Review article

# The effect of Si species released from bioactive glasses on cell behaviour: A quantitative review

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## ARTICLE INFO

## ABSTRACT

Despite over 50 years of silicate bioactive glass (SBG) research, commercial success, and 6000+ published articles, there remains a lack of understanding of how soluble silicate (Si) species released from SBGs influences cellular responses. Using a systematic approach, this article quantitatively compares the *in vitro* responses of cells to SBG dissolution products reported in the literature and determines if there is a Si concentration ([Si]) dependent effect on cell behaviour. Cell behavioural responses to SBGs [Si] in dissolution products included metabolic activity (reported in 52 % of articles), cell number (24 %), protein production (22 %), gene expression (22 %) and biomineralization (24 %). There was a difference in the [Si] reported to cause increased (desirable) cellular responses (median = 30.2 ppm) compared to the [Si] reported to cause decreased (undesirable) cellular responses (median = 52.0 ppm) ($P \leq 0.001$). The frequency of undesirable outcomes increased with increasing [Si], with ∼3 times more negative outcomes reported above 52 ppm. We also investigated the effect of [Si] on specific cellular outcomes (e.g., metabolic activity, angiogenesis, osteogenesis),if cell type/species influenced these responses and the impact of other ions (Ca, P, Na) within the SBG dissolution media on cell behaviour. This review has, for the first time, quantitatively compared the cellular responses to SBGs from the literature, providing a quantitative overview of SBG *in vitro* practices and presents evidence of a range of [Si] where desirable cellular responses may be more likely (30-52 ppm). This review also demonstrates the need for greater standardisation of *in vitro* methodological approaches and recommends some minimum reporting standards.

### Statement of significance

This systematic review investigates the relationship between the concentration of Si released from Si-bioactive glasses (SBG) and in vitro cellular responses. Si releasing materials continue to be of considerable scientific, commercial, and medical interest (with 1500+ articles published in the last 3 years) but there is considerable variation in the reported biologically effective Si concentrations and on the importance of Si on cell behaviour. Despite the variation in methodological approaches, this article demonstrated statistical commonalities in the Si concentrations that cause desirable and undesirable cellular behaviours, suggesting a window where positive cellular outcomes are more likely. This review also provides a quantitative analysis of in vitro practices within the bioactive glass field and highlights the need for greater standardisation.

## 1. Introduction

Despite the commercial and clinical success of silicate-containing bioactive glasses (SBGs) and a buoyant research com-

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munity, with nearly 1000[1] research publications acknowledging the importance of glass dissolution products (DPs) on cellular response [1,2], there remains a lack of understanding of how the Si species released from the SBGs interact with cells. It is unclear how Si species are internalised by cells, or how they influence intracellular mechanisms to influence gene and protein expression. There is also a lack of agreement in literature regarding the relationship between Si concentration and desirable cellular behaviour. A better understanding of the concentrations of Si that produce desirable and undesirable cellular responses, would enable a more targeted approach to the synthesis of new SBGs and other Si releasing materials to improve clinical outcomes. This study investigates if there is commonality in the experimental literature regarding the concentration of Si released from SBGs and cellular response *in vitro*. Furthermore, to improve translation of BGs, this article also highlights the variance in approaches to characterise cellular responses to BGs (which are also relevant to the development of other biomaterials).

The majority of publications investigating SBGs have been in relation to bone repair and regeneration (63 % of 6320 articles[2]) but SBGs have also been reported to have favourable interactions in other tissues including cartilage (9 % of articles) and in wound healing (21 % of articles). The importance of bioactive glass DPs (and the silicate ions within them) was highlighted more than 20 years ago by Xynos et al., who demonstrated favourable osteogenic gene expression by primary human osteoblasts exposed to DPs of 45S5 Bioglass® (46.1 mol % $SiO_2$, 24.4 mol % $Na_2O$, 26.9 mol % CaO and 2.6 mol % $P_2O_5$) [3]. The rational for the addition of Si to hydroxyapatite (HA)-based materials [4,5] and bioceramics [6,7], is often attributed to dietary studies, where Si-deprivation in diets of animals has been shown to decrease bone collagen content, resulting in poor skeletal formation in chickens [8–11] and other animals [11–13]. Human clinical trials have also identified positive correlations between Si dietary intake and spinal bone mineral density in men and pre-menopausal women [14–16]. While these Si-dietary studies demonstrate a role of Si within bone metabolism, there is a difference between the deficiency of a necessary trace element (Si) and the possible benefits of additional ion release (either systemically or locally from SBGs).

A number of publications have also demonstrated that Si species alone (not derived from SBG dissolution) can promote (*in vitro)*, cell proliferation [17–20], osteogenic differentiation [17,20–22], and promote desirable gene expression for bone regeneration [19–23]. For example, Zhou et al. observed that sodium silicate increased alkaline phosphatase (ALP) activity in osteoblasts whilst inhibiting NF-kB activity [21] (a factor known to regulate osteoclastogenesis [24] and osteoblast function [25]). Other studies observed that Si species can increase the differentiation of osteoblast-like cells [18,26], cause a significant decrease in osteoclast-like cell differentiation (100 µM Si) and regulate osteocyte/osteoblast and osteoclast crosstalk by modifying OPG-RANKL expression [23]. Whilst these studies demonstrate that Si species affect bone cell behaviour, there is considerable variation within the literature in terms of both [Si] concentrations used, the cell model and whether different [Si] concentrations cause desirable or undesirable cellular outcomes.

A broad range of [Si] released from bioactive glasses DPs have been used to investigate cellular interactions *in vitro* (from ~1 ppm [27,28] to over 200 ppm [7]), with concentration dependant effects reported. For example, a concentration dependant increase in

ALP activity in MC3T3-E1 cells was reported with SBG-conditioned medium containing [Si] ~10 [29], 30 [30] and 50 [31] ppm. Similarly, Tsigkou et al. observed that [Si] between 15-50 ppm could increase the metabolic activity [32] and cell proliferation [33] in foetal osteoblasts. Bielby et al., reported [Si] as high as 160 ppm could increase bone formation and cell proliferation of murine and human osteoblasts [7]. Other studies have, however, shown adverse cellular outcomes with increasing [Si], in SBG conditioned media [27,34] or found increased [Si] to have no significant effect [35].

It is unclear if the cellular interactions reported from SBG DPs are due to the Si ions acting independently or in combination with the other ions released from SBGs (e.g., Ca, P, Na). Different types or compositions of SBGs have different types of ions released and different ion release profiles. For example, 58S sol-gel derived glasses (60 mol % $SiO_2$, 36 mol % CaO and 4 mol % $P_2O_5$) do not contain sodium, whilst most melt derived bioactive glasses do. Obata et al., [36] directly compared the effect of varying the Si-concentration without altering the concentration of other ions and showed that increasing the concentration of Si species from 10 to 50 ppm in media conditioned with 45S5 Bioglass® increased metabolic activity, ALP activity and calcium deposition in osteoblast-like (SaOS-2) cells [36]. The same study, however, reported a significant reduction in metabolic activity when using MC3T3-E1 cells, suggesting cell-type specific responses to [Si]. Beilby et al., also observed that whilst 58S (sol-gel) BG DPs (163 and 203 ppm Si) caused an increase in mouse-derived cell numbers, no significant differences were found in human-derived osteoblast cultures [7]. The systematic approach used within this study will help determine if there is commonality in cellular response to [Si] released from SBGs, if there are differences between cellular responses to different types of glasses (e.g., sol gel or melt-derived) and the *in vitro* model parameters used (e.g., cell type/species and outcome measurements).

*In vitro* testing of BGs is important for ensuring safe translation and for understanding SBG-biological interactions, leading to the development of new BGs for specific applications. Compared to *in vivo* studies, *in vitro* SBG analysis allows for greater control over the experimental parameters, has lower variability, costs less, and often allows for greater resolution/characterisation and flexibility in analysis parameters. There is, however, considerable variation in the approaches used to evaluate SBGs *in vitro*, making comparative analysis from the literature difficult. While some of this experimental variation is undoubtably due to differing applications of the SBG (e.g., bone or wound healing) and experimental goals, there remains considerable variation in the experimental approach for SBG used for the same application and for similar investigational objectives. Jablonska et al. provide an excellent overview of the approaches and assays used to evaluate SBGs *in vitro* and discusses the need for standardisation [37]. The focus of this review is to quantitatively compare *in vitro* outcomes of SBG dissolutions products and cellular interactions reported within the literature, and thereby facilitating the discussion on strategies for standardisation.

In addition to differing biological assays, there is also variation in the methodology used to obtain SBG conditioned media, the approach used to measure [Si] within the media, the reporting of methodological outcomes, and the respective assessment of successful cellular outcomes. This is perhaps best highlighted by the 22 % of publications studying cellular response to SBG but not reporting the concentration of ions in the DPs used in their *in vitro* study (Fig. 1). Or the variable terminology used within the literature (viability, proliferation, and toxicity) to describe metabolic assays outcomes when studying SBG-cell interactions.

Using a systematic analysis of the literature, this study aims to determine if there is relationship between [Si] released from SBGs and cell behaviour *in vitro*. The systematic approach also allows for

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[1] Obtained by a WOS search using search term: Topic: "bioactive glass*" AND ("Extract* OR "conditioned medi*" OR "dissolution product*" OR "Ionic product*" OR "degradation product*").

[2] Obtained by a WOS search using search term: Topic: "bioactive glass*" OR "bioglass*".

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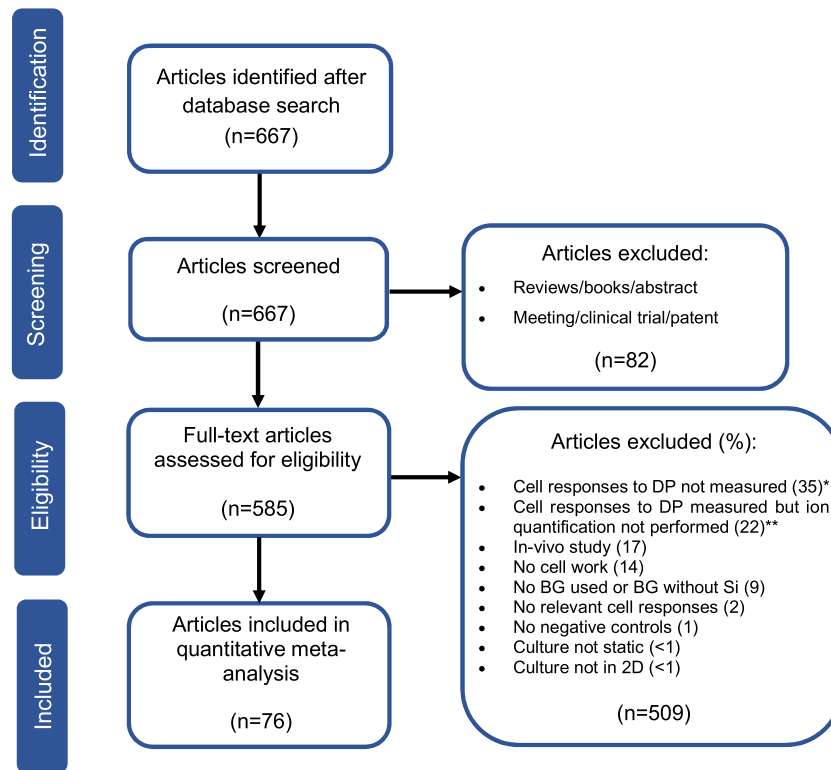
**Fig. 1.** Prisma chart describing the process of article selection and the article exclusion criteria. *Includes cell cultures on scaffolds, ion quantification not performed in culture mediums and cells directly exposed to BG particles. **includes studies that quantified ion concentrations in DPs but did not report [Si].

a reflective discussion on the methodological practices within the bioactive glass field to investigate biological response *in vitro*.

