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The effect of ionic dissolution products of Ca–Sr–Na–Zn–Si bioactive glass on in vitro cytocompatibility

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Abstract Many commercial bone grafts cannot regenerate healthy bone in place of diseased bone. Bioactive glasses have received much attention in this regard due to the ability of their ionic dissolution products to promote cell proliferation, cell differentiation and activate gene expression. Through the incorporation of certain ions, bioactive glasses can become therapeutic for specific pathological situations. Calcium–strontium–sodium–zinc–silicate glass bone grafts have been shown to release therapeutic levels of zinc and strontium, however the in vitro compatibility of these materials is yet to be reported. In this study, the in vitro cytocompatibility of three different calcium–strontium–sodium–zinc–silicate glasses was examined as a function of their ion release profiles, using Novabone® bioglass as a commercial comparison. Experimental compositions were shown to release Si^{4+} ranging from 1 to 81 ppm over 30 days; comparable or enhanced release in comparison to Novabone. The maximum Ca^{2+} release detected for experimental compositions was 9.1 ppm, below that reported to stimulate osteoblasts. Sr^{2+} release was within known therapeutic ranges, and Zn^{2+} release ranged from 0.5 to 1.4 ppm, below reported cytotoxic levels. All examined glass compositions show equivalent or enhanced in vitro compatibility in comparison to Novabone. Cells exposed to BT112 ionic products showed enhanced cell viabilities indicating cell proliferation was induced. The ion release profiles suggest this effect was due to a synergistic interaction between certain combinations and concentrations of ions. Overall, results indicate that the calcium–strontium–sodium–zinc–silicate glass compositions show equivalent or even enhanced in vitro compatibility compared to Novabone®.

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1 Introduction

An ideal bone graft material is one that resorbs by releasing its constituents at a rate and level commensurate to healthy bone regeneration. In this respect, bioactive glasses have become a key focus. An essential part of their functionality is the release of constituent ions upon implantation or exposure to the physiological environment [1]. This leads to some unique characteristics including their ability to form a surface hydroxy-carbonate apatite layer and bond directly to bone [2, 3]. Recently this cation release has been directly linked to bone cell proliferation and differentiation, leading to stimulation of bone growth and repair [4, 5]. The clinical success of commercial bioactive glasses such as Perioglas® (Novabone Products, US) and Novabone® (Novabone Products, US) can be attributed to the controlled release of active ions including calcium (Ca) and silicon (Si) [6]. Critical levels of these ions are not only biocompatible but also up regulate and activate 7 families of genes in osteogenic cells, steering the tissue towards regeneration and self-repair [7, 8]. Unfortunately, though biocompatible, osteoconductive and osteointegrative, commercial bioactive glasses cannot promote healthy bone regeneration [9] in frequently encountered pathologies in

orthopaedic patients; osteoporosis being one of the most common [10]. In such situations where the regenerative capacity of the host bone is compromised, a bone graft with the inherent ability to promote healthy bone regeneration in place of diseased bone is highly sought after. The biological properties of bioactive glasses have the potential to be enhanced by varying the chemical composition and concentration of the glass constituents, allowing for tailorability for specific pathological applications and provision of a therapeutic effect [11, 12].

