Cobalt-Containing Spherical Glass Nanoparticles for Therapeutic Ion Release

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Abstract Bioactive glass nanoparticles (BGNPs) can be internalized by cells, allowing the intracellular release of dissolution products with therapeutic benefit. Different therapeutic ions can be incorporated into the glass network that can promote angiogenesis via simulation of hypoxia conditions and consequent activation of pro-angiogenic genes. Here, novel monodispersed spherical dense BGNPs were obtained by a modified Stöber method with the SiO_2-CaO-CoO composition, with diameters of 92 ± 1 nm**,** with cobalt as the pro-angiogenic ion. The presence of Co^{2+} species and the role of Co and Ca as network modifiers in the silica glass were confirmed by X-ray photoelectron spectroscopy (XPS) and ^{29}Si solid-state magic-angle spinning nuclear magnetic resonance (MAS NMR), respectively. Controlled Co^{2+} ion release was observed in culture media, and no cytotoxicity was observed by MTT cell viability assay on human osteosarcoma cells (SAOS) in direct contact with the nanoparticles. This study demonstrated that Co^{2+} ions can be incorporated into dense and spherical BGNPs, and these materials exhibit great potential as intracellular ion delivery systems with therapeutic properties.

Keywords: bioactive glass, cobalt, sol-gel, nanoparticles, materials characterisation

1 Introduction

First reported in 1971 by Hench and collaborators[1], bioactive glasses (BGs) have shown bone repair ability due to the strong bonding with the host tissue after being implanted, stimulating new tissue formation[2]. The osteogenic potential is related to the glass dissolution products, such as calcium ions and soluble silica that stimulate bone matrix production by activating osteoblasts[3, 4]. The concept of the ions having a bioactive effect led to evaluations of new glass compositions that could be delivery vehicles for different ions with therapeutic properties, such as copper (known to stimulate angiogenesis) and silver (antimicrobial), while maintaining the BGs bioactivity and biocompatibility, allowing applications ranging from hard to soft tissue repair[5–8]. The amorphous structure of glass allows the incorporation and delivery of the ions, so it is essential to evaluate the effect of those ions on the glass structure to ensure controlled ion delivery.

Biodegradable bioactive glass nanoparticles (BGNPs) have been investigated for their ability to deliver ions inside cells following internalisation[9, 10], which could improve the therapeutic benefits. These particles could also be incorporated into nanocomposites and injectable biomaterials for different applications in tissue engineering. Previous work has reported the internalization of spherical SiO_2-CaO particles up to 215 ± 20 nm by adipose-derived stem cells and human bone marrow-derived cells[9, 11]. Intracellular delivery of ions was also reported for Sr-containing BGNPs internalized by MC3T3-E1 cells, showing enhanced osteogenic response[9]. Monodispersed nanoparticles of ~80 nm in diameter containing Sr (SiO_2-CaO-SrO) were found to be internalised by bone marrow-derived stem cells and promote differentiation down an osteogenic pathway, but when the same cells were exposed to similar particles without Sr (SiO_2-CaO), the cells remained stem-like[12], confirming the improved potential of BGNPs containing therapeutic ions.

BGNPs can be obtained by modified Stöber methods, a sol-gel based process that allows the synthesis of spherical silica particles with controlled particle size in a wide size range. In this process, a silicon alkoxide precursor, such as tetraethyl orthosilicate (TEOS) is hydrolysed under basic conditions[13, 14]. The reaction parameters influence the particle morphology, size and distribution. For instance, when ammonia is used as the basic catalyst, particle size can be reduced with the ammonia concentration, producing spherical silica particles ranging from 10 to 500 nm[10, 15]. Nonetheless, when additional ions are incorporated into the silica network, the morphology of the particles may become irregular, as particles tend to agglomerate and a polydispersed particle size distribution can occur[10, 14], therefore is necessary to control the process variables. Greasley et al. reported a method for successfully obtaining monodisperse spherical bioactive dense glass nanoparticles (DBGNPs), but found that the amount of Ca that could be incorporated was limited to 10 mol% CaO[10]. A similar procedure was also applied to obtain DBGNPs of SiO_2-CaO-SrO[9, 14] and SiO_2-CaO-MnO [16], confirming that the modified Stöber method can be used to obtain monodisperse spherical DBGNPs containing different ions. Interestingly, more Sr could be incorporated into the DBGNPs than Ca[14].

Co ions have been shown to induce angiogenesis when released from biomaterials. This process occurs due to the Co stabilization effect on the hypoxia-inducible factor (HIF-1α) and subsequent activation of pro-angiogenic genes, such as vascular endothelial growth factor (VEGF)[6, 17]. Melt and sol-gel derived bioactive glasses containing Co have been reported and reports confirm an increase in the expression of genes that are linked to the HIF-1α pathway and angiogenesis due to Co release[18–22]. Silica microspheres loaded with Co^{2+} ions showed stimulation of endothelial cells, which play an important role in angiogenesis, through the upregulation of key angiogenic genes, polarization, migration and tubular formation[23]. The *in vivo* biocompatibility of Co-containing glasses and a high angiogenesis level was also reported after implantation of BGs in rats[24]. However, a controlled Co release is needed, as cell toxicity was shown to increase once the Co

concentration reaches a critical value, as evaluated during *in vitro* studies in human mesenchymal stem cells[18, 25].

Here, novel DBGNPs were obtained by the incorporation of Co into SiO_2-CaO derived particles, and the effect of this incorporation on the glass structure, composition, morphology, ion release and cytotoxicity were evaluated.

2 Materials and Methods

2.1 Bioactive glass dense nanoparticles synthesis

A previously reported modified Stöber method[10, 14] was applied to obtain DBGNPs containing calcium and cobalt. All the chemicals described here were obtained from *Sigma-Aldrich* unless otherwise stated. First, distilled water, ammonium hydroxide (28 – 50% basis) and ethanol (99.5%) were mixed for 10 min, followed by TEOS (tetraethyl orthosilicate, 98%) by slow addition. The sol was left stirring overnight. Silica nanoparticles obtained were centrifuged and collected, followed by washing with ethanol three times and drying at 60°C for 24 h. The following concentrations were used to obtain silica particles: 6 M H_2O, 0.23 M NH_3, and 0.28 M TEOS. Dense silica nanoparticles (nominal composition of 100% SiO_2 (DNP)) were obtained after thermal treatment of the dried silica particles at 680°C/3 h/3°C.min^{-1}.

To incorporate Ca and obtain compositions in the SiO_2 – CaO system, the dried silica nanoparticles were dissolved and dispersed in water and mixed in the ultrasonic bath for 30 min with a calcium nitrate tetrahydrate (*Acros Organics,* 99%) solution. After that, the solution was centrifuged, and the particles collected were dried at 60°C/24 h. The thermal treatment was set to 680°C/3 h/3°C.min^{-1}, and after cooling, samples were washed with ethanol three times, removing the excess of ions that were not incorporated into the glass network. A 1:1.3 Si:Ca molar ratio was used as the optimal ratio for maximum Ca incorporation, as previously reported[10]. The SiO_2 – CaO sample was named DNP-0Co.

