# Osteoblast-like cell responses to silicate ions released from 45S5-type bioactive glass and siloxane-doped vaterite

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## Abstract

**Purpose:** Silicate ions released from bioactive glasses and ceramics have been reported to stimulate osteogenic cell functions. Here, we evaluated osteoblast-like cell reactions to silicate ions released from two different types of materials, 45S5 bioactive glass (BG) and siloxane-doped vaterite (SiV), to investigate the influence of the ionic structure of silicate ions on osteoblast-like cell properties.

**Methods:** BG and SiV powders were prepared by using melt-quenching and carbonation methods, respectively. Aminopropyltriethoxysilane was used as a siloxane source of SiV. MC3T3-E1 and SaOS-2 cells were cultured in media containing dissolved BG or SiV ions (10–50 ppm of Si). Cell proliferation (metabolic activity), differentiation (alkaline phosphatase activity) and mineralisation (Ca deposition) were examined.

**Results:** $^{29}$Si NMR spectra demonstrated that $Q^{0,1}$ species and $T^{0–3}$ species were released from BG and SiV, respectively. Proliferation and mineralisation of the two types of cells were influenced by silicate ions released from BG and SiV in a concentration-dependent manner. In particular, there were significant differences ($P < 0.05$) in the degree of proliferation and Ca deposition levels in SaOS-2 cells treated with dissolved BG and SiV ions. Furthermore, Ca deposition in SaOS-2 cells was influenced by both the presence of silicate ions and the duration of exposure of cells to them.

**Conclusions:** The structure of silicate ions influenced the proliferation and mineralisation of SaOS-2 cells incubated for different time periods in culture media containing different Si concentrations. Understanding the effect of Si on bone cell behaviour will enable a design-led approach to further BG optimisation.

**Keywords**

Silicate ions, 45S5-type bioactive glass, Vaterite, Osteoblast-like cells

## Introduction

Silicate-based bioactive glasses, such as 45S5-type bioactive glass (BG), have been clinically used for bone regeneration [1,2]. Silicate ion species in such glasses have been regarded to be one of the important factors contributing to glass bioactivity, possibly because silicate ions play a role as nucleation sites for hydroxyapatite (the inorganic phase of natural bone) [3,4] and as stimulators of osteogenic cell properties, such as proliferation, differentiation and mineralisation [5,6]. In addition, silicate ions were recently found to enhance angiogenesis and to inhibit osteoclastogenesis [7,8].

As has been previously reviewed [9], silicate-based bioactive glass ions have also been shown to regulate gene expression during repair of both hard and soft tissues. Although the mechanisms underlying the regulation of gene expression by bioactive glasses are not fully understood, several possible pathways have been suggested. Zhou *et al*. (2016) reported uptake of orthosilicic acid, $Si(OH)_4$, by human mesenchymal stem cells (hMSCs), which reduced NF-κB and Runt-related transcription factor 2 (RUNX2) activity via the up-regulation of interfering RNA (microRNA-146a) [10]. RUNX2 is the primary transcription factor necessary for osteoblast precursor differentiation. Furthermore, Han *et al*. (2013) reported that silicate ions stimulated sonic hedgehog homolog (SHH)-signalling pathway of bone marrow stromal cells [11]. SHH is one of the ligands of the hedgehog-Gil signalling pathway, which plays an important role in cell proliferation and differentiation. They, however, also mentioned that ion channels on the plasma membrane may be another possible target of stimulation by silicate ions. Interference with ion channels may also trigger events leading to changes in cellular functions and ion intake. We hypothesised that ionic structures of silicate ions are important factors influencing such stimulatory pathways. Silica phase in BG consists of 67.2% $Q^2$, 22.3% $Q^3$ and 10.1% $Q^1$ species [12]. $Q^n$ corresponds to silicon atom bonding to *n* other silicon atoms via bridging oxygen bonds ($Si(OSi)_n(OH)_{4-n}$). Therefore, BG is likely to release silicate ions with $Q^n$ species.