Calcium–strontium–sodium–zinc–silicate (Ca–Sr–Na–Zn–Si) glass has potential in this regard. Sr^{2+} has been shown to have a beneficial, dual mode of action on bone. It enhances the replication of preosteoblastic cells and stimulates bone formation, while simultaneously decreasing osteoclast activity [13, 14]. This ability to act as an uncoupling agent, diminishing bone resorption while maintaining bone formation, has rendered it effective in the treatment of osteoporosis in the pharmaceutical form of strontium ranelate (Protelos, Servier Laboratories, Dun Laoghaire, Ireland) [15, 16]. Zn^{2+} has also been known to play an important role in bone metabolism—stimulating bone formation and promoting the expression and maintenance of osteoblastic phenotypes in vitro [17]. Na^{+} has been shown to impart degradability upon silicate glass networks providing a certain amount of control over constituent ion release [18] Previous work has shown that the levels of both Sr^{2+} and Zn^{2+} released from Ca–Sr–Na–Zn–Si glass compositions under simulated physiological conditions are within reported therapeutic ranges [10]. In order to further assess the feasibility of these glasses as therapeutic bone grafts, determination of their biocompatibility in cell culture is warranted. Therefore the aim of this work is to relate the in vitro cytocompatibility of Ca–Sr–Na–Zn–Si glass compositions to their ion release profiles. L929 mouse fibroblast cells and the MTT assay are employed as they are a standard model recommended for cytotoxicity testing by measuring cell viability and proliferation [19]. Furthermore, the cytocompatibility of the ionic dissolution products from the commercially available Novabone, a bioactive glass based on the original bioglass composition, is used as a state-of-the-art comparison.

2 Materials and methods

2.1 Glass synthesis and characterisation

Three experimental glass formulations were synthesized (Table 1). Glasses were prepared by weighing out the appropriate amounts of analytical grade reagents (Sigma-Aldrich, Wicklow, Ireland); silicon dioxide, zinc oxide, calcium carbonate, strontium carbonate and sodium carbonate into a plastic container. Each formulation was thoroughly mixed in the closed container for 30 min. Compositions were then fired (1480°C for 1 h) in platinum crucibles and the glass melts shock quenched into water. The resulting frit was dried in an oven at 100°C for 1 day, ground and then sieved to retrieve a glass powder with a particle size in the range 90–710 μm. To confirm the amorphous nature of the glasses, X-ray diffraction (XRD) analysis was performed on each composition using a Philips Xpert MPD Pro 3040/60 X-ray Diffraction (XRD) Unit (Philips, Netherlands). Zero background nickel coated sample holders were used for analysis of the 90–710 μm glass particles with Cu kα radiation (at 40 kV and 35 mA). Diffractograms were collected in the range $5° < 2\theta < 80°$, at a scan step size 0.0083° and a step time of 10 s.

Table 1 Glass compositions (mol. fraction)

Glass designation	SiO_2	ZnO	CaO	SrO	Na_2O	P_2O_5
BT110	0.4	0.2	0.1	0.2	0.1	–
BT111	0.4	0.1	0.1	0.2	0.2	–
BT112	0.4	0	0.1	0.2	0.3	–
Novabone	0.461	–	0.269	–	0.244	0.026

2.2 Glass sterilisation

Each glass was sterilised using γ irradiation (Isotron, Westport, Co. Mayo, Ireland) in accordance with 'ISO11137: 2006; Sterilisation of healthcare products' [20]. The minimum and maximum doses recorded during sterilisation were 30.5 and 30.8 kGy, respectively. Sterilised glass was subsequently used for all experimentation.

In order to provide a commercial comparison, 15.5 cc of the pre-sterilised glass bone graft Novabone® (Lot No.0403C1) was purchased from the Musculoskeletal Transplant Foundation (New Jersey, US). The composition is presented in Table 1.

2.3 Preparation of glass extracts

The specific surface areas of the glasses were determined using the advanced surface area and porosimetry, ASAP 2010 System analyzer (Micrometrics Instrument Corporation, Norcross, USA). 100–300 mg of glass was used in a nitrogen atmosphere to calculate the specific surface areas using the Brunauer–Emmett–Teller (BET) method [10]. Subsequently, an equivalent surface area of 1 m^2 of glasses BT110, BT111, BT112 and Novabone were immersed in 10 ml of sterile tissue culture water (Sigma-Aldrich, Ireland) for 1, 7 and 30 Days ($n = 3$) at 37°C in a biological incubator (Binder, Germany) [9]. After each storage period, samples were filtered using a sterile 0.20 μm filter

(Sarstedt, Ireland), and filtrates stored at 4°C prior to in vitro evaluation.