Calcium was partially replaced by cobalt in the glass network to obtain particles in the SiO_2 – CaO – CoO system. The same process described before for calcium incorporation was applied, however, a solution containing both calcium nitrate tetrahydrate and cobalt nitrate tetrahydrate was used. Samples with 25% substitution of the total calcium content (DNP-25Co, Si:Ca:Co molar ratio of 1:0.98:0.35) and 50% substitution (DNP-50Co, Si:Ca:Co molar ratio of 1:0.65:0.65) were obtained.

2.2 DBGNPs characterization

Chemical composition:

First, 50 mg of each sample was mixed in a platinum crucible with 250 mg of anhydrous lithium metaborate (80% w/w) and lithium tetraborate (20% w/w), followed by fusion at 1050°C for 30 minutes in a furnace. Then, samples were dissolved in 2 M nitric acid, and the Si, Ca and Co concentrations were determined by inductively coupled plasma-optical emission spectrometer (ICP-OES - Thermo Scientific iCaP 6000series equipment). Finally, SiO_2, CaO and CoO proportions were calculated.

Morphological and textural properties

A Malvern Instrument 200 dynamic light scattering (DLS) equipment was used to confirm the particle size, whereas the transmission electron microscopy (TEM) was carried out on a JEOL 2100 Plus microscope operating at 200 kV. For DLS and TEM, samples were dispersed in ethanol (1 $mg.mL^{-1}$) and maintained for 15 min in a sonication bath (Elmasonic S180H, 37 kHz). For TEM, particles were collected on a copper grid (400 mesh) coated with carbon film (*TAAB,* UK). A Zetasizer Malvern Instrument 2000 was used for zeta potential evaluation, with samples dispersed in water.

A Quantachrome Autosorb AS-6 multi-station was used for nitrogen sorption analysis (40 absorption points and 40 desorption points). Samples were previously degassed

(200°C/24 h), and the specific surface area was analysed by the BET (Brunauer-Emmett-Teller) method, using the absorption points at 0.01 – 0.30 P/P_0 relative pressure.

Structural evaluation

FTIR (Fourier Transform Infrared Spectroscopy) was performed on a Thermo Scientific Nicolet iS10 equipped with Attenuated Total Reflectance Accessory, in the 400 to 4000 cm^{-1} range (32 scans per spectrum, 4 cm^{-1} resolution). X-Ray Diffraction (XRD) data were obtained on a Bruker D2 desktop equipment, using 0.02 step size without spinning, CuKα radiation source and data collected from 7 to 70° (2θ). A Thermo Scientific K-Alpha$^+$ system was used for X-Ray Photoelectron Spectroscopy (XPS) analysis. The equipment operated at $2x10^{-9}$ bar, microfused and monochromated Al Kα X-ray source (hv = 1486.6 eV), 2D detector and 180° double-focusing hemispherical analyser, 6 mA X-ray source emission current and 12 kV anode bias. Binding energy (BE) was corrected using the C 1s core line at 285.0 eV, and a flood gun was used to reduce sample charging. A 400 µm X-ray spot size was used for data collection and 200 eV pass energy for the survey, whereas a 20 eV pass energy was applied for core level scan. The obtained data were evaluated using the Avantage XPS software. High-resolution core-level scans were performed (Si 2p, O 1s, Co 2p).

Solid-state ^{29}Si magic-angle-spinning nuclear magnetic resonance (MAS NMR) measurements were performed at 7.05 T using a Varian/Chemagnetics InfinityPlus-300 spectrometer operating at a ^{29}Si Larmor frequency (ν_o) of 59.6 MHz. For all measurements, a Bruker 7 mm HX probe enabled a 5 kHz MAS frequency. The ^{29}Si pulse length was calibrated using solid kaolinite ($Al_2O_3 \cdot 2SiO_2 \cdot 2H_2O$) where a π/2 pulse time of 4.25 µs was measured. For all single-pulse experiments, a π/4 nutation angle (2.12 µs pulse duration) was implemented in conjunction with a long recycle delay of 240 s and strong heteronuclear ^1H decoupling (80 kHz) during data acquisition. All ^{29}Si chemical shifts are reported against the IUPAC recommended primary Me_4Si reference

(1 % in $CDCl_3$, δ_{iso} = 0.0 ppm) via a secondary solid kaolinite reference (δ_{iso} = −92 ppm). Equation 1 was used to obtain the degree of condensation (D_C)[26]:

$$D_c = (Q^1\% + 2*Q^2\% + 3*Q^3\% + 4*Q^4\%) / 4 \qquad \text{(Eq. 1)}$$

The bridging oxygen (BO) fraction from the total Q-species was also calculated considering that Q^0, Q^1, Q^2, Q^3 and Q^4 contribute respectively with 0.0, 0.5, 1.0, 1.5 and 2.0 bridging oxygen atoms per silica tetrahedron, whereas the total oxygen contributed is 4.0, 3.5, 3.0, 2.5 and 2.0 for each Q-species, respectively, in accordance to previously reported methods[27]. The BO fraction over the total oxygen contributed was calculated by using these values, as shown in Equation 2, and the NBO fraction was obtained by NBO = 1.0 – BO.

$$\text{BO fraction} = (0.5*Q^1\% + 1.0*Q^2\% + 1.5*Q^3\% + 2.0*Q^4\%) / (4.0*Q^0\% + 3.5*Q^1\% + 3.5*Q^2\% + 2.5*Q^3\% + 2.0*Q^4\%) \qquad \text{(Eq. 2)}$$

Ion release study in DMEM

The Si, Ca and Co dissolution rate was evaluated by immersing the samples in Dulbecco's Modified Eagle's medium (DMEM, *Gibco*, UK) at a 1.5 $mg.mL^{-1}$ ratio[9, 28]. Samples were suspended in DMEM and placed in a dialysis tubing (SnakeSkin, 3.5 K MWCO, 16 mm dry I.D; *ThermoFisher Scientific,* UK), followed by immersion in the final solution volume (45 mL) in an airtight polyethylene container. After that, the containers were placed on an incubator set to 37°C equipped with an orbital shaker (120 rpm). After different periods (4, 8, 24, 48 and 72 h), 1 mL solution aliquots were collected and then replaced by fresh DMEM (1 mL). The collected aliquots were dissolved in nitric acid solution (2 M) to allow ICP-OES evaluation of the Si, Ca and Co concentrations in the solution. Experiments were performed in triplicate, and a DMEM solution with no particles was also incubated following the same procedure described, to be used as a control sample.