Siloxane-doped $CaCO_3$ with vaterite crystalline phase (SiV) has been developed for bone regeneration in our previous work [13]. SiV has spherical particles of 1–2 μm in diameter. Vaterite exhibits higher solubility in aqueous solutions than other $CaCO_3$ polymorphs, such as calcite and aragonite. Siloxane in SiV possesses hydrophilic properties because it is derived from aminopropyltriethoxysilane (APTES). Thus, SiV has the ability to release Ca and Si in aqueous solutions. Composite materials consisting of SiV and biodegradable polymers, such as poly(L-lactic acid) (PLLA) and poly(lactic-*co*-glycolic acid), were developed as fibre mats and cotton wool-like structures by electrospinning [14-17]. Proliferation and bone-like properties of mouse osteoblast-like cells (MC3T3-E1 cells) were enhanced to a larger extent on SiV-PLLA composites (releasing both Si and Ca) than on vaterite-PLLA composites (releasing only Ca) [14]. Products of SiV dissolution were also found to enhance the differentiation of MC3T3-E1 cells [17]. Mineralised tissue formation in bone defects modelled in rabbit calvarias was also up-regulated by implanting SiV-PLLA electrospun fibre mats [15]. Silicate ions released from SiV, therefore, appear to have an ability to stimulate osteoblast functions, like the ions released from BG, whereas their ionic structures

are likely to be different. SiV probably releases silicate ions that include aminopropyl groups. It is, however, unclear whether silicate ions with different ionic structures affect osteogenic cells functions in distinct ways and whether this influence is concentration-dependent. The aim of the present work was, therefore, to compare the influence of silicate ions released from BG and SiV on osteoblast-like cell functions. Whereas others have investigated the role of BG ions on osteoblast behaviour [5–11], the differences between Si species has not been studied. This study demonstrates for the first time that Si ion species influence ion uptake and cellular properties of osteoblast-like cells, depending on the stage of osteoblast differentiation and duration of exposure.

## Materials and Methods

### Synthesis of BG and SiV

BG containing 46.1 $SiO_2$, 26.9 CaO, 24.4 $Na_2O$ and 2.5 $P_2O_5$ mol% was prepared using $SiO_2$ (Kishida Chemical Co., Ltd, Japan), $CaHPO_4/2{\cdot}H_2O$ (Wako Pure Chemical Industries, Ltd., Japan), $CaCO_3$ (Wako Pure Chemical Industries, Ltd., Japan) and $Na_2CO_3$ (Kishida Chemical Co., Ltd, Japan) by using melt-quenching method. All chemicals were weighed in a Pt crucible and heated to 1400 °C for 1 h. The melted solution was rapidly cooled by pouring onto a stainless steel plate, followed by pressing with an iron press. The resulting glass plates were pulverised with an alumina mortar and then passed through a sieve. The glass powders with a particle size less than 53 μm were used for preparing Si-containing culture media.

SiV containing 2.6 wt% of Si was synthesised as reported previously [13,14]. Briefly, 150 g of $Ca(OH)_2$ (Kishida Chemical Co., Ltd, Japan), 60 mL of aminopropyltriethoxysilane (APTES, Sigma, USA) and 2 L of methanol were mixed by bubbling $CO_2$ gas for 75 min at a rate of 2 L/min at room temperature, which resulted in the formation of a slurry. SiV powders with a particle size of approximately 1 μm were obtained by drying the resulting slurry at 110 °C.

### Preparation and characterisation of culture media containing BG and SiV extracts

BG and SiV powders were sterilised by heating at 180 °C for 90 min. Five hundred milligrams of each powder was soaked in 15 mL of regular culture medium, namely α-MEM (Wako Pure Chemical Industries, Ltd., Japan) or McCoy's 5A (Gibco, Thermo Fisher Scientific Inc., US), both supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin solution (Wako Pure Chemical Industries, Ltd., Japan). Powder-containing media were incubated at 37 °C for 24 h in a $CO_2$ incubator in the atmosphere of 95% air and 5% $CO_2$. The resulting supernatant solution was filtrated through a 0.2-μm polytetrafluoroethylene (PTFE) filter, and its ionic concentration was determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, ICPS-7000, Shimadzu, Japan). Media containing 10, 20, 30, 40 and 50 ppm of Si were prepared by diluting the obtained filtrate with the regular medium. The

prepared Si-containing media are referred to as *x*-SiV or *x*-BG (where *x* indicates Si concentration, 10–50 ppm). Regular (non-supplemented) culture medium was used as a control sample.

For differentiation and mineralisation tests, osteogenic differentiation medium was prepared by supplementing regular culture medium with 1 μM dexamethasone, 284 mM L(+)-ascorbic acid and 10 mM disodium glycerophosphate 5.5 hydrate. All chemicals were purchased from Wako Pure Chemical Industries, Ltd., Japan. Si-containing media were prepared as reported above, using the osteogenic differentiation medium in lieu of the regular culture medium. These media were then used in measurements of alkaline phosphatase (ALP) activity and amounts of Ca deposition in cells. The control samples in the differentiation and mineralisation tests were exposed to osteogenic differentiation medium (without Si) unless otherwise specified.