## 2. Materials and methods

### 2.1. Systematic search strategy and exclusion criteria

Web of science and Pubmed search engines were used to search for articles involving the use of both Si-containing BGs and their respective DPs on cells *in vitro* using the following search terms: ("bioactive glass*") OR bioglass AND (osteo* OR Macrophage* OR Fibroblast* OR endothelial* OR chondro* OR monocyte* OR ("stem cell*")) AND ((Extract* OR ("conditioned Medi*") OR ("dissolution product*") OR ("Ionic product*") OR ("degradation product*")). A total of 665 articles were collected. Reviews, conference abstracts, book chapters and duplicates were removed. Articles were also excluded if results did not quantitatively assess cellular responses to DPs or measure [Si] in the cell culture media used for their *in vitro* experiments. To reduce the number of possible confounding factors (e.g., topography, porosity, surface area, surface chemistry and mechanical properties), only cell responses to DPs on tissue culture plastic were considered and not those of cells in direct contact with BG surfaces.

Following the exclusion of these and others that did not meet the exclusion criteria (Fig. 1), a total of 76 articles were analysed. The [Si], glass composition, cell types, species, and changes to cellular behaviour (as determined by the percentage difference relative to the control without SBG DPs) were then collected from each article. To avoid bias during data extraction from studies, all collected values from studies had to be agreed by a second author. In the event of disagreement, a third author would be consulted and a decision for inclusion reached.

To determine the relationship between cellular response and [Si], at each [Si] data point, it was recorded if there was a signif-

icant increase ($P \leq 0.05$), a significant decrease, or no significant difference (P>0.05) in cell behaviour compared to a non-treated control (without Si species or SBG DPs) as reported by the article. Quantitative cell responses to SBGs were recorded and grouped according to the type of behaviour (Fig. 2c). For comparison of the effects of [Si] on cell behaviour, a minimum of 5 articles and 3 data points (e.g., 3 different [Si] or time points compared to control) were required for a response to be included for analysis. Cell behavioural outcomes in response to [Si] were then grouped according to different cell responses, along with their respective cell type, cell species and type of SBG. In addition to extracting cell responses to individual cell behaviours (e.g., biomineralization or metabolic activity), we also investigated if there were overarching commonalities in cellular response to [Si]. This allowed the inclusion of cell behavioural assays where there was limited data and a larger data pool to minimise the impact of variations in paper specific experimental approaches (time points, cell type, media, cell seeding density etc.). Cell responses were combined as nominally desirable for regenerative medicine/material-interactions (e.g., increased proliferation, metabolic activity, expression of angiogenic factors, extracellular matrix production and decreased cell death), or undesirable (increased cell death, decreased proliferation, decreased biomineralization etc.). For gene expression, due to the desirability of up- or down-regulation being dependent on the specific gene/inflammatory factor expressed/experimental aims, this data was analysed separately to other cellular responses but using the same methodological approach. The cell behavioural assays included in the combined figures are detailed in the result tables.

Protein production and enzyme activity (e.g., ALP activity and osteocalcin) data included a mixture of non-normalised data and data normalised to cell number (e.g. to DNA) or protein content. Normalised, non-normalised and combined data were analysed separately and together. For this review, stem cells were categorised as: a) undifferentiated primary cells, b) capable of
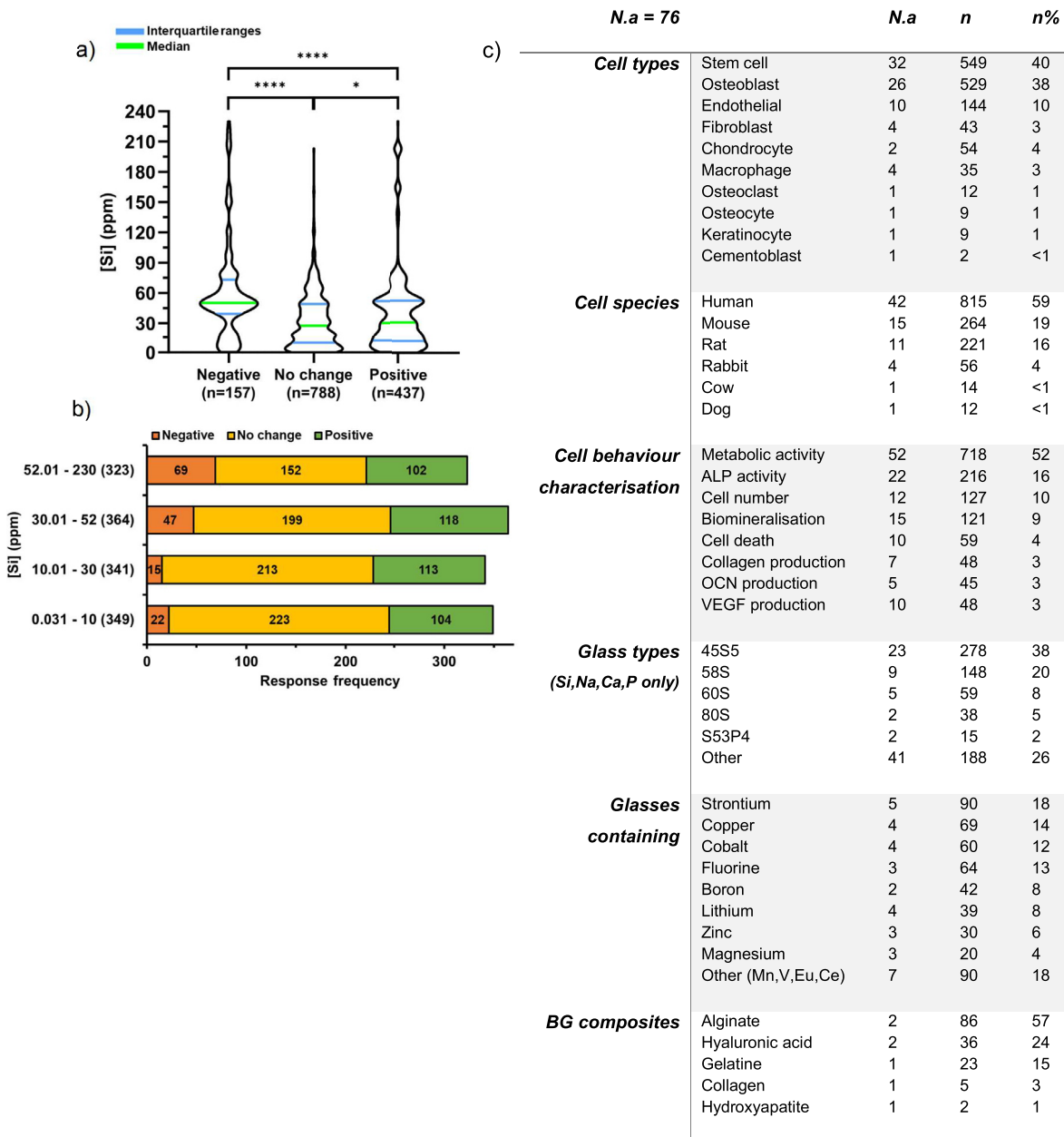
| N.a = 76 | | N.a | n | n% |
|---|---|---|---|---|
| **Cell types** | Stem cell | 32 | 549 | 40 |
| | Osteoblast | 26 | 529 | 38 |
| | Endothelial | 10 | 144 | 10 |
| | Fibroblast | 4 | 43 | 3 |
| | Chondrocyte | 2 | 54 | 4 |
| | Macrophage | 4 | 35 | 3 |
| | Osteoclast | 1 | 12 | 1 |
| | Osteocyte | 1 | 9 | 1 |
| | Keratinocyte | 1 | 9 | 1 |
| | Cementoblast | 1 | 2 | <1 |
| **Cell species** | Human | 42 | 815 | 59 |
| | Mouse | 15 | 264 | 19 |
| | Rat | 11 | 221 | 16 |
| | Rabbit | 4 | 56 | 4 |
| | Cow | 1 | 14 | <1 |
| | Dog | 1 | 12 | <1 |
| **Cell behaviour characterisation** | Metabolic activity | 52 | 718 | 52 |
| | ALP activity | 22 | 216 | 16 |
| | Cell number | 12 | 127 | 10 |
| | Biomineralisation | 15 | 121 | 9 |
| | Cell death | 10 | 59 | 4 |
| | Collagen production | 7 | 48 | 3 |
| | OCN production | 5 | 45 | 3 |
| | VEGF production | 10 | 48 | 3 |
| **Glass types (Si,Na,Ca,P only)** | 45S5 | 23 | 278 | 38 |
| | 58S | 9 | 148 | 20 |
| | 60S | 5 | 59 | 8 |
| | 80S | 2 | 38 | 5 |
| | S53P4 | 2 | 15 | 2 |
| | Other | 41 | 188 | 26 |
| **Glasses containing** | Strontium | 5 | 90 | 18 |
| | Copper | 4 | 69 | 14 |
| | Cobalt | 4 | 60 | 12 |
| | Fluorine | 3 | 64 | 13 |
| | Boron | 2 | 42 | 8 |
| | Lithium | 4 | 39 | 8 |
| | Zinc | 3 | 30 | 6 |
| | Magnesium | 3 | 20 | 4 |
| | Other (Mn,V,Eu,Ce) | 7 | 90 | 18 |
| **BG composites** | Alginate | 2 | 86 | 57 |
| | Hyaluronic acid | 2 | 36 | 24 |
| | Gelatine | 1 | 23 | 15 |
| | Collagen | 1 | 5 | 3 |
| | Hydroxyapatite | 1 | 2 | 1 |

**Fig. 2.** The effect of [Si] released from bioactive glasses on cell behaviour. The median [Si] (a) and frequency (b) reported to cause a significant negative (decrease), no significant difference (no change) or a positive significant (increase) in cell responses in vitro, following exposure to Si released from bioactive glasses. The median [Si] that was reported to have negative or undesirable cellular outcomes (e.g. decreased metabolic activity compared to controls) was 52 ppm and higher than the [Si] reported to have positive or desirable cellular outcomes (30 ppm). Positive cellular responses occurred at a higher [Si] than outcomes that were not significantly different to non-Si controls (25 ppm). There was an increased frequency of negative outcomes above 52 ppm (b). Quantification of number of articles (N.a), number of data points (n), percentage of total data points (n %) is presented in (c). *=$P \leq 0.05$, ****=$P \leq 0.0001$.

self-renewal without differentiation and c) could differentiate into more specialised cell types. Stem cell types included were derived from, adipose, mesenchyme, urine, dental pulp, and deciduous teeth.

The effect of calcium, phosphorus and sodium ion concentration ([Ca], [P] and [Na] respectively) released from SBG DPs on cell responses was also evaluated. When comparing individual experimental parameters (e.g., type of SBG or cell type) the [Si] range was also determined as a possible confounder. For example, if the range of [Si] for articles investigating human cells was significantly different to the [Si] range used in articles investigating non-human cells. Unless stated in the results, the range of [Si] were not significantly different between the parameters studied.