2.4 Ionic content analysis

2.4.1 Atomic absorption spectroscopy

The Si^{4+} Zn^{2+}, Ca^{2+}, Sr^{2+} and Na^{+} ionic concentration of each glass extract was analysed using a Varian SpectraAA 240. An acetylene-nitrous oxide flame was used for Si^{4+} Ca^{2+} and Sr^{2+} measurements and an acetylene-air flame for Na^{+} and Zn^{2+} measurements. Si, Zn, Ca, Sr and Na hollow cathode lamps were used at wavelengths 251.6, 213.9, 422.7, 460.7 and 589.0 nm, respectively. In order to eliminate interferences when measuring Sr^{2+} and Ca^{2+} levels 0.5 g of KCl and $LaCl_3$ respectively were added to each filtrate.

2.4.2 Inductively coupled plasma–OES spectroscopy

The phosphorus (P) ionic concentration of Novabone extracts was analyzed using an ICP-OES Agilent 7500 A. The absorption wavelength used was 880 nm.

2.5 Cell culture of mouse fibroblast cell line L929

The established mouse fibroblast cell line L929 (American Type Culture Collection CCL 1 fibroblast, NCTC clone 929) was cultured in M199 media (Sigma-Aldrich, Ireland) supplemented with 10% foetal bovine serum (Sigma-Aldrich, Ireland) and 1% (2 mM) L-glutamine (Sigma-Aldrich, Ireland). Cells were grown in T-75 flasks (Sarstedt, Ireland) at 37°C in a 5% CO_2 incubator. When the cells reached confluence, they were chemically removed using 0.25% trypsin (Sigma-Aldrich, Ireland), centrifuged and re-suspended in fresh culture media to create a new single cell suspension for further inoculation.

2.6 Cell viability assay

L929 cells were seeded at a density of 1×10^4/ml in 24-well plates (Sarstedt, Ireland). M199 culture media only was used as a negative control and culture media plus cells used as a positive control. Plates were then incubated for 24 h in a cell culture incubator at 37°C (5% CO_2/95% air atmosphere). After 24 h, 100 µl of sterile tissue culture water was added to control wells. 100 µl of relevant experimental extracts (Sect. 2.3) were added to appropriate wells for testing. The plate was then incubated again for 24 h in a cell culture incubator at 37°C (5% CO_2/95% air atmosphere) [9]. After 24 h incubation, each well was exposed to MTT (Sigma-Aldrich, Ireland) at an amount equal to 10% of the culture media volume (100 µl) [9]. Plates were then returned to the incubator for 3 h. After incubation, MTT solubilisation solution was added to each well at a volume equal to the original culture media volume (1 ml). Each well was titrated using a pipette in order to enhance dissolution of the crystals, after which the absorbance of each well was spectrophotometrically measured (TriStar LB 941, Berthold Technologies, US) at a wavelength of 570 nm [9]. Cell positive control wells were assumed to have 100% metabolic activity corresponding to cellular viability of 100% [9] and the percentage cell viability of the cells exposed to experimental extracts were calculated relative to this.

2.7 Statistical analysis

Each experiment was performed in triplicate and analysed using Graphpad Prism 4 software (Graphpad software Inc.) Results are expressed as mean ± standard error of the mean of triplicate determinations. Analysis of the results was carried out using Students's *t*-test, with a significance level of $P < 0.05$.

3 Results

3.1 Glass characterisation

Characterisation of each glass was performed using XRD to determine if crystalline species were present. Figure 1 shows the X-ray diffractograms collected from BT110, BT111 and BT112, confirming their amorphous nature.

3.2 Ionic content of extracts

3.2.1 Si^{4+} content

Si^{4+} content of BT111, BT112 and Novabone extracts for Day 1, Day 7 and Day 30 are shown in Fig. 2. No Si^{4+} content was detected for all BT110 extracts.