Si concentration in the obtained filtrate and prepared Si-containing media was measured by ICP-AES. Reported values represent the mean of measurements from three samples. pH of the Si-containing medium in the $CO_2$ incubator with 5% of $CO_2$ was measured with a pH meter (TPX-999i, Toko Chemical Laboratories Co. Ltd., Japan).

**Characterisation of silicate ions released from BG and SiV**

Twenty grams of SiV or BG were immersed in 20 mL of ultrapure water and incubated at 37 °C for 24 h, followed by filtration through a 0.2-μm PTFE filter. $^{29}$Si NMR spectra of silicate ions released from SiV or BG were obtained with a Bruker Avance 400 Plus NanoBay spectrometer (Bruker, US). Ten grams of SiV or 15 g of BG powders were immersed in 10 mL of regular culture medium and kept in a $CO_2$ incubator for 24 h, followed by filtration through a 0.2-μm PTFE filter. The obtained Si-containing media were mixed with KBr, then dried and pressed into pellets, before their analysis with Fourier transform infrared spectroscopy (FTIR; FTIR-4000, JASCO, Japan).

**Effect of Si-containing media on cell cultures**

Mouse and human osteoblast-like cells, represented by MC3T3-E1 and SaOS-3 cells, respectively, were cultured in Si-containing media based on α-MEM and McCoy's 5A, correspondingly. For the proliferation, differentiation and mineralisation tests, MC3T3-E1 and SaOS-3 cells were cultured at the densities of 30,000 cells/well and 100,000 cells/well, respectively. Different starting cell densities were used due to dissimilar cell proliferation profiles observed in our preliminary tests.

To measure cell proliferation, cells were seeded in a regular medium prior to the addition of the treatment (Si-containing) medium. The number of live cells at each time point (1, 3, 5, 7 and 14 days) was measured with a metabolic activity assay using a Cell Counting Kit-8 (Dojindo, Japan) following the procedure recommended by the manufacturer. MC3T3-E1 and SaOS-2 cells were cultured for 7 and 14 days, respectively, because culture wells got confluent with respective cells at these time points in our experiments. The cells were incubated in the reaction solution of the kit for 2 h in a $CO_2$ incubator.

Absorbance from the medium at a wavelength of 450 nm was measured with a microplate reader (SUNRISE Remote, Tecan Group Ltd., Switzerland).

The differentiation and mineralisation tests in Si-containing media were carried out using SaOS-2 cells. The cells were seeded and proliferated using regular medium without osteogenic supplement for 8 days and then cultured using Si-containing medium with osteogenic supplement for another 16 days. ALP activity of the cells was measured with *p*-nitrophenyl phosphate (*p*NPP) tablets (Sigma, USA) dissolved in 20 mL of ultrapure water. At each time point, the cells were washed with PBS twice and then reacted with the prepared *p*NPP solution for 6 min in a $CO_2$ incubator. The *p*NPP solution in the well was sampled and mixed with the same amount of 0.2 M NaOH (stop solution) as that of the sampled *p*NPP solution, following measurements of absorbance at 405 nm with the microplate reader. To calculate live cell numbers, the Cell Counting Kit-8 assay was conducted, and a calibration curve was obtained by measuring the absorbance at 450 nm from media containing SaOS-2 cells at different densities.

Cell cultures for mineralisation tests were grown by using nearly the same method as that for differentiation tests, except for the time point when the culture medium was changed from the regular medium to Si-containing medium with an osteogenic supplement. To estimate the influence of the duration of cell exposure to Si-containing media on their mineralisation ability, culture tests were carried out with four different methods (Table 2). Cells were cultured using Si-containing media from Day 1 to Day 30 (method 1, all culture stages), from Day 1 to Day 7 (method 2, proliferation stage), from Day 8 to Day 39 (method 3, differentiation stage) and from Day 15 to Day 39 (method 4, mineralisation stage). The culture periods for each method were decided based on the results of preliminary tests undertaken in cells grown in control medium. Si-containing media based on the regular medium and osteogenic differentiation medium were used for methods 1, 2 and methods 3, 4, respectively. Control media with osteogenic supplement were used for other days, when Si was provided. For example, in the case of method 2, Si-containing medium without osteogenic supplement was used from Day 1 to Day 7, and then control medium with the supplement was used from Day 8 to Day 39. At the end of the culturing period, the cells were treated with a 2 M HCl solution for 1 h at 4 °C to dissolve intracellular Ca deposits. The amount of Ca in the well was measured using Calcium E test (Wako Pure Chemical Industries, Ltd., Japan), following the procedure recommended by the manufacturer. Briefly, the solution in the well was removed after the treatment with HCl solution, mixed with a buffer solution and then mixed with a methyl xylenol blue solution. The absorbance of the resulting solution at 620 nm was measured with a microplate reader. All samples in cell culture tests were run in triplicate for statistical analysis.