To investigate whether Si species (not derived from SBG DPs) could influence cell behaviour directly, additional articles were collected (22 articles total). Articles were collected from WOS and Pubmed according to the following search terms: *"orthosilicic acid" OR orthosilicate OR "soluble silica" OR "silicate ions" OR "soluble silicon" OR "ionic silicon" OR "biological silicon" (Topic) AND osteoblast\* OR Macrophage\* OR Fibroblast\* OR endothelial\* OR chondrocyte\* OR monocyte\* OR "stem cell\*" OR osteoclast\* OR myocyte\* (Topic)*. Full dissolution of Si in these studies was assumed and therefore quan-

titative analysis of [Si] in Si-conditioned media was not recorded. All other exclusion criteria were used as in Fig. 1 for SBGs.

### 2.2. Data analysis methodology

More than 1375 data points (excluding gene expression) were extracted from 76 SBG and Si articles. Once groups containing different cell responses, cell type and species group were formulated, each were then individually assessed for normal distribution using a Shapiro-Wilks test. Normally distributed significant differences between the [Si] that caused increased, decreased or no changes in cell behaviour was determined by a one-way analysis of variance (ANOVA) and post-hoc Tukey's test for multiple comparisons. Differences in non-normally distributed datasets were determined by a Kruskal-Wallis test, followed by a Dunn's test for multiple comparisons. Bin widths for plots assessing the frequency of cells responses compared to [Si] were chosen based on quartiles of [Si] distribution to ensure a near equal number of data points in each. The correlation of [Si] and percentage differences in cellular response (compared to the control) was also evaluated using a Spearman's correlation test. All results were statistically significant if they exceeded 95 % confidence. Average [Si] is presented as median ($M_d$) 'X' ppm or mean ($M_n$) as 'X' ppm ± 'X' standard deviation.

## 3. Results

### 3.1. Descriptive comparison of SBGs compared with the wider field

The search criteria initially identified 665 *in vitro* research, articles involving *in vivo* data only (20 %), no quantitative assessment of cellular responses to DPs (39 %, e.g., cell morphology comparisons, or lacking an untreated control) or did not report [Si] in the media (22 %) were excluded (Fig. 1). Research articles used for analysis were found to be dominated by those investigating bone (63 %) followed by wound healing (17 %), dental applications (12 %),immunological responses (5 %) and cartilage (3 %) (Fig. S1a). A similar distribution of BG applications was found in both the articles collected during initial searches (n=665), and the articles selected for analysis, indicating that the analysed articles were representative of the wider SBG field (Fig. S1b).

### 3.2. Methodology used to analyse in vitro responses to SBG dissolution products

Human cells (59 % of articles), stem cells (39 %) and osteoblasts (34 %) were the most commonly used cells (respective to species and cell type) to investigate SBG DPs and their cellular interactions. Metabolic activity assays were the most frequently reported cellular outcome (68 % of articles), which could be due to 10993 ISO standard, which suggests the reporting of cellular metabolic activity in response to medical devices. There was high variance in the type of metabolic assays used (Fig. 5g), with both MTT and WST-8 assays the most frequently observed (51 % and 29 % of articles respectively). Metabolic assays that produce soluble products e.g., WST-1, WST-8, Presto and Alamar blue, as opposed to MTT which requires a solvent to dissolve insoluble formazan crystals, were more frequently used in the last five years. The use of MTT in the last 5 years (33 % of articles investigating metabolic activity) was lower than that of the previous 5 years (2016-2011, 67 % of articles). Whilst most articles used metabolic activity assays, normalisation of this data (metabolic activity per cell number) was observed in only ~2 % of collected articles. Only 24 % of articles used a direct measure of cell number (as opposed to metabolic activity) and of these, 85 % quantified DNA from lysed cells (Fig. 5g). The most common cell-culture time points used to study SBG DP

interactions were for 3 time points (when evaluating metabolic activity) (1, 3, 7 days accounted for 24 % of articles). Approximately 25 % of articles used 2 time points or less (1 and 2 days accounted for 15 % of papers). For studies that investigated *in vitro* bone nodule formation or biomineralization (12 % of articles), quantification of calcium deposition was the most common outcome measurement (90 % of articles), compared with nodule area (7 %) and nodule count (3 %).

The most common bioactive glass composition (within the search criteria) was 45S5 Bioglass® (30 % of articles), whilst the commercial composition S53P4 (Bonalive®) accounted for 3 % of articles. Sol-gel glasses of the composition 58S and 70S30C were investigated *in vitro*, 12 % and 1 % of articles respectively, and melt-derived 13-93 composition (1 % of articles). Approximately 54 % of articles, reported on SBGs compositions that were unique to the individual article within our search criteria, often these articles were investigating the effect of additional therapeutic ions (e.g., Co, Sr, Li) added to the base SBG composition. Magnesium and strontium (both 10 % of articles) were the most common additional ion investigated.

The mean [Si] within the cell culture media used to investigate SBG DPs interactions with cells (*in vitro*) was 37.8 ppm ± 36.6 (Fig. S2a). Interestingly, for some cell and species types, researchers used (on average) different [Si] concentrations (Fig. 3 and 4). A higher [Si] was used in human cells ($M_n$ = 41.5 ppm ± 37.3) compared to non-human cells ($M_n$ = 32.5 ppm ± 34.8) and studies using stem cells ($M_n$ = 41.1 ppm ± 34.3) used a higher [Si] range compared to non-stem cells ($M_n$ = 35.6 ppm ± 37.8).

### 3.3. Does Si concentration influence cell responses?

When all cellular responses were combined, there was a [Si] concentration dependent effect on cell behaviour, where the [Si] that caused increased (positive) effects ($M_n$ = 40.3 ppm ± 41.2), was higher than the [Si] that caused no significant difference ($M_n$ = 32.2 ppm ± 28.3), but lower than the [Si] that caused decreased (negative) outcomes ($M_n$ = 59.8 ppm ± 48.2) (Fig. 2a, $P \le 0.001$). The most frequently reported outcome, on the effect of [Si] on cellular responses, were not significantly different (no change) to untreated controls without SBGs (788 data points). This compared with 437 [Si] reported data points that caused significantly increased outcomes and 157 data points reporting significantly decreased outcomes.

The frequency of negative outcomes was also observed to increase with increasing [Si] (Fig. 2b). Negative outcomes were found to occur ~3 times more frequently above 52 ppm [Si]. The frequency of reported significantly positive outcomes were found to be most common in the [Si] range 30-60 ppm. There was, however, only a very weak negative correlation between [Si] and the magnitude of cell response (percentage difference to the untreated control without SBG DPs), (R=-0.08) (Fig. S2b).

### 3.4. Does cell type and species influence overall responses to dissolution products?

The [Si] reported to cause decreased (negative) outcomes in human cells ($M_d$ = 54.3 ppm) was, on average, higher than non-human cells ($M_d$ = 31.4 ppm), suggesting that human cells can tolerate higher [Si] levels (Fig. 3a). There was no difference between human and non-human cells in the [Si] that was reported to cause positive outcomes. The median [Si] in studies using human cells ($M_n$ = 41.5 ppm ± 37.3) were found, however, to be significantly higher than those using non-human cells ($M_n$ = 32.5 ppm ± 29.6). This higher [Si] range in human cell studies, may therefore, account for the difference between human and non-human cells, rather than cell specific responses.
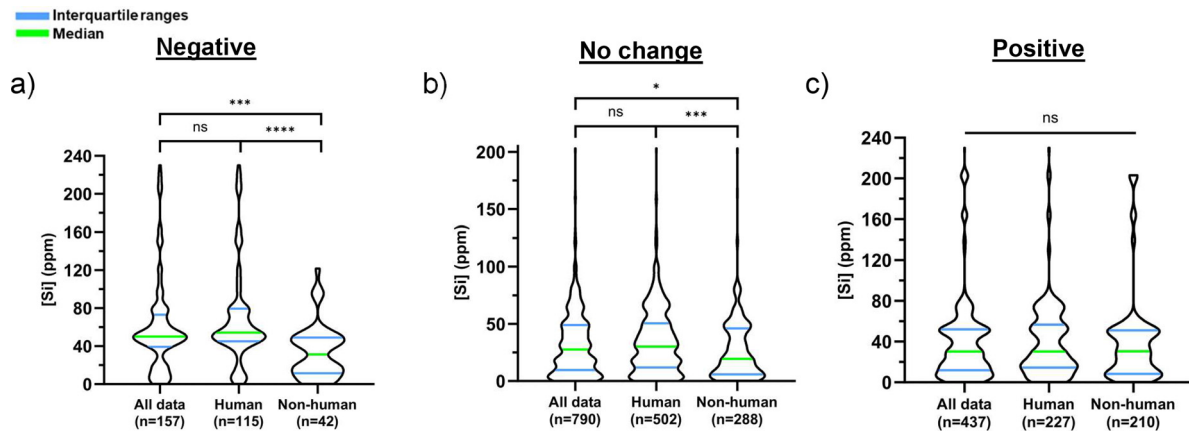
**Fig. 3.** Species specific cell responses to [Si] released from bioactive glasses and ceramics. Comparisons between cell species in response to [Si], negative or undesirable cellular responses (a) or no significant change (b) were reported at lower [Si] in no-human cells compared to human cells (a) *=$P \leq 0.05$, **=$P \leq 0.01$, ***$P = \leq 0.001$, ****=$P \leq 0.0001$.
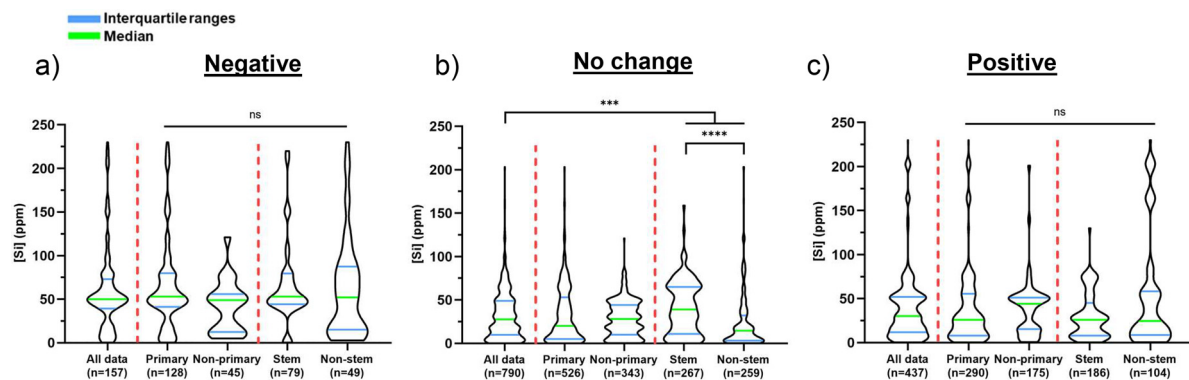


**Fig. 4.** Cell type specific responses to [Si] released from bioactive glasses. A higher median [Si] was reported to cause a positive or desirable cellular response in non-stem cells compared to stem cells ($p<0.005$). 'Primary' contains all in-vitro studies and responses from non-cancerous or immortalised cell lines. 'Stem' contains cells that adhere to characteristic definitions 'a', 'b', and 'c', as described in methods and materials. ***=$P \leq 0.001$, ****=$P \leq 0.0001$ when compared with 'all data'. Red dotted lines highlight that primary cells were compared to non-primary, and stem to non-stem cells.