In vitro cytotoxicity assay

Human osteosarcoma cells (SAOS, ATCC HTB-85) were obtained from the Rio de Janeiro cell bank (BCRJ, Federal University of Rio de Janeiro, Brazil). Cells were grown in DMEM with the addition of fetal bovine serum (FBS, 10%, v/v), streptomycin sulfate (10 $mg.mL^{-1}$), sodium penicillin G (10 $units.mL^{-1}$), and amphotericin-b (0.025 $mg.mL^{-1}$), all supplied by Gibco BRL (NY, USA), in an oven under 5% CO_2 and 37 °C, in accordance to previously reported works[29, 30]. Cells at passage 25 were used for this study.

In vitro cytotoxic assay was performed by MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. The assay was conducted following the 10993-5:2009 standards (Biological evaluation of medical devices: Tests for *in vitro* cytotoxicity). SAOS cells were plated (1 x 10^4 cells/well) in 96-well plates in a serum-free medium for 24 h. The media was then aspirated and replaced by conditioned media, prepared at nanoparticles to DMEM/10% FBS ratio of 0.1 $mg.mL^{-1}$. The following reference controls were used: DMEM/10%FBS and DMEM/10%FBS with sterile polypropylene Eppendorf tubes chips (1 $mg.mL^{-1}$, Eppendorf, Hamburg, Germany) as negative controls; Triton X-100 (1% v/v in phosphate-buffered saline solution (PBS), Gibco BRL, New York, USA) as a positive control for toxicity. After 24 h and 72 h, the media were aspirated and replaced by 60 µL of DMEM with 10% FBS in each well. MTT 40 µL (5 $mg.mL^{-1}$; Sigma-Aldrich, MO, USA) was added to each well and incubated in an oven for 4 h at 37 °C/5% CO_2. After that, 40 µL of Isopropanol/4% HCl solution were placed in each well. Finally, 100 µL were removed from each well and transferred to a 96-well plate to quantify the absorbance (Abs) in a spectrophotometer (I-Mark, Bio-Rad; 595 nm filter). The percentage viable cells were calculated according to Eq. 2 after blank corrections, and control group values were set to 100% cell viability.

Cell Viability (%) = Abs (sample) x 100 % / Abs (control) (Eq. 2)

2.3 Statistical analysis

Results shown for the bioactive glasses chemical compositions, textural properties, ion release in DMEM and MTT cell viability assay were performed in triplicate and expressed as mean ± standard deviation. For MTT cell viability assay, results were considered significant if $p < 0.05$ (One-way ANOVA followed by Tukey posthoc test).

3 Results and Discussion

Compositional, morphological and textural analysis

Table **1** shows the chemical compositions of the obtained DBGNPs. A 100% SiO_2 particle (DNP) was obtained and used to incorporate calcium and cobalt. Co was incorporated in partial replacement for the nominal Ca content. DNP-0Co chemical analysis shows a total of 8.0 ± 1.2 mol% CaO, in agreement with previously reported data, in which the nominal Si:Ca ratio of 1:1.3 was defined as the optimal ratio for Ca incorporation into the dense silica nanoparticles[10]. Previous work has shown that no further increase on the Ca incorporation into the DBGNPs was achieved when higher calcium concentrations were used during the glass synthesis, therefore the same Si:Ca ratio was maintained in this work. For DNP-25Co, a 3.1 ± 0.2 mol% CoO was measured, whereas DNP-50Co (50% substitution of the total Ca content by Co) had 4.6 ± 0.4 mol% CoO, with a consequent reduction on the total CaO content for both samples. Variations in the SiO_2 concentration were within the values of uncertainty, as shown in Table **1**. In this method, silica nanoparticles were firstly obtained, and then calcium and cobalt precursors were added. The Ca and Co precursors deposited on the particle surface during the drying process, and diffused into the glass network during the thermal treatment at higher temperatures[10, 31]. A subsequent washing step was needed to remove the excess of ions not incorporated in the glass network, avoiding the formation of Ca and Co-rich phases around the particles and also an overestimation of the glass chemical composition[10, 14].

Chemical composition analysis showed that Co was successfully incorporated into the DBGNPs at different concentrations in partial replacement of the Ca content.

Table 1 also reports the mean particle size, as evaluated by DLS, and similar results were obtained as the amount of Co increased, with no statistical differences. Previous work reported that different BGNPs of similar particle size were internalized by various cell types, e.g. MC3T3-E1, human bone marrow-derived stem cells (hMSCs), adipose-derived stem cells (ADSCs)[9, 11, 12] and epithelial cells[32], therefore the obtained particles may also be suitable for intracellular delivery of ions. Also, from Table 1 it is possible to observe that the DBGNPs displayed a relatively high negative surface charge, as evaluated by the zeta potential, which is favourable for the attachment of biomolecules on nanoparticles[33, 34], due to hydroxyl groups that may be present on the glass surfaces[35]. Nanoparticles tend to coagulate or flocculate when the surface charge is within the 5 to -5 mV range[34], whereas it shows good stability when the zeta potential values are around 30 or -30 mV, beneficial for therapeutic nanoparticles.

TEM images (Figure 1) showed that particles maintained their monodispersed spherical morphology despite Ca or Co incorporation, and similar particle size, in agreement with the observed by DLS. Furthermore, the washing steps were efficient in removing unincorporated Ca and Co of the particle surface[14], which may be formed as small clusters deposited around the nanoparticles, increasing particles agglomeration, as previously related for SiO_2-CaO nanoparticles systems before the washing step [10, 11]. The particles were confirmed as being dense (non-porous) by TEM and through their nitrogen sorption isotherms. Specific surface areas of 39 to 48 $m^2.g^{-1}$ were determined by BET for the obtained particles (Table 1), which were similar to those previously reported for similar non-porous silica particles[10, 16].

Structural characterization

FTIR spectra of the obtained DBGNPs (Figure 2a) contained the typical bioactive glass absorption bands: e.g. Si-O-Si asymmetric stretch (1000 – 1300 cm^{-1})[36, 37]; Si-O-Si symmetric stretching at 800 cm^{-1} [36] and Si-O groups bending at 450 cm^{-1} [38, 39]. No qualitative difference can be observed between the spectra of the different samples, indicating that the amorphous glass network structure was maintained even after Ca and Co incorporation at different concentrations. The XRD patterns (Figure 2b) featured a broad halo for all particles characteristic of amorphous structures, although a small shoulder between 35° and 37° for the DNP-50Co sample may indicate an initial formation of crystalline cobalt (II, III) oxide (Co_3O_4) when higher Co concentrations are present[40, 41]. Nonetheless, glasses remained mainly amorphous even after calcium and cobalt incorporation into the silica network[38]. When compared to crystalline materials, amorphous structures usually present a higher dissolution rate, which could also be beneficial to the glass bioactivity.