**Measurement of Si amount in the cells and media after culturing**

SaOS-2 cells were seeded at a density of 100,000 cells/mL in an 8000-$mm^2$ flask (1,500,000 cells/flask) using regular medium. The number of seeded cells was modified, as a higher concentration of cells was needed to be within ICP-AES detection limits. The cells were then cultured for one day and then

for another five days using 50-BG and 50-SiV. The media before and after these five culture days were filtrated through a 0.2-μm PTFE filter, and their Si concentrations were subsequently measured by ICP-AES. To measure the amount of Si in cells after culturing, cells cultured in 50-BG and 50-SiV were harvested and centrifuged. The supernatant solution was removed, resulting in the formation of cell pellets. These cell pellets were dissolved in a 1 M $HNO_3$ solution and then diluted with ultrapure water. Si concentration of the resulting solution was measured by ICP-AES. To measure Si amount in cells before culturing in Si-containing media, cell pellets were prepared from suspensions of cells not treated with Si-containing media. Then, the above-mentioned steps were repeated, yielding a solution containing cell lysate. Measured Si amounts in cells after culturing in Si-containing media were normalised by the amounts of Si determined in cells before culturing. All samples were run in triplicate for statistical analysis.

**Statistical Analysis**

Data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using the Student's *t*-test. Differences were considered to be significant if $P < 0.05$.

**Results**

**Characterisation of Si-containing media**

Table 1 shows ion concentrations in each medium after exposure to SiV or BG powders for 24 h. Si concentrations in SiV and BG samples were 323.9 and 146.3 ppm for α-MEM medium and 405.3 and 151.3 ppm for McCoy's 5A medium, respectively. SiV released more silicate ions than did BG. As for P, its concentration in all solutions after addition of SiV and BG was lower than that in control samples. In contrast, Ca concentration in α-MEM after exposure to BG was higher than that in control, although Ca concentrations in other solutions after exposure were lower. Fig. 1 shows concentrations of Ca, P and Na in media containing 10, 30 and 50 ppm Si and in control media. There were no significant differences in concentrations of these elements between Si-containing and control media. This implies that the introduction of Si-containing medium affects only Si concentration.

Fig. 2 (a,b) shows $^{29}$Si NMR spectra of silicate ions released from BG and SiV. The spectrum of BG medium indicates the presence of $Q^0$ (-70 $cm^{-1}$) and $Q^1$ (-80 $cm^{-1}$) species, whereas the spectrum of SiV medium shows the presence of $T^0$ (-40 $cm^{-1}$), $T^1$ (-50 $cm^{-1}$), $T^2$ (-60 $cm^{-1}$) and $T^3$ (-70 $cm^{-1}$) species. This implies that BG and SiV released silicate ions of different ionic structures. $Q^n$ corresponds to Si atom bonding to *n* other Si atoms via bridging oxygen bonds (Si(OSi)*n*(OH)4-*n*). $T^n$ sites indicate Si atom bonding to one carbon atom (Si-C) and to *n* bridging oxygen bonds (CSi(OSi)*n*(OH)3-*n*). All of the silicate ions released from SiV included aminopropyl groups, because APTES was used as a siloxane source in SiV.

Fig. 2(c,d) shows FTIR spectra of Si-containing media. Although shoulder peaks corresponding to Si-OH stretching (3300 $cm^{-1}$, 940 $cm^{-1}$) and a peak assigned to Si-O-Si *linear* stretching (1040 $cm^{-1}$) were observed in the spectra of the BG sample as well as in spectra of SiV samples in both α-MEM and McCoy's

5A media, an additional band corresponding to Si-O-Si *cyclic* stretching (1130 $cm^{-1}$) was observed for SiV samples.