Cell type (primary vs non-primary, or stem cells vs non-stem cells) did not influence the [Si] reported to cause negative or positive cellular responses (Fig. 4a/c). Whilst the range of [Si] used to investigate primary and non-primary cells was not significantly different, there was a difference between the average [Si] used to investigate stem cells ($M_n = 41.3$ ppm $\pm 34.2$) and those using non-stem cells ($M_n = 35.6$ ppm $\pm 37.8$) ($P \leq 0.001$). Considering that higher [Si] is associated with increased negative effects (Fig. 2), the lack of j differences between stem cells and non-stem cells, may suggest increased tolerance to a higher [Si] range in stem cells (compared to non-stem cells).

*3.5. Does [Si] affect cell metabolic activity, cell number, and death?*

Metabolic activity (Fig. 5a) was the most common cellular assay used to analyse cell responses to SBG DPs (52 % of articles) and produced similar trends to those in overall cellular response (Fig. 2a) where an increase in the frequency of negative responses was observed in metabolic activity responses with increasing [Si] (Fig. 5b). Studies that used [Si] at or above 90 ppm, did not report increased metabolic activity (Fig. 5a). It is interesting to note that the [Si] that was reported to cause decreased metabolic activity in human cells was higher than the [Si] that caused positive outcomes, but this was not observed in non-human cells (Fig. S3c). There were no other species or cell type specific differences in the metabolic activity, cell number or cell death responses to [Si] observed.

Cell number data had a similar trend to metabolic activity, with [Si] above 40 ppm associated with an increase in the frequency of undesirable cellular interactions (Fig. 5c/d). There was, however, no difference between the [Si] reported to increase or decrease cell number, with the most reported outcome that [Si] did not significantly change cell number. There was also no significant difference observed in the mean [Si] reported to increase cell death (Fig. 5e/f), but this may reflect the limited data points (4 % of articles - Fig. 2). The frequency of reported significant cell death outcomes did, however, increase with increasing [Si], with 44 % of data points above 60 ppm reported to cause a cell death compared to 2 % below 60 ppm.

*3.6. Osteogenic and angiogenic responses to [Si]*

No significant differences were observed between [Si] and osteoblast differentiation markers (osteocalcin and ALP activity), biomineralization, or VEGF production(Fig. 6a-d). When considering the differentiation of stem cells alone (9 articles), the same was true i.e. there was no difference in the [Si] that caused increased or decreased osteogenesis/angiogenesis. Given the difference in ALP expression at different stages of osteogenic differentiation, the effect of cell culture time was also considered. No difference in ALP activity were observed at any time point analysed (one, two or three weeks). The frequency of articles reporting an increase in ALP activity in response to [Si], is, however, 3 times more common than decreased ALP activity. Most studies (70 % of
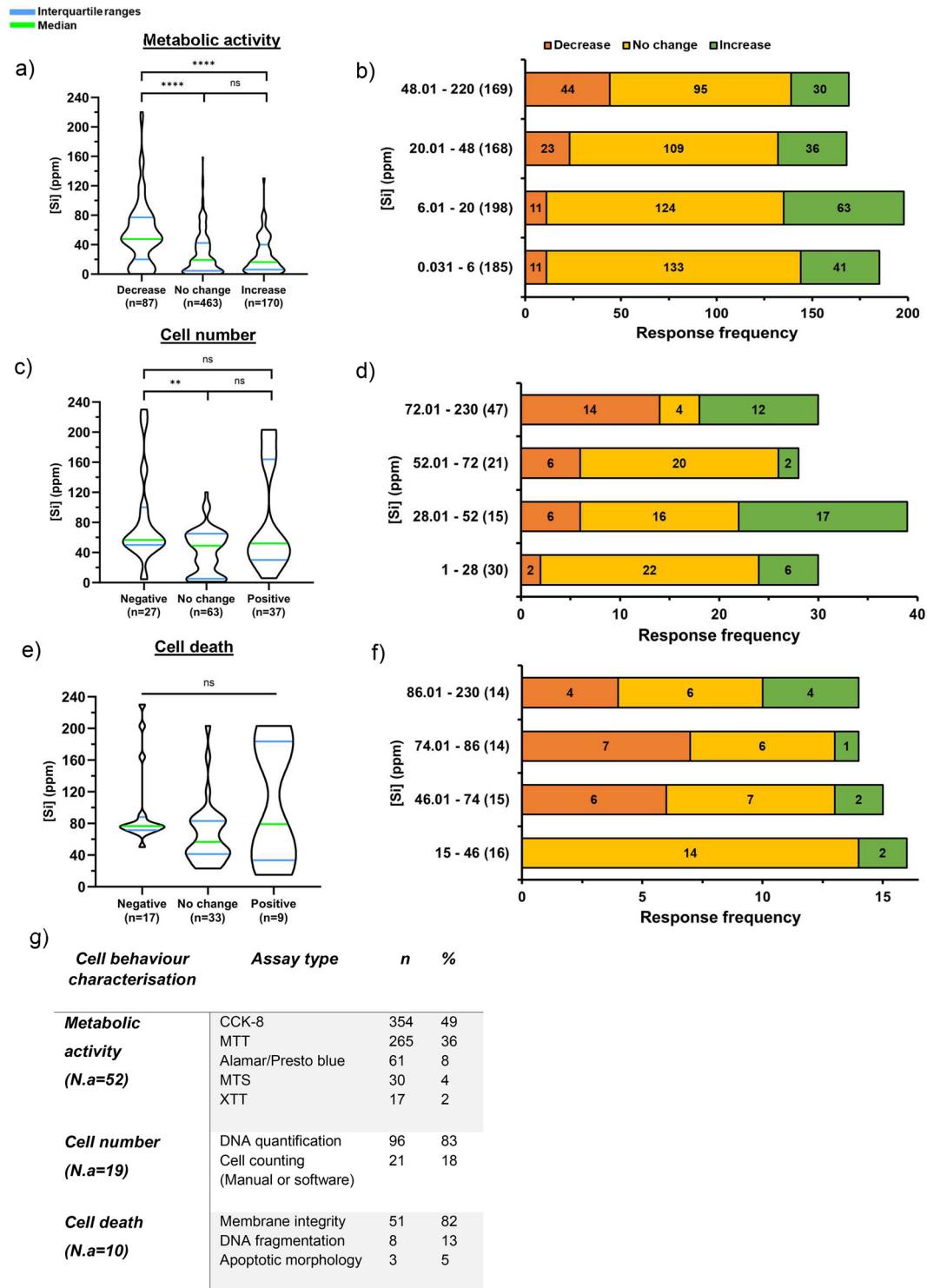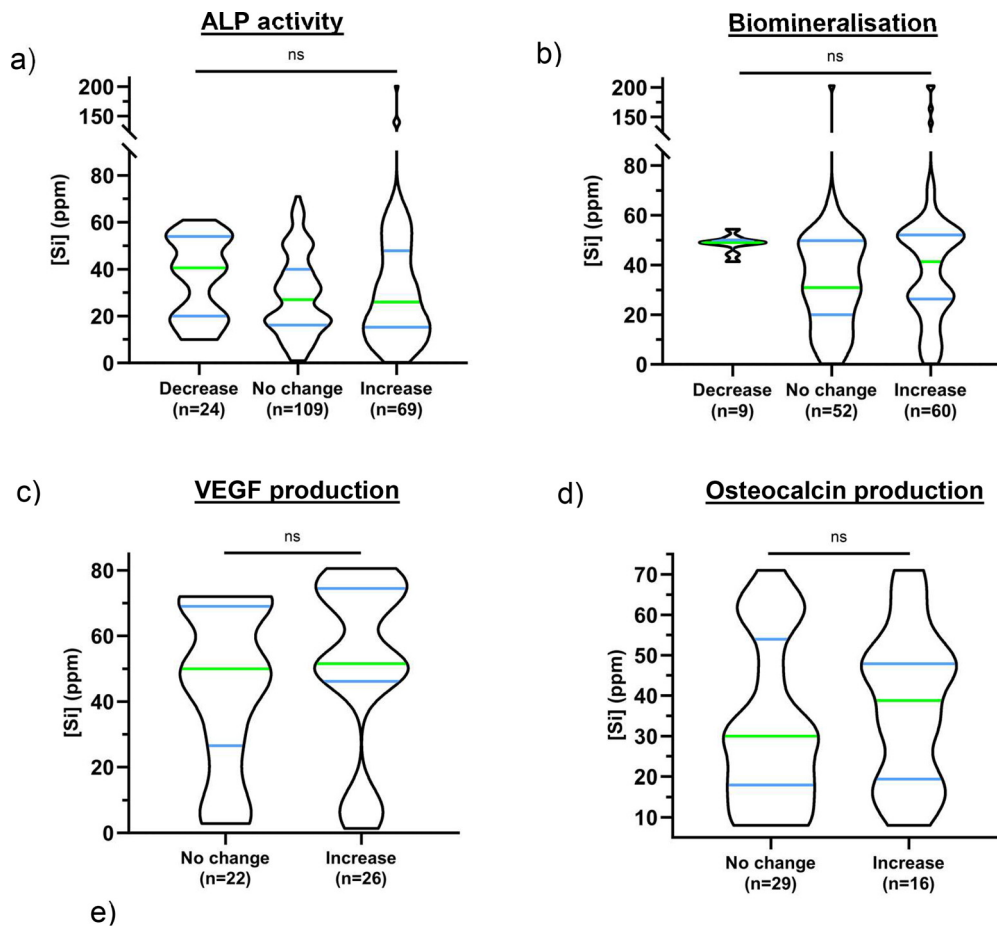
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**Fig. 5.** The influence of [Si] released from bioactive glasses on cell metabolic activity, proliferation, and cell death. *Median [Si] and frequency of negative, no change and positive proliferative cell responses of metabolic activity (a/b), cell number (c/d) and cell death (e/f) following culture with bioactive glasses. Quantification of frequency and percentage of assay types used in studies are presented in (g). Significant differences were observed in negative metabolic cell responses above ~50ppm compared to no change and positive data. No significant trends were found in proliferation or cell death responses, but an increased frequency of negative outcomes was observed with increase [Si]. \*\*=P ≤ 0.01, \*\*\*\*=P ≤ 0.0001, N.a=number of articles, n=number of data points, n %=percentage of total data points.*

articles, 75 % of data points) normalised ALP enzyme activity to either cell number (total DNA) or total protein, but only 33 % of articles (30 % of data points and articles) normalised osteocalcin or VEGF production to cell number. Without normalisation it is difficult to determine whether the influence of [Si] on ALP activity is due to cell number differences or individual cell response. No dif-ferences, however, were found on the effects of [Si] on either the ALP normalised or non-normalised data. Several different methods were used to determine biomineralization, with Alizarin Red stain-ing being used most common, followed by elemental analysis and nodule (number and area) quantification. For articles that quan-tified calcium amount by cetylpyridinium chloride extraction (the

**Fig. 6.** The influence of [Si] in cell type specific responses. Median [Si] and frequency of negative, no change and positive ALP activity (a), biomineralisation (b) and osteocalcin (c) and VEGF protein production (d) cell responses following culture in mediums containing Si released from bioactive glasses and ceramics. Quantification of frequency (n) and percentage of cell, species and material types used in studies are presented in (e). No differences were found in any response type. N.a=number of articles, n=number of data points, n %=percentage of total data points, 'data normalised' refers to data that has normalised to cell numbers.

most common quantification approach) there was no relationship between [Si] and calcium deposition.

