The development and characterisation of gallium doped bioactive glasses for potential bone cancer applications.

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ABSTRACT

In this study we have developed a series of novel gallium oxide doped bioactive glasses to specifically target osteosarcoma cells whilst aiding new bone formation. The results show that osteosarcoma (Saos-2) cell death is induced through the addition of gallium oxide. Relative to the gallium-free control glass (0% Ga) glasses containing 1, 2 and 3% Ga decreased Saos-2 cell viability in a dose dependant manner. After 72 hours in media preconditioned with 3% Ga Saos-2 cell viability was reduced by over 50%. Corresponding studies undertaken on primary normal human osteoblast cells (NHOst) demonstrated no adverse effects to the gallium containing glasses. Hydroxyapatite formation was observed for all glasses when exposed to simulated body fluid.

1. INTRODUCTION

Survival for osteosarcoma patients is poor despite the aggressive use of surgery, chemotherapy, and/or radiotherapy[1]. Therefore, to improve the clinical outcome safe and effective therapeutic materials are required. The minimum key requirements for an effective biomaterial targeted towards osteosarcoma therapy are (1) to successfully eradicate any residual tumour not excised during the surgery without being cytotoxic to the surrounding tissue and (2) to provide a suitable platform for the regeneration of new bone. A potential solution to this problem is to

engineer materials capable of replacing damaged tissue whilst simultaneously preventing reoccurrence and/ or metastases of tumours after surgery. The development of synthetic alternatives that help regenerate bone by acting as active temporary scaffolds is associated with considerable research activity. However there have been very few reports of synergistic scaffolds which can help manage cancer and simultaneously promote wound healing.

Bioactive glasses are one of the most promising bone replacement/ regeneration materials because they bond to existing bone, are degradable and can stimulate new bone growth by the action of their dissolution products on cells[2,3]. Bioglass®, $(SiO_2)_{46.1}(CaO)_{26.9}(Na_2O)_{24.4}(P_2O_5)_{2.6}$, exhibits class A bioactivity meaning it bonds to bone and stimulates new bone growth even away from the glass–bone interface[4-6]. Direct comparisons with hydroxyapatite *in vivo* have shown that 45S5 Bioglass® forms a more rapid and stronger bond with bone[7,8]. Bioglass® was originally developed to provide a controlled release of calcium and phosphorous ions under physiological conditions[5]. The calcium and phosphorous ions precipitate into amorphous calcium phosphate (acp) which then crystallises into hydroxyapatite to form new bone mineral[9-11]. Bioglass® has received FDA approval and has been in clinical use since 1985. Whilst there has been considerable interest in developing and optimising bioactive glasses for bone regeneration little or no research has been undertaken on bioactive glasses specifically for bone cancer applications.

Gallium (Ga) is the most widely used metal ion for cancer treatment with the exception of platinum[12]. Gallium ions are known to be effective against cancer even in simple forms such as nitrates or chlorides whereas platinum is most effective in complexes such as cisplatin which would not survive the high temperatures involved in glass manufacturing. Gallium has the ability to localise in tumour cells via surface transferrin receptors[13]. Based on its clinical efficacy, gallium nitrate (Ganite™) is used as a treatment for cancer-associated

hypercalcaemia[14]. Furthermore, gallium nitrate has been shown to inhibit increased bone turnover and to decrease osteolysis in patients with bone metastases from a variety of different cancers[14]. Bockman *et al* (1995)[15] demonstrated that administrating low, non-toxic doses of gallium nitrate to patients with Paget's disease of bone, a disease characterised by abnormal bone remodelling and increased bone resorption, rendered bone more resistant to resorption by blocking osteoclast function without affecting their viability. Several studies indicate that gallium nitrate may have an application in diseases associated with increased bone loss such as osteoporosis, multiple myeloma and bone metastases. Uptake of bioavailable gallium is however low when administered orally as a salt. The recommended mode of administration for gallium nitrate is therefore via a continuous intravenous infusion for 5–7 days. However, this treatment is very inconvenient since patients receive this drug either intravenously in hospital or as an outpatient via a pump device. A scaffold that can provide a site specific controlled delivery of gallium would therefore be highly advantageous. Gallium ions have previously been incorporated into phosphate based glasses to deliver a controlled antimicrobial effect[16,17]. Wren *et al.* and Towler *et al.* incorporated Ga into bioactive glasses for bone cement applications and for antimicrobial functionality[18-20]. More recently, Frachini *et al*. and Lusvardi *et al.* have incorporated gallium oxide into bioactive glasses for antimicrobial applications[21,22]. However no studies have been undertaken on osteosarcoma cells. The present study therefore investigates the potential of incorporating gallium into bio-degradable bioactive glasses specifically targeted towards bone cancer applications.

2. MATERIALS AND METHODS

2.1. Glass synthesis. Melt-quench derived 45S5 Bioglass, $(SiO_2)_{46.1}(CaO)_{26.9}(Na_2O)_{24.4}(P_2O_5)_{2.6}$, and the gallium doped analogues were prepared using SiO_2 (Alfa Aesar, 99.5%), $CaCO_3$ (Alfa Aesar, 99.95–100.5%) and Na_2CO_3 (Sigma-Aldrich, ≥99.5%), $NH_4H_2PO_4$ (Sigma-Aldrich, ≥99.5%), and Ga_2O_3 (Alfa Aesar, 99.99%)[23]. In brief, the precursors were weighed in the appropriate molar ratio to give

$(Ga_2O_3)_X(SiO_2)_{46.1-3X}(CaO)_{26.9}(Na_2O)_{24.4}(P_2O_5)_{2.6}$ where X=1, 2 and 3%. The compositions were carefully selected to maintain a network connectivity of 2.11 which exhibits optimal bioactivity (see supplementary section). Precursors were thoroughly mixed and placed into a 90% platinum - 10% rhodium crucible. The crucible was placed into a furnace at room temperature and heated at a rate of 10°C min^{-1} to 1450 °C and held at this temperature for 90 min. The melt was then poured into a graphite mould which had been preheated to 370 °C to produce glass rods ~ 10 long and 10mm in diameter. The rods were annealed at this temperature overnight before being allowed to cool slowly to room temperature.

Glass rods were cut in discs using an IsoMet™ 1000 Precision Diamond Saw (Buehler). Discs, 10mm diameter and 2mm thick, were polished using a MetaServ® (Buehler) polishing machine to a finish of 0.06μm using colloidal silica. Glass particles were prepared using a planetary ball mill (PM100, Retsch) and the particles were sieved to give a size distribution between 40 to 60 microns.

