 rpm) at 37 °C for 24 h. After incubation the solutions were filtered using a 0.4 μm syringe filter to remove the glass particles from the solution. Nitric acid was added to all the samples to ensure that the elemental component remained in the solution. Experiments were undertaken using an ICP-OES (iCAP 7000 Plus Series). Reference standards of Ga^{3+}, Si^{4+}, Ca^{2+}, and Na^{+} were diluted with distilled water at 1, 10, 20, 100 ppm to create calibration curves. The concentrations of ions were calculated using the linear portion of the plotted standard curve.

2.2.3. Fourier transform infrared (FTIR) analysis

FTIR spectroscopy was performed using a PerkinElmer Frontier FTIR equipped with PEAK technologies GladiATR sampling accessory. The FTIR measurements were undertaken with a spectral resolution of 4 cm^{-1} from 4000 to 400 cm^{-1} at room temperature and a mean of 32 scans were recorded per sample. The data was plotted as percentage of transmission.

2.2.4. XRD analysis

X-ray diffraction measurements were undertaken using a Bruker D8 diffractometer operating at the copper kα wavelength of 1.54 Å. Finely powdered samples were measured over a 2θ range of 10–90° in 0.02° steps at 1 s per point.

2.2.5. Sample preparation for bioactivity testing

SBF was prepared as outlined by Macon *et al* [22]. Bioactive glass samples were placed in SBF (10 mg ml^{-1}) and incubated in a shaking incubator operating at 200 rpm and 37 °C for a period of 7 d. After 7 d the glass powders were removed from the solution using filter paper, washed with distilled water, and then rinsed with acetone to halt any further reactions and eliminate the residual elements of SBF. The samples were then dried at 60 °C overnight and were used for characterisation analysis including EDS elemental analysis and FTIR as described in sections 2.2.1 and 2.2.3 respectively.

2.3. Cell culture

Saos-2 cells were purchased from the American Tissue Culture Collection and grown according to manufacturer's instructions in McCoy's 5A media containing 1.5 mM L-glutamine and 2200 mg l^{-1} sodium bicarbonate. Media was supplemented with 15% foetal bovine serum (FBS). Primary NHOst cells were purchased from Lonza and grown according to manufacturer's instructions in Clonetics OGM Osteoblast growth media which was supplemented with 10% FBS, gentamicin sulphate-Amphotericin (GA-1000) 0.50 ml, and ascorbic acid 0.50 ml. The cells were kept at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Cells were seeded in T-75 flasks and the media was replenished every 2–3 d and split upon 80% of confluency.

2.4. Conditioned media

Conditioned media was prepared by mixing the glass particles (particle size range from 40 to 63 μm) at concentrations of 10 and 20 mg ml^{-1} with serum free McCoy's 5A media to treat Saos-2 cells and OGM™ media for NHOst cells. The media was incubated in a shaker incubator at 200 rpm and 37 °C for 24 h. After incubation, the conditioned media was filtered with 0.2 μm syringe filter to remove the glass particles and to also sterilise the stock solution. The conditioned media for Saos-2 was supplemented with 15% of FBS while 10% FBS was added to the conditioned media for NHOst as per the supplier's instructions. Media was then incubated overnight with 5% CO_2/95% air

at 37 °C prior to use. This buffers the pH and ensures false positive results are not present due to the elevated pH of the conditioned media [23].

2.5. Cell cytotoxicity and proliferation

Cell viability assays were conducted using the MTT viability assay kits (Fisher Scientific) to investigate the cytotoxic effect of the Ga doped bioactive glasses *in vitro* to Saos-2 (OS) cells with NHOst as the non-cancerous control cells. Cells were seeded into 48 well plates, with a density of 5000 cells per cm^2, in complete growth media. Twenty-four hours after seeding, the growth media was replaced with media conditioned using the Ga doped bioactive glass. MTT assays were performed at day 1, 3, 5, 7 and 10. The media was changed on day 3 and 7. Ethanol 70% (v/v) was used to represent the positive control while the cells grown in normal growth media represents negative control. For the MTT assay, a 12 mM stock solution of MTT was prepared as per manufacturer's instructions and diluted 1/10 in phenol-red free Dulbecco's Modified Eagle Media (DMEM) before being added to the cells and incubated for 4 h at 37 °C. After removing the MTT reagent, the resulting formazan derivatives were dissolved in dimethyl sulfoxide and incubated at 37 °C for 10 min. Metabolically active cells reduce MTT to formazan and after formazan extraction, the optical density was measured using Ascent MultiScan GO spectrophotometer (Fisher Scientific, Leicester, UK) at 570 nm. This assay was performed in 5 replicates. Cell free blank samples containing DMEM media and MTT reagent were used for detection and background subtraction.

2.6. Live/Dead cell viability

Cell viability was investigated using a LIVE/DEAD™ Viability assay Kit (Thermo Fisher Scientific, MA, USA). The assay contains two fluorescent nucleic acid stains: Calcein-acetoxymethyl (AM) which is green, and ethidium homodimer-1 (EthD-1) which is red. Calcein-AM permeates living cells, upon entering the cell the intracellular esterases cleave the AM ester group trapping the Calcein fluorescent green dye within the living cell. EthD-1, the fluorescence red dye, can only penetrate cells with damaged membranes, when it then binds with nucleic acid. Therefore, live cells with undamaged cell membrane appear green while the dead cells with damaged cell membranes appear red.

Saos-2 and NHOst cells were seeded at 5000 cm^{-2} in 48 well plates and the viability of the cells was evaluated post exposure to the conditioned media. The LIVE/DEAD assay was performed at time-points of 1, 3, 5, 7 and 10 d with media changes on day 3 and 7. Calcein-AM at 2 μM and EthD-1 at 4 μM were mixed into a single solution with Dulbecco's phosphate-buffered saline (D-PBS) and 200 μl of this master mix was added to each well and incubated at the room temperature (under dark conditions) for 1 h. Cells were photographed using a Leica fluorescent microscope at 10× magnification.

2.7. Cell proliferation and migration

Cell proliferation and cell migration studies were performed using label-free, non-invasive cellular confluence assay by IncuCyte Live-Cell Imaging Systems (Essen Bioscience, Ann Arbor, MI, USA). Saos-2 cells were seeded into 48 well plates with the density of 5000 cells per well in McCoy's 5A medium and supplemented with 15% FBS and maintained in a cell culture incubator at 37 °C with 5% CO_2/95% air overnight. The next day the growth medium was replaced with conditioned media and the plate was transferred to the IncuCyte imaging system for a period of 10 d. Images were captured every 2 h for up to 10 d using 10X objective to monitor the cell proliferation in real time. Cell confluence was calculated using Fiji image analysis.