When all gene expression responses to [Si] were combined, in a similar manner to other cellular responses, the mean [Si] associated with a decrease in gene expression was higher than the [Si] reported to cause an increase in gene expression (Fig. S5a). The

most common genes studied were associated with osteogenic differentiation (e.g., RUNX-1, OSX, BSP, ALP, BMP), extracellular matrix (ECM) production (collagen type I) or angiogenesis (VEGF), where an increase gene expression would normally be described as desirable for tissue regeneration. Decreased gene expression for VEGF, ALP, OCN, OSN, RUNX-2, collagen T1, were observed at
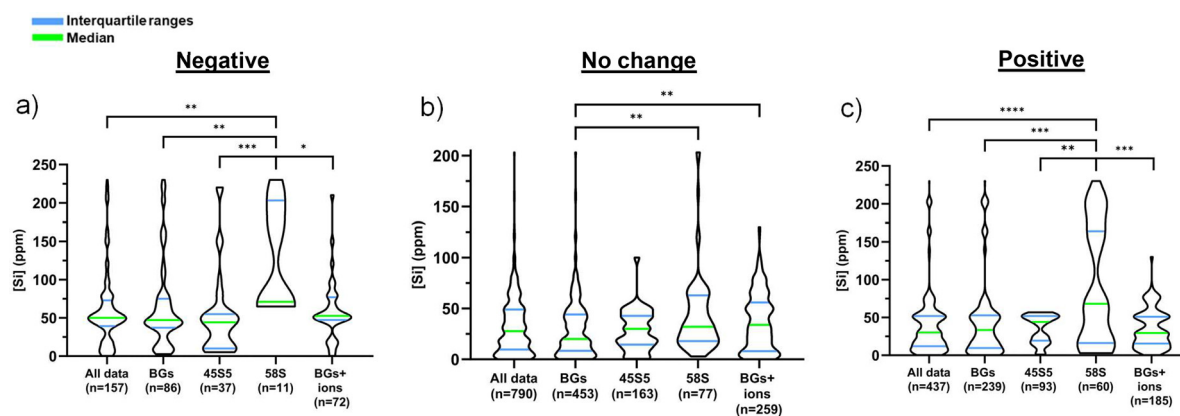
**Fig. 7.** Glass types and [Si]. Median [Si] of negative (a), no change (b) and positive (c) cell responses to all observed in-vitro studies when cultured with Si released from 45S5 bioactive glass ('45S5'), BGs containing only silica, calcium, phosphorus and sodium ('BGs') and the addition of other therapeutic ions ('BG+ions'). An increase in [Si] was observed to cause both negative and positive outcomes when released from 58S. Different material categories are compared with combined cell responses ('all data'). *=$P \leq$ 0.05, **=$P \leq$ 0.01, ***=$P \leq$ 0.001, ****=$P \leq$ 0.0001, n=number of data points.
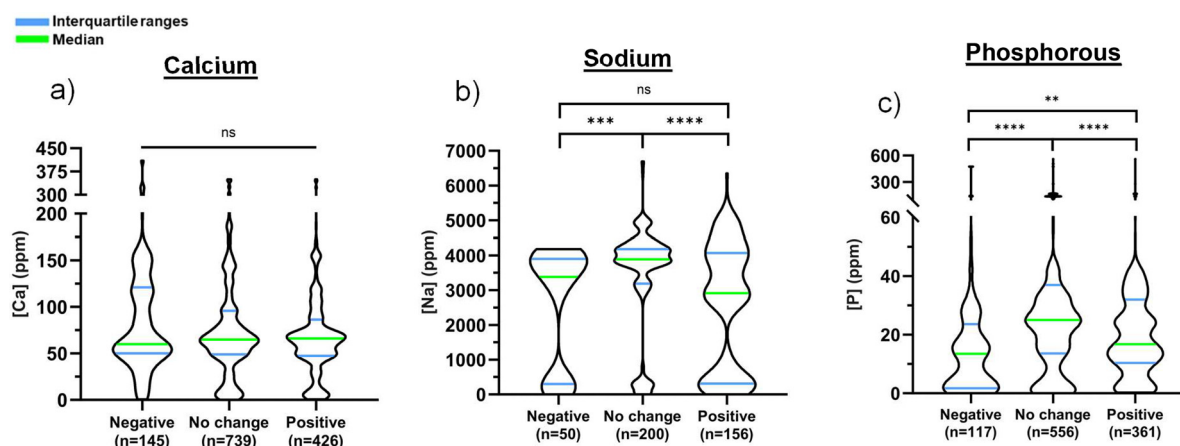


**Fig. 8.** The influence of [Ca], [Na] and [P] on overall cell responses. Cell responses in all observed in vitro studies when cultured with dissolution products containing calcium (a), sodium (b) and phosphorus (c). Overall responses to sodium at between 3500 and 4500 produced significant no change data compared to negative and positive data. Concentration between 18 and 25 ppm in phosphorus were found to cause positive responses whilst no significant trends were observed in either calcium. **=$P \leq$ 0.01, ***=$P \leq$ 0.001, ****=$P \leq$ 0.0001, n=number of data points.

higher [Si], but the reverse was also observed for OSX and BSP (Fig. S5b).

### 3.7. Does the type of SBG type affect the cellular response?

Different SBG compositions have been produced for various medical applications, but it is unclear if there is commonality between the type of BG and *in vitro* cellular outcomes. The most common SBG composition used within our data set was melt-derived 45S5 Bioglass® (23 articles), followed by sol-gel derived 58S (9 articles). We compared cellular outcomes between all melt-derived BGs (containing just Si, Ca, P, Na), with 45S5 Bioglass®, 58S glasses and glasses containing additional therapeutic ions (e.g., Sr, Co, Mg). Interestingly, negative, and positive cellular responses to 58S DPs, were observed to occur at a higher [Si] than for the other glass types (Fig. 7b). The range of [Si] in 58S studies is, however, higher ($M_n$ = 69.0 ppm ± 65.0) than the other SBGs ($P \leq$ 0.01). Additional therapeutic ions were found to alter the cellular response to [Si] concentrations, where positive outcomes occurred at lower [Si] compared to 45S5, and negative outcomes occurred at higher [Si] compared to 45S5. Considering Si and the therapeutic ions are released proportionally, this may indicate the influence of the therapeutic ions on cellular response.

### 3.8. The effect of calcium, sodium, and phosphorus ions in BG dissolution products

The concentration of other ions released from SBGs, apart from [Si], may also influence cellular interactions. The concentration dependent effects of calcium, phosphorus, and sodium ([Ca], [P], [Na] respectively) on cell behaviour were, therefore, also compared (Fig. 8). Contrary to the effects of [Si] concentrations on cell behaviour, the concentration of [P] that is reported to have negative cellular outcomes ($M_d$ = 13.5 ppm) was lower ($P \leq$ 0.001) than the [P] reported to have positive outcomes ($M_d$ = 16.8 ppm). A similar effect of [Na] on cell was reported, with negative outcomes reported at a lower median [Na] ($M_n$ = 1963 ppm ± 1599) compared to those producing positive effects ($M_n$ = 2673 ppm ± 1630). No significant differences were identified in the effect of [Ca] on cellular behaviour.

We also investigated whether these ions influence the concentration at which Si affects cell behaviour. Concentration ranges of each ion ([Ca], [P], [Na]) were split into equally sized 'high', 'medium' and 'low' groups (Fig. S4). A similar cellular response to [Si], as observed in all data (Fig. 2), was found in SBG with higher [Si] concentrations associated with negative outcomes. The difference between the mean [Si] causing negative and positive outcomes, was however, greater in the 'low' [Ca] group (41.6-0 ppm),
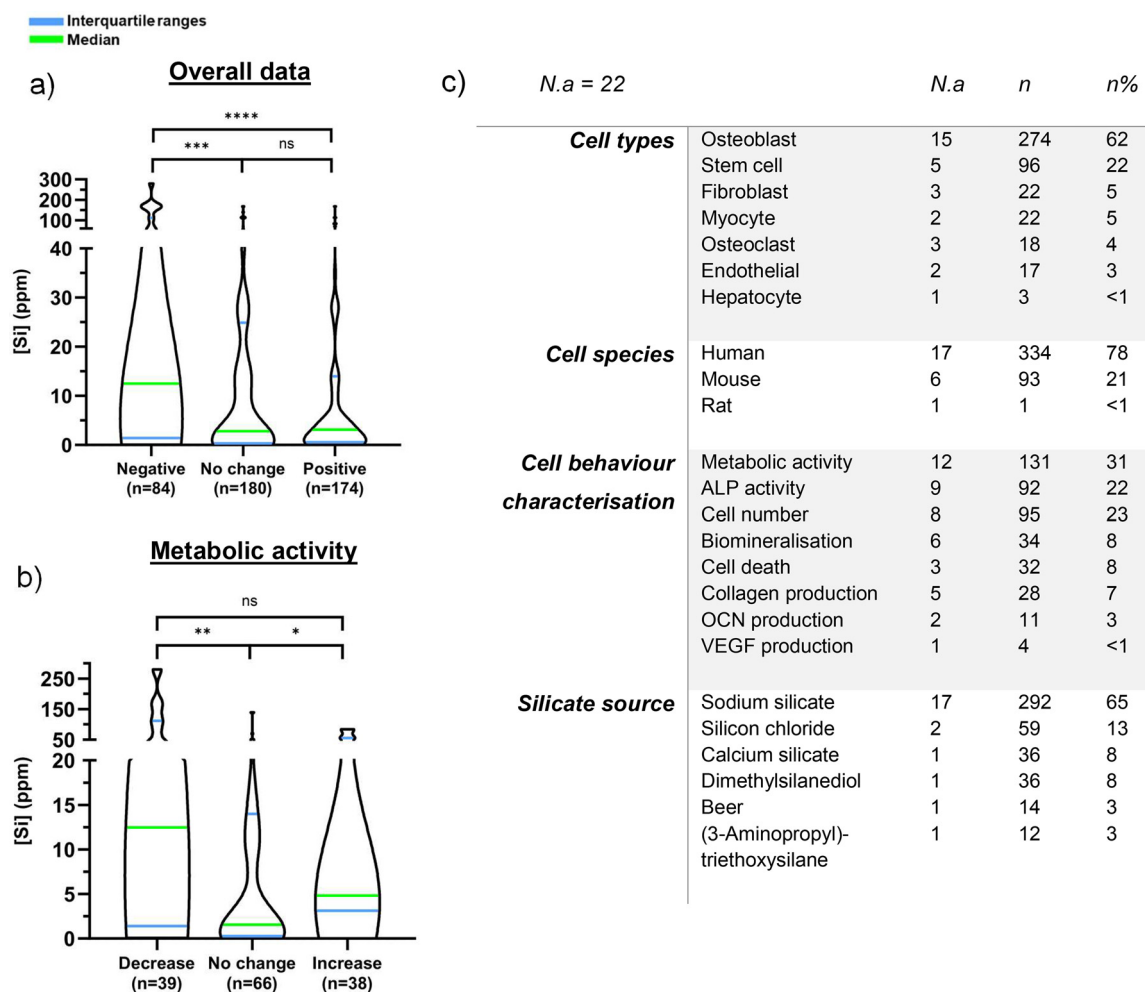
**Fig. 9.** The effect of Si derived from non-dissolution products on cell behaviour. The median [Si] that is reported to cause a negative, no change or positive responses in all observed cell (a) and metabolic activity (b) responses in-vitro following exposure to silicate species derived from non-dissolution product. Quantification of number of articles (N.a), number of data points (n), percentage of total data points (n %) is presented in (c). [Si] above ~12 ppm was observed to produce negative responses when compared to no change and positive outcomes in overall cell responses. In contrast, [Si] below ~2 ppm was associated with no change responses in metabolic activity. $^{*}$=$P \leq 0.05$, $^{**}$=$P \leq 0.01$, $^{***}$=$P \leq 0.001$, $^{****}$=$P \leq 0.0001$.

compared with higher [Ca] (125-83 ppm), indicating that calcium may influence the effect of [Si] (Fig. S4) on cell behaviour. Interestingly, [P] appeared to have the opposite effect, where a greater difference between cellular responses to [Si] (the difference in the reported negative or positive effects) was present in the groups containing more phosphorus within the SBG media, and there was no difference in responses to [Si] in the group with the 'low' [P] concentration (13.3-0 ppm) (Fig. S4). No significant trends were observed between [Si] and [Na] in any group, which may reflect the much fewer number of papers that measure Na in the medium.

