In vitro osteogenesis by intracellular uptake of strontium containing bioactive glass nanoparticles

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Abstract

Monodispersed strontium containing bioactive glass nanoparticles (Sr-BGNPs) with two compositions were synthesised, through a modified sol-gel Stöber process, wherein silica nanoparticles (SiO2-NPs) were formed prior to incorporation of calcium and strontium, with diameters of 90 ± 10 nm. The osteogenic response of a murine preosteoblast cell line, MC3T3-E1, was investigated in vitro for a nanoparticle concentration of 250 µg/mL with compositions of 87 mol% SiO2, 7 mol% CaO, 6 mol% SrO and 83 mol% SiO2, 3 mol% CaO, 14 mol% SrO. Dissolution studies in minimum essential media (α-MEM) at pH 7.4 and artificial lysosomal fluid (ALF) at pH 4.5 showed that the particles dissolved and that Sr2+ ions were released from Sr-BGNPs in both environments. Both particle compositions and their ionic dissolution products enhanced the alkaline phosphatase (ALP) activity of the cells and calcium deposition. Immunohistochemistry (IHC) staining of Col1a1, osteocalcin (OSC) and osteopontin (OSP) showed that these proteins were expressed in the MC3T3-E1 cells following three weeks of culture. In the basal condition, the late osteogenic differentiation markers, OSC and OSP, were more overtly expressed by cells cultured with Sr-BGNPs with 14 mol% SrO and their ionic release products than in the control condition. Col1a1 expression was only slightly enhanced in the basal condition, but was enhanced further by the osteogenic supplements. These data demonstrate that Sr-BGNPs accelerate mineralisation without osteogenic supplements. Sr-BGNPs were internalised into MC3T3-E1 cells by endocytosis and stimulated osteogenic differentiation of the pre-osteoblast cell line. Sr-BGNPs are likely to be beneficial for bone regeneration and the observed osteogenic effects of these particles can be attributed to their ionic release products.

Keywords: Bioactive glass nanoparticles; osteogenic; strontium; intracellular uptake; endocytosis
1. Introduction

Bioactive glass-based products are used for several orthopaedic and dental applications [1] because they form strong bonds with host bone [1-4] and the ions released from these products stimulate osteogenic gene expression, leading to rapid bone regeneration [5-9]. Recently, the development of new formulations by introduction of other therapeutic metallic cations, such as copper, zinc, cobalt into the silica network, and using co-networks of borate, have widened the potential therapeutic applications to angiogenesis, wound healing, antimicrobial and anti-cancer [9-12]. Strontium (Sr) has been clinically used as pharmaceutical agent for osteoporosis treatment [13, 14] because of its ability to activate osteoblasts and inhibit osteoclast activities [15-18]. Strontium ions (Sr\(^{2+}\)) have a similar charge and ionic radius to calcium ions (Ca\(^{2+}\)) [15]. SrO can therefore replace some CaO in the bioactive glass structure [15, 19-21]. Sr is slightly larger and heavier compared with Ca, therefore the substitution decreases the connectivity of the silica network by further disrupting the network [11, 22].

Bioactive glass nanoparticles (BGNPs) are promising injectable biomaterials for bone regeneration applications [12, 23, 24]. The potential benefits of nanoparticles (NPs) over microparticles are their high surface to volume ratios, which increases their dissolution rate [17, 25, 26], and their small size allows them to be internalised into cells to deliver their therapeutic ions intracellularly [26, 27]. The benefit of the glass is that it can provide a sustained delivery of the therapeutic cations from their amorphous structure during dissolution [6, 16, 26, 28].

The size and shape of NPs is known to affect the endocytosis pathway [29-33]. NPs internalise and localise within the cells through different uptake pathways, including phagocytosis, macropinocytosis, clathrin-independent endocytosis, and clathrin-dependent endocytosis [34, 35]. To explore the potential utility of Sr-BGNPs as therapeutic cation carriers, it is critical to understand the different mechanisms of Sr-BGNPs uptake by cells. SiO\(_2\)-CaO NPs with diameters of 215 ± 20 nm were previously found to be internalised into human bone marrow and adipose derived stem cells and internalization by the stem cells was not affected following inhibition of clathrin- or caveolin-mediated endocytosis [35].

A previous study [36] showed that cation incorporation into the silica network was not trivial, indicating that previous studies had overestimated the amount of calcium incorporated into nanoparticles, without checking composition. The most effective method to incorporate calcium ions into the NPs is by adding calcium nitrate, after the monodispersed SiO\(_2\)-NPs has already been formed [36]. This modified Stöber method was adapted to produce SiO\(_2\)-CaO-SrO NPs [37]. In each case, not all of the nominal Ca\(^{2+}\) and Sr\(^{2+}\) ions were incorporated into the silica network [36, 37]. There is an upper limit of network modifier incorporation into the dense SiO\(_2\)-NPs. A maximum of 10% mol CaO incorporation into the binary BGNPs (90% mol SiO\(_2\) and 10% mol CaO), using a nominal ratio of Si:Ca of 1:1.3, was possible using a modified Stöber process [36]. The upper limit of Sr\(^{2+}\) ion incorporation into the binary BGNPs was approximately 17 mol% (83% mol SiO\(_2\) and 17% mol SrO), using the nominal ratio of Si: Sr of 1:1.3 [37]. Substituting Sr\(^{2+}\) for Ca\(^{2+}\) ions on a molar basis had no effect on the size and morphology of the particles. The in vitro cell viability also showed that the Sr-BGNPs had low cytotoxicity (> 70% viability) for particle concentrations of up to 250 µg/mL and the ions
released from these particles significantly increased the viability of the MC3T3-E1 osteoblast-like cells at concentrations of 200 and 250 μg/mL [37]. Only cell viability was assessed.