To investigate cell migration, a scratch wound assay was performed on Saos-2 cells. Saos-2 cells were seeded onto a 6-well plate with 5×10^5 cells per well in complete growth medium. Cells were grown for 24 h to form a 90% confluent monolayer. A scratch ∼3 μm wide was made using a micropipette tip. Media was removed and the residue of the lifted cells was washed twice with PBS and the cells were cultured in absence and presence of conditioned medium containing gallium compounds. Images were acquired immediately following media replacement and then every 2 h for a period of 10 d using IncuCyte Live-Cell imaging system (Essen Bioscience, Ann Arbor, MI, USA) at 10× magnification. The final images were analysed using ImageJ (National Institutes of Health) to examine the wound area distance. Cells were kept under sterile culture conditions at 37 °C in an atmosphere containing 5% CO_2 during image collection. The percentage of wound area was plotted over the time for each concentration. Data are presented as mean ± SD. The percentage (%) of wound closure was calculated using,

$$\text{Wound closure}\ (\%) = \frac{W_0 - W_t}{W_0} \times 100 \qquad (1)$$

where W_0 is the initial width of the scratch wound at day zero and W_t is the width of the scratch after time t.

2.8. Statistical analysis

All the experiments described were performed in at least three independent experiments with typically 4 replicates per experiment. The data were analysed using GraphPad Prism 8 software. The results are expressed as the mean ± standard deviation. Two-way ANOVA and Tukey's multiple comparisons test was performed to test for significance with statistically significant values defined as $p < 0.05$ for a precise comparison between the viability of the cell's expose to different glass compositions at different time points.

Table 1. Compositional values obtained from EDS for the as prepared bioactive glasses, nominal calculated values are given in parentheses.

Glass code	Composition (mol%)				
	SiO_2	CaO	Na_2O	P_2O_5	Ga_2O_3
45S5	43.6 ± 1.0 (46.1)	30.1 ± 0.8 (26.9)	24.0 ± 0.7 (24.4)	2.2 ± 0.4 (2.6)	0 (0)
1% Ga	41.9 ± 1.1 (44.0)	30.1 ± 0.8 (27.5)	24.4 ± 0.7 (24.9)	2.7 ± 0.5 (2.7)	0.9 ± 0.7 (1.0)
2% Ga	37.9 ± 1.5 (41.9)	32.1 ± 1.2 (28.0)	25.6 ± 1.1 (25.4)	2.1 ± 0.6 (2.7)	2.2 ± 1.2 (2.0)
3% Ga	36.9 ± 0.9 (39.8)	32.3 ± 0.8 (28.6)	25.2 ± 0.7 (25.9)	3.0 ± 0.4 (2.8)	2.6 ± 0.8 (3.0)
4% Ga	32.1 ± 1.5 (37.7)	34.8 ± 1.4 (29.1)	26.5 ± 1.2 (26.4)	3.1 ± 0.8 (2.8)	3.5 ± 1.5 (4.0)
5% Ga	32.6 ± 1.4 (35.5)	33.2 ± 1.2 (29.7)	26.5 ± 1.1 (26.9)	2.5 ± 0.7 (2.9)	5.2 ± 1.5 (5.0)

3. Results

3.1. Physical characterisation of the bioactive glasses

Six bioactive glasses containing 0–5 mol% of Ga_2O_3 were successfully fabricated. The glasses all looked optically transparent (by eye) and showed no signs of crystallisation or phase separation. When incorporating 5 mol% Ga_2O_3 into the glass a tendency for crystallisation to occur during air quenching was noticed. Consequently, this composition was manufactured by quenching directly into distilled water thereby achieving a faster quenching rate and avoiding crystallisation.

Glass compositional values obtained from EDS are given in table 1 (raw EDS spectra are given in the supplementary information). These values are in agreement with the expect theoretical values, within experimental errors. Nominal Ga_2O_3 values are 0–5 mol% in increments of 1%. The average variation between the theoretical and measured value is just 0.1 mol% with a maximum difference of 0.5 mol% observed for the 4 mol% Ga glass. Sodium is within one % of the expected nominal values and Ca within 4%. SiO_2 and CaO values are ∼10% below and above the theoretical values respectively. However, this is a consistent and systematic difference across all samples and is attributed to an error in the calibration standards rather than a deviation from the expected nominal theoretical values.

Figure 1 shows the dissolution profile of the Ga doped glass compounds after incubating in distilled water at the concentration of 10 mg ml^{-1} as a function of time. Dissolution values for the 24 h timepoint, which is the timepoint used for cellular studies, is given in table 2. The release rate of ions is broadly as expected. A systematic increase is observed for Ga with the increasing Ga_2O_3 content, Si content decreases as expected as the concentration of SiO_2 is reduced by 3 times the concentration of Ga_2O_3. Ca is reasonably stable whilst Na is seen to increase significantly. CaO and Na_2O concentrations are expected to increase as the glass composition is renormalised following the reduction is SiO_2. At first inspection it appears as though the glass dissolves more rapidly for the 5% Ga glass than expected however the value (after normalising by composition) is still within 2 standard deviations of the expected trend.

3.2. Bioactivity, apatite formation

Figure 2 shows the FTIR spectra for the gallium doped bioactive glasses after being immersed in SBF for 7 d. IR absorption bands are clearly visible ∼565 and 605 cm^{-1} and are attributed to the PO_4^{-3} tetrahedra. The peak ∼1030 cm^{-1} is assigned to the P–O bending [22]. Bands detected ∼875, 1420 and 1460 cm^{-1} are assigned to CO_3^{-2}. These CO_3^{-2} bands have previous been identified and attributed to carbonated apatite precipitation ($Ca_9(HPO_4)_{0.5}(CO_3)_{0.5}(PO_4)_5OH$) which preferentially forms rather than pure hydroxyapatite. Note there is no evidence of CO_3^{-2} or PO_4^{-3} IR bands in the unreacted '*as prepared*' bioactive glasses.

The formation of an apatite layer is further supported by compositional data obtained from EDS after incubating the glasses for 7 d in SBF (see table 3). As shown the SiO_2 content has increased by on average 14 mol% across the whole series. This to be expected as Na_2O, P_2O_5 and to a lesser extent CaO are known to leach rapidly from the glass. Consequently, after the removal of these elements from the glass followed by renormalising the remaining components back to 100% a significant increase is observed for the remaining SiO_2 [24]. The Na_2O content has significantly decreased as expected. Initially it is expected that CaO and P_2O_5 would both leach out of the glass however by 24 h the formation of an amorphous calcium phosphate/carbonate apatite layer begins to occur. As seen the P_2O_5 content has doubled after exposure to SBF (table 3) compared to the unreacted glasses (table 1). This is further evidence of the formation of an amorphous calcium phosphate/apatite layer on the glass surface indicating the initial stages of bone regeneration.

Figure 3 shows the x-ray diffraction spectra obtained for the unreacted bioactive glasses. As shown the peaks are very broad confirming the amorphous nature of these glasses. A small peak is observed for the 5% glass indicating a small amount of crystallisation. Calculating the relative area under the peaks indicates that less than 5% of the sample is the crystallised.