### 3.9. Investigating differences between Si species alone and BG dissolution products on cell behaviour

To investigate whether Si species could influence cell behaviour independently of other ions released from BG (Na, Ca and P), additional articles focusing on the individual effect of Si species (not resulting from BG DPs) were collected (22 articles). In a similar manner to BG conditioned medium, Si ions alone also caused increasingly negative outcomes with increasing [Si]. The median [Si] reported to cause a negative outcome ($M_n = 56.6 \pm 77.6$ ppm) was significantly different ($P \leq 0.001$) to that reported to cause no change ($M_n = 18.7 \pm 33.9$ ppm) and positive outcomes ($M_n = 15.4 \pm 30.5$ ppm). These Si concentrations were significantly

lower when compared to responses to [Si] in SBG DPs ($P \leq 0.001$). There was, however, a difference in the [Si] range used in Si ions used alone ($M_n = 24.5$ ppm $\pm 47.0$) compared to those in DPs ($M_n = 37.8$ ppm $\pm 36.5$) ($P \leq 0.0001$), which may be a confounding factor in interpreting the difference between SBGs DPs and Si alone. Whilst sodium silicate was the most common Si precursor for conditioning cell culture medium (65 % of articles), silicon chloride (13 %) and calcium silicate (8 %) have also been studied (Fig. 9c). No differences were found between Si source and cell outcomes.

## 4. Discussion

By using a systematic quantitative approach, this review compared cellular responses to [Si] released from SBGs within the literature. Despite different experimental models (cell types, seeding densities, media, outcome measurements etc.) and different compositions of SBGs, commonalities were still observed on the effect of [Si] on cell behaviour. This is important for developing our understanding of how soluble Si effects cell behaviour (which remains poorly understood), and for the optimisation of Si releasing materials with tailored ion release profiles for specific applications. By collating the *in vitro* methodology used in SBG research, this review also provides a comprehensive overview of practices

used to investigate SBGs interactions with cells and reflects on the methodological approaches that provide comparable results.

### 4.1. The concentration dependent effect of Si on cell behaviour

A concentration dependent effect of Si (both within SBG dissolution media and Si alone) was observed when all cell responses were combined. In SBG dissolution media the frequency of negative outcomes reported in the literature was approximately 3 times higher above 52 ppm Si. This result identifies commonalities in the cellular response to Si, despite the variance in methodological approaches. Limited quantities of data or a lack of standardisation in experimental approaches for specific cell outcomes (e.g. VEGF expression or cell death), necessitated the grouping together cellular responses into "desirable" or "undesirable" cell responses. Whilst increased proliferation/metabolic activity/VEGF expression is usually described as a positive/desirable outcome in biomaterials *in vitro* research, this is clearly not always the case *in vivo* e.g., increased myo-fibroblast proliferation could be associated with contractile fibrotic membrane formation [38] or undifferentiated cells (e.g., osteoblasts) often having a period of proliferation followed by differentiation where there is a decrease in proliferation [39]. Combining the experimental data (as in Fig. 2) loses this nuance but provides a larger data pool to observe commonalities, that would not be present in smaller data sets, where the variances in experimental approaches between studies masked the overarching trends.

How Si influences cell behaviour remains unclear and is likely to depend upon both the concentration, form (soluble or insoluble) and possibly the Si species (e.g., ortho- di- or polysilicates). Studies by Iler et al., suggested that below pH 9, [Si] at approximately 2 - 3 mM (56 - 84 ppm) in water begins to polymerise, where below this concentration Si is thought to be predominately the monomer orthosilicate ($Si(OH)_4$) [40]. The [Si] that initiates polymerisation in body fluids or cell culture media, however, remains unclear. The formation of silica nanoparticles from the polymerisation of orthosilicate units, is likely to alter Si uptake and cellular interactions. Silica nanoparticles have been associated with cellular toxicity [41,42], for example Gong et al., found that silica nanoparticles (15, 30 and 100 nm in diameter) caused apoptosis and that this was likely caused by increased reactive oxygen species (ROS) damage [43]. Si has, however, also been reported to protect cells from oxidative stress [44,45].

If ROS is important in cellular response to [Si], part of cellular variance observed in negative outcomes to [Si] (above 50 ppm, Fig. 2 and 3) may be due to variance in each different cell type's ability to resist oxidative stress, in addition to differences in the amount of free radicals scavengers present in cell culture media. For example, in osteoblast mineralisation studies, additional free radical scavengers such as ascorbic acid (~50 μg/ml) are commonly added to osteogenic media [46], and this may explain osteoblast viability in the presence of high levels of [Si] (112 ppm [19]) where Si particles are more likely. Other cell types like endothelial cells have been reported to show increased cell death in response to [Si] as low as 14 ppm [45]. Stem cells have been shown to resist oxidative damage via increased intracellular antioxidant concentrations compared to terminally differentiated cells [47]. This may explain why there is no difference in the medium [Si] that causes negative outcomes in stem cells compared to non-stem cells (Fig. 3b) despite a higher [Si] range used (on average) to treat stem cells.

Undesirable cellular outcomes, with increasing Si (non-dissolution products) were also found when Si ions alone were used (not from SBGs) although at lower [Si] compared to those from DPs (Fig. 9a). The release of other BG ions (e.g., phosphorus, calcium, and sodium) may account for this difference. Both calcium and phosphate ions have been reported to increase cell

metabolic activity, proliferation [48,49] and mineralisation [50,51], possibly by increasing the rate of oxidative phosphorylation and thus ATP production [52,53]. Alternatively, higher [Ca] and [P] may also increase apatite formation, silicate-calcium or possibly Si-HA complexes, thereby lowering the availability of soluble ions within the media during cell culture. Other ions may also have an effect on pH in culture media, a factor that has been shown to affect [Si] polymerisation and cell behaviour [54].

The number of different glass types (in the collected data set) is also likely to contribute towards the variance in cellular responses observed. Positive and negative outcomes were observed in sol-gel 58S at higher [Si] than in other BGs. This is likely to be due to higher ion ranges used in studies in sol-gel studies (due to the increased surface area, higher mol % Si and the absence of sodium in 58S networks increasing the rate of Si release) [55]. Despite evidence suggesting that Si could increase mineralised ECM in osteoblast cells [19,20,22,23,56], no correlations were found in either ALP activity or biomineralization. A lack of differences in the effect of [Si] on ALP activity, may be due to variance in the respective cell seeding density which will likely influence the stage of osteoblast differentiation in each study [57,58]. The influence of time on the effect of [Si] on ALP activity was therefore investigated (<1, 2 and 3 weeks) but did produce any significant differences.

### 4.2. Methodological approaches and the need for standardisation

There is considerable variance in methodology used to evaluate cell viability and functionality in response to SBG *in vitro*. Previous reviews have compared the methodological approaches used to assess cellular outcomes with BGs and thus the difficulties in comparing outcomes from different studies [37]. Qualitative responses (e.g., morphological changes), although valuable to understand material/DPs interactions, were not included in this study as they are rarely quantified and therefore difficult to quantitatively compare outcomes from different studies. If researchers within the field generated data that allows for greater comparison between studies, it would enable greater progress in the field, more impactful research and preserve resources (increased sustainability). For these reasons we recommend a minimum reporting standard for *in vitro* studies with SBG DPs (Table 1).

Our results identified metabolic activity assays to be the most common assessment of cell viability in response to DPs. This is perhaps unsurprising given ISO standard 10993-5 suggest these metabolic assays (MTT/XTT in particular) to be the primary method of evaluating cell behaviour in response to medical devices. ISO 10993-5 was, however, developed for assessing whether leachates from a device could cause toxicity rather than assessing an intentionally biodegradable device. Considerable variation in metabolic responses to [Si] was also observed (Fig. 5) which may in part be due to variance in metabolic assay used (e.g., MTT, WST-8 and Alamar blue). MTT was the most used metabolic assay to measure cell responses to SBG DPs, which may be due to ISO10993-5 recommending this assay. The MTT assay requires solvents (such as DMSO) to dissolve the formazan product, which therefore necessitates the termination of the cell culture [59]. Similar assays that produce soluble products (and thus don't require additional solvents) such as MTS and Alamar blue were more commonly used within the last 5 years (2016-2021) compared to the previous 5 years (2016-2011) possibly for sensitivity [60] and resource preservation reasons. Media composition (e.g., pH buffers, serum type or percentage) and type (e.g., DMEM, RPMI or MEM-$\alpha$) have also been shown to influence the outcome of metabolic assays [61,62], further contributing to variance observed in metabolic activity responses. Compared to metabolic assays, less variance, however, was found in direct measures of cell number with most articles selecting total DNA quantification (95 % or articles) over other ap-

**Table 1**
Suggested minimum reporting standard for studies investigating the effect of SBG in vitro.

1. **Quantification of BG dissolution products in cell culture media:** Ion concentrations of SBG DPs should quantified in the cell culture media used for *in vitro* studies (approximately 40 % of articles in our search did not report/quantify ion concentrations in media). Different media contains different ion concentrations (e.g., [Ca], [Na], [P]) and thus the total ion availability will vary in different media.

2. **Untreated controls**: Cells cultured in normal media on standard cell culture plastic should be compared to treated cells and included in the results for comparison purposes.

3. **Normalisation of protein production/activity**: To determine if the BG PDs are influencing cell phenotype or influencing cell number, the protein of interest should be normalised (e.g., to total DNA, or a protein that is not influenced by the treatment). The total amount of protein should also be reported.

4. **Cell number vs metabolic activity:** SBG DPs can change the cellular metabolic activity (Fig. 5a). Metabolic activity may, therefore, equate to increased cell number or increased metabolic activity/cell. Cell number should, therefore, be reported separately to metabolic activity. For clarity, metabolic assays should be referred to as metabolic assays as opposed to proliferation or cytotoxicity assays*.

5. **Stem cell source:** Isolation method, passage number and source should be included. Authors should state whether the stem cells were a single population (e.g., immunochemically isolated using FACs) or a mixed population of cells (using adhesion and centrifugation approaches).

6. **Availability of data.** Data should be available online, for scrutiny, further analysis and for comparison purposes.

*Commercially available metabolic activity assays often have confusing marketing terminology (e.g., Cell Counting Kit-8, CCK-8) and often assume that cellular metabolic activity is unaffected by conditions/treatments.

proaches e.g., manual or imaging software analysis of cell number [57].