Changes in pH of the media containing 30 and 50 ppm of Si as well as those of control media in a $CO_2$ incubator are shown in Fig. 3. At the early stage of the incubation, media exposed to BG series exhibited higher pH values than those detected in control and SiV series experiments in both α-MEM and McCoy's 5A media. In particular, pH of 50-BG medium was around pH 9 at the early stage of the incubation. In contrast, SiV series media possessed almost the same pH values as control media. Over the 12 h following the start of incubation, pH values of all tested media became lower. Whereas pH values of the SiV series media reached the same level as those of control media, pH values of the BG series media were systematically higher, except for pH of the 30-BG-containing α-MEM medium.

**Cell responses to Si-containing media**

Metabolic activity of live MC3T3-E1 cells and SaOS-2 cells after culturing in Si-containing media is shown in Fig. 4. The activity of MC3T3-E1 cells was dependent on BG and SiV concentrations in the media at Day 7: activity levels of 20-BG and 20-SiV samples were higher than activity in other media, including control one. However, these activity levels became lower in media with higher Si concentration. Notably, activity levels at earlier time points, Day 1 to Day 5, showed no obvious dose-dependence, and their values in the majority of Si-containing media were lower than those of control samples. In the case of SaOS-2 cells, dose-dependence of metabolic activity levels, similar to that detected in MC3T3-E1 cell culture tests, was observed only in BG series samples. SiV series samples of SaOS-2 cells displayed a dose-dependent response different from that of MC3T3-E1 cell samples. Interestingly, when the cells were cultured for longer time, *i.e.*, 14 days, the differences in activity levels between BG and SiV series became more obvious: activity of BG series samples was similar to or lower than that of the control, whereas activity levels of SiV series samples were significantly higher. Different patterns of cell proliferation upon exposure to silicate ions were therefore observed, depending on the type of cells (MC3T3-E1 or SaOS-2 cells) and source of silicate ions (BG or SiV).

Fig. 5 shows the results of ALP activity tests in SaOS-2 cells. ALP activity levels in all samples peaked at Days 15–18. The same pattern was observed in control samples. Although some of the samples showed higher ALP activity levels than those of control samples after this peak period, no significant up-regulation of ALP activity by Si-containing media was observed before and at the peak point.

Fig. 6 shows the results of the mineralisation test (method 1) in SaOS-2 cells. Cells were exposed to silicate ions from their proliferation stage to their mineralisation stage. In contrast to the observations in previous studies, silicate ions released from both sample series did not up-regulate the extent of mineralisation. In particular, in SiV series samples, dose-dependent down-regulation of mineralisation was observed. These results led us to formulate the following hypothesis: mineralisation is positively influenced particularly after longer exposures of cells to Si-containing medium. To confirm this hypothesis, further

experiments were performed by using methods 2–4.

Results of mineralisation tests in SaOS-2 cells exposed to BG and SiV throughout different cell development stages (proliferation, differentiation and mineralisation) are illustrated in Fig. 7. In the case of method 2, the amounts of deposited Ca in 10-BG, 30-BG, 10-SiV and 30-SiV samples were significantly higher than those in control samples. In contrast, Ca depositions in 50-BG and 50-SiV samples were similar to those in control cells. The outcomes of experiments carried out by using methods 3 and 4 exhibited a different pattern: the amounts of Ca depositions in both BG and SiV series samples tended were positively related to Si concentration.

Fig. 8 shows Si concentrations in 50-BG and 50-SiV culture media before and after five days of culturing as well as Si concentrations in SaOS-2 cells after five days of culturing in the presence of 50-BG and 50-SiV. As shown in Fig. 8(a), Si concentration in the 50-BG sample slightly decreased after culturing, whereas in the 50-SiV sample, Si concentration was not significantly different before and after culturing. According to Fig. 8(b), Si concentration in 50-BG samples was significantly higher than that in 50-SiV samples.

**Discussion**

Our previous work revealed that composite materials containing SiV up-regulated proliferation and differentiation of mouse osteoblast-like cells *in vitro* and mineralisation of surrounding tissues *in vivo* [14,15]. Therefore, silicate ions released from SiV may play an important role in this up-regulation. In addition, it is well-established that BG and its composite materials enhance osteogenesis, and silicate ions released from BG have been reported to be an important factor in this phenomenon. It remains, however, unclear if the two types of silicate ions released from BG and SiV exert the same effect on bone cells. Several studies have compared cellular responses to different types of silica-based bioactive glasses and ceramics, such as a sol-gel glass with 70 mol% $SiO_2$ and 30 mol% CaO or calcium silicate cement [18,19]. Silicate ions released from the materials used were not, however, analysed and were deemed to be similar based on the assumption that they were prepared by similar methods and using the same chemicals.