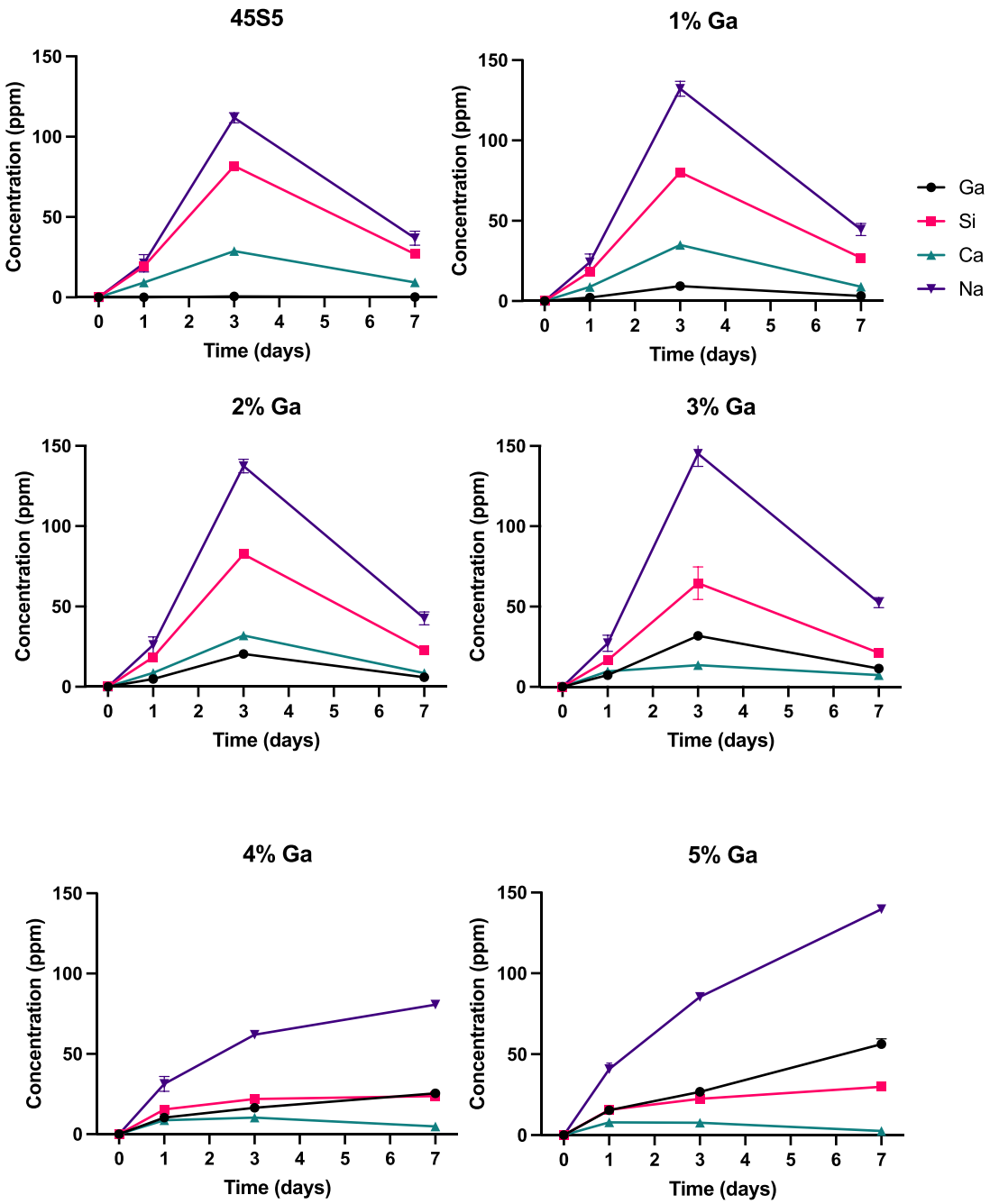

Figure 1. Elemental concentration release for Ca, Na, Ga, and Si as determined by ICP analysis of solutions resulting from the dissolution of 45S5, 1%–5% Ga doped BG in dH_2O for a period of 7 d.

Table 2. ICP data after 24 in distilled water (ppm).

	Concentration (ppm)			
Glass code	Na	Ca	Si	Ga
45S5	21.1	9.1	19.2	0.1
1% Ga	24.2	8.9	18.2	2.2
2% Ga	26.0	8.6	18.5	4.8
3% Ga	27.2	9.7	16.5	7.3
4% Ga	31.4	8.7	15.5	10.3
5% Ga	40.8	8.0	15.6	15.4

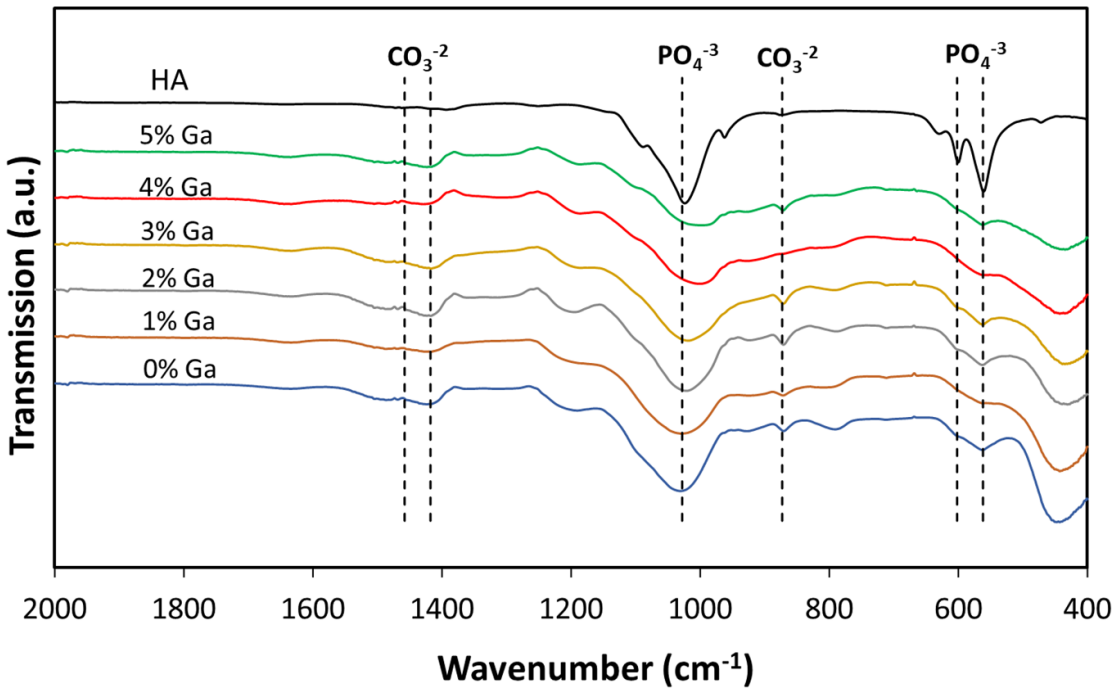

Figure 2. FTIR bands for bioactive glasses following 7 d immersion in simulated body fluid. Hydroxyapatite (HA) is shown for reference. Data sets are offset for clarity.

Table 3. Composition of bioactive glass surface after immersion in simulated body fluid for 7 d.