XPS analysis was performed to identify the role of cobalt on the glass network. The XPS wide scan of the DBGNPs are shown in Figure 3 and high-resolution spectra are shown in Figure 4. Auger and XPS lines typical of all constituent elements (Si, O, Ca and Co), were identified in Figure 3. Also, the C 1s peak, commonly named “adventitious carbon”, was observed for all samples, attributed to hydrocarbon impurities absorbed by the particle surface[37]. The C 1s peak was used to calibrate the binding energy (BE) due to sample charging by setting it to 285.0 eV.

The Si 2p core level high-resolution spectra are shown in Figure 4(a). The BE of all samples were observed in the range typical of SiO_2 structures[42]. The Si 2p peak was narrower for DNP, and also slightly shifted to higher BE (maximum at 103.8 eV) when compared with nanoparticles containing Ca and Co (103.7 eV). This could indicate changes in the proportion of silicate species in the glass network due to the addition of

network modifier ions[27]. When an oxygen atom is bonded with two Si atoms together in the silicate network (Si-O-Si), a bridging oxygen bond (BO) forms, but the incorporation of modifiers ions can disrupt the silica network, forming a non-bridging oxygen (NBO), in which Si atoms are bonded to a metal cation[43]. Different species can be formed according to the number of BO and NBO units, and are represented by Q^n, in which *n* is the number of BO units on a silica tetrahedral structure[44]. The incorporation of network modifiers can alter the proportions of BO and NBO oxygens, consequently affecting the proportion of Q^n species on the silica network. The BE can be shifted for different Q^n species, as the electron density over each Si atom can also differ depending on the number of BO or NBO bonded to it[27]. Structures that contain a higher NBO content present lower BE values for the Si 2p core level[27], indicating that both Ca and Co ions are playing a network modifier role on the silica structure, leading to an increase in the number of NBO units and consequently shifting the Si 2p core-level spectra. Figure 4(b) shows the O 1s high-resolution spectra, and similar behaviour to that of Si 2p spectra was observed. DNP O 1s peak maxima (533.1 eV) was slightly shifted to higher values when compared to the DBGNPs containing Ca and Co. Also, DNP presented a narrower peak. NBO units in silicate glasses are usually observed at lower BE values[27, 43, 45], and it is also indicative that Co and Ca may be disrupting the silica network with consequent formation of an increased number of NBO structures. However, surface hydroxyls may also contribute to the binding energy, usually at higher values[46].

Ca 2p XPS spectra of the DBNGPs are shown in Figure 4(c), and well-distinguished pairs from the spin-orbit splitting (Ca $2p_{1/2}$ and Ca $2p_{3/2}$), typical of calcium compounds[47], were observed at around 351.3 eV and 347.8 eV, respectively, for all samples. DBGNPs Ca 2p spectra showed 3.5 eV spaced spin-orbit components, which is in the range reported for calcium compounds, and it indicates that the calcium chemical environment was similar for all samples[47, 48]. Finally, high-resolution Co 2p XPS spectra (Figure 4(c)) for DNP-25Co and DNP-50Co had multiple peaks. Previous work[45] shows that cobalt

ions can be found as Co^{3+} and Co^{2+} in the glass network, however, the Co^{3+} 2p binding energies (779 – 780 eV) are usually lower than those for Co^{2+} 2p (781 – 783 eV); spin-orbit splitting separation of Co $2p_{3/2}$ and Co $2p_{1/2}$ for Co^{2+} species is usually between 15.7 and 16.0 eV, whereas for Co^{3+} species the spin-orbit splitting is usually in the range of 15.0 to 15.5 eV[49–51]. Also, strong satellite shake-up structures are usually related to high-spin Co^{2+} species[50, 51]. Here, the Co $2p_{3/2}$ and Co $2p_{1/2}$ spin-orbit splitting peak pairs, typical of Co 2p spectra, were observed at around 782 eV and 798 eV respectively, for both samples, with a split-orbit splitting of 16.0 eV. These results are often attributed to the presence of Co^{2+} species in the glass network. Strong shake-up satellite features were also observed at their higher BE side, also indicating the presence of Co^{2+} [45, 52]. Both samples presented similar behaviour, although the Co 2p peaks intensities were higher for DNP-50Co, likely due to the higher Co content for that particle composition. Therefore, XPS evaluation indicated that cobalt is present mainly as Co^{2+} in the glass network. Both Si 2p and O 1s XPS spectra indicated that Ca and Co are acting mainly as a network modifier on the glass structure, reducing the number of bridging oxygen units.

Solid-state ^{29}Si MAS NMR spectra are shown in Figure 5 and Table 2 reports the relative percentage of Q^n species, as obtained by the deconvolution of the ^{29}Si MAS NMR spectra. A mixture of Q^4, Q^3 and Q^2 species were observed from the Si speciation, showing characteristic chemical shifts at around -111, -102 and -92 ppm, respectively [53, 54]. The highest Q^4 species fraction was observed for DNP (87.5 %), corresponding to a high degree of condensation (96.4%), in agreement with previously reported silica dense nanoparticles[10]. As cations were introduced (8 mol%), the network connectivity decreased to 75.7%. Introducing 3 mol% CoO in place of CaO did not appear to affect the network connectivity. As the Co content increased to 4.6 mol%, the Q^4 fraction reduced to 69.4%, and Q^3 and Q^2 increased for the nanoparticles, reducing the degree of condensation and the BO fraction. These ^{29}Si MAS NMR observations corroborated

the XPS results, thus indicating that Co acts as a network modifier in the glass structure, increasing the formation of NBO structures. The decrease in connectivity for DNP-50Co is more significant considering the total cation content was 0.8% lower for DNP-50Co compared to DNP-25Co. Since the divalent Co^{2+} speciation exhibits a significantly smaller ionic radius than its corresponding divalent Ca^{2+} counterpart within the total inventory, it can be rationalised that the Co^{2+} species is more easily incorporated into the glass network. Furthermore, Ca^{2+} incorporation has been previously demonstrated to exhibit simultaneous network forming and network modifying properties, and this observation indicates that the Co^{2+} speciation imparts immediate impact towards the network disruption[10, 55]. Glasses containing a less polymerized network usually show higher dissolution rates[56], therefore it is also an important aspect of the glass bioactivity and release of therapeutic ions.