2.2. Hydroxyapatite formation. A simulated body fluid (SBF) solution was prepared using the method outlined by Saravanapavan and Hench [24]. SBF emulates the salt ion concentrations found in human blood plasma and can be used to test for the formation of hydroxyapatite *in vitro*. Twenty five mg of each of the three powdered (40-60 μm) gallium containing glasses 1%, 2% and 3% together with the control glass (45S5) were placed in separate plastic containers and 37.5 ml of SBF salt ion solution was added (1.5 mm/ml). The samples were sealed and maintained at 37 °C for 7 days. After reacting in SBF the samples were removed and rinsed with distilled water and acetone to remove any residual salts and halt reactions. Samples were dried at 60 °C and then assessed for apatite formation using X-ray diffraction.

2.3. X-ray diffraction experiments were conducted using a Bruker D8 diffractometer operating at the copper k_α wavelength of 1.54 Å. Finely powdered glasses were measured over a two theta range of 10 to 80° in 0.02° steps. Measurements were taken at one second per point and no smoothing was undertaken.

2.4 Fourier transform infrared spectroscopy (FT-IR) spectra were recorded using a Thermo Nicolet IS50 infrared spectrometer fitted with a single bounce diamond ATR crystal. Spectra were recorded

from 400 to 4000 cm^{-1} with a step size of 0.05 cm^{-1}. All measurements were undertaken at room temperature and a total of 64 scans were recorded per sample. Spectral processing was performed using OMNIC version 9.

2.5 X-ray photoelectron spectroscopy (XPS) measurements were performed using a Kratos axis HSi XP spectrometer fitted with a charge neutraliser and Mg kα anode (1253.6 eV) and a base pressure of $5x10^{-9}$ Torr. Samples were loaded via adhesion onto carbon tape and spectra were calibrated to adventitious carbon (284.8 eV). Spectra were fit using CASA v2.3.15 using Gaussian-Lorentz (30) peak shapes and background subtracted with a Shirley background. Calcium peaks were fit with a doublet separation of 3.5 eV and phosphorous peaks with a doublet separation of 0.87 eV.

2.6 Scanning Electron Microscopy (SEM) images were recorded using a Carl Zeiss EVO MA10 scanning electron microscope operating at 10 kV and a working distance of 12.5 mm. The bioactive glass discs were immersed in SBF and maintained at 37 °C for 1 week. The samples were then removed, rinsed with water and acetone to remove residual salts and halt any further reactions. The samples were mounted onto SEM stubs using conductive carbon tape and splutter coated with gold. Images were recorded at magnifications of 500x and 3000x.

2.7. Dissolution. Stock solutions of 10mg/ml of ground glass in ultra-pure water were prepared for quantitative ionic profile using inductively coupled plasma optical emission spectrometry ICP-OES (iCAP™ 7000 Plus Series). Solutions were maintained at 37 °C for up to 3 days. Reference standards were used to calibrate the concentrations and the amount of each ion was calculated from the linear portion of the generated standard curve.

2.8. Preparation of conditioned media. Stock solutions of conditioned media were prepared by dissolving glass particles in complete McCoy's 5A medium to treat Saos-2 cells and Clonetics OGM Osteoblast complete growth media to treat osteoblasts. Stock solutions were prepared at a concentration of 10mg/ml and left to incubate in a shaker incubator at 250rpm at 37 ºC for 24 hours. Following the 24 hour incubation period, stock solutions were filtered using 0.2 micron syringe filter. 3ml of each media was taken to record stock solution pH values following glass dissolution. To allow for the natural

buffering of the body, pH neutralisation was completed by preparing conditioned media and storing it in humidified atmosphere of 5% CO_2/95% air and 37 ºC overnight to allow CO_2 mediated buffering.

2.9. Cell culture. Human osteosarcoma (Saos-2) cells were purchased from the American Tissue Culture Collection and maintained in McCoy's 5A medium containing 1.5mM L-glutamine and 2200mg/L sodium bicarbonate. Media was supplemented with 1% penicillin, streptomycin and 15% fetal bovine serum (FBS). Primary Normal Human Osteoblasts (NHOst) were purchased from Lonza and cultured in Clonetics OGM Osteoblast growth media supplemented with 10% FBS, 1% L-glutamine and 1% penicillin, streptomycin. Both cell lines were maintained at 37 ºC in a humidified atmosphere of 5% CO_2/95% air.

2.10. Live/Dead cell viability assay. 10,000 cells per well were seeded of both Saos-2 and NHOst cells and treated for 72 hours with conditioned media containing the dissolution products of 45S5, Ga 1, 2 and 3% at a concentration of 10mg/ml. Following the incubation period, a positive control of dead cells was established by incubating cells with 70% ethanol for 30 minutes. Cells treated with regular growth media served as a negative control. The polyanionic dye calcein-green is retained within live cells, producing an intense uniform green fluorescence. Ethidium homodimer-1 (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells. To stain Saos-2 cells working concentrations of calcein-green at 0.5µM and EthD-1 at 2.5µM were combined into one solution and used to treat cells, and to treat NHOst cells both calcein-green and EthD-1 were both prepared at a concentration of 0.5µM. Cells were overlaid with 100µl of staining solution and left to incubate for 45 minutes at room temperature. Cells were photographed using a fluorescent microscope at 100X magnification.

2.11. Cell cytotoxicity and proliferation. Cytotoxicity was analysed using MTT viability assay (Thermo Fisher), where 5000 cells per well of both Saos-2 and NHOst cells were cultured using conditioned media containing 10mg/ml respective glass compositions, for a period of 72 hours. 10µM Etoposide was used as a positive control, while cells grown using normal cell media served as a negative control. For the MTT assay, a 12mM stock solution of 3-(4.5-dimethylthiazol-2-yl)-2.5-

diphenyltetrazolium bromide (MTT) was prepared as per manufacturer's instructions and diluted 1/10 in phenol red free media before being added to the cells, which were incubated for 4 hours at 37 ºC. Following the incubation 75µl of treatment was removed and 50µl of Dimethyl sulfoxide (DMSO) was added and left to incubate at 37 ºC for 10 minutes. Metabolically active cells reduce MTT to formazan and after formazan extraction the optical density was measured using a spectrophotometer (at 570 nm). This assay was performed in quintuplicate.