Glass code	Composition (mol%)				
	SiO_2	CaO	Na_2O	P_2O_5	Ga_2O_3
45S5	60.1 (±2.5)	34.3 (±1.9)	0.9 (±0.4)	4.7 (±1.3)	0.0 (0)
1% Ga	57.6 (±1.8)	29.3 (±1.3)	5.9 (±0.6)	5.5 (±1.0)	1.8 (±0.9)
2% Ga	57.1 (±2.5)	32.6 (±1.8)	2.3 (±0.7)	5.0 (±1.3)	2.9 (±1.4)
3% Ga	47.8 (±1.2)	38.8 (±1.0)	4.5 (±0.4)	4.8 (±0.7)	4.1 (±0.9)
4% Ga	46.8 (±1.9)	32.2 (±1.4)	10.5 (±0.9)	4.3 (±1.0)	6.2 (±1.6)
5% Ga	44.5 (±2.0)	38.2 (±1.7)	5.8 (±0.8)	4.9 (±1.1)	6.7 (±1.7)

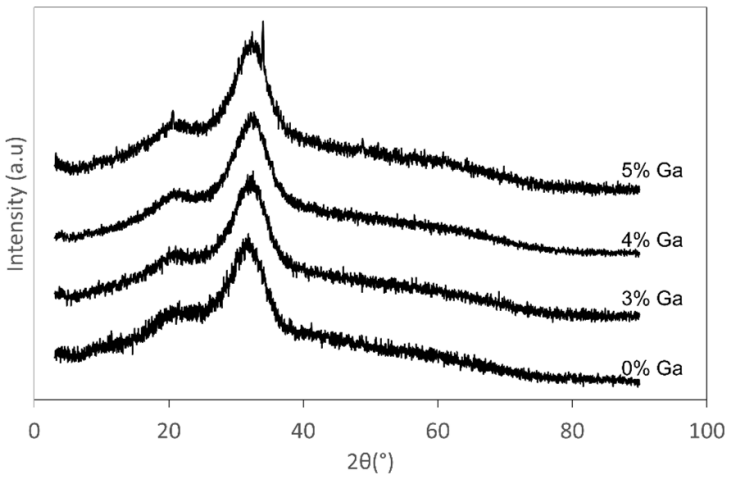

Figure 3. X-ray diffraction spectra of the unreacted bioactive glasses.

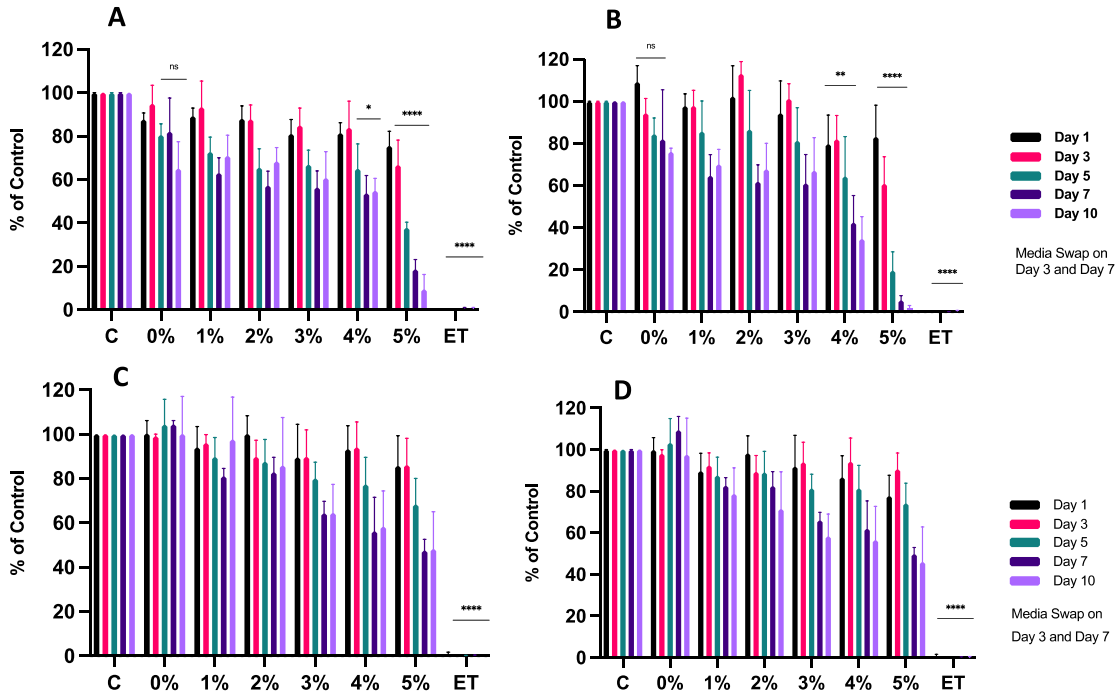

Figure 4. The effect of the dissolution products of Bioglass 45S5 and Ga doped silica based bioactive glasses (1–5 mol%) on the cellular metabolic activity using MTT viability assay. The data is represented as Mean ± SD ($N = 5$). (A) Saos-2 cells exposed to the 10 mg ml^{-1} conditioned media. (B) Saos-2 cells exposed to the 20 mg ml^{-1} conditioned media. (C) NHOst cells exposed to the 10 mg ml^{-1} conditioned media. (D) NHOst cells exposed to the 20 mg ml^{-1} conditioned media.

3.3. Cytotoxic analysis of Ga doped bioactive glasses *in vitro*

The cytotoxic effect of Ga doped bioactive glasses was investigated against Saos-2 and NHOst cells using an MTT viability assay. Cells were exposed to the conditioned media containing extracts from the 0, 1, 2, 3, 4, 5 mol% Ga doped powder at 10 and 20 mg ml^{-1} and the results are shown in figure 4. Cell viability assessed as percentage of negative control (unconditioned media) shows a clear dose dependant inhibition of Saos-2 cell growth with the increase in the accumulation of Ga ions in media with both concentrations of the glass ($p < 0.0001$). A significant cytotoxic effect was observed in the Saos-2 cells exposed to the conditioned media containing 20 mg ml^{-1} Ga glass with increasing Ga ions content comparing to the control ($p < 0.0001$). Viability of cells exposed to 10 mg ml^{-1} also demonstrated a steady and significant decrease comparing to the control group ($p < 0.0001$) but in comparison to the 20 mg ml^{-1} Ga glass concentration, cell cytotoxicity was less pronounced.

After a period of 10 d, Saos-2 cells exposed to conditioned media for 4 and 5 mol% Ga_2O_3 doped bioactive glasses (10 mg ml^{-1}) showed a significant decrease in cell viability with ∼60% and <10% viability respectively ($p = 0.0206$ and $p < 0.0001$ in order). Saos-2 cells treated with 20 mg ml^{-1} of 4% of Ga_2O_3 conditioned media showed less than 40% of Saos-2 cell viability whilst over 99.9% cell death was observed for cells treated with the media containing 20 mg ml^{-1} of 5% Ga glasses after 10 d. There was no significant reduction in Saos-2 cell viability, when treating with either 10 or 20 mg ml^{-1} of Ga free (Bioglass 45S5) conditioned media, even after 10 d when compared to the negative control cells (figures 4(A) and (B)).

The MTT results showed some mild cytotoxic effects against NHOst cells after treatment with Ga containing conditioned media for a period of 10 d. Cells treated with 10 and 20 mg ml^{-1} of 4% and 5% Ga content conditioned media had over 60% and 50% viability post 10 d of treatment. Cells treated with undoped conditioned media (45S5) showed no reduction in cell viability after 10 d. The group of cells treated with 70% ethanol showed complete cell death as expected ($p < 0.0001$). Two-way ANOVA and Tukey's multiple comparisons test were performed to test for significance and the data are presented as mean ± SD. Significance was set at $p \leqslant 0.05$, $N = 5$.