Here, the aim was to investigate the effect of Sr-BGNPs on the osteogenic response of MC3T3-E1 cells in vitro using Sr-BGNPs, with diameters within the range of 80-100 nm, and the influence of mol% of SrO. The mechanism of incorporation of the nanoparticles into the cells was also investigated. To understand whether or not the therapeutic effect is dependent on intracellular release of therapeutic ions from the particles, the cells were exposed to both the particles and their dissolution products. Ion release from the Sr-BGNPs was also measured in three different conditions: phosphate-buffered saline (PBS), minimum essential media (α-MEM) at pH 7.4 and artificial lysosomal fluid (ALF) at pH 4.5.

2. Materials and methods

2.1 Sr-BGNPs synthesis

All reagents were from Sigma–Aldrich (Dorset, UK) unless stated otherwise. SiO₂-NPs and Sr-BGNPs, with diameters of ~90 nm, were prepared using the modified Stöber method described previously [36, 37]. A diameter of 90 nm was chosen because of previous work on size dependence on internalisation of Stöber-like particles, where particles of < 200 nm were internalised into cells [38, 39] and particles of 60 nm caused higher toxicity than 100 nm particles [40], which did not cause toxicity up to the concentration of 500 μg/mL [41].

For their synthesis, first, 32.92 mL of ethanol (99.5%), 4.11 mL of distilled water, and 0.48 mL of ammonium hydroxide were mixed in an ultrasonication bath for 10 min. Then, 2.50 mL of tetraethyl orthosilicate (TEOS) was gently added to the mixed solution and left in the ultrasonication bath at least 6 h to complete hydrolysis and polycondensation reactions [37]. Hydrolysis and condensation reactions of TEOS occurred simultaneously to form the silica network (Si-O-Si). SiO₂-NPs were centrifuged for collection and then were washed with ethanol (two times) and distilled water.

For Ca and Sr incorporation, calcium nitrate tetrahydrate (99%) and strontium nitrate tetrahydrate (99%) were added. Based on previous work [37], a nominal molar ratio of 1:1.3 ratio of Si:total cations was selected. Binary BGNPs (SiO₂-CaO) and ternary BGNPs (SiO₂-CaO-SrO) compositions were synthesised, with 25 mol% and 75 mol% calcium nitrate tetrahydrate being replaced with strontium nitrate tetrahydrate, leaving SiO₂-CaO-SrO compositions containing 6.2 mol% SrO (6%Sr-BGNPs) and 14.2 mol% SrO (14%Sr-BGNPs) respectively (measured by acid digestion, Section 2.2). The resulting primary particle suspension was then dried at 60°C overnight to remove excess water following with thermal treatment at 680°C for 3 h at a heating rate 3 °C/min in order to produce Sr-BGNPs. These particles were then washed with ethanol two times.

2.2 Acid digestion compositional analysis

Acid digestion compositional analysis was carried out to measure the composition of the BGNPs by the lithium metaborate fusion dissolution method. 50 mg of finely ground particles
was mixed carefully with 250 mg of anhydrous lithium metaborate (80% w/w) and lithium tetraborate (20% w/w) (Spectroflux 100B, Alfa Aesar, Lancashire, UK) in a clean and dry platinum crucible using a glass rod [42]. The mixture was fused in a furnace for 20 min at 1050 °C and later dropped to room temperature. The mixture was subsequently dissolved in 2 M nitric acid [43]. The elemental concentration in the solution was measured using inductively coupled plasma optical emission spectroscopy (ICP-OES, Thermo Scientific iCAP 6000 series).

2.3 Particle characterization

Particle size was investigated using Dynamic Light Scattering (DLS, Malvern instrument 2000) and Transmission Electron Microscopy (TEM, JEOL 2100 Plus microscope operated at 200 kV). To prepare samples, the dried particles were dissolved in ethanol and sonicated in the sonication bath for 15 min before conducting the DLS measurements. Particles were collected on 400 mesh copper transmission electron microscopy (TEM) grids, coated with holey carbon film (TAAB, Berkshire, UK). TEM images were used to confirm particles’ size and morphology.

To indicate the stability of particles in solutions, Zeta (ζ) potential values were measured in distilled water in three different pH: 3.0, 7.4, and 11.0 using Zeta sizer (Malvern instrument 2000).

X-ray Diffraction (XRD) patterns were collected with a Philips PW1700 series automated powder diffractometer using Cu Kα radiation (1.54 Å) at 40 kV/40mA. Data was collected in the 10-70° 2θ range with a step size of 0.04° and a dwell time of 1.0 second to identify the crystallised pattern of the particles.

2.4 Dissolution study

To compare the release rate of ions from the Sr-BGNPs with different mol% SrO, the release of Si, Ca and Sr ions from Sr-BGNPs was evaluated as a function of time in three different solutions: minimum essential media (α-MEM) medium (Thermo Fisher Scientific, Hemel Hempstead, UK) at pH 7.4, artificial lysosomal fluid (ALF) at pH 4.5 (Supplementary Information Table S1) and phosphate-buffered saline (PBS) at pH 7.4. 75 mg of BGNPs were suspended with 5 mL of media in dialysis tubing, that had a molecular weight cut-off of 10 kDa, and immersed into 45 mL of media [42]. All samples were incubated at 37 °C with continuous shaking at 120 rpm for 1, 2, 3, 4, 5, 6, 7, 24, 48, 72, 96, 168, 240 h in PBS and ALF and for 1, 2, 3, 4, 5, 6, 7, 24 h in α-MEM medium. At each of the time intervals, 1 mL of the bulk solution was collected and then immediately replaced with 1 mL of the fresh solution. The pH of solution was monitored at each specific interval over a period of 240 h in PBS and ALF and for 24 h in α-MEM.