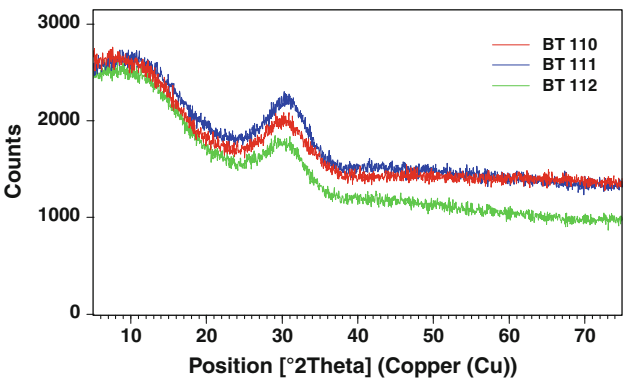

Fig. 1 X-ray diffractograms for BT110, BT111 and BT112

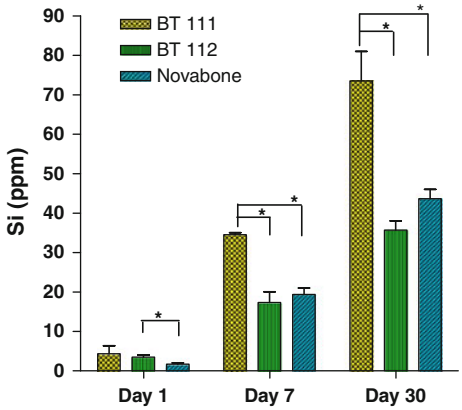

Fig. 2 Si ionic content of BT111, BT112 and Novabone for Day 1, Day 7 and Day 30 extracts. Results represent mean ± SEM of triplicate determinations (* $P < 0.05$)

In Day 1 extracts, BT112 contained statistically higher levels of Si^{4+} compared to Novabone (3.5 ± 0.5 and 1.6 ± 0.3 ppm, respectively). Both Day 7 and Day 30 extracts for BT111 contained significantly larger quantities of Si^{4+} in comparison to BT112 and Novabone. When Si^{4+} content is assessed as a function of incubation time, glass extracts BT111, BT112 and Novabone contain significantly higher levels of Si^{4+} by Day 7 when compared to their Day 1 counterpart ($P = 0.0014$, $P = 0.0281$ and $P = 0.0005$, respectively), and by Day 30 when compared to Day 7 ($P = 0.0352$, $P = 0.0066$ and $P = 0.0011$ respectively).

3.2.2 Ca^{2+} content

Ca^{2+} content of BT111, BT112 and Novabone extracts for Day 1, Day 7 and Day 30 are shown in Fig. 3. No Ca^{2+} was detected for all BT110 extracts and for Day 1 and Day 7 BT111 extracts.

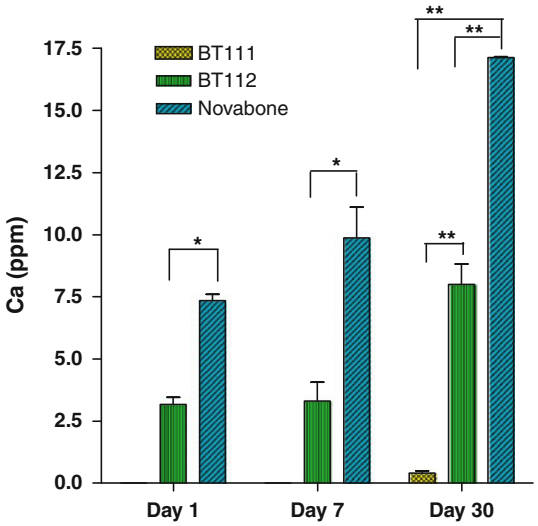

Fig. 3 Ca ionic content of BT111, BT112 and Novabone for Day 1, Day 7 and Day 30 extracts. Results represent mean ± SEM of triplicate determinations (* $P < 0.05$, ** $P < 0.005$)

At each timepoint Novabone released statistically higher levels of Ca^{2+} than the other examined glasses. By Day 30, low concentrations of Ca^{2+} were detected for BT111 (0.4 ± 0.1 ppm). When Ca^{2+} release is assessed as a function of incubation time, both BT112 and Novabone show significantly higher levels of Ca^{2+} at Day 30 when compared to their Day 1 counterpart ($P = 0.0051$ and $P < 0.0001$, respectively).