3.4. Investigation of cell viability by Live/Dead assay

Live/Dead images for Saos-2 and NHOst are shown in figures 5 and 6 respectively. Live cells are illustrated in green while the dead cells are in red. Saos-2 and NHOst cells were exposed to the conditioned media containing 10 and 20 mg ml^{-1} of the glass dissolution product for a period of 10 d and the cytotoxic effect

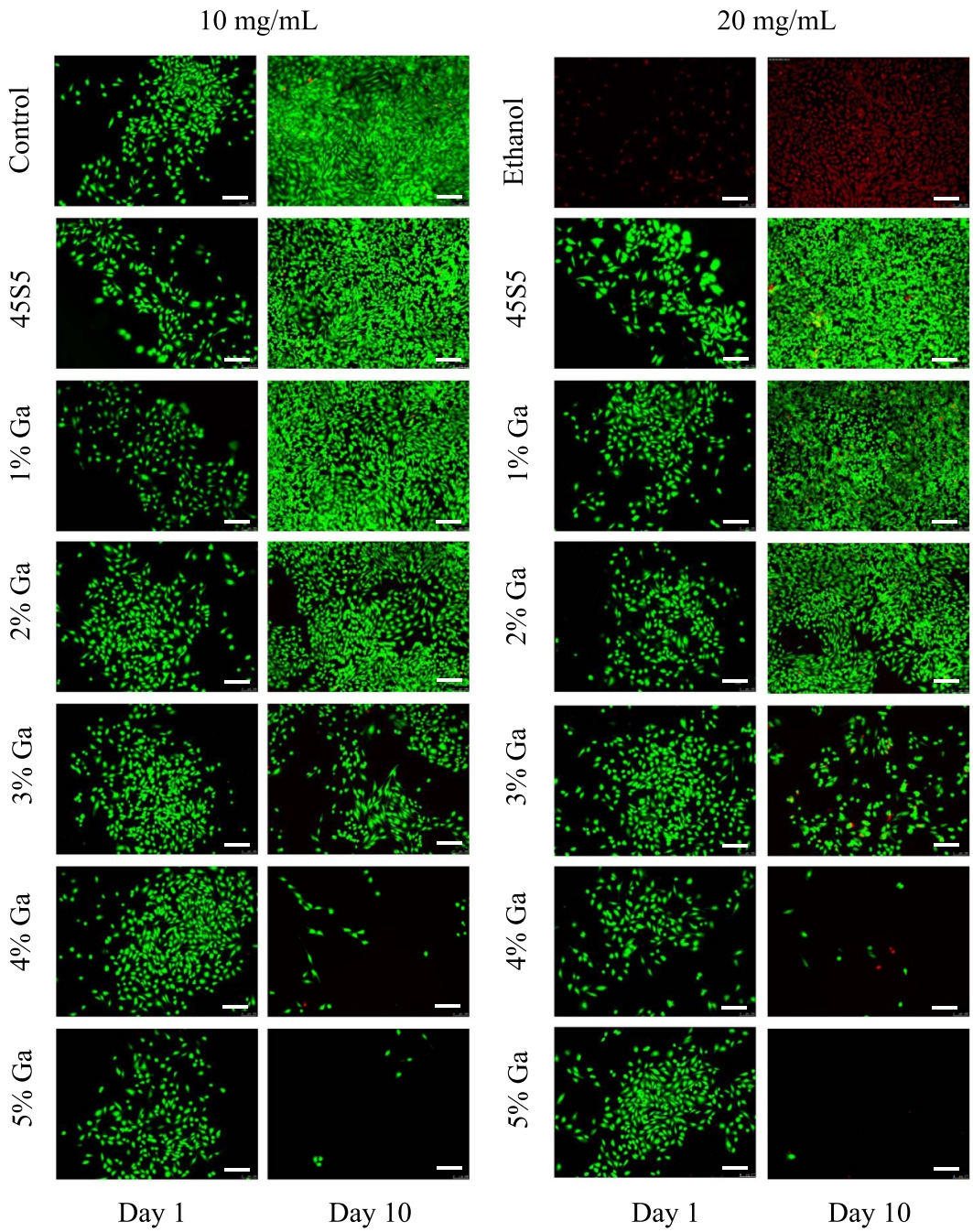

Figure 5. Live/Dead fluorescence images for Saos-2 cells treated with 10 and 20 mg ml^{-1} conditioned media (10× magnification). Green (live), red (dead), scale bar equals 100 μm.

was evaluate at the time points day 1, 3, 5, 7, and 10 however, only the time points of day 1 and 10 have been illustrated for clarity (figures 5 and 6).

Saos-2 cells treated with dissolution products of bioactive glass 45S5 at 10 and 20 mg ml^{-1} for 10 d demonstrated similar viability as the cells cultured in normal OS cell media i.e., negative control. Saos-2 cells showed the lowest susceptibility towards the conditioned media of 1 and 2 mol% Ga glass over the course of 10 d at both concentrations of 10 and 20 mg ml^{-1}. However, cancer cell death started to become more pronounced from glasses containing 3% Ga_2O_3 and above. Dissolution products of Ga doped glass containing 4% and 5% of Ga demonstrated a significant reduction of the Saos-2 cell viability at both concentrations of 10 and 20 mg ml^{-1}. The cytotoxic effect of Ga ions is obvious from day 3 (data not shown) for 4 and 5 mol% of Ga containing media as the cell growth is inhibited alongside with cell death. Saos-2 cells treated with conditioned media containing 5% Ga demonstrated a significant decrease in the cell viability. The viability of cancers cells was reduced over the course of 10 d and on the time point of day 10 less than 1%