In the present study, we exposed osteoblast-like cells in culture to the extracts of BG and SiV, because these two materials were assumed to release silicate ions with different ionic structures based on their chemical composition. In fact, as shown in Fig. 2(a, b), the ions indeed had different ionic structures: BG and SiV released $Q^n$ and $T^n$ species, respectively. The two types of silicate ions possessed different chemical reactivity in culture media: the ions released from BG and SiV formed linear-shaped and linear/cyclic-shaped silica, respectively (Fig. 2(c, d)). In addition, pH values of Si-containing media were different in BG and SiV series samples. pH values of BG series samples were higher than those of SiV series and control samples. In particular, high pH values in 50-BG samples persisted even after a 12-h incubation (Fig. 3). Shen *et al*. (2012) revealed that the proliferation of MC3T3-E1 cells was inhibited in

culture media at pH levels more basic than pH 9.0 at the early stage of the culture test, *i.e.*, Day 1 to Day 3 [20]. Therefore, in the case of 50-BG samples, cell responses must have been influenced not only by the presence of silicate ions but also by high pH, especially during early responses, within the first three days in culture.

Proliferation of MC3T3-E1 and SaOS-2 cells after seven days of culturing was up-regulated by 20-BG but became significantly lower at higher Si concentrations (Fig. 4). MC3T3-E1 cells cultured in the presence of SiV showed a similar tendency. However, the proliferation of SaOS-2 cells cultured in the presence of SiV displayed a different tendency: media containing higher amounts of silicate ions, 30–50 ppm, up-regulated the extent of the proliferation. In particular, when SaOS-2 cells were cultured for 14 days, which was the time point of cell confluence in our experiments, the cells were highly stimulated to proliferate at high Si concentrations in the experiments with SiV series. In contrast, in the BG series, their proliferation was progressively hindered by higher Si concentrations. One of the possible reasons for this inhibition is that cells were stimulated by the silicate ions released from BG to differentiate and calcify, but not to proliferate. However, as shown in Figs. 5 and 6, no up-regulation by silicate ions was observed during the differentiation and mineralisation stages. Therefore, it could be concluded that a large amount of silicate ions released from BG hindered cell proliferation. There were significant differences in the proliferation of SaOS-2 cells in the presence of BG and SiV, as well as in the proliferation tendency with respect to Si concentration between MC3T3-E1 and SaOS-2 cells in the SiV series. This indicates that the influence of silicate ions on cellular functions depends on the ionic structures of silicate ions and the type of cells.

de la Concepción Matesanz *et al*. (2012) carried out proliferation tests exposing three different types of cells, SaOS-2, MC3T3-E1 and murine L929 fibroblasts, to silicon-substituted hydroxyapatite (Si-HA) and HA. The number of SaOS-2 cells cultured in the presence of Si-HA powder for four days was significantly larger than in the presence of HA powder, whereas experiments with MC3T3-E1 cell cultures led to the opposite result. Furthermore, neither Si-HA nor HA affected the numbers of L929 cells [21]. Because in that study no data on Si concentration and pH value of the media were reported, it is unclear if Si released from the powders solely contributed to the modulation of proliferation in SaOS-2 and MC3T3-E1 cells. Nonetheless, those results indicate that cellular responses to extracts of bioactive glasses and ceramics are likely to depend on the type of cells.

ALP activity is one of the functional markers of osteoblastic differentiation in the early stage of cell development. ALP activity levels in SaOS-2 cells were similarly unaffected by BG and SiV series (Fig. 5). Such absence of ALP activity up-regulation by the silicate ions contradicts many reports about Si effects on osteogenic differentiation. However, some studies also failed to detect up-regulation of ALP activity by silicate ions, especially in the case of culture tests that employed osteogenic supplements, dexamethasone, L(+)-ascorbic acid and disodium glycerophosphate 5.5 hydrate. For example, primary osteoblasts derived from human foetal long bone cultured with osteogenic supplement exhibited similar ALP activity levels in cultures with and without BG extract, whereas ALP activity in cells cultured without a supplement (normal

culture medium) was higher in the medium containing the extract than in the medium without it [22]. In our previous studies, MC3T3-E1 cells were stimulated to differentiate when cultured on composite materials releasing silicate ions without osteogenic supplement, whereas they showed no differentiation on materials that did not release silicate ions [14]. ALP activity levels of hMSCs were significantly elevated by culturing in the absence of osteogenic supplement on composite materials that released silicate ions. However, there were no significant differences in ALP activity levels between materials with and without silicate ion releasing ability, when osteogenic supplement was present [23]. Thus, the ALP activity level of SaOS-2 cells in the present study could have been potentially increased by Si-containing media if osteogenic supplement was absent. Further experiments without osteogenic supplements will be needed to confirm this assumption.