Ion release study in DMEM

To assess the bioactive glass particles degradation behaviour and therapeutic ions release capability in a cell culture medium relevant for *in vitro* studies[57], samples were immersed in DMEM for different periods, and ion release profiles for all samples are shown in Figure 6. A control group was also evaluated, in which no particles were added to the DMEM, to verify possible fluctuations in the concentrations of the elements over time. No Si and Co were detected throughout the study in the Control, as expected. Calcium in the DMEM varied between 61 – 64 $\mu g.mL^{-1}$ during the time of the experiment. For all samples, Si release rapidly increased during the first 24 h, stabilizing thereafter. Si release was higher for nanoparticles containing Co reaching 51 $\mu g.mL^{-1}$ after 72 h of immersion compared to 39 $\mu g.mL^{-1}$ for particles without Co. Figure 6 also shows the Ca release profile for all samples, and it is possible to observe that Ca concentration for DNP sample remained similar to the observed for the control group, whereas samples containing Ca showed an initial fast release during the first 8 hours of immersion in DMEM, reducing the release rate thereafter, reaching a maximum of 77 $\mu g.mL^{-1}$ for DNP-

25Co after 72 hours of study. DNP-0Co showed slightly lower Ca release in DMEM when compared to DNP-25Co and DNP-50Co, samples with lower Ca content within the glass composition as evaluated by acid digestion method and ICP-OES, which may be related to the deposition of a calcium phosphate layer on the glass surface[58, 59], consequently reducing the Ca concentration in DMEM. Nonetheless, further evaluation is needed to confirm it, and it was not the focus of this work. Previous work has shown that Si and Ca release can stimulate osteoblast cells, and shows an important role in bone mineralization[60, 61].

Cobalt release for DNP-25Co and DNP-50Co was sustained. As expected, the glass with higher Co content (DNP-50Co) produced higher Co release, reaching a maximum of 2.9 $\mu g.mL^{-1}$ after 72 h. It is not clear what would be the optimal Co release from these particles as they would release the ions inside cells. Bioactive glass and glycosaminoglycan scaffolds that released cobalt extracellularly, in the range of 3 – 12 $\mu g.mL^{-1}$, provoked high ALP activity and VEGF expression in endothelial cells[62]. However, a reduction in the chondrogenic differentiation of human mesenchymal stem cells (hMSCs) was seen when they were exposed to a Co concentration of 7 $\mu g.mL^{-1}$ [25]. Co-releasing bioceramics have been shown to support the proliferation of human bone marrow stromal cells (HBMSCs) and stimulate VEGF expression at lower concentrations (0.059 $\mu g.mL^{-1}$)[63]. In any case, the Co cytotoxicity is time and dose-dependent. Fleury et al.[64] reported a 40% reduction in the cell viability after 72 h exposure of MG-63 osteoblast-like cells to 10 $\mu g.mL^{-1}$ Co^{2+}, therefore a controlled ion release is needed below 7 $\mu g.mL^{-1}$. The Co release showed in this work is within the range that could present therapeutic effects according to the literature without leading to cell toxicity, and the controlled release capability of the dense spherical nanoparticles could be beneficial for tissue repair.

In vitro cytotoxic assay

Measuring the toxicological impact *in vitro* is an initial step in testing their safety[65]. In this work, cell viability was evaluated by MTT assay using SAOS cells, an osteoblast-like cell line, a widely available cell line[30, 66, 67] that show high repeatability of results[30, 68]. Figure 7 presents the MTT assay results for the dense nanoparticles after 24 and 72 h of contact with the SAOS cells. The mitochondrial metabolic activity was not reduced after cells were exposed to the DBGNPs. DNP-50Co particles caused an increase in the cell viability was observed after 72 h seeding. This is the first step in assessing the safety of the particles. Future work should investigate particle internalization by different cell types.

4 Conclusions

Novel monodisperse dense spherical bioactive glasses nanoparticles containing cobalt (SiO_2 – CaO – CoO) were obtained with diameters of 92 ± 1 nm. Introducing Co did not affect the diameter and Co was confirmed as a network modifier role, as confirmed by ^{29}Si MAS-NMR and XPS analysis. A sustained Co release was observed after samples were immersed in DMEM during the dissolution study, and the MTT assay showed no reduction in the cell viability when osteoblast-like SAOS cells were exposed to the nanoparticles. This work shows that Co can be incorporated into DBGNPs with controlled morphology and particle size at different concentrations, and the supported Co release features as a potential strategy to obtain bioactive glasses with improved therapeutic properties. Future work should further confirm particle internalization by different types of cells and the Co release effect when released within the cells.

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Conflict of interest

The authors declare that there is no personal or financial conflict of interests in the current paper.

5 References

1. Hench LL, Splinter RJ, Allen WC, Greenlee TK. Bonding mechanisms at the interface of ceramic prosthetic materials. *J Biomed Mater Res*. 1971;5(6):117–141. https://doi.org/10.1002/jbm.820050611

2. Hench LL, Jones JR, El-fiqi A, Jones JR. Bioactive Glasses : Frontiers and Challenges. *Front Bioeng Biotechnol*. 2015;3(November):1–12. https://doi.org/10.3389/fbioe.2015.00194

3. Jones JR. Review of bioactive glass: From Hench to hybrids. *Acta Biomater*. 2013;9(1):4457–4486. https://doi.org/10.1016/j.actbio.2012.08.023

4. Baino F. Bioactive glasses – When glass science and technology meet regenerative medicine. *Ceram Int*. 2018;44(13):14953–14966. https://doi.org/10.1016/j.ceramint.2018.05.180

5. Baino F, Novajra G, Miguez-Pacheco V, Boccaccini AR, Vitale-Brovarone C. Bioactive glasses: Special applications outside the skeletal system. *J Non Cryst Solids*. 2015. https://doi.org/10.1016/j.jnoncrysol.2015.02.015

6. Miguez-Pacheco V, Hench LL, Boccaccini AR. Bioactive glasses beyond bone and teeth: Emerging applications in contact with soft tissues. *Acta Biomater*. 2015;13:1–15. https://doi.org/10.1016/j.actbio.2014.11.004

7. Bari A, Bloise N, Fiorilli S, *et al.* Copper-containing mesoporous bioactive glass nanoparticles as multifunctional agent for bone regeneration. *Acta Biomater*. 2017;55:493–504. https://doi.org/10.1016/j.actbio.2017.04.012

8. Kargozar S, Montazerian M, Hamzehlou S, Kim HW, Baino F. Mesoporous bioactive glasses: Promising platforms for antibacterial strategies. 2018 https://doi.org/10.1016/j.actbio.2018.09.052

9. Naruphontjirakul P, Porter AE, Jones JR. In vitro osteogenesis by intracellular uptake of strontium containing bioactive glass nanoparticles. *Acta Biomater*. 2018;66:67–80. https://doi.org/10.1016/j.actbio.2017.11.008

10. Greasley SL, Page SJ, Sirovica S, *et al.* Controlling particle size in the Stöber process and incorporation of calcium. *J Colloid Interface Sci*. 2016;469:213–223. https://doi.org/10.1016/j.jcis.2016.01.065

11. Tsigkou O, Labbaf S, Stevens MM, Porter AE, Jones JR. Monodispersed bioactive glass submicron particles and their effect on bone marrow and adipose tissue-derived stem cells. *Adv Healthc Mater*. 2014;3(1):115–125. https://doi.org/10.1002/adhm.201300126

12. Naruphontjirakul P, Tsigkou O, Li S, Porter AE, Jones JR. Human mesenchymal stem cells differentiate into an osteogenic lineage in presence of strontium containing bioactive glass nanoparticles. *Acta Biomater*. 2019. https://doi.org/10.1016/j.actbio.2019.03.038

13. Stober W, Fink A, Bohn E. Controlled growth of monodisperse silica spheres in the micron size range. *J Colloid Interface Sci*. 1968;26(1):62–69.