The most common timepoints that articles observed cell response to DPs, were over 3 time points (1, 3 and 7 days – 24 % of articles), and approximately 25 % of these studies reported only 1 or 2 time points (commonly up to 3 days). Although ISO10993-5 recommends cells be in contact with conditioned media for 1 day, a culture of 1 week or longer may be necessary to observe other important cell behavioural changes (e.g. differentiation or ECM production).

In terms of protein production (largely with ELISAs - enzyme-linked immunosorbent assay), no clear trends in response to [Si] within DPS were found (Fig. 6). However, only 30 % of observed studies using ELISA kits were found to be normalised to cell numbers or total protein concentrations. Without normalisation comparisons between studies on SBG DPs is difficult, as it is unclear if the protein production is due to cell number or cellular phenotypic changes (increased expression of the protein per cell) Gene expression (where expression is relative to a house-keeper gene) did, however, demonstrate a [Si] dependent response, where the [Si] that caused a decrease in target gene expression was significantly higher than the [Si] that increased target gene expression (Fig. S5a). There is also not always a direct correlation between gene transcription and functional protein expression [63].

The majority of SBG studies were focused on bone regeneration, but the quantification and characterisation of *in vitro* bone formation largely relied upon calcium staining (Alizarin Red) and quantification of calcium. Calcium deposition assays, however, can be influenced by the calcium ions present in the SBG extracts, differing Ca present in the media and spontaneous formation of apatite. Experiments involving more than one type of bone nodule quantification, including area or volume were observed in less than 10 % of studies. Other physicochemical approaches to characterise *in vitro* bone formation (e.g., mineralised collagen fibres) and comparison to native bone including biochemical (Raman/FTIR), ultrastructure (TEM), elemental (EDX/ICP-OES/MS) and mechanical characterisation, were rarely studied. Previous studies have also shown that different cell types and species alter the amount mineral deposited during bone formation *in vitro* [64–66].

## 5. Conclusion

This review has, for the first time, attempted to quantify the effect of Si species released from bioactive glasses on *in vitro* cellular responses. The quantitative analysis reveals that higher [Si] within SBG DPs has an increased the frequency of negative outcomes reported, this is perhaps not unexpected, but the specific concentrations where this more likely and the window of [Si] where de-

sirable cell responses are more likely to occur, is useful in tailoring the Si release from novel SBGs. The systematic approach used in this study also presents a novel approach to compare complex studies with high methodological variance (e.g., different cell types, experimental conditions and methods used to evaluate cellular responses). Standardisation of *in vitro* characterisation approaches to BGs, may provide more insight into the concentration dependent effects of [Si] and understanding of its various roles in cell behaviour.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2023.09.012.

## References

[1] A. Hoppe, N.S. Güldal, A.R. Boccaccini, A review of the biological response to ionic dissolution products from bioactive glasses and glass-ceramics, Biomaterials 32 (2011) 2757–2774, doi:10.1016/j.biomaterials.2011.01.004.

[2] L.L. Hench, J.R. Jones, Bioactive glasses: frontiers and challenges, Front. Bioeng. Biotechnol. 3 (2015), doi:10.3389/fbioe.2015.00194.

[3] I.D. Xynos, A.J. Edgar, L.D.K.K. Buttery, L.L. Hench, J.M. Polak, Gene-expression profiling of human osteoblasts following treatment with the ionic products of Bioglass® 45S5 dissolution, J. Biomed. Mater. Res. 55 (2001) 151–157, doi:10.1002/1097-4636(200105)55:2≤151::AID-JBM1001≥3.0.CO;2-D.

[4] E.S. Thian, J. Huang, S.M. Best, Z.H. Barber, W. Bonfield, Novel silicon-doped hydroxyapatite (Si-HA) for biomedical coatings: An *in vitro* study using acellular simulated body fluid, J. Biomed. Mater. Res. B 76B (2006) 326–333, doi:10.1002/JBM.B.30368.

[5] F.J. Martínez-Vázquez, M.V. Cabañas, J.L. Paris, D. Lozano, M. Vallet-Regí, Fabrication of novel Si-doped hydroxyapatite/gelatine scaffolds by rapid prototyping for drug delivery and bone regeneration, Acta Biomater. 15 (2015) 200–209, doi:10.1016/J.ACTBIO.2014.12.021.

[6] I.D. Xynos, A.J. Edgar, L.D.K.K. Buttery, L.L. Hench, J.M. Polak, Ionic products of bioactive glass dissolution increase proliferation of human osteoblasts and induce insulin-like growth factor II mRNA expression and protein synthesis, Biochem. Biophys. Res. Commun. 276 (2000) 461–465, doi:10.1006/bbrc.2000.3503.

[7] R.C. Bielby, I.S. Christodoulou, R.S. Pryce, W.J.P.P. Radford, L.L. Hench, J.M. Polak, Time- and concentration-dependent effects of dissolution products of 58S sol-gel bioactive glass on proliferation and differentiation of murine and human osteoblasts, Tissue Eng. 10 (2004) 1018–1026, doi:10.1089/ten.2004.10.1018.

[8] E.M. Carlisle, Silicon: An essential element for the chick, Science 178 (1972) 619–621, doi:10.1126/science.178.4061.619.

[9] M.A. Elliot, H.M. Edwards, Effect of dietary silicon on growth and skeletal development in chickens, J. Nutr. 121 (1991) 201–207, doi:10.1093/jn/121.2.201.

[10] E.M. Carlisle, Silicon: A requirement in bone formation independent of vitamin D1, Calcif. Tissue Int. 33 (1981) 27–34, doi:10.1007/BF02409409.

[11] E.M. Carlisle, Silicon as an essential trace element in animal nutrition, Ciba Found. Symp. 121 (1986) 123–139, doi:10.1002/9780470513323.ch8.

[12] M. Hott, C. de Pollak, D. Modrowski, P.J. Marie, Short-term effects of organic silicon on trabecular bone in mature ovariectomized rats, Calcif. Tissue Int. 53 (1993) 174–179, doi:10.1007/BF01321834.

[13] K. Schwarz, D.B. Milne, Growth-promoting effects of silicon in rats, Nature 239 (1972) 333–334, doi:10.1038/239333a0.

[14] J. Eisinger, D. Clairet, Effects of silicon, fluoride, etidronate and magnesium on bone mineral density: a retrospective study, Magn. Res. 6 (1993) 247–249 http://www.ncbi.nlm.nih.gov/pubmed/8292498. (Accessed 23 April 2018).

[15] M.K. Choi, M.H. Kim, Dietary silicon intake of Korean young adult males and its relation to their bone status, Biol. Trace Elem. Res. 176 (2017) 89–104, doi:10.1007/s12011-016-0817-x.

[16] R. Jugdaohsingh, K.L. Tucker, N. Qiao, L.A. Cupples, D.P. Kiel, J.J. Powell, Dietary silicon intake is positively associated with bone mineral density in men and premenopausal women of the framingham offspring cohort, J. Bone Miner. Res. 19 (2004) 297–307, doi:10.1359/JBMR.0301225.

[17] S. Zou, D. Ireland, R.A. Brooks, N. Rushton, S. Best, The effects of silicate ions on human osteoblast adhesion, proliferation, and differentiation, J. Biomed. Mater. Res. B 90 B (2009) 123–130, doi:10.1002/jbm.b.31262.

[18] A. Obata, T. Ogasawara, T. Kasuga, Combinatorial effects of inorganic ions on adhesion and proliferation of osteoblast-like cells, J. Biomed. Mater. Res. A (2019) 1042–1051, doi:10.1002/jbm.a.36623.

[19] M.Y. Shie, S.J. Ding, H.C. Chang, The role of silicon in osteoblast-like cell proliferation and apoptosis, Acta Biomater. 7 (2011) 2604–2614, doi:10.1016/j.actbio.2011.02.023.

[20] P. Han, C. Wu, Y. Xiao, The effect of silicate ions on proliferation, osteogenic differentiation and cell signalling pathways (WNT and SHH) of bone marrow stromal cells, Biomater. Sci. 1 (2013) 379–392, doi:10.1039/c2bm00108j.

[21] X. Zhou, F.M. Moussa, S. Mankoci, P. Ustriyana, N. Zhang, S. Abdelmagid, J. Molenda, W.L. Murphy, F.F. Safadi, N. Sahai, Orthosilicic acid, Si(OH)4, stimulates osteoblast differentiation *in vitro* by upregulating miR-146a to antagonize NF-κB activation, Acta Biomater. 39 (2016) 192–202, doi:10.1016/j.actbio.2016.05.007.

[22] P. Uribe, A. Johansson, R. Jugdaohsingh, J.J. Powell, C. Magnusson, M. Davila, A. Westerlund, M. Ransjö, Soluble silica stimulates osteogenic differentiation and gap junction communication in human dental follicle cells, Sci. Rep. 10 (2020), doi:10.1038/s41598-020-66939-1.

[23] H.C. Schröder, X.H. Wang, M. Wiens, B. Diehl-Seifert, K. Kropf, U. Schloßmacher, W.E.G. Müller, Silicate modulates the cross-talk between osteoblasts (SaOS-2) and osteoclasts (RAW 264.7 cells): Inhibition of osteoclast growth and differentiation, J. Cell. Biochem. 113 (2012) 3197–3206, doi:10.1002/jcb.24196.

[24] J. Chang, Z. Wang, E. Tang, Z. Fan, L. McCauley, R. Franceschi, K. Guan, P.H. Krebsbach, C.Y. Wang, Inhibition of osteoblastic bone formation by nuclear factor-B, Nat. Med. 15 (2009) 682–689, doi:10.1038/nm.1954.

[25] D.V. Novack, L. Yin, A. Hagen-Stapleton, R.D. Schreiber, D.V. Goeddel, F.P. Ross, S.L. Teitelbaum, The IκB function of NF-κB2 p100 controls stimulated osteoclastogenesis, J. Exp. Med. 198 (2003) 771–781, doi:10.1084/jem.20030116.

[26] V.G. Varanasi, K.K. Leong, L.M. Dominia, S.M. Jue, P.M. Loomer, G.W. Marshall, Si and Ca individually and combinatorially target enhanced MC3T3-E1 subclone 4 early osteogenic marker expression, J. Oral Implantol. 38 (2012) 325–336, doi:10.1563/AAID-JOI-D-11-00108.

[27] W. Dang, X. Wang, J. Li, C. Deng, Y. Liu, Q. Yao, L. Wang, J. Chang, C. Wu, 3D printing of Mo-containing scaffolds with activated anabolic responses and bilineage bioactivities, Theranostics 8 (2018) 4372–4392, doi:10.7150/thno.27088.

[28] M.S. Kang, N.-H. Lee, R.K. Singh, N. Mandakhbayar, R.A. Perez, J.-H. Lee, H.-W. Kim, Nanocements produced from mesoporous bioactive glass nanoparticles, Biomaterials 162 (2018) 183–199, doi:10.1016/j.biomaterials.2018.02.005.

[29] A. Moghanian, S. Firoozi, M. Tahriri, A. Sedghi, A comparative study on the *in vitro* formation of hydroxyapatite, cytotoxicity and antibacterial activity of 58S bioactive glass substituted by Li and Sr, Mater. Sci. Eng. C 91 (2018) 349–360, doi:10.1016/j.msec.2018.05.058.