3.2.3 Na^{+} content

Na^{+} content of BT110, BT111, BT112 and Novabone extracts for Day 1, Day 7 and Day 30 are shown in Fig. 4.

In Day 1 extracts, there was no significant difference in Na^{+} concentration between each glass. However, by Day 7 BT111 released statistically higher amounts of Na^{+} (317.7 ± 8 ppm) than all other compositions. Regarding Day 30 extracts, Novabone released a significantly higher amount of Na^{+} than BT110 and BT112 ($P = 0.0101$ and $P = 0.0135$, respectively) but not compared to BT111. When Na^{+} release is assessed as a function of incubation time, BT110 extracts show no difference between each incubation time, Day 7 release for BT111 was found to be significantly higher than Day 1 ($P = 0.0006$), and Day 30 was found to be statistically higher than Day 1 for Novabone ($P = 0.0398$).

3.2.4 Sr^{2+} content

Sr^{2+} content of BT110, BT111 and BT112 extracts for Day 1, Day 7 and Day 30 are shown in Fig. 5.

In both Day 1 and Day 7 extracts, BT112 released significantly higher concentrations of Sr^{2+} than BT110 and

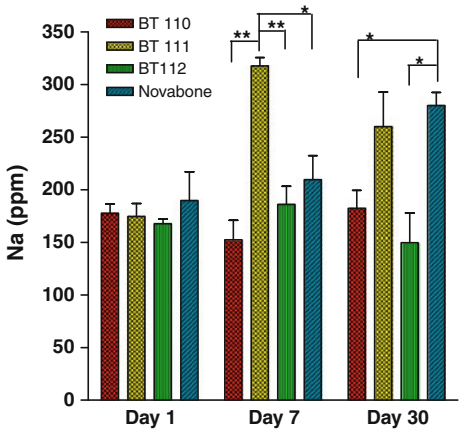

Fig. 4 Na ionic content of BT110, BT111, BT112 and Novabone for Day 1, Day 7 and Day 30 extracts. Results represent mean ± SEM of triplicate determinations (* $P < 0.05$, ** $P < 0.005$)

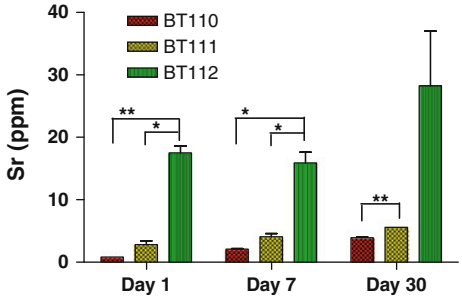

Fig. 5 Sr ionic content of BT110, BT111 and BT112 for Day 1, Day 7 and Day 30 extracts. Results represent mean ± SEM of triplicate determinations (* $P < 0.05$, ** $P < 0.005$)

BT111. By Day 30, no statistical difference was found, though a large error bar is associated with BT112. At Day 30 BT111 released significantly higher levels of Sr^{2+} than BT110 ($P = 0.0034$). When Sr^{2+} release is assessed as a function of incubation time, BT112 extracts show no difference between each time period. In contrast, both BT110 and BT111 extracts show a significantly higher level of Sr^{2+} by Day 30 when compared to Day 1 ($P = 0.001$ and $P = 0.0428$ respectively).