14. Naruphontjirakul P, Greasley SL, Chen S, Porter AE, Jones JR. Monodispersed strontium containing bioactive glass nanoparticles and MC3T3-E1 cellular response. *Biomed Glas*. 2016;2(1):72–81. https://doi.org/10.1515/bglass-2016-0009

15. Han M-Y, Wang D, Guo Z, *et al.* Unraveling the Growth Mechanism of Silica Particles in the Stöber Method: In Situ Seeded Growth Model. *Langmuir*.

2017;33(23):5879–5890. https://doi.org/10.1021/acs.langmuir.7b01140

16. Barrioni BR, Naruphontjirakul P, Norris E, *et al.* Effects of manganese incorporation on the morphology, structure and cytotoxicity of spherical bioactive glass nanoparticles. *J Colloid Interface Sci*. 2019;547:382–392. https://doi.org/10.1016/j.jcis.2019.04.016

17. Namiki a, Brogi E, Kearney M, *et al.* Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. *J Biol Chem*. 1995;270(52):31189–31195. https://doi.org/10.1074/jbc.270.52.31189

18. Azevedo MM, Tsigkou O, Nair R, Jones JR, Jell G, Stevens MM. Hypoxia Inducible Factor-Stabilizing Bioactive Glasses for Directing Mesenchymal Stem Cell Behavior. *Tissue Eng Part A*. 2014;00(00):1–8. https://doi.org/10.1089/ten.TEA.2014.0083

19. Azevedo M, Jell G, O'Donnell M, Law R, Hill R, Stevens M. Synthesis and characterization of hypoxia-mimicking bioactive glasses for skeletal regeneration. *J Mater Chem*. 2010;20:8854–8864. https://doi.org/10.1039/c0jm01111h

20. Wu C, Zhou Y, Fan W, *et al.* Hypoxia-mimicking mesoporous bioactive glass scaffolds with controllable cobalt ion release for bone tissue engineering. *Biomaterials*. 2012;33(7):2076–2085. https://doi.org/10.1016/j.biomaterials.2011.11.042

21. Hoppe A, Jokic B, Janackovic D, *et al.* Cobalt-releasing 1393 bioactive glass-derived scaffolds for bone tissue engineering applications. *ACS Appl Mater Interfaces*. 2014;6(4):2865–2877. https://doi.org/10.1021/am405354y

22. Solanki AK, Lali F V, Autefage H, *et al.* Bioactive glasses and electrospun composites that release cobalt to stimulate the HIF pathway for wound healing applications. 2021;1–16.

23. Boldbaatar K, Dashnyam K, Knowles JC, Lee HH, Lee JH, Kim HW. Dual-ion delivery for synergistic angiogenesis and bactericidal capacity with silica-based microsphere. *Acta Biomater*. 2019;83:322–333. https://doi.org/10.1016/j.actbio.2018.11.025

24. Barrioni BR, de Laia AGS, Valverde TM, *et al.* Evaluation of in vitro and in vivo biocompatibility and structure of cobalt-releasing sol-gel bioactive glass. *Ceram*

Int. 2018;44(16):20337–20347. https://doi.org/10.1016/j.ceramint.2018.08.022

25. Littmann E, Autefage H, Solanki AK, *et al.* Cobalt-containing bioactive glasses reduce human mesenchymal stem cell chondrogenic differentiation despite HIF-1α stabilisation. *J Eur Ceram Soc*. 2018;38(3):877–886. https://doi.org/10.1016/j.jeurceramsoc.2017.08.001

26. Ting H-K, Page S, Poologasundarampillai G, Chen S, Hanna J V., Jones JR. Phosphate content affects structure and bioactivity of sol-gel silicate bioactive glasses. *Int J Appl Glas Sci*. 2017. https://doi.org/10.1111/ijag.12322

27. Nesbitt HW, Bancroft GM, Henderson GS, *et al.* Bridging, non-bridging and free (O2–) oxygen in Na2O-SiO2 glasses: An X-ray Photoelectron Spectroscopic (XPS) and Nuclear Magnetic Resonance (NMR) study. *J Non Cryst Solids*. 2011;357(1):170–180. https://doi.org/10.1016/j.jnoncrysol.2010.09.031

28. Li S, Maçon AL, Jacquemin M, Stevens MM, Jones JR. Sol–gel derived lithium-releasing glass for cartilage regeneration. *J Biomater Appl*. 2017;32(1):104–113. https://doi.org/10.1177/0885328217706640

29. dos Santos DMM, de Carvalho SM, Pereira MM, Houmard M, Nunes EHM. Freeze-cast composite scaffolds prepared from sol-gel derived 58S bioactive glass and polycaprolactone. *Ceram Int*. 2019;45(8):9891–9900. https://doi.org/10.1016/j.ceramint.2019.02.030

30. Moreira CDF, Carvalho SM, Mansur HS, Pereira MM. Thermogelling chitosan–collagen–bioactive glass nanoparticle hybrids as potential injectable systems for tissue engineering. *Mater Sci Eng C*. 2016;58:1207–1216. https://doi.org/10.1016/j.msec.2015.09.075

31. Yu B, Turdean-Ionescu C a, Martin R a, *et al.* Effect of Calcium Source on Structure and Properties of Sol–Gel Derived Bioactive Glasses. *Langmuir*. 2012;28(50):17465–17476. https://doi.org/10.1021/la303768b

32. Chen S, Greasley SL, Ong ZY, *et al.* Biodegradable zinc-containing mesoporous silica nanoparticles for cancer therapy. *Mater Today Adv*. 2020;6:100066. https://doi.org/10.1016/j.mtadv.2020.100066

33. de Oliveira AAR, de Souza DA, Dias LLS, de Carvalho SM, Mansur HS, de Magalhães Pereira M. Synthesis, characterization and cytocompatibility of

spherical bioactive glass nanoparticles for potential hard tissue engineering applications. *Biomed Mater*. 2013;8(2):025011. https://doi.org/10.1088/1748-6041/8/2/025011

34. Doostmohammadi A, Monshi A, Salehi R, Fathi MH, Golniya Z, Daniels AU. Bioactive glass nanoparticles with negative zeta potential. *Ceram Int*. 2011;37(7):2311–2316. https://doi.org/10.1016/j.ceramint.2011.03.026

35. El-Fiqi A, Kim T-H, Kim M, *et al.* Capacity of mesoporous bioactive glass nanoparticles to deliver therapeutic molecules. *Nanoscale*. 2012;4(23):7475. https://doi.org/10.1039/c2nr31775c