The majority of studies that examined the effects of silicate ions on cultured cells demonstrated that modulation of cellular functions, such as proliferation, differentiation or mineralisation, was dose-dependent. However, some studies revealed that higher concentrations of ions did not systematically stimulate such cellular functions to a greater degree. According to the authors of those latter studies, two factors possibly explained such a lack of dose-dependent effects on cellular responses: i) differences in the period of exposure of cells to silicate ions and ii) the type of cells used for the experiments. For example, Gough *et al*. (2004) reported that 58S type bioactive glass, which is composed of 60 mol% $SiO_2$, 36 mol% CaO and 4 mol% $P_2O_5$, enhanced the mineralisation (bone nodule formation) of human primary osteoblasts derived from femoral heads. The enhancement was observed for cells cultured on glass surfaces for 10 days and also, in the culture media containing glass extracts for 24 h [24]. Christodoulou *et al*. (2005) revealed that the extract of 58S type bioactive glass had no significant effect on the expression of osteoblastic marker genes in primary osteoblasts derived from human foetal long bone after culturing for 7–14 days [25]. However, they also reported that glass extracts affected gene expression, including that of osteoblastic markers, in the same cells when they were cultured with the extract for 24 h [26]. The authors suggested that the variation in the time of cell exposure to the extract was a possible reason for the differences in gene expression observed between their two studies. In the case of 45S5 type bioactive glass (BG), Xynos *et al*. (2000, 2001) reported that the expression of osteoblastic markers in human primary osteoblasts derived from femoral heads was enhanced when they were cultured for 48 h with BG extracts and that the mineralisation (bone nodule formation) of the cells was also enhanced after four days of culturing with the same extract [5,27]. Tsigkou *et al*. (2009) revealed that gene expression in primary osteoblasts derived from the human foetal long bone was also influenced by the presence of extracts and showed dependence on Si concentration in the extract [22].

Mineralisation (deposition of Ca) of SaOS-2 cells in the present study was dependent on both the period of exposure of the cells to Si-containing media and ionic structures of the silicate ions (Figs. 6 and 7). With respect to the exposure duration, although a similar period was set for methods 1 and 3 (approximately 30 days), the amounts of deposited Ca were significantly different between the two

conditions. The results obtained by methods 3 and 4 exhibited a similar tendency even though the exposure period was different in these two conditions: 30 and 15 days, respectively. Interestingly, the largest amounts of Ca deposition were observed when the cells were cultured in 30-BG and 30-SiV by using method 2. This indicates that the shortest exposure term, seven days, in our experiment was the most optimal for observing up-regulation. Thus, we expect that the mineralisation of cells depends not only on the exposure period but also on the cell stage—namely proliferation, differentiation or mineralisation—during the exposure to Si-containing media. Regarding the influence of the ionic structure of silicate ions on mineralisation, significant differences in the levels of Ca deposition between BG and SiV series were observed only in experiments conducted according to method 1. Although the origins of different mineralisation patterns observed between the two series are still unclear, and no theory can be put forward to explain the discrepancy between the outcomes of experiments according to method 1 and results of other methods, the present study clearly demonstrates that the modulation of cellular functions depends on the ionic structure of silicate ions, which are used to treat the cells.

Some studies suggested that silicate ions enter the cells and modulate cellular functions by affecting gene expression [10,11]. Therefore, we hypothesise that ionic structure defines the efficiency of uptake of silicate ions by the cell. Another hypothesis that can be put forward is that the amount of silicate ions inside cells was different in experiments with BG and SiV series. In fact, as shown in Fig. 8(b), intracellular Si concentration in BG-treated cells was significantly higher than that in SiV samples. Si concentration in 50-BG medium after 5 days of culturing was lower than before culturing, whereas there was no significant difference between Si concentrations before and after culturing in SiV medium (Fig. 8(a)). These results indicate that Si ions released from BG entered the cells, whereas ions released from SiV did not. Entry pathways of Si ions were probably different for the two materials and this might have contributed to differences in cellular responses to these materials observed in the present study. We, however, think that further research on this topic is necessary.