The collected solution was diluted in distilled water (for the α-MEM medium) and 2 M nitric acid (for ALF and PBS) with a 10-fold dilution factor. The elemental concentrations of Si, Ca, and Sr were measured using ICP-OES (Thermo Scientific iCAP 6000 series).
At the end of incubation period, the Sr-BGNPs were washed with ethanol and acetone to terminate any reactions [42] and then collected on 400 mesh copper TEM grids, coated with a holey carbon support film and bright field TEM imaging was conducted.

2.5 In vitro cytotoxicity assay

The next step was to evaluate whether the therapeutic effect of the Sr-BGNPs could be linked directly to an ionic effect of the Sr and Ca ions which are released from the particles. A murine pre-osteoblast cell line, MC3T3-E1 cells (ATCC) was incubated with both the particles and media containing only their dissolution products. MC3T3-E1 cells were routinely cultured under standard condition in a humidified atmosphere at 37°C and 5% CO₂ in basal α-MEM media. These media were supplemented with 10% fetal bovine serum (FBS) (v/v), 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific, Hemel Hempstead, UK). Cells were seeded in flat-bottomed 96-well plates, at a seeding density of 5x10⁴ cells/mL, and incubated at 37°C and 5% CO₂ for 24 h to allow cells to attach in a monolayer.

Based on our previous report [37], 6%Sr-BGNPs and 14%Sr-BGNPs did not cause toxicity to the MC3T3-E1 cells up to 250 μg/mL. Here, effects of the of 6%Sr-BGNPs and 14%Sr-BGNPs on cell viability was investigated with the extended NP concentration range from 0 to 1000 μg/mL (0.01, 0.1, 1, 10, 100, 150, 200, 250, 500, 1000) using a pulse-chase exposure, where cells were exposed to the pulse phase for 24 h, followed by chase period of 1, 3 and 7 days. The effect of the ions released from the 6%Sr-BGNPs and 14%Sr-BGNPs on cell viability was measured after cells were incubated with the media retrieved from the dissolution experiments for 1, 3, and 7 days.

Cell viability was determined using the MTT colorimetric assay (Thermo Fisher Scientific, Hemel Hempstead, UK) based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into formazan. The formazan is soluble in dimethyl sulfoxide (DMSO) and the concentration of soluble formazan was determined at 570 nm using a microplate reader (SpectraMax M2®, Molecular device).

2.6 Effect of Sr-BGNPs and their ionic release products on MC3T3-E1 differentiation

MC3T3-E1 cells were cultured, using a cell density at 5x10⁴ cells/mL, in a flat-bottomed 24-well plate and incubated at 37°C and 5% CO₂ for 24 h to allow cells to attach to the plate. Cells were cultured either in basal α-MEM or osteogenic medium (α-MEM supplemented with 100 μM L-ascorbic acid (Sigma-Aldrich, UK), 10 mM β-glycerophosphate (Sigma-Aldrich, UK) and 10 nM dexamethasone (DEX, Sigma-Aldrich, UK)).

Cells were exposed to Sr-BGNPs or the media containing the 6% Sr-BGNP’s and 14%Sr-BGNP’s ionic release products both in the basal and osteogenic conditions. Media containing the ionic release products were made by immersing Sr-BGNPs in the media at concentration of 250 μg/mL for 24 h with continuous shaking at 120 rpm. The culture media were changed every three days to ensure a high nutrient concentration. The NPs were incubated with the cells with every media change. Cells were fixed with 4% paraformaldehyde in PBS at time intervals of up to 21 days. Cells were stained with an alkaline phosphate (ALP) detection kit (Merck Millipore, Middlesex, UK) according to the manufacturer’s instructions.
Key osteoblastic differentiations marker staining was carried out for Collagen type 1 (Col1a1), osteocalcin (OSC), and osteopontin (OSP). After fixation, cells were permeabilised with a permeability buffer for 30 min and then blocked with 1% BSA in PBS for 5 min. The cells were stained with a rabbit IgG primary antibody (Abcam, Cambridge, UK) at 4°C overnight. The secondary antibody used for immunofluorescence was goat anti-rabbit IgG H&L conjugated with Alexa Fluor 455 (Abcam, Cambridge, UK). 4',6-Diamidino-2-Phenylindole (DAPI, Thermo Fisher Scientific, Hemel Hempstead, UK) was used to stain nuclei.

Cells were stained with 1% Alizarin Red S in PBS at pH 4.2 to detect calcified tissue formation.

2.7 Endocytosis study

2.7.1 Fluorescent labelling of nanoparticles

14%Sr-BGNPs were functionalised using a modified method from previous work [44]. 50 mg of 14%Sr-BGNPs was re-dispersed in 10 mL absolute ethanol (5% w/v) followed by careful addition of 500 μL of 28% NH₄OH. Then, 2 mL of (3-aminopropyl) triethoxysilane (APTES) was mixed on a shaker at 200 rpm overnight to complete reactions. Finally, amine functionalised Sr-BGNPs were completely washed with absolute ethanol two times to remove the excess components.

After Sr-BGNPs were functionalised with amine groups, the particles were labelled with fluorescein coupling using a modified method from previous research [45]. For 1% w/v fluorescein 5(6)-isothiocyanate bioreage: FITC, 20 mg of FITC was dissolved in absolute ethanol and gently mixed (200 rpm) in dark conditions. Next, 20 mg of amine functionalised Sr-BGNPs was added and stirred for 16 h to complete the reaction. Lastly, FITC-14%Sr-BGNPs were washed with absolute ethanol (2 times) and D.I. water (once) (Scheme 1). To investigate the effect of the FITC conjugated BGNPs (FITC-14%Sr-BGNPs) on the viability of the MCT3T-E1 cells, an MTT assay was performed.

2.7.2 Endocytosis mechanism inhibition study

To understand the mechanism by which the NPs were internalised by the cells, MCT3T-E1 cells (cell density at 5x10⁴ cells/mL) were seeded on 6-well plates. After the cells were cultured under standard conditions, in a humidified atmosphere at 37°C and 5% CO₂ in basal α-MEM media, the cells were subsequently incubated with different endocytosis inhibitors for 2 h.