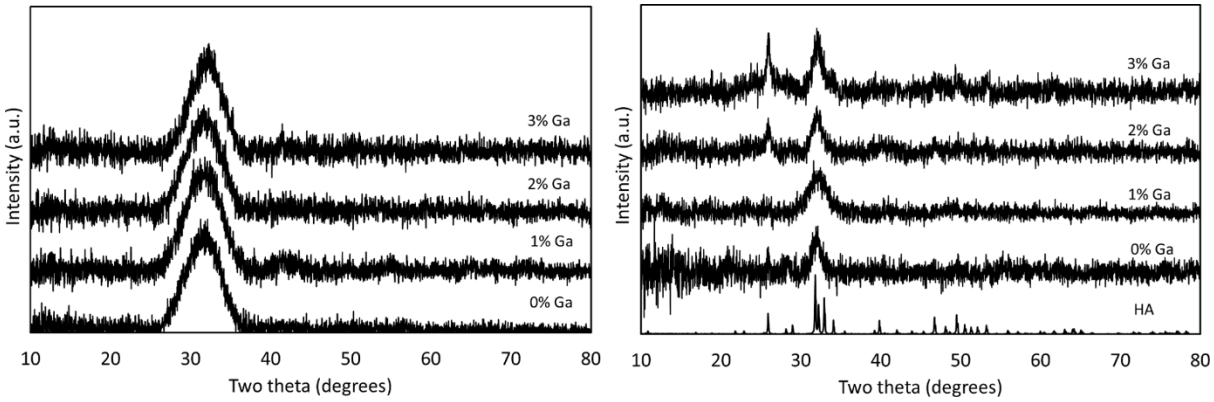
2.12. Statistical analysis.

Experiments described above were performed with at least three independent samples per data point. Data were analysed using SPSS version 21 and GraphPad Prism 6. MTT, ICP and pH results are expressed as the mean ± standard deviation. To compare cell viability between different time points and glass compositions Two-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance with statistically significant values defined as $P < 0.05$. Fluorescent microscopy and Live/Dead staining was used to distinguish between viable and non-viable cells results are qualitative and clearly indicate cell cytotoxicity or lack of.

3. RESULTS

3.1. Glasses and hydroxyapatite formation. A series of gallium doped bioactive glasses were successfully prepared. 45S5 Bioglass was used as the control glass and gallium oxide was incorporated into the remaining bioactive glasses with increasing concentrations (1, 2 and 3% Ga_2O_3). As shown in Figure 1(a) the glasses were completely amorphous with no visible signs of Bragg peaks when examined using X-ray diffraction. A broad peak has previously been reported for Bioglass[10] and corresponds to the calcium silicate[25]. After placing the glasses in a simulated body fluid (salt ion solution at 37 °C) for 7 days a visible layer of amorphous calcium phosphate /apatite was observed to have formed on the glass surface. X-ray diffraction confirmed the surface layer was an amorphous calcium phosphate / poorly defined highly substituted apatite[26] (Figure 1b) which is consistent with results typically observed for bioactive glasses[10]. The feature ~ 26° corresponds to the 002 reflection which preferentially occurs and has been previously reported for bioactive glasses[27]. The second feature ~ 32° whist still broad (FWHM of 1.5°) is significantly narrower that the unreacted glass (FWHM 5.4°). This

feature is assigned to overlapping 211, 112 and 300 reflections (Franchini *et al.*[22]). No reduction in apatite formation was observed for the gallium containing glasses compared to the 45S5 standard.



(a) (b)

Figure 1 (a) X-ray diffraction spectra of the unreacted bioactive glasses, (b) XRD spectra of bioactive glasses after exposure to simulated body fluid for 7 days and hydroxyapatite is shown for reference.

FTIR spectra the gallium doped bioglasses, after immersion in SBF for 1 week, are shown in Figure 2 together with a spectra of pure hydroxyapatite for reference. Each of the spectra exhibit the characteristic hydroxyapatite bands at 560, 600 and 1018 cm^{-1} [28,29]. Bands ~ 1410 and 1480 cm^{-1} are characteristic of carbonate groups as typically reported for apatite formations on bioglass [27,28].

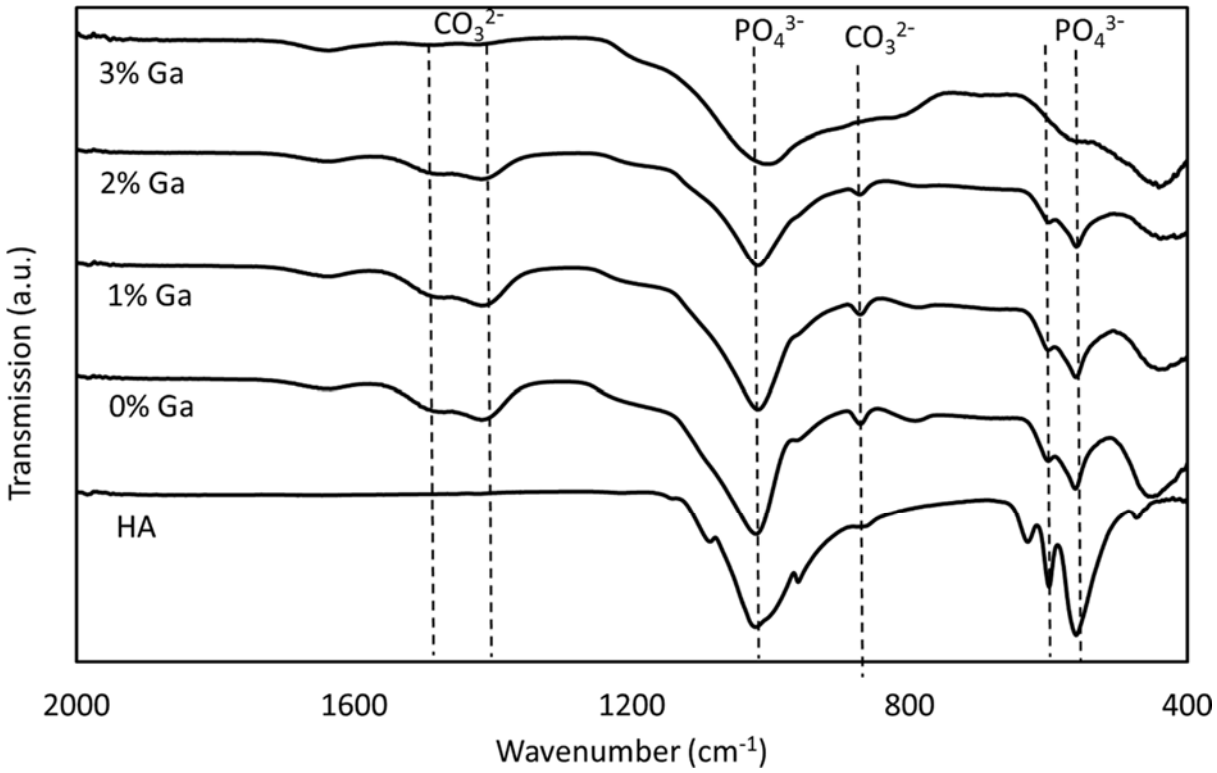


Figure 2. FT-IR spectra of the bioactive glasses following immersion in SBF for 7 days.