[30] V.G. Varanasi, E. Saiz, P.M. Loomer, B. Ancheta, N. Uritani, S.P. Ho, A.P. Tomsia, S.J. Marshall, G.W. Marshall, Enhanced osteocalcin expression by osteoblast-like cells (MC3T3-E1) exposed to bioactive coating glass (SiO2-CaO-P2O5-MgO-K2O-Na2O system) ions, Acta Biomater. 5 (2009) 3536–3547, doi:10.1016/j.actbio.2009.05.035.

[31] J. Liu, S.C.F. Rawlinson, R.G. Hill, F. Fortune, Fluoride incorporation in high phosphate containing bioactive glasses and *in vitro* osteogenic, angiogenic and antibacterial effects, Dent. Mater. 32 (2016) e221–e237, doi:10.1016/j.dental.2016.07.003.

[32] O. Tsigkou, J.R. Jones, J.M. Polak, M.M. Stevens, Differentiation of fetal osteoblasts and formation of mineralized bone nodules by 45S5 Bioglass® conditioned medium in the absence of osteogenic supplements, Biomaterials 30 (2009) 3542–3550, doi:10.1016/j.biomaterials.2009.03.019.

[33] I. Christodoulou, L.D.K. Buttery, P. Saravanapavan, G. Tai, L.L. Hench, J.M. Polak, Dose- and time-dependent effect of bioactive gel-glass ionic-dissolution products on human fetal osteoblast-specific gene expression, J. Biomed. Mater. Res. B 74 (2005) 529–537, doi:10.1002/jbm.b.30249.

[34] R.L. Du, J. Chang, S.Y. Ni, W.Y. Zhai, J.Y. Wang, Characterization and *in vitro* bioactivity of zinc-containing bioactive glass and glass-ceramics, J. Biomater. Appl. 20 (2006) 341–360, doi:10.1177/0885328206054535.

[35] E. Gentleman, Y.C. Fredholm, G. Jell, N. Lotfibakhshaiesh, M.D. O'Donnell, R.G. Hill, M.M. Stevens, The effects of strontium-substituted bioactive glasses on osteoblasts and osteoclasts *in vitro*, Biomaterials 31 (2010) 3949–3956, doi:10.1016/j.biomaterials.2010.01.121.

[36] A. Obata, N. Iwanaga, A. Terada, G. Jell, T. Kasuga, Osteoblast-like cell responses to silicate ions released from 45S5-type bioactive glass and siloxane-doped vaterite, J. Mater. Sci. 52 (2017) 8942–8956, doi:10.1007/s10853-017-1057-y.

[37] E. Jablonská, D. Horkavcová, D. Rohanová, D.S. Brauer, A review of: In vitro cell culture testing methods for bioactive glasses and other biomaterials for hard tissue regeneration, J. Mater. Chem. B 8 (2020) 10941–10953, doi:10.1039/d0tb01493a.

[38] N. Noskovicova, B. Hinz, P. Pakshir, Cells implant fibrosis and the underappreciated role of myofibroblasts in the foreign body reaction laboratory of tissue repair and regeneration, Cells (2021), doi:10.3390/cells10071794.

[39] S. Ruijtenberg, S. van den Heuvel, Coordinating cell proliferation and differentiation: antagonism between cell cycle regulators and cell type-specific gene expression, Cell Cycle 15 (2016) 196–212, doi:10.1080/15384101.2015.1120925.

[40] R.K. Iler, The Chemistry of Silica : Solubility, Polymerization, Colloid and Surface Properties, and Biochemistry, Wiley, 1979.

[41] F. Zhou, F. Liao, L. Chen, Y. Liu, W. Wang, S. Feng, The size-dependent genotoxicity and oxidative stress of silica nanoparticles on endothelial cells, Environ. Sci. Pollut. Rese. 26 (2019) 1911–1920, doi:10.1007/s11356-018-3695-2.

[42] S.E. Lehman, A.S. Morris, P.S. Mueller, A.K. Salem, V.H. Grassian, S.C. Larsen, Silica nanoparticle-generated ROS as a predictor of cellular toxicity: mechanistic insights and safety by design, Environ. Sci. Nano 3 (2016) 56–66, doi:10.1039/c5en00179j.

[43] C. Gong, G. Tao, L. Yang, J. Liu, H. He, Z. Zhuang, The role of reactive oxygen species in silicon dioxide nanoparticle-induced cytotoxicity and DNA damage in HaCaT cells, Mol. Biol. Rep. 39 (2012) 4915–4925, doi:10.1007/s11033-011-1287-z.

[44] K. Awad, N. Ahuja, M. Fiedler, S. Peper, Z. Wang, P. Aswath, M. Brotto, V. Varanasi, Ionic silicon protects oxidative damage and promotes skeletal muscle cell regeneration, Int. J. Mol. Sci. 22 (2021) 1–25, doi:10.3390/ijms22020497.

[45] F. Monte, T. Cebe, D. Ripperger, F. Ighani, H.V. Kojouharov, B.M. Chen, H.K.W. Kim, P.B. Aswath, V.G. Varanasi, Ionic silicon improves endothelial cells' survival under toxic oxidative stress by overexpressing angiogenic markers and antioxidant enzymes, J. Tissue Eng. Regen. Med. 12 (2018) 2203–2220, doi:10.1002/term.2744.

[46] H.K. Choi, G.J. Kim, H.S. Yoo, D.H. Song, K.H. Chung, K.J. Lee, Y.T. Koo, J.H. An, Vitamin C activates osteoblastogenesis and inhibits osteoclastogenesis via Wnt/β-catenin/ATF4 signaling pathways, Nutrients 11 (2019), doi:10.3390/nu11030506.

[47] T. He, T.E. Peterson, E.L. Holmuhamedov, A. Terzic, N.M. Caplice, L.W. Oberley, Z.S. Katusic, Human endothelial progenitor cells tolerate oxidative stress due to intrinsically high expression of manganese superoxide dismutase, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 2021–2027, doi:10.1161/01.ATV.0000142810.27849.8f.

[48] A.M.C. Barradas, H.A.M. Fernandes, N. Groen, Y.C. Chai, J. Schrooten, J. Van de Peppel, J.P.T.M. Van Leeuwen, C.A. Van Blitterswijk, J. De Boer, A calcium-induced signaling cascade leading to osteogenic differentiation of human bone marrow-derived mesenchymal stromal cells, Biomaterials 33 (2012) 3205–3215, doi:10.1016/j.biomaterials.2012.01.020.

[49] S. An, J. Ling, Y. Gao, Y. Xiao, Effects of varied ionic calcium and phosphate on the proliferation, osteogenic differentiation and mineralization of human periodontal ligament cells *in vitro*, J. Periodontal Res. 47 (2012) 374–382, doi:10.1111/j.1600-0765.2011.01443.x.

[50] M.N. Lee, H.S. Hwang, S.H. Oh, A. Roshanzadeh, J.W. Kim, J.H. Song, E.S. Kim, J.T. Koh, Elevated extracellular calcium ions promote proliferation and migration of mesenchymal stem cells via increasing osteopontin expression, Exp. Mol. Med. 50 (2018), doi:10.1038/s12276-018-0170-6.

[51] H. Lin, Y. Zhou, Q. Lei, D. Lin, J. Chen, C. Wu, Effect of inorganic phosphate on migration and osteogenic differentiation of bone marrow mesenchymal stem cells, BMC Dev. Biol 21 (2021), doi:10.1186/s12861-020-00229-x.

[52] A. Rossi, P. Pizzo, R. Filadi, Calcium, mitochondria and cell metabolism: A functional triangle in bioenergetics, Biochim. Biophys. Acta. Mol. Cell Res. 1866 (2019) 1068–1078, doi:10.1016/j.bbamcr.2018.10.016.

[53] P.R. Angelova, A.Y. Baev, A.V. Berezhnov, A.Y. Abramov, Role of inorganic polyphosphate in mammalian cells: From signal transduction and mitochondrial metabolism to cell death, Biochem. Soc. Trans. 44 (2016) 40–45, doi:10.1042/BST20150223.

[54] M. Dietzel, Dissolution of silicates and the stability of polysilicic acid, Geochim. Cosmochim. Acta 64 (2000) 3275–3281, doi:10.1016/S0016-7037(00)00426-9.

[55] L.L. Sepulveda, P. Jones, J.R. Hench, In vitro dissolution of melt derived 45 S5 and sol–gel derived 58 S bioactive glasses, Biomed. Mater. Res. 61 (2002)

301–311 https://onlinelibrary.wiley.com/doi/pdf/10.1002/jbm.10207. (Accessed 8 May 2018).

[56] M. Dong, G. Jiao, H. Liu, W. Wu, S. Li, Q. Wang, D. Xu, X. Li, H. Liu, Y. Chen, Biological silicon stimulates collagen type 1 and osteocalcin synthesis in human osteoblast-like cells through the BMP-2/Smad/RUNX2 signaling pathway, Biol. Trace Elem. Res. 173 (2016) 306–315, doi:10.1007/s12011-016-0686-3.

[57] K. Kim, D. Dean, A.G. Mikos, J.P. Fisher, Effect of initial cell seeding density on early osteogenic signal expression of rat bone marrow stromal cells cultured on cross-linked poly(propylene fumarate) disks, Biomacromolecules 10 (2009) 1810–1817, doi:10.1021/bm900240k.

[58] H. Zhou, M.D. Weir, H.H.K. Xu, Effect of cell seeding density on proliferation and osteodifferentiation of umbilical cord stem cells on calcium phosphate cement-fiber scaffold, Tissue Eng. Part A 17 (2011) 2603–2613, doi:10.1089/ten.tea.2011.0048.

[59] J. Galvao, B. Davis, M. Tilley, E. Normando, M.R. Duchen, M.F. Cordeiro, Unexpected low-dose toxicity of the universal solvent DMSO, FASEB J. 28 (2014) 1317–1330, doi:10.1096/FJ.13-235440.

[60] B. Luzak, P. Siarkiewicz, M. Boncler, An evaluation of a new high-sensitivity PrestoBlue assay for measuring cell viability and drug cytotoxicity using EA.hy926 endothelial cells, Toxicol. in vitro 83 (2022) 105407, doi:10.1016/J.TIV.2022.105407.

[61] M. Ghasemi, T. Turnbull, S. Sebastian, I. Kempson, Molecular sciences the MTT assay: utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis,Int. J. Mol. Sci. (2021). https://doi.org/10.3390/ijms222312827.

[62] A. Van Tonder, A.M. Joubert, A.D. Cromarty, Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays, BMC Res. Notes 8 (2015) 1–10, doi:10.1186/S13104-015-1000-8/FIGURES/3.

[63] I. Kosti, N. Jain, D. Aran, A.J. Butte, M. Sirota, Cross-tissue analysis of gene and protein expression in normal and cancer tissues, Sci. Rep. 6 (2016), doi:10.1038/srep24799.

[64] E.M. Czekanska, M.J. Stoddart, J.R. Ralphs, R.G. Richards, J.S. Hayes, A phenotypic comparison of osteoblast cell lines versus human primary osteoblasts for biomaterials testing, J. Biomed. Mater. Res. A 102 (2014) 2636–2643, doi:10.1002/jbm.a.34937.

[65] C. Pautke, M. Schieker, T. Tischer, A. Kolk, P. Neth, W. Mutschler, S. Milz, Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts, Anticancer Res. 24 (2004).

[66] V. Kartsogiannis, K.W. Ng, Cell lines and primary cell cultures in the study of bone cell biology, Mol. Cell. Endocrinol. 228 (2004) 79–102, doi:10.1016/j.mce.2003.06.002.