The MC3T3-E1 cells were then pre-treated at 37°C for 2 h with five different endocytosis chemical inhibitors including wortmannin (wor, an inhibitor of phagocytosis); amiloride hydrochloride hydrate (ami, an inhibitor of macropinocytosis); chlorpromazine hydrochloride USP (chlor, an inhibitor of macropinocytosis); genistein (gen, an inhibitor of clathrin-independent endocytosis) and cytochalasin D (cytD, an inhibitor of clathrin-dependent endocytosis). The NPs were replaced at concentration 250 µg/mL and were then incubated for 24 h. The concentration of endocytosis inhibitors was: 23 μM of wor; 1 mM of ami; 37 μM of gen (VWR International Ltd, Lutterworth, UK); 4 μM of cytD; and 30 μM of chlor. These concentrations were selected using previous work as a guide [46]. After 24 h cells were fixed with 4% paraformaldehyde in PBS.
2.8 Statistics

Statistical analyses were performed by one-way analysis of variance (ANOVA) in Minitab with the appropriate post hoc comparison test (Tukey's test). A p-value < 0.05 was considered significant. The graphs shown present the results as the mean value with the standard deviation (SD) as the error bars.

3. Results

3.1 Material characterization

Table 1 shows the compositions of the Sr-BGNPs. Elemental analysis confirmed that the binary glass had a composition of 93 mol% SiO₂, 7 mol% CaO. Interestingly, adding a third component (Sr), while keeping the nominal ratio of Si:total modifying cations constant, increased the overall amount of network modifiers in the stabilised NPs (13 mol% and 17 mol% CaO + SrO), compared with the binary NPs containing only CaO (7 mol% CaO). This increase might be due to the larger ionic radius of the Sr compared to Ca, causing the silicate network to be more open, allowing diffusion of both cations into the NPs during thermal stabilisation. However, this does not explain the reduction in Ca content.

The morphology of the batches and particle diameters agreed with that found previously [37]. The different compositions of the BGNPs, for the different Ca and Sr contents, did not influence the size and morphology of the particles. TEM images of dense monodisperse Sr and Si-BGNPs (Fig. 1) showed that they all had spherical morphology. Fig. 2 shows their size distribution measured using DLS with modal diameters of 90 ± 10 nm.

As an indicator of the stability of particles in water, and the effect of changing pH, the ζ-potential of the particles of were measured by suspending them in DI water at three different pH values: 3.0, 7.4 and 11. (Table 2). The ζ-potential values ranged from 1.5±0.1 to 4.6±0.3 mV (pH 3.0), -22.6±0.6 to -29.7±1.3 mV (p7.4) and 32.6±1.6 to -41.6±1.0 mV (pH 11.0). As expected, the ζ-potential shifted when the pH changed, being positive in the acidic condition and negative in the neutral and basic conditions.

XRD patterns (Fig. 3) of the Sr-BGNPs after drying at 130°C (but prior to thermal stabilization) showed the presence of the cation precursors. The XRD patterns showed amorphous halos once the Sr-NPs were heated to 680°C, indicating that calcium oxide (CaO) and strontium oxide (SrO), were incorporated into the silica networks after thermal stabilisation, agreeing with previous studies on cation incorporation [36, 47-50]. Previous slice and view studies, using FIB and TEM, on 500 nm SiO₂-CaO NPs made using the same method, showed a homogeneous distribution of Ca throughout the NP [36]. As diffusion distances are smaller here, it is likely that the Sr-BGNPs also have a homogeneous distribution, although their small size made it impossible to do slice and view imaging. Sr-BGNPs are likely to have the potential to generate a sustained release of therapeutic cations because of the Sr being part of an amorphous silica network.
3.2 Ion release profiles

Fig. 4 shows the dissolution profiles of each of the Sr-BGNPs in PBS, ALF, and α-MEM media. The results demonstrate that the Si content of the α-MEM media, ALF, and PBS increased as the incubation period increased, confirming that soluble silica was released from the BGNPs at a sustained rate over the immersion periods. Si release was slowest in PBS, not reaching 20 µg/mL at 3 days. Si release in ALF and α-MEM was faster and exceeded 50 µg/mL at 3 days. In ALF, the concentration of Sr increased sharply during the first 24 h of immersion, after which it increased more slowly, reaching a plateau at 48 h; this increase was significantly higher for the 14% Sr-BGNPs. In α-MEM, the Sr-BGNPs rapidly released Sr ions during the first 4 h, followed by a gradual release for a prolonged period. In PBS, the concentration of Sr increased slightly as a function of time for the first 24 h of immersion before levelling off. The medium type and pH affected Sr release: the total amount of Sr release was greatest in the neutral α-MEM medium and was similar for the two NP compositions (Table 3), which could be due to the amino acids present in the medium chelating the Sr²⁺ ions. In contrast, the amount of Sr released in ALF was significantly higher for the 14% Sr-BGNPs and similar to that measured in α-MEM. The Ca ion concentrations in all three solutions remained approximately constant over time, after rising in the first 4 h of immersion. Interestingly, in ALF solution, there was an increase in the Ca ion concentration for the 6%Sr-BGNPs which was pronounced at 24 and 48 h. It is well known that low pH accelerates cation exchange in aqueous media for all glasses. The reduction in the stability of the NPs in an acidic environment is likely to be beneficial by triggering the release of cations inside the lysosomes following cellular uptake of the NPs. Table 3 showed that the total amount of Ca and Sr release from Sr-BGNPs in ALF and α-MEM were 3-times higher than the amount released in the PBS. None of the Ca profiles show a reduction in Ca content of the media within the 24 h that the cells were exposed to the nanoparticles, indicating that there was no calcium phosphate deposition.