3.2.5 Zn^{2+} content

Zn^{2+} content of BT110 and BT111 extracts for Day 1, Day 7, and Day 30 are shown in Fig. 6.

At all time periods BT110 released statistically higher concentrations of Zn^{2+} than BT111. It should be noted though that the overall level of Zn^{2+} release was much lower than that of other examined ions (maximum of 1.36 ppm for BT110 at Day 30). When assessed as a function of incubation time, both BT110 and BT111 extracts show statistically higher levels of Zn^{2+} by Day 30 when compared to Day 1 counterparts ($P < 0.001$ and $P = 0.0006$, respectively).

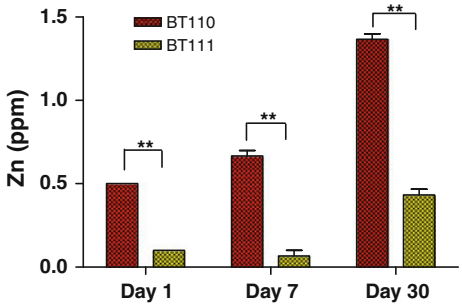

Fig. 6 Zn ionic content of BT110 and BT111 for Day 1, Day 7 and Day 30 extracts. Results represent mean ± SEM of triplicate determinations (** $P < 0.005$)

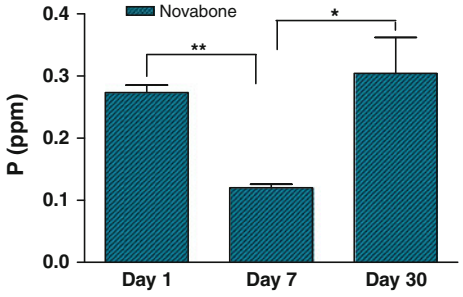

Fig. 7 P ionic content of Novabone Day 1, Day 7 and Day 30 extracts. Results represent mean ± SEM of triplicate determinations (* $P < 0.05$, ** $P < 0.005$)

3.2.6 P content

The P ionic content of Novabone extracts for Day 1, Day 7 and Day 30 are shown in Fig. 7, and range from 0.11 to 0.4 ppm.

Both Day 1 and Day 30 extracts contained statistically higher levels of P than Day 7 extracts ($P = 0.0003$ and $P = 0.034$, respectively). Similarly to Zn^{2+}, the overall level of P release was much lower than that of other examined ions.

3.3 Cell viability

The viability of cells exposed to BT110, BT111, BT112 and Novabone Day 1, Day 7 and Day 30 extracts are presented in Fig. 8.

Upon comparing the cell viabilities after exposure to Day 1 extracts, BT112 and Novabone both had viabilities statistically higher than BT111 but no significant difference was found when they were compared to BT110 and to each other. BT110 showed no significant difference when

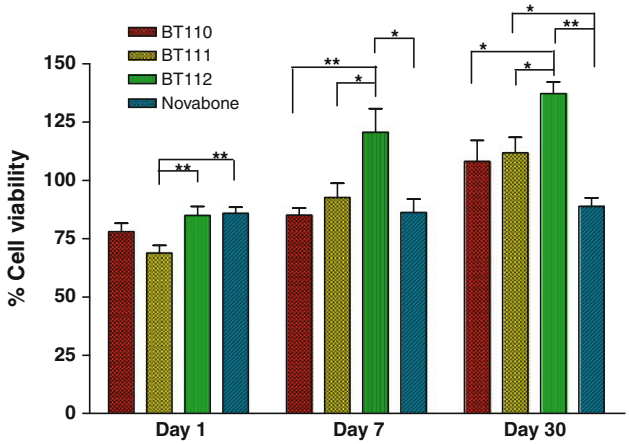

Fig. 8 Cell viability in the presence of BT1110, BT111, BT112 and Novabone for Day 1, Day 7 and Day 30 extracts. Results represent mean ± SEM of triplicate determinations. (* $P < 0.05$, ** $P < 0.005$)

compared to BT111. On comparing Day 7 extracts, BT112 shows significantly higher cell viabilities than all other glass extracts. BT112 also shows % cell viability (120.6 ± 10.1%) higher than the control used (100%). On comparing Day 30 extracts, BT112 again shows significantly higher cell viability than all other extracts. Similarly at Day 30, BT112 and BT111 show higher than 100% viability (137.2 ± 4.9 and 111.8 ± 6.7%, respectively).