**Conclusion**

To investigate the influence of the ionic structure of silicate ions on osteoblast-like cell properties, MC3T3-E1 and SaOS-2 cells were cultured in media containing dissolved 45S5 bioactive glass and siloxane-doped vaterite ions, with Si concentration ranging from 10 to 50 ppm. Silicate ions released from BG and SiV formed Q and T species, respectively. Proliferation and mineralisation of both types of cells were influenced by the silicate ions released from BG and SiV in a concentration-dependent manner. In addition, these cellular properties were differentially affected by BG and SiV. Mineralisation of SaOS-2 cells was influenced by both the duration of exposure to silicate ions and the cell development stage, during which the cells were exposed to silicate ions. The variation of ionic structure of silicate ions was found to influence osteoblast-like cell properties.

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**Conflict of Interest**

The authors declare no conflict of interest associated with this manuscript.

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**Figure captions**

Fig. 1. Concentrations of Ca, P and Na in control medium and media containing 10, 30 or 50 ppm Si. (a, c, e) α-MEM-based media and (b, d, f) McCoy's 5A-based media. Data are presented as the mean ± standard deviation, $n$ = 3. Control indicates non-supplemented media. No significant differences ($P < 0.05$) in the concentrations of Ca, P and Na were detected between control medium and any of the Si-containing media.

Fig. 2. $^{29}$Si NMR spectra of silicate ions released from (a) BG and (b) SiV. Fourier transform infrared spectroscopy spectra of Si-containing and control media. Results obtained in (c) α-MEM-based media and (d) McCoy's 5A -based media are illustrated. Control indicates non-supplemented media. BG and SiV released $Q^n$ and $T^n$ species, respectively. The released ions formed linear-shaped silica in BG-treated samples and linear/cyclic-shaped silica in SiV-treated samples. These results indicate that ionic structures of Si released by BG and SiV were different.

Fig. 3. Changes in pH of Si-containing and control media in a $CO_2$ incubator. Results obtained in (a,c) α-MEM-based media and (b,d) McCoy's 5A-based media are illustrated ($n$ = 1). Control indicates non-supplemented media. pH values in samples of BG series were higher than those in samples of SiV series or in control samples.

Fig. 4. Metabolic activity of live cells after culturing in Si-containing and control media. Results obtained in experiments with (a) BG and (b) SiV series in MC3T3-E1 cells and with (c) BG and (d) SiV series in SaOS-2 cells are illustrated. Control indicates non-supplemented media. *: $P < 0.05$ vs. control. Data are presented as the mean ± standard deviation, $n$ = 3. The numbers of both types of cells were in a dose-dependent relationship with Si concentration. Different patterns of dose-dependency were observed in experiments with MC3T3-E1 and SaOS-2 cells.

Fig. 5. ALP activity of SaOS-2 cells after culturing in Si-containing and control media. Results obtained in experiments with (a) BG and (b) SiV series are illustrated. Control indicates non-supplemented media. *: $P < 0.05$ vs. control. Data are presented as the mean ± standard deviation, $n$ = 3. Changes in ALP activity values in Si-containing media showed time courses similar to that in control samples, peaking at Day 18. No up-regulation of ALP activity by Si-containing media was observed.

Fig. 6. Amounts of Ca deposition in SaOS-2 cells after culturing in Si-containing and control media (method 1). Results obtained in experiments with (a) BG and (b) SiV series are illustrated. Control indicates non-supplemented media. *: $P < 0.05$ vs. control. Data are presented as the mean ± standard deviation, $n$ = 3.

No significant differences in amounts of deposited Ca were observed between BG-treated and control samples. Ca deposition amounts exhibited an inverse dose-dependent relationship to Si concentration in experiments with SiV series.

Fig. 7. Amounts of Ca deposition in SaOS-2 cells after culturing in Si-containing and control media. Results obtained in experiments using (a,b) method 2, (c, d) method 3 and (e, f) method 4 are illustrated. Control indicates non-supplemented media. *: $P < 0.05$ vs. control. Data are presented as the mean ± standard deviation, $n = 3$. Mineralisation levels were dependent on both the duration of exposure of cells to Si-containing media and ionic structures of silicate ions. Data obtained by methods 3 and 4 had similar relationships even though the exposure periods were different in these two conditions. The largest amounts of Ca deposition were observed for 30-BG and 30-SiV cultured by using method 2.

Fig. 8. Si concentration in (a) supplemented McCoy's 5A medium before and after five days of culturing SaOS-2 cells and (b) inside SaOS-2 cells after five days of culturing (following medium removal). *: $P < 0.05$ vs. control. Data are presented as the mean ± standard deviation, $n = 3$. Si concentration inside the cells was significantly higher after BG treatment than after SiV treatment. Silicate ions released from BG may have been taken up by the cells.