The XPS spectra are shown in Figure 3. XPS analysis of the glass surface following submersion in simulated body fluid for 1 week revealed Ca:P ratios consistent with hydroxyapatite (Ca:P atomic ratio = ~1.64±0.02) for all samples, corroborating the findings from X-ray diffraction. The Ca $2p_{3/2}$ peak binding energy was found to be ~347.5 eV and phosphorous $2p_{3/2}$ ~133.5 eV, again consistent with previously reported literature values for Ca2+ and P5+ within an apatite formation[30].

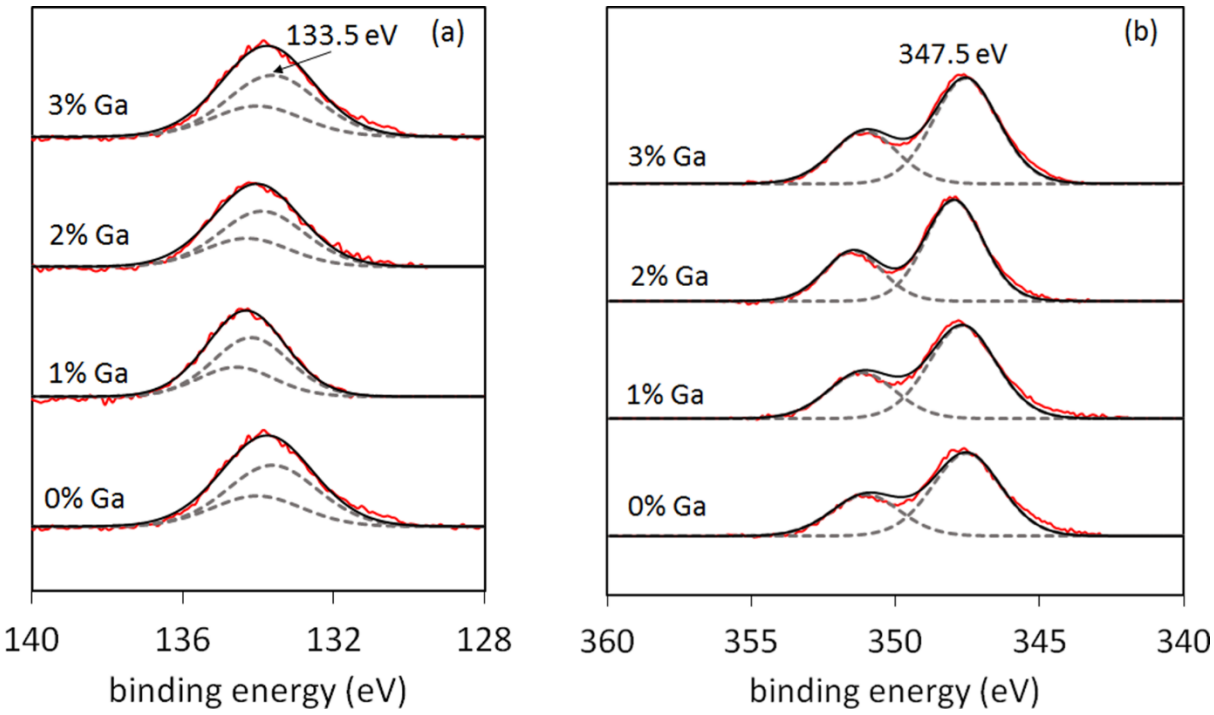


Figure 3. Phosphorous (a) and calcium (b) XPS spectra of the bioactive glasses following immersion in SBF for 7 days.

Figure 4 shows the glass surface following immersion in SBF for 1 week. As shown there is full surface coverage after 1 week. All samples show similar features, a relative smooth surface layer composed of smaller fused spherical apatite precipitates is clearly visible. Termination of the surface reaction using acetone caused dehydration and cracking of the apatite layer. Larger fused apatite spheres, as typically observed for bioglass[31], are seen on top of this initial layer. There is no indication of reduced acp/apatite formation on the gallium doped glasses.