When each glass extract is assessed as a function of incubation time, all experimental glass compositions (BT110, BT111 and BT112) show significantly increased cell viability by day 30 when compared to Day 1 ($P = 0.0054$, $P < 0.0001$ and $P < 0.0001$, respectively). Contrastingly, Novabone shows no statistical difference in cell viability between Day 1, Day 7 and Day 30 extracts.

4 Discussion

The aim of this work was to evaluate the ion release profiles of Ca–Sr–Na–Zn–Si glass and to relate them to cytocompatibility using Novabone® as a commercial control. The authors have previously examined the release of the therapeutic ions Sr^{2+} and Zn^{2+} in pH 3 and pH 7.4 simulated physiological environments from such glass compositions [10]. However, this study did not quantify the entire ion release profiles of these glasses or relate such to in vitro compatibility. In order to fully evaluate the potential of these glass compositions as therapeutic bone grafts, quantifying and analysing their entire ion release profiles over time, and an understanding of how such ion levels effect in vitro compatibility is warranted.

Si^{4+} release from experimental glasses in this study ranged from 1 to 81 ppm, and from 1 to 48 ppm for Novabone®. BT111, BT112 and Novabone® increased in Si^{4+} release with respect to time (Fig. 2). Previous studies have shown that Si^{4+} release levels in the range 0.1 to 100 ppm [21, 22] from bioactive glass and other biomaterials show stimulatory effects on osteoblasts, even at the genetic level. Furthermore, gradual release of soluble Si^{4+} over time may not only increase cytocompatibility but may also enhance bone bonding due to the increased formation of surface Si–OH (Silanol) groups, which are known to play an active role in the precipitation of calcium phosphate [1]. Thus the comparable or enhanced release of Si^{4+} for BT111 and BT112 in relation to Novabone® indicates their potential in a bone graft application. No Si^{4+} release was detected for BT110, which may be as a result of its lower Na and increased Zn content compared to other examined glasses. Na is known to impart degradability on SiO_2 glassy networks by depolymerising Si–O–Si bridges [18], and Zn has been shown to retard degradation [23]. Thus the low Na/high Zn content of BT110 may lead to decreased degradation of the glass network, and consequently Si^{4+} release at levels too low to reach the minimum detection limit of the AAS.

Ca^{2+} release from experimental glasses in this study ranged from 0.2 to 9.1 ppm and from 7.1 to 17.2 ppm for Novabone®. BT112 and Novabone® increased in Ca^{2+} release over time (Fig. 3) The release of higher Ca^{2+} levels from Novabone® is not unexpected as its initial Ca^{2+} content is much higher than that of the experimental glass compositions (0.269 and 0.1 mol fraction, respectively). Previous studies have shown that Ca^{2+} in the range 13.1 to 90 ppm has stimulatory effects on osteoblast activity [4, 6, 24] and its gradual release over time may enhance therapeutic efficacy. Thus the lower levels of Ca^{2+} released by experimental compositions here may, in isolation, have little stimulatory effect on bone formation. No Ca^{2+} release was detected for BT110 and for Day 1 and Day 7 BT111 extracts. Similarly to Si^{4+} release, this may be as a result of their Zn:Na ratio.