TEM images (Fig. 5) show the morphological changes to the 14%Sr-BGNPs following incubation in the three different media. When the 14%Sr-BGNPs were immersed in PBS for 10 days, salt precipitation on the surface of the particles was considerable in PBS (Fig. 5(a)). Fig. 5(b) shows that in ALF the surfaces of the particles changed, developing a mottled morphology, indicating that the silicate network of Sr-BGNPs became more unstable and degraded in the acidic environment. In α-MEM (Fig. 5(c)), a shell with reduced contrast was observed around the particles, the particles had a mottled appearance and necks were observed between the particles, following 24 h of incubation. These features all indicated that the particles had undergone degradation.

3.3 In vitro cytotoxicity

Our previous work showed that submicron BGNPs can be internalised by cells and degrade inside the cells [35, 43]. Therefore, the effect of NPs themselves (direct method) was evaluated, in which the cell monolayer was exposed to NPs directly. A reduction in cell viability by more than 30% is considered a cytotoxic effect (cell viabilities less than 70%) and this was used as a cut-off value to evaluate cytotoxicity of these particles (ISO 10993-5). To evaluate the effect of the 6%Sr-BGNPs and 14%Sr-BGNPs on the viability of the MC3T3-E1 cells, both NP compositions were introduced to MC3T3-E1 cells for 24 h (pulse period), i.e. the media
containing non-internalised particles was removed and the cell culture was continued for 0, 1, 3, and 7 days (chase period). Cells cultured on tissue culture plates (TCP) served as controls. The results were consistent with our previous report, which showed no toxicity at all concentrations up to 250 µg/mL, but also little difference between samples (Fig. 6 (a)). A significant reduction in cell viability was observed with NP concentrations greater than 500 µg/mL for both compositions of Sr-BGNPs. When cells were exposed to the Sr-BGNPs with concentrations ranging from 0.01–250 µg/mL, the cell viability did not decrease significantly compared to the control (TCP). The Stöber SiO2-NPs without Ca or Sr did reduce the cell viability after 24 h at a concentration of 200 µg/mL.

The effect of the BGNP ionic release products were also evaluated (Fig. 6 (b)). The cell viability of the MC3T3-E1 cells increased significantly \( p<0.05 \) when treated with the Sr-BGNP ion release products with 87 mol% SiO2, 7 mol% CaO, 6 mol% SrO2 (6% Sr-BGNPs) and 83 mol% SiO2, 3 mol% CaO, 14 mol% SrO2 (14% Sr-BGNPs) at 200 and 250 µg/mL after 3 and 7 days, compared to the cells in the other groups and compared to cells under the basal condition. The concentration of Si, Ca and Sr ions released into the media is shown in Supplementary Information (Table S2). The concentration of Sr required to enhance the activity of the MC3T3-E1 cells was in the range of 2 to 6 µg/mL.

### 3.4 Osteogenic differentiation

To evaluate therapeutic efficacy of Sr-BGNPs and the ion release products for bone regeneration, several early and late bone formation markers were investigated. Fig. 7 shows the expression of ALP activity in the extracellular matrix of the MC3T3-E1 cells treated with 6%Sr-BGNPs and 14%Sr-BGNPs, and their ionic release products, following 21 days of culture (media contained particles or dissolution ions at every media change), compared to cells cultured under the basal and osteogenic media (basal media plus osteogenic supplements). There was a significant increase in the ALP activity of the MCT3T-E1 cells treated with 14%Sr-BGNPs compared to the 6%Sr-BGNPs in the basal medium. No difference was seen for the different Sr contents when osteogenic supplements were used. The ionic release products from both the 6%Sr-BGNPs and 14%Sr-BGNPs also stimulated the ALP activity of the cultured MC3T3-E1 cells in the basal condition, suggesting that the ALP simulation resulted from an effect of the ions released from the BGNPs as they dissolved.

To illustrate the effect of the NPs on osteogenic differentiation of the MC3T3-E1 cells in vitro, markers associated with early and late osteogenic differentiation, including Col1a1, OSC and OSP were investigated using immunohistochemistry (IHC) staining. Fig. 8 shows that Col1a1, OSC, and OSP were expressed on the MC3T3-E1 cells following 21 days of culture. In the basal condition, the late osteogenic differentiation markers, OSC and OSP, were more overtly expressed by the 14%Sr-BGNPs and their ionic release products. In contrast, Col1a1 expression was only slightly enhanced in the basal condition, but was enhanced by the osteogenic supplements. Both OSP and Colla1 are proteins associated with extracellular matrix formation [51, 52]. Thus, the data show that the Sr-BGNPs and their dissolution products could affect both mineralisation and extracellular matrix formation, but highlight that osteogenic supplements are also essential for early extracellular matrix formation.
Calcified nodule formation was observed using Alizarin Red S staining at 7, 14 and 21 days. MC3T3-E1 cells were periodically incubated with 6% Sr-BGNPs and 14% Sr-BGNPs and their ionic release products. After 7 days, no difference was observed between the basal and osteogenic conditions, with either the 6% Sr-BGNPs or 14% Sr-BGNPs or the ionic release products (Fig. 9). After two and three weeks in culture, mineralization of the MC3T3-E1 cells treated with 6% Sr-BGNPs and 14% Sr-BGNPs and also their ionic release products, increased significantly compared with the cells cultured in the control conditions (basal without BGNPs (Fig. 9(A)) and osteogenic condition without BGNPs (Fig. 9(B)). This effect of the BGNPs on mineralisation was significantly more pronounced in the cells cultured with osteogenic medium. Quantitative analysis of the Alizarin Red S staining confirmed an increase in calcium formation of the treated MC3T3-E1 cells exposed to both the 6% Sr-BGNPs and 14% Sr-BGNPs compared to the control cells (untreated cells in both the basal and osteogenic conditions) (Fig. S3, Supplementary Information). This increase in calcium formation was also seen for the Sr-BGNP ionic release products. These results indicate that both the 6% Sr-BGNPs and 14% Sr-BGNPs enhanced mineralisation without the osteogenic supplements, and that this effect can be attributed to their ionic release products.