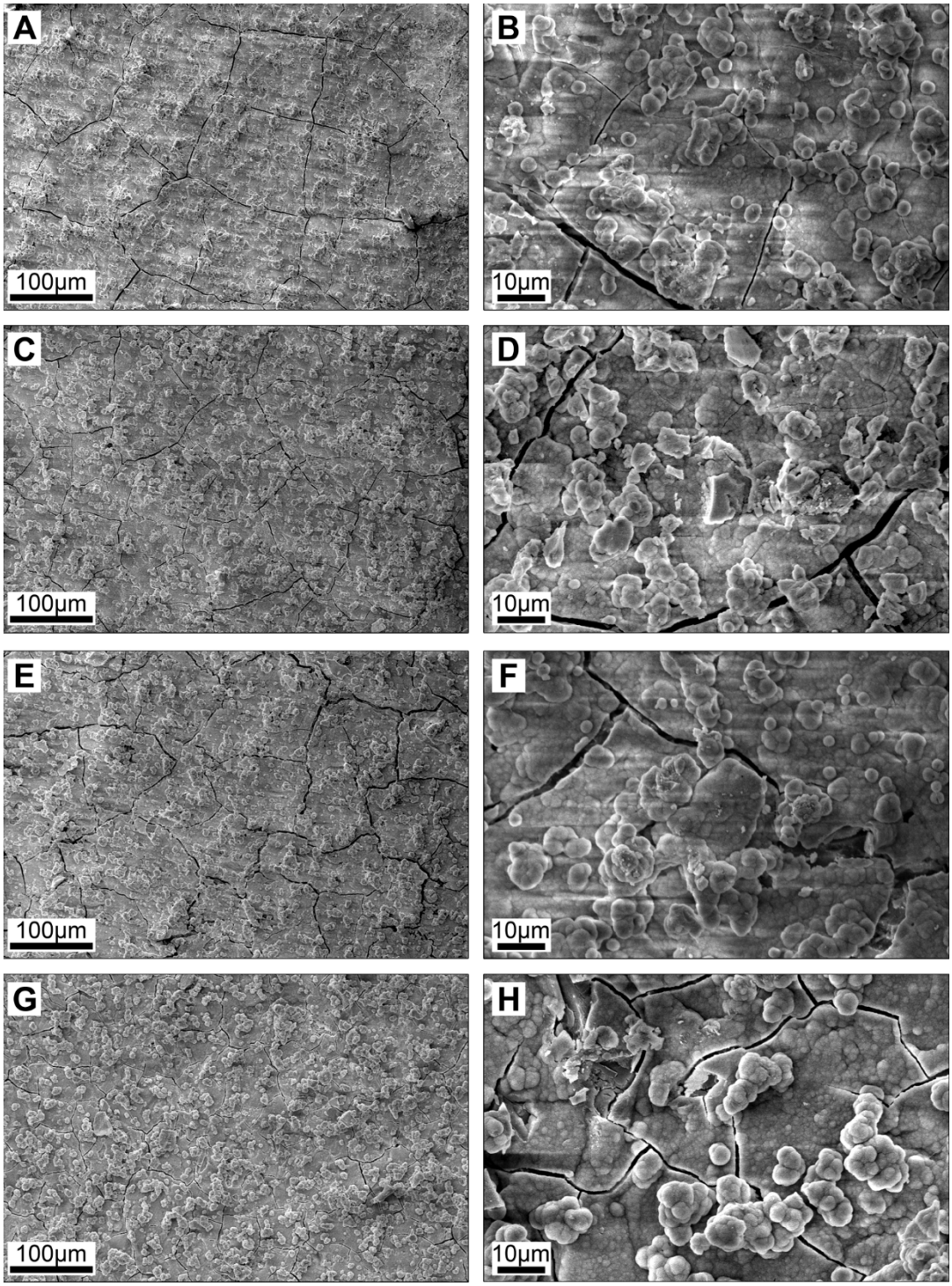


Figure 4. SEM images of the bioactive glasses after immersion in SBF for 1 week. A, C, E and G represent the 0, 1, 2 and 3% Ga glass surfaces at lower magnification (500x). B, D, F and H represent the 0, 1, 2 and 3% Ga glass surfaces at higher magnification (3000x).

3.2. ICP analysis of dissolution products. To determine the concentration of ions released from the bioactive glasses ICP analysis was conducted. The concentration of gallium ions released in distilled water as a function of time for 10mg/ml solutions are given in Figure 5. A rapid release of ions was observed during the first few hours followed by a slower more gradual increase in ion concentration.

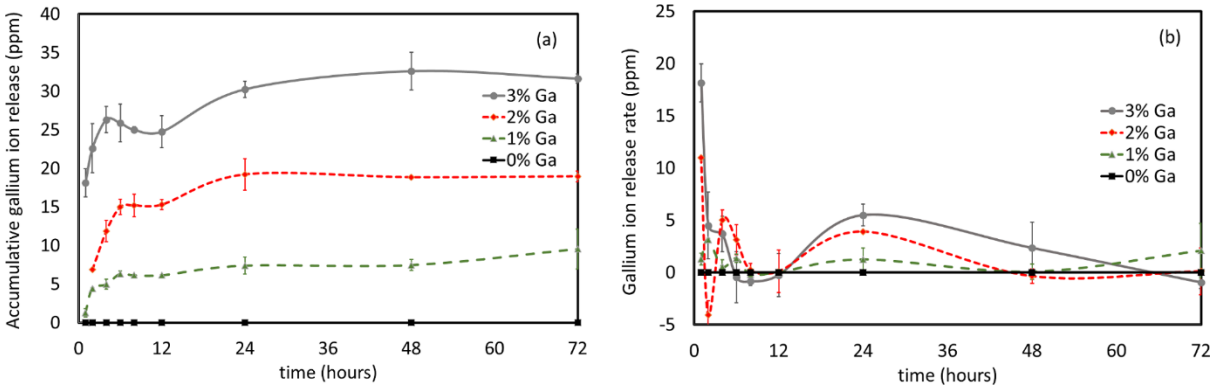


Figure 5. Release of Gallium ions from 45S5, 1, 2 and 3% Ga doped bioactive glasses. (a) accumulative release, (b) absolute release.

As expected the concentration of gallium ions increases in an approximately linear trend with increasing gallium oxide content. At 24 hours the average gallium ion concentrations were 7.3, 18.8 and 30.2 ppm for the Ga1%, Ga2% and Ga3% respectively. The gallium concentration approximately stabilised after 24 hours and minimal increases in concentrations were observed at 48 and 72 hours. The release rate of Ca, P and Si is essential for upregulating gene expression[3]. The ion concentrations are not significant for altered Ca, P and Si due to the incorporation of gallium oxide into the glasses. For example, at 24 hours the concentration of Si is 65, 70, 61 and 67 ppm for 45S5, 1%, 2% and 3% respectively.

3.3. Conditioning media and pH neutralisation. During this study experiments were performed using indirect methods as direct contact measurements can cause false positive results due to the rapid increase in pH *in vitro* which is not representative of the *in vivo* environment. For example *in vitro* studies have indicated that bioglass is antimicrobial however *in vivo* studies by Xie *et al* have shown that this result is a false positive since pH in the body at the site of bioglass implantation cannot increase to the level necessary to inhibit bacterial growth[32]. Allan *et al* and Begum *et al* have also

shown that following pH neutralisation bioglass-conditioned media loses it antimicrobial efficacy[33,34]. Is it also known that pH fluctuations have the same effect on cells, which is why preconditioning is sometimes required for bioactive glasses[35].