Na^{+} release from experimental glasses in this study ranged from 101 to 333 ppm and from 191 to 305 ppm for Novabone® (Fig. 4). Naturally occurring Na^{+} plasma levels are above 3200 ppm [25], thus it can be inferred that the small levels released here are of little physiological significance [26]. One of the main roles of Na^{+} in bioactive glass compositions is not to induce biological effects itself but to impart degradability and control over the release of other constituent ions [10]. This effect is particularly evident upon examining Sr^{2+} and Ca^{2+} release from the experimental compositions. As the Na_2O concentration is increased (Table 1), the release of both Sr^{2+} and Ca^{2+} increased (Figs. 2, 5). For BT110 and BT112 Na^{+} release was independent of incubation time. This result is not unexpected as the first step upon immersion of a bioactive glass in solution is the rapid exchange of Na^{+} for protons in solution.

Sr^{2+} release from experimental glasses in this study ranged from 0.8 to 38 ppm, and Zn^{2+} release from 0.5 to 1.4 ppm. Sr^{2+} concentrations from 8.7 to 87.6 ppm have shown stimulatory effect on osteoblasts [10] both in vitro and in vivo, again suggesting potential of the examined glasses in bone grafting. Zn^{2+} release above 6.5 ppm has shown cytotoxic responses [27], so it can be expected that the much lower levels released here are tolerable in vitro.

Results from the cell viability assay indicate that the experimental groups show either no difference or significantly increased cell viability when compared to the commercial control Novabone®. This demonstrates the equivalent or even enhanced in vitro compatibility of these glass compositions in relation to a current state of the art material. The only exception to this is for the Day 1 extract for BT111 (glass with a ZnO:Na_2O compositional ratio of 0.5); the reason for its reduced performance is currently

unclear. Experimental extract BT112 in particular showed enhanced performance at Day 7 and Day 30 illustrated by % cell viabilities higher than the control used. This indicates that the ions present induced proliferation and growth of the cells. When related to the ion release profiles, the only ion to significantly change level between all time-points is Si^{4+}. (Day 1—3.5 ± 0.5 ppm, Day 7—17.33 ± 2.67 ppm and Day 30—36.67 ± 2.33 ppm). As mentioned, Si^{4+} release from biomaterials ranging from 0.1 to 100 ppm has previously been shown to induce proliferation of other cell types, suggesting that Si^{4+} levels here are playing a role in the induced cell proliferation. However, Novabone® Day 7 and Day 30 extracts show statistically similar Si^{4+} levels to BT112 (Fig. 2) with no corresponding proliferation or increase in cell viability (Fig. 8). Previous studies have indicated that specific combinations and concentrations of ions enhance cell proliferation of various cell types in comparison to exposure of individual ions of equal concentration [24, 28]. In particular, stimulation of cells has occurred in the presence of previously inactive levels of Ca^{2+} and Si^{4+} when Sr^{2+} is also present [28]. Thus it is plausible to suggest that the enhanced cell viabilities of BT112 exposed cells seen in this study are due to a synergistic effect between certain concentrations of ions. However, further investigation is required to identify the mechanisms of the specific stimulatory effect of ion combinations.

Of note is the presumption that enhanced cell viability evident by the induced cell proliferation corresponds to a beneficial result. Genotoxic effects can cause cell DNA damage, resulting in malfunctions within the regulation of the cell cycle and subsequent uncontrolled proliferation of mutated cells [29]. Classic cytotoxicity tests, such as that used in this study, do not differentiate between normal and aberrant cell growth. In order to fully assess the materials examined here and to support the findings that the levels of ions released enhance biocompatibility, relevant genotoxicity testing is necessary.

5 Conclusion

All examined Ca–Sr–Na–Zn–Si glass compositions show equivalent or enhanced in vitro compatibility in comparison to the commercially available bioactive glass Novabone®. When related to the ion release profiles, the induced cell proliferation indicated by enhanced cell viability for BT112 exposed cells suggests a possible synergistic effect between certain combinations and concentrations of ions. In order to eliminate the possibility of any genotoxic effects leading to misinterpretation of results, relevant gentoxicity testing is warranted.

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