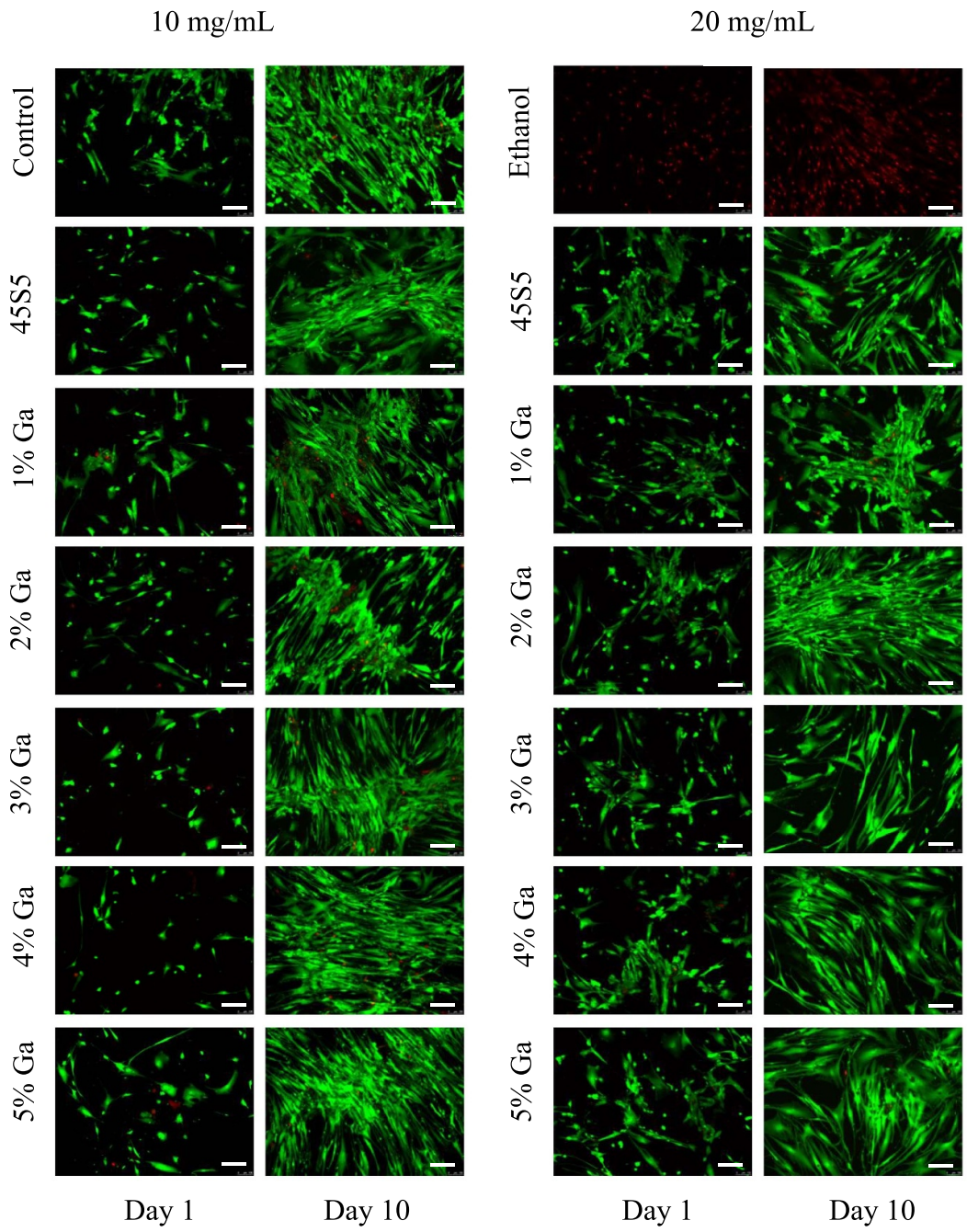

Figure 6. Live/Dead fluorescence images for NHOst cells treated with 10 and 20 mg ml^{-1} conditioned media (10× magnification). Green (live), red (dead), scale bar equals 100 μm.

viable cells were observed. Unlike for Saos-2 cells, all conditioned media including 5% Ga content did not induced severe cytotoxicity for NHOst cells. None of the conditioned media at 10 mg ml^{-1} demonstrated any significant detrimental effect on the viability of NHOst cells even after 10 d of treatment. Nevertheless, at the concentration of 20 mg ml^{-1}, conditioned media showed a mild growth inhibition for NHOst cells for 3%–5% Ga containing bioactive glasses.

3.5. Cell proliferation and migration

Cell proliferation data is shown in figure 7. As expected, a significant increase in cell numbers is observed for both the control media and the 45S5 (0% Ga) control glass after 10 d at both 10 and 20 mg ml^{-1}. A significant increase in cell numbers is also clearly visible for cells exposed to media conditioned with the 1%, 2% and 3% Ga doped glasses at 10 mg ml^{-1}, where the cell density is approaching 100% confluency. Cells treated with 4% Ga doped bioactive glass

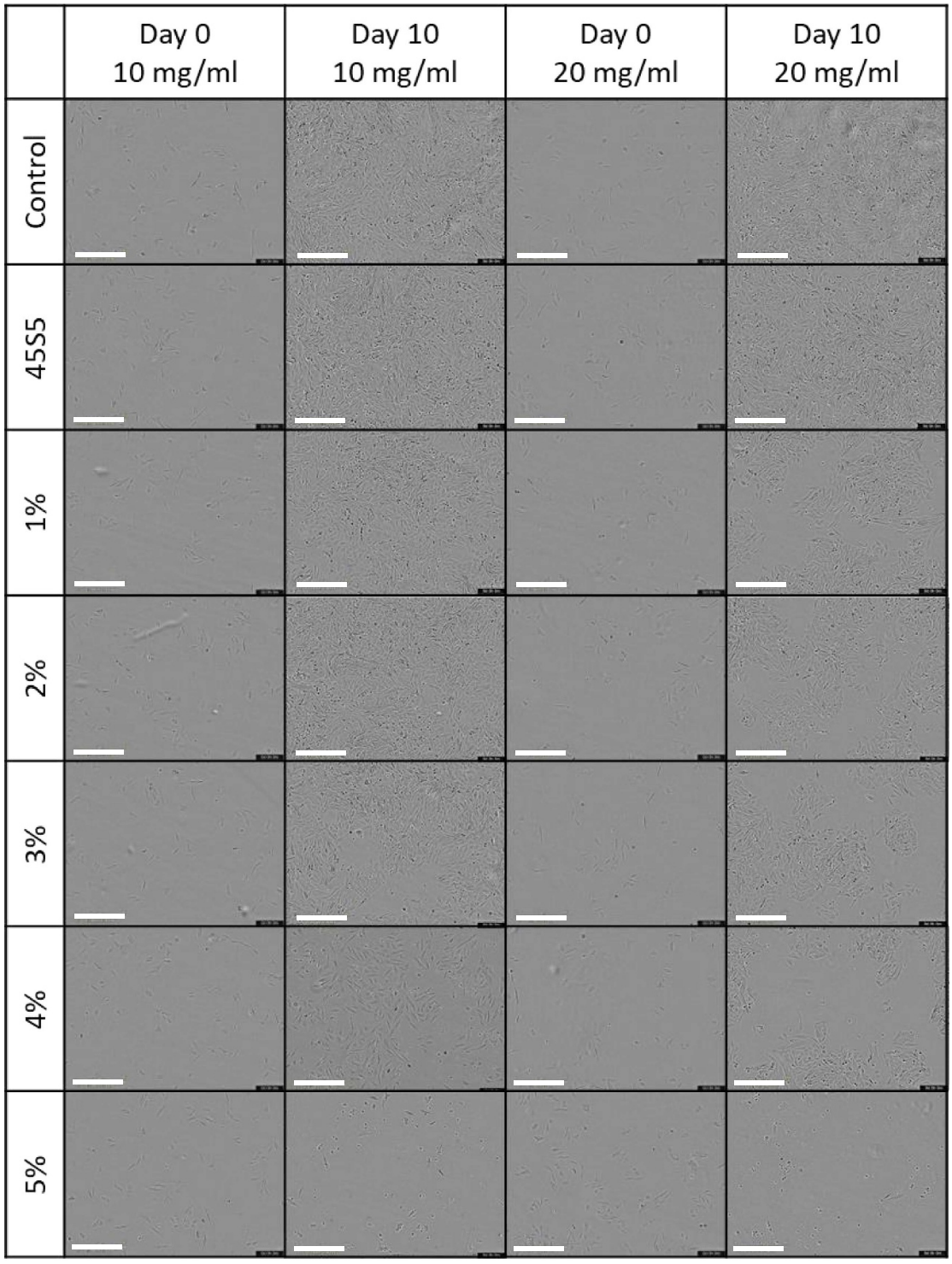

Figure 7. Cell proliferation images for Saos-2 cells at time point at day 0 and day 10. Scale bar equals 400 μm.