The final aim was to track whether BGNPs had been internalised by the cells and to understand the mechanism by which the BGNPs were internalised. Cell viability was not affected, remaining above 70% of the positive control, by functionalization of the Sr-BGNPs with APTES and fluorescently labelling with FITC (Fig. S4, Supplementary Information).

Confocal microscopy images (2D in Fig. 10 and z-stacks shown in the video in Supplementary Information) showed that the FITC-14% Sr-BGNPs were internalised by the cells and were present in the cytoplasm of the MCT3T-E1 after 24 h of incubation. Fig. 11 shows the fluorescence intensity of the FITC-14% Sr-BGNPs in the MC3T3-E1 cells, confirming that the NPs were internalised by the cells. There was a significant decrease in uptake of the FITC-14% Sr-BGNPs by the cells treated with CytD, suggesting that a clathrin-dependent endocytosis pathway was responsible for uptake of the FITC-14% Sr-BGNPs by the MC3T3-E1 cells. However, some particles were still internalised in the cells treated by CytD and uptake of FITC-14% Sr-BGNPs was nearly completely suppressed when the MC3T3-E1 cells were treated with all five endocytosis inhibitors, indicating that the MCT3T3-E1 cells also used alternative pathways to uptake particles. These data indicate that the FITC-14% Sr-BGNPs enter the cells via a number of endocytosis pathways.

4 Discussion

Monodispersed Sr-BGNPs were successfully synthesised using the modified Stöber process [36, 37]. These nanoparticles were amorphous and spherical with diameters of 90 ± 10 nm. To improve the osteogenic response to the BGNPs, additional cations, such as Sr2+ were introduced during the sol-gel process [16, 53, 54]. An important step is to incorporate cations, by calcination above 450°C, while maintaining particle morphology and diameter [36, 49]. Here, Ca(NO₃)₂ and Sr(NO₃)₂ were used as the cation precursors to incorporate Ca²⁺ and Sr²⁺ into the silica networks. During drying, Ca(NO₃)₂ and Sr(NO₃)₂ deposits onto the secondary
particle surface of the silica [49]. During thermal stabilisation, Ca and Sr were incorporated into the silica network as network modifiers, as confirmed by XRD (Fig. 3), without affecting particle size, distribution and morphology (Table 1, Fig. 1 and Fig. 2). When the percentage of Sr substitution increased from 0 to 14 mol%, the total amount of network modifiers increased from approximately 7% to 17%, even though the nominal concentration of potential network modifiers was constant. The reason behind this is that the ionic radii of Sr (1.12 Å) is larger than Ca (0.99 Å), leading to a more open silica network, which also leads to more rapid dissolution (Fig. 4).

Delivery of Sr inside cells may form part of a therapeutic treatment for osteoporosis, so it is important to understand how the particles are internalised and the behaviour of the particles in physiological solutions outside and inside cells. One hypothesis was that when cells uptake Sr-BGNPs, they form lysosomal vesicles that encapsulate the particles. Sr-BGNPs would therefore be subjected to acidic conditions inside the cell lysosome. Here, in vitro dissolution showed that more cations dissolved from the Sr-BGNPs in ALF solution at pH 4.5 than in the media at pH 7.4, because cation exchange is promoted under the low pH environment (Fig. 4). Dissolution in α-MEM was more rapid than in PBS at the same pH, which is likely due to organic molecules having an affinity (chelation) to cations in the glass.

The in vitro cell viability of MC3T3-E1 cells treated with Sr-BGNPs was not significantly changed when the particle concentration increased up to 250 μg/mL, but Sr-BGNPs concentrations at or above 500 μg/mL caused cells death after 3 days in the direct method. The results suggested BGNPs containing cations had higher cellular biocompatibility compared to the control of the much less degradable SiO₂-NPs (Fig. 6). The relative cell viability increased when the Sr concentration in the media was in a range of 2 to 6 μg/mL. Following on from previous research on 45S5 Bioglass ionic release products, which induce osteogenic differentiation of osteoblasts without adding osteogenic supplements [8, 55-57], we hypothesised that the media containing the ionic release products of the Sr-BGNPs would stimulate osteogenic differentiation of MC3T3-E1 with no added osteogenic supplements. MC3T3-E1 cells treated under basal conditions with particles or their ionic release products did indeed show an increase in ALP activity (Fig. 7) and calcium deposition compared to the control (Fig. 9). Osteogenesis associated markers, including Col1a1, OSC, and OSP, were also evaluated (Fig. 8). MC3T3-E1 cells treated with the Sr-BGNPs and their ionic release products under basal conditions showed a strong green fluorescent intensity of Col1a1, OSC, and OSP. The results indicate that Sr-BGNPs stimulate osteogenic differentiation and that this effect arises due to the ionic release products of the Sr-BGNPs.

To identify the uptake route used by the Sr-BGNPs, uptake by MC3T3-E1 cells was investigated under specific inhibitors using microplate reader and confocal fluorescent microscope, as suggested in previous studies [58, 59]. The 14%Sr-BGNPs were probably predominantly taken up via the clathrin-dependent endocytosis pathway in the MC3T3-E1 cells (Fig. 11), which is consistent with previous findings that clathrin-dependent endocytosis is the main pathway for particle size up to 200 nm [59]. However, the 14%Sr-BGNPs were also internalised into cells treated with all of the inhibitors. The possible explanation for this might be because either the concentrations of inhibitors were not suited for MC3T3-E1 cells, leading
to not completely blocked pathways, or that the uptake of these particles by the cells occurred through mixed pathways. The results agree with previous studies on SiO$_2$-CaO NPs in stem cells [35], and it can be speculated that 14% Sr-BGNPs entered into the cells through endocytosis pathways. The green fluorescent intensity of the confocal microscopy images (Fig. 10 and the 3D video in supplementary information) also showed internalisation of 14% Sr-BGNPs by the MC3T3-E1 cells, confirming intracellular ion delivery inside the cells.