Furthermore the dissolution products will circulate more widely though the body and are therefore more like to prevent metastasis compared to direct contact with the bioactive glass. The conditioned media was prepared by adding 10mg/ml of bioactive glass to McCoy's media and incubating at 37 °C for 24 hours in a shaking incubator. As shown in figure 6 the conditioned media shows a significant rise in pH as sodium and calcium ions rapidly leach from the glass in the surrounding media. pH readings taken directly after conditioning the media are designated as time point 0 in Figure 6. Deviations from neutral pH are known to have detrimental effects on cell viability/proliferation, furthermore it is known that the body naturally buffers pH. Therefore the conditioned media was incubated at 37 ºC in a humidified atmosphere of 5% CO_2 for up to 72 hours to buffer the pH. McCoy's media was used as a control and it is clearly evident over the course of 72 hours the pH of the media fluctuates marginally from 7.91 at time point 0 to 7.23 at 72 hours, both readings within the physiological pH range acceptable for cell growth and maintenance. In contrast 10mg/ml of 45S5 bio-active glass significantly elevates the pH of the media and at time point 0 a reading of 10.3 was recorded and a gradual decline was observed over the course of 72 hours (1 hour, 9.4, 2 hours, 8.65, 8 hours 7.85). Conditioned media generated by the addition of 10mg/ml of 1, 2 and 3% gallium doped bioactive glass also exhibits a similar trend, as a high pH was recorded for each glass composition at time point 0 (Ga1%, pH 9.45, Ga2% pH 9.26 and Ga3% pH 9.37). At 24 hours the pH of all gallium containing conditioned media had decreased to physiologically acceptable levels where cell growth is viable (Ga1%, pH 7.77, Ga2% pH 7.81 and Ga3% pH 7.87). Beyond 24 hours negligible changes in pH were observed. Therefore in following experiments the media was conditioned for 24 hours and then placed in the incubator for 24 hours to neutralise before being used. Neutralising the pH in this way eliminated any potential cytotoxic effects of the glass conditioned media due to lethal pH.

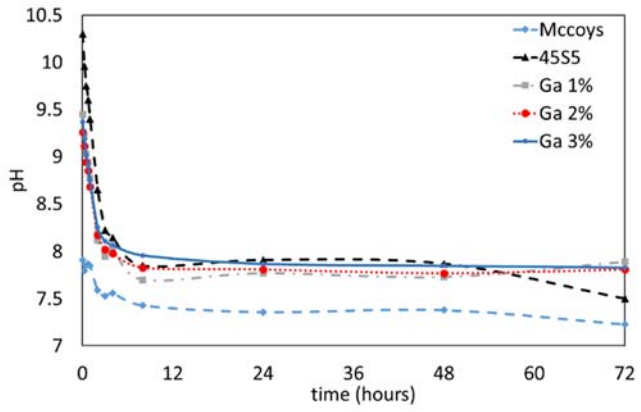


Figure 6. pH measurements of conditioned media cell treatments taken over the course of 72 hours.

Conditioned media containing respective bioactive-glass at 10mg/ml was incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. At timed intervals pH readings were recorded. McCoy's media was used a negative control.

3.4. Live/Dead Staining for cell cytotoxicity. Conditioned media containing 3% gallium oxide significantly increases osteosarcoma (tumorous) cell death relative to the 1% and 2% Ga glasses and 45S5 control as shown in Figure 7. A significant increase in dead cells was observed between the 45S5 control glass and the 1% and 2 % Ga glasses whilst at 3% all cells appeared dead. In contrast all the glasses including the 3% gallium remained non-toxic to primary normal human osteoblast (non-tumorous) cells (Figure 8).

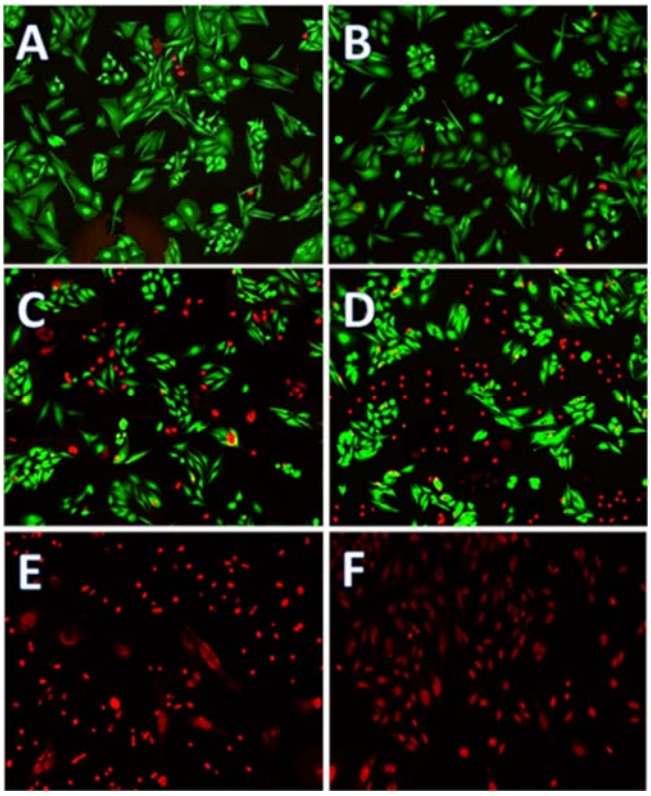


Figure 7. Fluorescence images of Live/dead staining of HTB-85 Saos-2 cells cultured for 72 hours in conditioned media containing bio-active glass after pH neutralisation.

5000 cells were seeded and left to attach for 24 hours. Following attachment cells were treated with A) Mccoy basal media, B) conditioned media containing 45S5 bioactive glass, C) conditioned media containing 1% gallium, D) conditioned media containing 2% gallium, E) conditioned media containing 3% gallium and F) 1mM Etoposide treatment as a positive inducer of cell death. All conditioned media treatments were prepared at 10mg/ml of the respective glass. Images were taken using a fluorescent microscope at 100x magnification.