36. El-Kady AM, Ali AF. Fabrication and characterization of ZnO modified bioactive glass nanoparticles. *Ceram Int*. 2012;38(2):1195–1204. https://doi.org/10.1016/j.ceramint.2011.07.069

37. Serra J, González P, Liste S, *et al.* FTIR and XPS studies of bioactive silica based glasses. *J Non Cryst Solids*. 2003;332(1–3):20–27. https://doi.org/10.1016/j.jnoncrysol.2003.09.013

38. Luz GM, Mano JF. Preparation and characterization of bioactive glass nanoparticles prepared by sol–gel for biomedical applications. *Nanotechnology*. 2011;22(49):494014. https://doi.org/10.1088/0957-4484/22/49/494014

39. Pirayesh H, Nychka J a. Sol-gel synthesis of bioactive glass-ceramic 45S5 and its in vitro dissolution and mineralization behavior. *J Am Ceram Soc*. 2013;96(5):1643–1650. https://doi.org/10.1111/jace.12190

40. Barrioni BR, Norris E, Jones JR, Pereira M de M. The influence of cobalt incorporation and cobalt precursor selection on the structure and bioactivity of sol–gel-derived bioactive glass. *J Sol-Gel Sci Technol*. 2018;88(2):309–321. https://doi.org/10.1007/s10971-018-4823-7

41. Musat V, Fortunato E, Botelho AM, Monteiro R. Sol – gel cobalt oxide – silica nanocomposite thin films for gas sensing applications. 2008;516:1499–1502. https://doi.org/10.1016/j.tsf.2007.07.197

42. Toufiq AM, Wang F, Javed Q, Li Y. Influence of SiO2 on the structure-controlled synthesis and magnetic properties of prismatic MnO2 nanorods. *Nanotechnology*. 2013;24(41):415703. https://doi.org/10.1088/0957-4484/24/41/415703

43. Dalby KN, Nesbitt HW, Zakaznova-Herzog VP, King PL. Resolution of bridging oxygen signals from O 1s spectra of silicate glasses using XPS: Implications for O and Si speciation. *Geochim Cosmochim Acta*. 2007;71(17):4297–4313. https://doi.org/10.1016/j.gca.2007.07.005

44. Shruti S, Salinas AJ, Malavasi G, *et al.* Structural and in vitro study of cerium, gallium and zinc containing sol–gel bioactive glasses. *J Mater Chem*. 2012;22(27):13698–13706. https://doi.org/10.1039/c2jm31767b

45. Mekki A, Salim M. XPS study of transition metal doped silicate glasses. *J Electron Spectros Relat Phenomena*. 1999;101(October):227–232. https://doi.org/10.1016/S0368-2048(98)00450-2

46. Verné E, Bretcanu O, Balagna C, *et al.* Early stage reactivity and in vitro behavior of silica-based bioactive glasses and glass-ceramics. *J Mater Sci Mater Med*. 2009;20(1):75–87. https://doi.org/10.1007/s10856-008-3537-8

47. Ni M, Ratner BD. Differentiation of Calcium Carbonate Polymorphs by Surface Analysis Technique - An XPS and TOF-SIMS study. *Natl Institue Heal*. 2014;40(10):1356–1361. https://doi.org/10.1002/sia.2904.Differentiation

48. Demri B, Muster D. XPS study of some calcium compounds. *J Mater Process Technol*. 1995;55(3–4):311–314. https://doi.org/10.1016/0924-0136(95)02023-3

49. Biesinger MC, Payne BP, Grosvenor AP, Lau LWM, Gerson AR, Smart RSC. Resolving surface chemical states in XPS analysis of first row transition metals, oxides and hydroxides: Cr, Mn, Fe, Co and Ni. *Appl Surf Sci*. 2011;257(7):2717–2730. https://doi.org/10.1016/j.apsusc.2010.10.051

50. Elkabouss K, Kacimi M, Ziyad M, Ammar S, Bozon-Verduraz F. Cobalt-exchanged hydroxyapatite catalysts: Magnetic studies, spectroscopic investigations, performance in 2-butanol and ethane oxidative dehydrogenations. *J Catal*. 2004;226(1):16–24. https://doi.org/10.1016/j.jcat.2004.05.007

51. Chuang TJ, Brundle CR, Rice DW. Interpretation of X-Ray Photoemission Spectra of Cobalt Oxides and Cobalt Oxide Surfaces. *Surf Sci*. 1976;59:413–429.

52. Kramer E, Itzkowitz E, Wei M. Synthesis and characterization of cobalt-substituted hydroxyapatite powders. *Ceram Int*. 2014;40(8 PART B):13471–13480. https://doi.org/10.1016/j.ceramint.2014.05.072

53. Poologasundarampillai G, Wang D, Li S, *et al.* Cotton-wool-like bioactive glasses for bone regeneration. *Acta Biomater*. 2014;10(8):3733–46. https://doi.org/10.1016/j.actbio.2014.05.020

54. Lin Z, Jones JR, Hanna J V, Smith M. A multinuclear solid state NMR spectroscopic study of the structural evolution of disordered calcium silicate sol-gel biomaterials. *Phys Chem Chem Phys*. 2015;17(4):2540–9. https://doi.org/10.1039/c4cp04492d

55. Maçon ALB, Jacquemin M, Page SJ, *et al.* Lithium-silicate sol–gel bioactive glass and the effect of lithium precursor on structure–property relationships. *J Sol-Gel Sci Technol*. 2016;(June). https://doi.org/10.1007/s10971-016-4097-x

56. Haimi S, Gorianc G, Moimas L, *et al.* Characterization of zinc-releasing three-dimensional bioactive glass scaffolds and their effect on human adipose stem cell proliferation and osteogenic differentiation. *Acta Biomater*. 2009;5(8):3122–3131. https://doi.org/10.1016/j.actbio.2009.04.006

57. Nawaz Q, Atiq M, Rehman U, *et al.* Synthesis and characterization of manganese containing mesoporous bioactive glass nanoparticles for biomedical applications. *J Mater Sci Mater Med*. 2018;5. https://doi.org/10.1007/s10856-018-6070-4

58. Clupper DC, Gough JE, Hall MM, Clare AG, Lacourse WC, Hench LL. In vitro bioactivity of S520 glass fibers and initial assessment of osteoblast attachment. *J Biomed Mater Res*. 2003;(67A):285–294.