5 Conclusions

This study reports the osteogenic response of MC3T3-E1 cells treated with monodispersed Sr-BGNPs SiO$_2$-NPs synthesised using the modified Stöber process and incorporating Ca and Sr. Incorporation of Ca and Sr into the silica network did not affect the size and shape of the particles. Culture of the Sr-BGNPs with MC3T3-E1 cells did not alter the viability of the cells up to concentration 250 µg/mL. The dissolution products of the Sr-BGNPs were non-toxic at all concentrations. Ion release products from the 6% Sr-BGNPs (87 mol% SiO$_2$, 7 mol% CaO, 6 mol% SrO) and 14% Sr-BGNPs (83 mol% SiO$_2$, 3 mol% CaO, 14 mol% SrO) at BGNP concentrations of 200 and 250 µg/mL enhanced MC3T3-E1 proliferation up to 7 days in vitro. Sr-BGNPs concentrations at or above 500 µg/mL had adverse effects on MC3T3-E1 cell viability after 3 days in the direct method. The 6% Sr-BGNPs and 14% Sr-BGNPs and their ionic release products had the ability to stimulate an osteogenic response without adding osteogenic supplements in the culture system and this effect could be attributed to their ionic release products. Crucially, the data show that the Sr-BGNPs could affect both mineralisation and extracellular matrix formation, but highlight that osteogenic supplements are also essential for early extracellular matrix formation. The results show both an increase in mineralisation and expression of proteins associated with collagen production. As Sr content in the NPs and in the dissolution products increased, ALP, OSC and OSP expression increased. The exact mechanisms for the cells to uptake Sr-BGNPs remained indefinable, but most likely based on the clathrin-dependent endocytosis pathway. These in vitro results revealed that 6% Sr-BGNPs and 14% Sr-BGNPs at a concentration of 250 µg/mL promoted osteogenic response which maintaining cell proliferation, which is likely to be beneficial to use as an inorganic drug delivery for bone regeneration applications.

6. Acknowledgements

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7. References


List of Figures

Fig. 1. TEM images of Sr-BGNPs: (a) SiO$_2$-NPs (100 mol\% SiO$_2$); (b) 0\%Sr-BGNPs (93 mol\% SiO$_2$, 7 mol\% CaO); (c) 6\%Sr-BGNPs (87 mol\% SiO$_2$, 7 mol\% CaO, 6 mol\% SrO); and (d) 14\%Sr-BGNPs (83 mol\% SiO$_2$, 3 mol\% CaO, 14 mol\% SrO).
Fig. 2. Dynamic Light Scattering (DLS) results of 0% Sr-BGNPs, 6% Sr-BGNPs and 14% Sr-BGNPs.

Fig. 3. XRD patterns of (a) SiO$_2$-NPs; (b) 0% Sr-BGNPs; (c) 6% Sr-BGNPs; and (d) 14% Sr-BGNPs before and after heat treatment process (heating at 680$^\circ$C).
Fig. 4. Dissolution profiles of 6%Sr-BGNPs and 14%Sr-BGNPs in three different solutions: (a) α-MEM media at pH 7.4; (b) ALF at pH 4.5; (c) PBS at pH 7.4.

Fig. 5. Bright-field TEM images of 14%Sr-BGNPs following immersion of the NPs in: (a) PBS for 10 days, (b) ALF for 10 days, and (c) α-MEM for 24 h (all scale bars 50 nm).
Fig. 6. Effect of (a) Sr-BGNPs and (b) their ionic release products, on the metabolic cell activity of the pre-osteoblastic cell line (MC3T3-E1) based on an MTT assay after 24 h pulse followed by chase period in culture of 0, 1, 3, and 7 days (n = 6 per group). The metabolism of the cells treated with different concentrations (0.01-1000 μg/mL). * (6%Sr-BGNPs) and + (14%Sr-BGNPs) were statistically different from the control (TCP), p<0.05. **(6%Sr-BGNPs) and ++ (14%Sr-BGNPs) were statistically decreased from the control (TCP), p<0.05.
Fig. 7. Staining for ALP activity, which indicates the differentiation of MC3T3-E1 cells grown in media containing 6% Sr-BGNPs and 14% Sr-BGNPs or their ionic release products (Sr-BGNP concentration at 250 µg/mL).

<table>
<thead>
<tr>
<th>ALP staining</th>
<th>Control</th>
<th>Direct NPs</th>
<th>Ion dissolution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal media</td>
<td>6% Sr BGNPs</td>
<td>14% Sr BGNPs</td>
</tr>
<tr>
<td></td>
<td>Osteogenic media</td>
<td>6% Sr BGNPs</td>
<td>14% Sr BGNPs</td>
</tr>
</tbody>
</table>
Fig. 8. Fluorescence image, DAPI (blue), Colla1, OSC and OSP staining (all green) of MC3T3-E1 cells exposure to 14%Sr-BGNPs and their ionic release products (Sr-BGNP concentration at 250 µg/mL) in basal and osteogenic conditions (3-week culture period). Scale bar 150 µm.
Fig. 9. Alizarin red staining for calcified nodule formation from MC3T3-E1 cells treated with 6% Sr-BGNPs and 14% Sr-BGNPs or their ionic release products (Sr-BGNP concentration at 250 µg/mL) under: (A) the basal condition and (B) the osteogenic condition. Scale bar = 400 µm.
Fig. 10. Confocal microscopy images showing internationalisation of the FITC-14%Sr-BGNPs (concentration at 250 µg/mL) by the MC3T3-E1 after pre-treatment with endocytosis inhibitors; Wor (wortmannin), Ami (amiloride hydrochloride hydrate), Chlor (chlorpromazine), Gen (genistein), CytD (cytochalasin D). Scale bar=20 µm. Nuclei were stained with DAPI. F-actin filaments were stained with rhodamine phalloidin.
Fig. 11 Effect of endocytosis inhibitors on FITC-14%Sr-BGNPs (concentration at 250 µg/mL) internalisation by MCT3T3-E1 cells; Wor (wortmannin), Ami (amiloride hydrochloride hydrate), Chlor (chlorpromazine), Gen (genistein), Cyt D (cytochalasin D).
List of Schemes

(a) Sr-BGNPs conjugation with FITC and

(b) indirect measurement of FITC-Sr-BGNPs endocytosis mechanism.