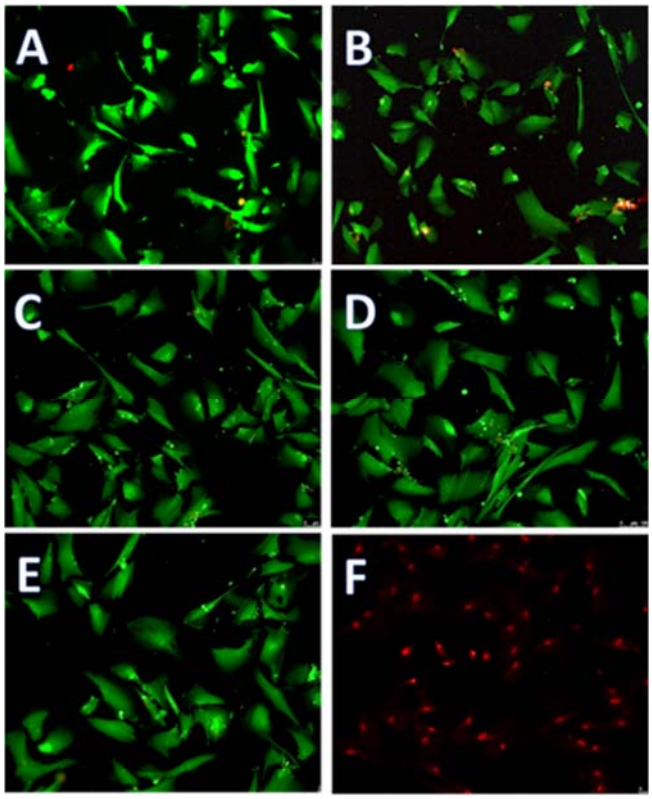


Figure 8. Fluorescence images of Live/dead staining of NHOst-Osteoblasts cultured for 72 hours in conditioned media containing bio-active glass.

5000 cells were seeded and left to attach for 24 hours. Following attachment cells were treated with A) OBM basal media, B) conditioned media containing 45S5 bioactive glass, C) conditioned media containing 1% gallium, D) conditioned media containing 2% gallium, E) conditioned media containing 3% gallium and F) 1mM Etoposide treatment as a positive inducer of cell death. All conditioned media treatments were prepared at 10mg/ml of the respective glass. Images were taken using a fluorescent microscope at 100x magnification.

3.5. Osteosarcoma and osteoblast cell viability. The cytotoxicity of conditioned media containing 1, 2, 3% gallium at 10mg/ml was assessed using the MTT assay on both osteosarcoma and osteoblast cells. No significant reduction in Saos-2 cell viability was observed between the positive control (unconditioned media) and the gallium free control glass (45S5). However a steady but significant decrease in Saos-2 cell viability was observed with increasing gallium oxide content in a dose response manner (Ga 2% $p < 0.01$ and Ga3% $p < 0.0001$) as shown in Figure 9. After 72 hours in conditioned 3% Ga conditioned media Saos-2 cell viability was less than 50%. In contrast conditioned media from the glasses showed no cytotoxic effects against osteoblast cells as shown in Figure 10. In each experiment 10nm etoposide was used as a positive control and a known inducer of cell death ($p < 0.0001$).

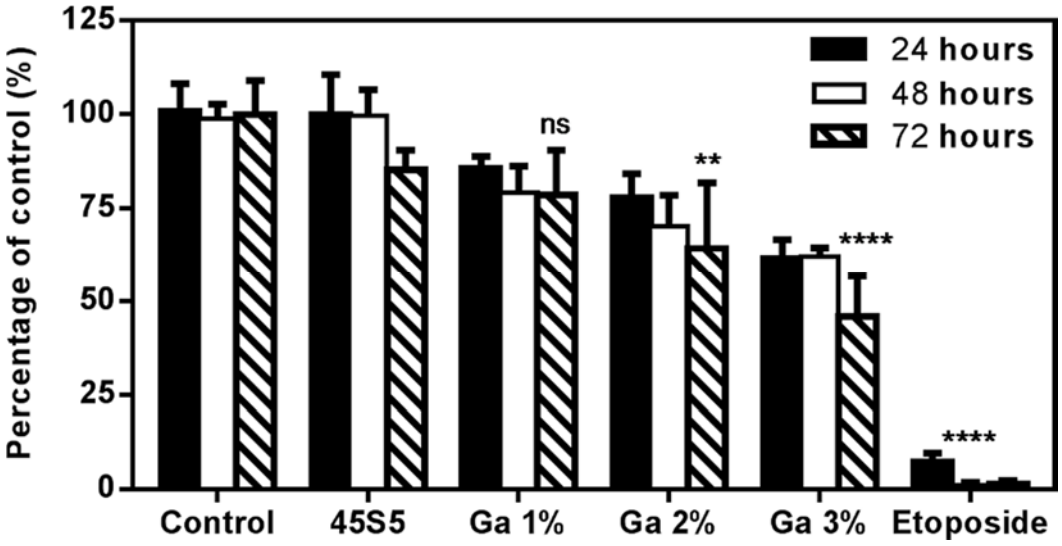


Figure 9. SOAS-2 cell viability following treatment with pH neutralised conditioned media containing 10mg/ml bio-active glass compositions over 72 hours.

SOAS-2 osteosarcoma cell viability was determined following treatment with conditioned media containing 10mg/ml 1% Gallium, 2% Gallium, 3% Gallium and compared to 45S5 as a control. 10µM Etoposide was used a positive control ($p = <0.0001$). Two-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as mean ± SD. Significance was set at $p = <0.05$, $N = 4$.

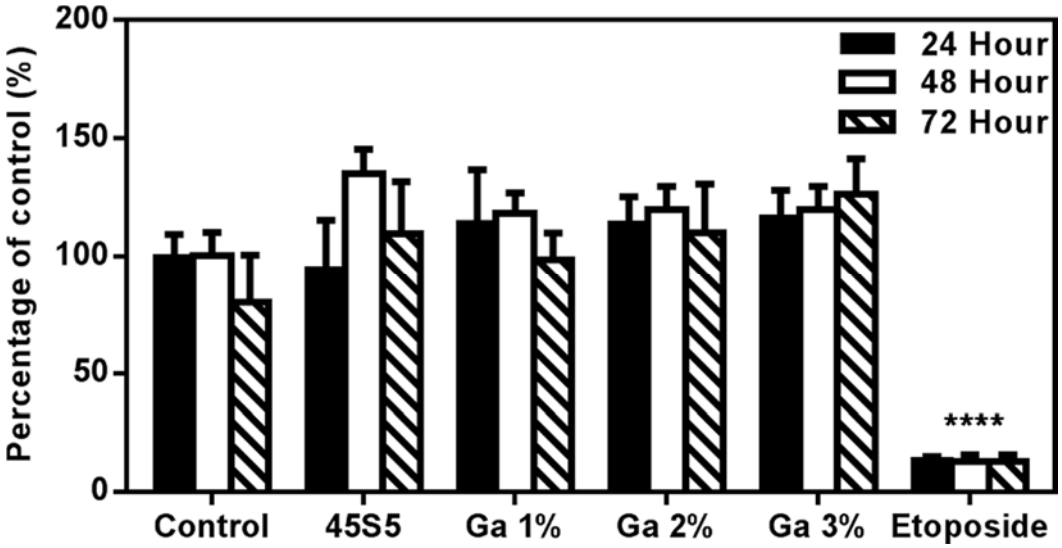


Figure 10. NHOst cell viability following treatment with conditioned media containing 10mg/ml of bio-active glass compositions over 72 hours.