at 10 mg ml^{-1} do show an increase in cell numbers, but the cells are clearly much less confluent. In contrast cells exposed to 5% Ga doped bioactive glass at 10 mg ml^{-1} do not show any increase in cell numbers confirming that these glasses completely inhibit cell proliferation when exposed to such a concentration of gallium. Results for cells exposed to 20 mg ml^{-1} show a similar but much more pronounced effect; cell numbers are significantly lower for all the Ga doped glasses compared to results at 10 mg ml^{-1} with the 4% and 5% glasses again showing the lowest number of cells.

Cell migration data for Saos-2 cells as a function of time is shown in figure 8(A). Quantitative data for the scratch wound as a percentage of the original scratch width is shown in figure 8(B). As shown the wound is almost completely closed for the negative control (∼70% closed) and the 45S5

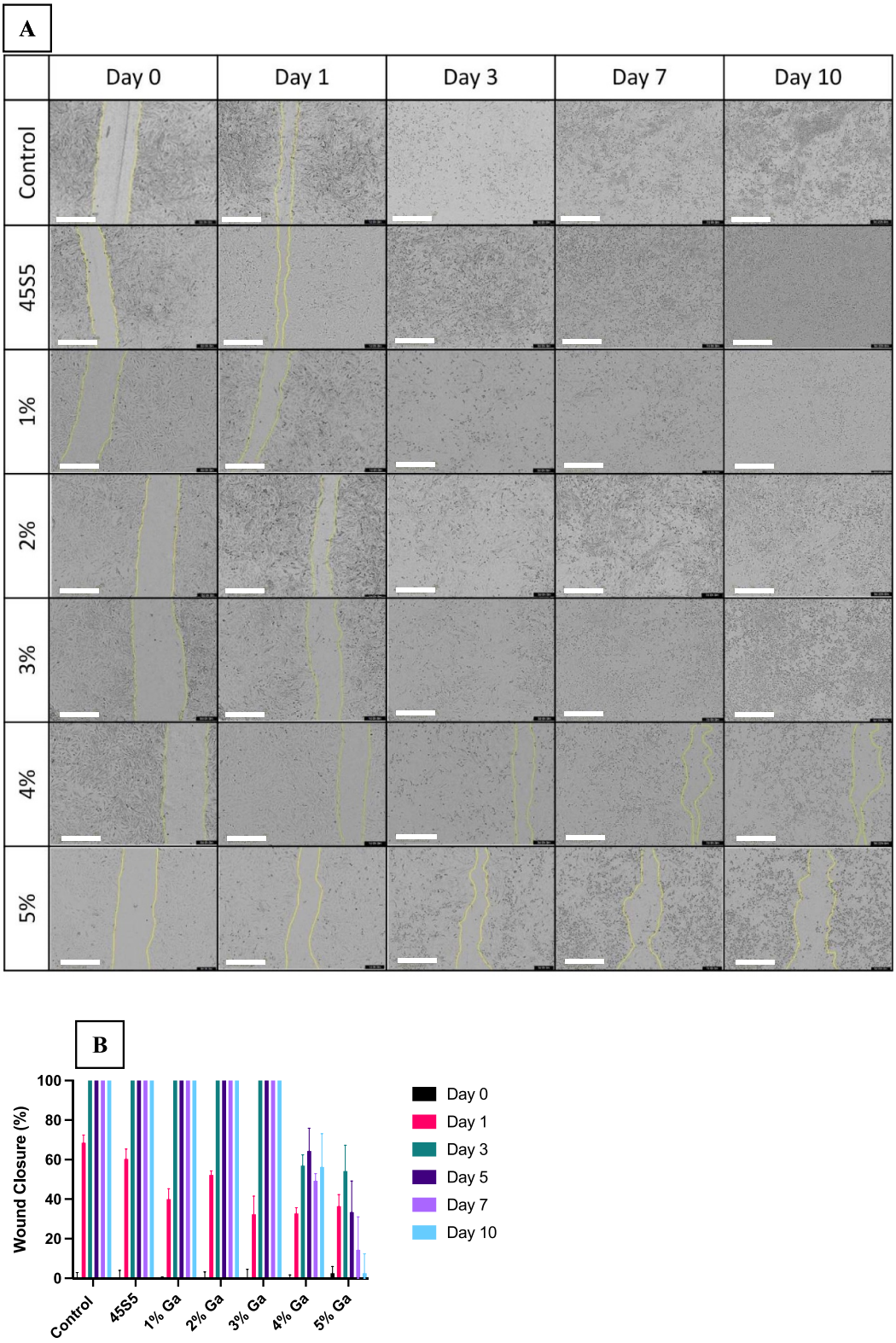

Figure 8. (A) Cell migration images for Saos-2 cells as a function of time, scale bar equals 400 μm. (B) Quantification of the scratch wound data.

bioactive glass control (∼60% closed) within 24 h. After 3 d the wound is completely closed for both controls and the 1%–3% gallium doped bioactive glasses. However, cells exposed to media conditioned using the 4% and 5% Ga still had clearly visible scratch wounds. This indicates that presence of gallium ions decreased cell mobility in the wound healing assay. The results shows that the number of cells moving into the scratched region was considerably lower in the groups exposed to 4% and 5% gallium containing media from time points 72 h onwards. After a period of 10 d, the wound size of the cells exposed to the

20 mg ml^{-1} of 5% gallium conditioned medium was almost the same as the initial scratch pre-treatment.

4. Discussion

OS is a highly malignant primary bone tumour. Patients typically undergo resection with adjuvant and neoadjuvant chemotherapy however local recurrence can still occur at the primary site. The rate of local recurrence reported in the literature varies from as high as 33% [25] down to ∼8% [26]. However, it widely agreed that patients with local recurrence have a much poorer chance of survival. Weedan *et al* reported that patients are more than four times likely to die if local recurrence happens within 18 months of surgery [26]. Current treatments are combinational and necessarily aggressive often with unpleasant side effects. However, survival rates need to be significantly improved and new therapies are needed. Furthermore, resistance has been reported for key chemotherapy drugs such as cisplatin [27].

Ga is the most commonly used metal ion for treatment of cancer after platinum [14, 18]. Unlike platinum which works best in complex molecules such as cisplatin (which would not survive the melting procedure required to prepare glasses) Ga is known to work in a variety of simple chemical forms such as gallium nitrate, gallium chloride or gallium oxides [14] and is therefore the ideal choice for incorporating into bioactive glasses. In addition to being antineoplastic gallium also possess potential antibacterial and anti-inflammatory characteristics, and has the capacity to control bone resorption [28].