Scheme 1 (a) Sr-BGNPs conjugation with FITC and (b) indirect measurement of FITC-Sr-BGNPs endocytosis mechanism.
List of Tables

Table 1. Composition of the synthesised Sr-BGNPs (mol%).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SiO₂</td>
</tr>
<tr>
<td>0% Sr-BGNPs</td>
<td>92.8 ± 1.3</td>
</tr>
<tr>
<td>6% Sr-BGNPs</td>
<td>87.1 ± 0.3</td>
</tr>
<tr>
<td>14% Sr-BGNPs</td>
<td>83.3 ± 0.2</td>
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Table 2 Size and Zeta potential of Sr-BGNPs in water at three different pH values: 3.0, 7.4 and 11.0.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DLS size distribution (nm)</th>
<th>TEM size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>pH 3.0</td>
</tr>
<tr>
<td>0% Sr-BGNPs</td>
<td>89.3 ± 2.9</td>
<td>75.1 ± 5.5</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>6% Sr-BGNPs</td>
<td>91.2 ± 10.6</td>
<td>79.4 ± 7.7</td>
<td>3.2 ± 2.1</td>
</tr>
<tr>
<td>14% Sr-BGNPs</td>
<td>98.0 ± 9.2</td>
<td>83.2 ± 7.7</td>
<td>4.6 ± 0.3</td>
</tr>
</tbody>
</table>

Table 3. Percentage elemental release from 6% Sr-BGNPs and 14% Sr-BGNPs after immersion in different media for 1 (α-MEM) and 10 days (PBS and ALF).

<table>
<thead>
<tr>
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<th>PBS</th>
<th>ALF</th>
<th>α-MEM</th>
<th>% release</th>
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<tr>
<td></td>
<td>Si</td>
<td>Ca</td>
<td>Sr</td>
<td>Si</td>
</tr>
<tr>
<td>6% Sr-BGNPs</td>
<td>2.32</td>
<td>1.50</td>
<td>8.17</td>
<td>4.62</td>
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<tr>
<td>14% Sr-BGNPs</td>
<td>3.20</td>
<td>0.97</td>
<td>3.68</td>
<td>4.71</td>
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Supplementary Information

Supplementary Information Table S1. ALF solution composition.

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<th>Chemicals (g/L)</th>
<th>Weight</th>
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<tr>
<td>Sodium chloride, NaCl</td>
<td>3.210</td>
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<tr>
<td>Disodium hydrogen phosphate, Na₂HPO₄</td>
<td>0.071</td>
</tr>
<tr>
<td>Sodium sulphate, Na₂SO₄</td>
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</tr>
<tr>
<td>Calcium chloride dihydrate, CaCl₂.2H₂O</td>
<td>0.128</td>
</tr>
<tr>
<td>Sodium citrate dihydrate, C₆H₅Na₃O₇.2H₂O</td>
<td>0.077</td>
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<tr>
<td>Sodium hydroxide, NaOH</td>
<td>6.000</td>
</tr>
<tr>
<td>Citric acid, C₆H₅O₇</td>
<td>20.800</td>
</tr>
<tr>
<td>Glycine, H₂NCH₂COOH</td>
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</tr>
<tr>
<td>Sodium tartrate dihydrate, C₄H₆O₆Na₂.2H₂O</td>
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<tr>
<td>Sodium lactate, C₃H₆NaO₃</td>
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<tr>
<td>Sodium pyruvate, C₃H₄O₄Na</td>
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Supplementary Information Table S2. Ion concentration of dissolution media.

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<th>Si (µg.mL)</th>
<th>Ca (µg.mL)</th>
<th>Sr (µg.mL)</th>
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</thead>
<tbody>
<tr>
<td>6% Sr BGNPs</td>
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<td>0.5</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.3</td>
<td>2.9</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8.4</td>
<td>5.4</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>19.6</td>
<td>5.3</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>27.2</td>
<td>6.0</td>
<td>3.0</td>
</tr>
<tr>
<td>14% Sr BGNPs</td>
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<td>0.9</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.8</td>
<td>0.6</td>
<td>1.2</td>
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<td></td>
<td>100</td>
<td>4.1</td>
<td>1.5</td>
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<td>200</td>
<td>8.9</td>
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<td>4.8</td>
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<tr>
<td></td>
<td>250</td>
<td>11.0</td>
<td>2.1</td>
<td>6.3</td>
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</table>
Fig. S3 Quantitative analysis of Alizarin red staining to show calcified nodule formation by MC3T3-E1 cells treated with 6% Sr-BGNPs and 14% Sr-BGNPs or ionic release products (Sr-BGNP concentration at 250 µg/mL) under both basal and osteogenic conditions.
Fig. S4 Effect of FITC conjugated 14%Sr-BGNPs on the metabolic cell activity of pre-osteoblastic cell line (MC3T3-E1) based on the MTT assay after 24 h pulse phase followed by chase period in culture for 0, 1, 3, and 7 days (n = 6 per group). Cells were treated with different concentrations (50-250 μg/mL).