NHOst osteoblast cell viability was determined following treatment with conditioned media containing 10mg/ml 1% Gallium, 2% Gallium, 3% Gallium and compared to 45S5 as a control. 10µM Etoposide was used a positive control ($p = <0.0001$). Two-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as mean ± SD. Significance was set at $p = <0.05$, $N = 4$.

4. DISCUSSION

Orthopaedic products based on Bioglass® 45S5 have been used in a wide range of bone regeneration applications. These applications include trauma arthroplasty, spine fusion and the general filling of bone defects after cyst or tumour removal[36,37]. In essence bioactive glasses are biodegradable controlled release materials. Bioglass 45S5 provides a controlled release of Ca and P ions which precipitates into hydroxyapatite, and through the controlled release of Ca, P and Si gene expressions are upregulated. Considerable research effort has been devoted to modifying and enhancing the delivery of ions. For example, cobalt ions have been added to bioactive glasses to enhance vascularisation[38,39] whilst silver ions have been incorporated to enhance the antimicrobial properties of bioglass[40]. To date there have been no reports of bioactive glasses being tailored specifically for bone cancer applications. Whilst there have been alternative bioactive glasses developed for treating deep seated soft tissue cancers[41,42] these materials do not work through the controlled dissolution of ions and are in fact highly insoluble as they are used to deliver controlled radiotherapy. Consequently this class of materials are unable to promote apatite formation or stimulate gene expression, in addition it is also difficult for the body to excrete these materials.

The present study has successfully incorporated gallium ions into bioactive glasses to specifically target bone cancer applications. Gallium ions have previously been incorporated into bioactive glasses for antimicrobial benefits. However the systems previously studied have been fundamentally different. For example the glasses developed by Wren and Towler were based on modifying glasses for bone cement applications which contained very high concentrations of zinc oxide[18-20]. Consequently those glasses are highly insoluble compared to the glasses developed here. The glasses developed by Franchini, Lusvardi *et al*. also found that when gallium oxide was incorporated the glasses became less soluble due to the stabilising

effect of gallium. It took 30 days for glasses with 1.0 and 1.6 mol % to be fully covered in homogenous Ca-P rich layer.

The glasses presented in this study show a rapid release of gallium within an hour of immersion in SBF. It is envisaged that the release of gallium ions will be reduced by the formation of the ACP/apatite layer and that the glasses will dissolve over an extended time period provide a long term therapeutic benefit. Gallium-containing hydroxyapatite has been previously reported where the substituted apatite can accommodate up to 11% Ga ions by mass[43]. In addition gallium is known to preferentially locate in bone[44], it is therefore likely that a significant fraction of the released gallium remains localises in the ACP /apatite layers.

Here through the addition of gallium and by tailoring the silicon content to maintain the network connectivity a series of dose dependant release glasses were manufactured. Live dead staining and MTT cell viability assays both revealed a significant reduction in cell viability for tumorous Saos-2 cells in the presence of bioactive glasses containing gallium. Glasses containing the highest concentration of gallium demonstrated the greatest reduction in sarcoma cells. Bioglass doped with 3% Ga showed a 50% reduction in viable Saos-2 cells. Gallium containing glasses were found to have no detrimental effects on the non-tumorous primary normal human osteoblast cells compared to the gallium free glass and control media. Gallium ions are known to localise in tumour cells via transferrin receptors[13]. It is noteworthy that tumour cells express a significantly higher number of transferrin receptors, relative to non-cancerous cell types. Existing literature has elucidated numerous mechanisms behind the anti-neoplastic properties of gallium; including altering the three-dimensional structure of DNA, inhibiting DNA polymerase function and dysregulating protein synthesis. Furthermore gallium has been reported to increase cell membrane permeability, thereby destabilising electric charges at the surface of the cell and inducing an increased efflux of calcium from mitochondria and initiating the preliminary steps involved in apoptosis. In addition gallium preferentially

locates in metabolically active regions where new bone formation and bone remodelling occurs[44].

Whilst it is envisaged that these glasses would be used as part of a combined therapy, including drug delivery, it is interesting to compare our results directly with recently reported controlled drug release calcium phosphates developed for osteosarcoma therapy. Recently Hess et al. have assessed the efficacy of calcium phosphates doped with cisplatin, doxorubicin (DOX) and co-doped with both drugs[43]. The viability of the osteosarcoma cell line MG-63 was assessed after exposure to the controlled release calcium phosphates for up to 7 days. For samples where DOX was incorporated into the matrix only a 9% kill was reported, Samples with cisplatin beads and DOX loaded in the matrix resulted in a 19% kill of MG-63 cells. The composites co-doped with both cisplatin and DOX loaded beads exhibited the lowest cell viability of 34%[43]. The gallium doped bioactive glasses developed in this work therefore exhibit a comparable toxicity to sarcoma cells compared to calcium phosphate scaffolds loaded with anti-cancer drugs.

Whilst this study has been careful to avoid exposing cells to un-physiologically relevant pH fluctuations, which are more likely to occur *in vitro* and could potentially lead to unrepresentative results it is worth noting that pH fluctuations caused by these bioactive glasses may be potentially beneficial *in vivo*. For example, human osteosarcoma sites can be acidic in nature, the release of sodium and calcium ions from these bioactive glasses will increase the pH and reducing the acidity. This can be potentially beneficial in two ways; firstly acidosis of intramedullary extracellular fluid can inhibit the proliferation of osteoblasts and secondly raising the pH can prevent spontaneous metastases.

5. CONCLUSIONS

In conclusion our data has demonstrated that gallium doped bioactive glasses can be designed to selective target bone cancer cell lines, reducing tumorous cell viability by over 50%, whilst still promoting normal human osteoblast cell viability and proliferation. Hydroxyapatite formation was observed when the glasses were placed in simulated body fluid and no reduction in formation was observed for the gallium containing glasses compared to the gallium free 45S5 bioglass control. The gallium doped bioactive glasses provide a localised controlled delivery of gallium ions at the surgical site of interest and has considerable potential clinical applications for bone cancer treatment.

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Notes

The authors declare no competing financial interests regarding this work.

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