The aim of this study was to investigate the bioactivity and cytotoxic effects of bioactive glasses doped with increasing Ga_2O_3 concentrations on viability and proliferative behaviour of OS Saos-2 with primary NHOst cells acting as the normal healthy non-cancerous control. Gallium oxide was successfully incorporated into the sodium, calcium phosphor-silica-based bioactive glasses over a much wider range of concentrations than previously reported and provided a controlled release of Ga ions as shown by the ICP results. Following exposure to SBF EDS results confirmed the formation of a calcium and phosphorous rich layer indicating bioactivity in the form of an amorphous calcium phosphate/hydroxyapatite layer. These results were further confirmed via FTIR which showed evidence of PO_4^{-3} bands which were not present in the unreacted bioactive glasses. In addition, there was evidence of CO_3^{-2} bands indicative of carbonated apatite formation.

In the current study, MTT results shows the Ga containing conditioned media generates a strong cytotoxicity for the OS Saos-2 cells and results in a significant reduction in the Saos-2 cell's viability. This reduction occurs in a dose dependant manner directly correlating to the concentration of Ga within the bioactive glasses. The non-toxicity of the gallium free bioactive glass (0% Ga) confirms that the toxicity is due to gallium and not the other components (Ca, Na, Si and P) of the glass or pH changes.

Conditioned media containing 20 mg ml^{-1} of 4% and 5% Ga doped bioactive glasses were the most toxic glass compositions for the Saos-2 cells with 60% and 99% reductions in viable Saos-2 cells respectively in MTT assay. The results from Live/Dead assay were in agreement with the MTT assay confirming the highly cytotoxic effect of Ga doped bioactive glass on the Saos-2 cells and relatively minor toxicity towards NHOst cells.

Numerous studies have proven the cytotoxic effect of the different Ga compounds on cancer cells [29–32]. However, the method of administration is a very important factor in the efficacy of Ga ions for treatment of cancer as well as the cytotoxic effects on different healthy tissues. It has been reported in many studies that Ga bioavailability is low following oral administration [33]. In addition, several studies stated that oral administration can have dose dependant repressing effect on biosynthesis of heme and hepatic oxidative stress along with primary immune response. On the other hand, parenteral route generates great bioavailability and efficacy, but renal toxicity is one of the limiting factors to achieve appropriate efficacy [34].

Kelsen *et al* [35] demonstrated that significant concentrations of Ga are excreted through kidneys which reduce the efficacy of Ga and also has potential implications for renal toxicity. Also, another negative aspect of intravenous injection is the requirement for the facility for gallium nitrate injection such as a pump device for continues infusion [35].

Bioactive glasses naturally release compositional ions and gradually degrade in aqueous media releasing calcium, sodium, phosphorous ions, and gallium ions in this present case. Ga doped bioactive glass provide a platform for safe delivery of Ga ions which specifically target cancer cells and stimulate the cell death for OS bone cancer cells. It has been shown that Ga ions localise into cancer cells via surface transferrin receptors (TfRs) [36]. It has also been shown that tumour cells possess significantly more TfR compared to non-cancer cells as TfR expression is extremely elevated in highly proliferative cells including cancer, intestinal epithelium, basal epidermis, and certain activated immune cells [37]. TfR is an ideal choice to target cancers cell due to its high proportion in malignant cells, its relevance in cancer, and its extracellular accessibility [18]. TfR is often overexpressed at levels several-fold higher than normal cells and that is the reason it has been recognised as a universal cancer marker [38]. The expression of iron metabolism genes was investigated in tumour tissues and adjacent tissue and was found to increase three-fold in cancer compared to the normal tissue which demonstrated higher iron intake in cancer progression. Also, the expression of *FTH1* which

is cellular iron utilization genes and *FLCVCR1*, iron efflux genes were increased in tumour tissue by more than five-fold [39]. High expression of TfR1 have been reported in 43.4% of patients with OS and considered as a prognostic factor for patients [40].

Ga mechanisms of action have been illustrated by recent research studies including 3D DNA structure alteration, DNA polymerisation inhibition and protein synthesis dysregulation. In addition, it has been observed that Ga induces apoptosis via the mitochondrial pathway [14]. Ga can activate Bax protein which translocate to mitochondria, and this results in piercing of the mitochondrial membrane and releasing mitochondrial cytochrome C and activation of caspase-3. These sets of events ultimately result in cell's apoptosis [18].

Ga doped bioactive glasses have been investigated for tissue engineering and antimicrobial properties [12, 20, 29]. Also, several studies have proven Ga ions play role in stimulation of osteogenesis [41–43]. Ga ions have been shown to decrease the activity of osteoclast cells which resorb bone cells. However, very few studies have investigated the potential of these materials to selectively kill bone cancer cells.

Rana *et al* [12] showed that bioactive glasses doped with 3 mol% Ga_2O_3 had a cytotoxic effect on Saos-2 cell. However, following treatment of the cells with Ga containing conditioned media for a period of 3 d only a 50% cell reduction was observed. Rahimnejad Yazdi *et al* incorporated up to 15 wt% Ga_2O_3 in borate glasses and assessed their toxicity against OS cells and preostoeblasts [20]. They concluded the optimal concentration of gallium oxide was 5 wt% Ga_2O_3 with the 15 wt% causing significant reduction in pre-osteoblast activity. However, after 28 d only modest reductions in OS cells were observed of 18% and 14% for the 5 wt% and 15 wt% Ga_2O_3 glasses respectively. Given how rapidly cancer cells can proliferate a much greater rate of kill is needed than previously provided in either of these studies. The present study shows that Ga doped bioactive glasses can achieve a 99+% reduction in OS cells and have the potential to prevent tumour recurrence due to controlled and targeted released of Ga ions.

5. Conclusion

The cytotoxicity of bioactive glasses containing 0–5 mol% Ga_2O_3 was investigated against OS Saos-2 cells with NHOst cells as the non-cancerous control group. MTT, Live/Dead assays, and image analysis all confirmed that OS cells are preferentially killed compared to the non-cancerous NHOst. Viability results showed an extremely high percentage of OS death in a dose dependant manner with the highest cell death observed for the bioactive glass composition containing 5% of G_2O_3 content (which achieved >99% kill after 10 d). The glasses only produced a mild cytotoxicity effect against healthy normal human derived osteoblast cells. Based on the results we conclude that the suppression of OS cell growth was due to the toxicity of the Ga ions within the bioactive glasses. OS Saos-2 cells were found to have significantly reduced proliferation and migration when exposed to gallium doped bioactive glasses.

Under physiological conditions these glasses provide a controlled release of calcium and phosphorous ions which were found to precipitate onto the glass surface forming an amorphous calcium phosphate/hydroxyapatite layer as confirmed using FTIR and EDS. This bioactivity will help generate new bone formation and prevent bone mass deficits and potential future fractures. The results when combined strongly suggest that Ga doped bioactive glasses have great potential for OS-related bone grafting applications.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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ORCID iDs

Shirin B Hanaei https://orcid.org/0009-0009-0237-9214
Lucas P Souza https://orcid.org/0000-0002-0188-5168
Juan I Cadiz-Miranda https://orcid.org/0000-0002-7620-6975
Richard A Martin https://orcid.org/0000-0002-6013-2334

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