59. Rohanová D, Boccaccini AR, Horkavcov D, Bozdechová P, Bezdicka P, Castorálová M. Is non-buffered DMEM solution a suitable medium for in vitro bioactivity tests ? *J Mater Chem B*. 2014;2:5068–5076. https://doi.org/10.1039/c4tb00187g

60. Christodoulou I, Buttery LDK, Saravanapavan P, Tai G, Hench LL, Polak JM. Dose- and time-dependent effect of bioactive gel-glass ionic-dissolution products on human fetal osteoblast-specific gene expression. *J Biomed Mater Res - Part B Appl Biomater*. 2005;74(1):529–537. https://doi.org/10.1002/jbm.b.30249

61. Goel A, Rajagopal RR, Ferreira JMF. Influence of strontium on structure, sintering and biodegradation behaviour of CaO-MgO-SrO-SiO2-P2O5-CaF2 glasses. *Acta Biomater*. 2011;7(11):4071–4080. https://doi.org/10.1016/j.actbio.2011.06.047

62. Quinlan E, Partap S, Azevedo MM, Jell G, Stevens MM, O'Brien FJ. Hypoxia-mimicking bioactive glass/collagen glycosaminoglycan composite scaffolds to enhance angiogenesis and bone repair. *Biomaterials*. 2015;52:358–366. https://doi.org/10.1016/j.biomaterials.2015.02.006

63. Zhang M, Wu C, Li H, Yuen J, Chang J, Xiao Y. Preparation, characterization and in vitro angiogenic capacity of cobalt substituted β-tricalcium phosphate ceramics. *J Mater Chem*. 2012;22(40):21686. https://doi.org/10.1039/c2jm34395a

64. Fleury C, Petit A, Mwale F, *et al.* Effect of cobalt and chromium ions on human MG-63 osteoblasts in vitro: Morphology, cytotoxicity, and oxidative stress. *Biomaterials*. 2006;27(18):3351–3360. https://doi.org/10.1016/j.biomaterials.2006.01.035

65. Vicente S, Moia C, Zhu H, Vigé X. In vitro evaluation of the internalization and toxicological profile of silica nanoparticles and submicroparticles for the design of dermal drug delivery strategies. *J Appl Toxicol*. 2017;37(12):1396–1407. https://doi.org/10.1002/jat.3507

66. Wang X, Tolba E, Der HCS, *et al.* Effect of bioglass on growth and biomineralization of saos-2 cells in hydrogel after 3d cell bioprinting. *PLoS One*. 2014;9(11):1–7. https://doi.org/10.1371/journal.pone.0112497

67. Kargozar S, Lotfibakhshaiesh N, Ai J, *et al.* Strontium- and cobalt-substituted bioactive glasses seeded with human umbilical cord perivascular cells to promote bone regeneration via enhanced osteogenic and angiogenic activities. *Acta Biomater*. 2017;58:502–514. https://doi.org/10.1016/j.actbio.2017.06.021

68. Mozafari M, Rabiee M, Azami M, Maleknia S. Biomimetic formation of apatite on the surface of porous gelatin/bioactive glass nanocomposite scaffolds. *Appl Surf Sci*. 2010;257(5):1740–1749. https://doi.org/10.1016/j.apsusc.2010.09.008

Figure Captions

Figure 1: Bright field TEM images of nanoparticles (a) DNP, (b) DNP-0Co, (c) DNP-25Co and (d) DNP-50Co. DNP = SiO_2; DNP-0Co = SiO_2-8CaO-0CoO; DNP-25Co = SiO_2-4.5CaO-3.1CoO; DNP-50Co = SiO_2-2.2CaO-4.6CoO (mol%).

Figure 2: (a) FTIR spectra and (b) XRD patterns of dense glass nanoparticles. DNP = SiO_2; DNP-0Co = SiO_2-8CaO-0Co; DNP-25Co = SiO_2-4.5CaO-3.1CoO; DNP-50Co = SiO_2-2.2CaO-4.6CoO (mol%).

Figure 3: X-Ray Photoelectron Spectroscopy (XPS) wide scan spectra of dense bioactive glass nanoparticles: DNP = SiO_2; DNP-0Co = SiO_2-8CaO-0Co; DNP-25Co = SiO_2-4.5CaO-3.1CoO; DNP-50Co = SiO_2-2.2CaO-4.6CoO (mol%).

Figure 4: X-Ray Photoelectron Spectroscopy (XPS) spectra of glass nanoparticles (a) Si 2p, (b) O 1s, (c) Ca 2p and (d) Mn 2p core level. DNP = SiO_2; DNP-0Co = SiO_2-8CaO-0Co; DNP-25Co = SiO_2-4.5CaO-3.1CoO; DNP-50Co = SiO_2-2.2CaO-4.6CoO (mol%).

Figure 5: ^{29}Si solid state MAS NMR spectra for dense bioactive glass nanoparticles samples. DNP = SiO_2; DNP-0Co = SiO_2-8CaO-0Co; DNP-25Co = SiO_2-4.5CaO-3.1CoO; DNP-50Co = SiO_2-2.2CaO-4.6CoO (mol%).

Figure 6: Si, Ca and Co concentrations ($\mu g.mL^{-1}$) in DMEM as measured by ICP-OES after immersion of dense bioactive nanoparticles during different soaking times. DNP = SiO_2; DNP-0Co = SiO_2-8CaO-0Co; DNP-25Co = SiO_2-4.5CaO-3.1CoO; DNP-50Co = SiO_2-2.2CaO-4.6CoO (mol%).

Figure 7: Cell viability of DBGNPs by MTT assay for 24 and 72 h at a significance level of 0.05% (* represents significant difference compared to DMEM control, in which ***$p<0.001$ and ****$p<0.0001$). Control = culture medium; Control+ = positive control for toxicity; Control- = negative control; DNP = SiO_2; DNP-0Co = SiO_2-8CaO-0Co; DNP-25Co = SiO_2-4.5CaO-3.1CoO; DNP-50Co = SiO_2-2.2CaO-4.6CoO (mol%).

Table 1: Measured composition (mol%) of dense bioactive glasses nanoparticles as determined by acid digestion method and ICP-OES; particle size distribution as measured by DLS, zeta potential and specific surface area (BET analysis).

Samples	Measured composition (mol %)			Particle size (nm)	Zeta potential (mV)	Specific Surface Area ($m^2.g^{-1}$)
	SiO_2	CaO	CoO			
DNP	100.0 ± 0.2	0 ± 0	0 ± 0	99.8 ± 10.2	-36.3 ± 0.6	42.9 ± 1.7
DNP-0Co	92.0 ± 1.1	8.0 ± 1.2	0 ± 0	112.1 ± 13.5	-35.1 ± 1.9	39.8 ± 0.7
DNP-25Co	92.4 ± 0.5	4.5 ± 0.3	3.1 ± 0.2	111.4 ± 14.1	-22.4 ± 0.4	42.5 ± 2.1
DNP-50Co	93.2 ± 0.6	2.2 ± 0.2	4.6 ± 0.4	109.1 ± 10.6	-22.4 ± 1.4	48.1 ± 2.8

Table 2: Percentage of Q^n species, as obtained by ^{29}Si MAS NMR (vr = 5kHz), and calculated degree of condensation (D_c), fraction of non-bridging oxygen (NBO) and fraction of bridging oxygen (BO) for the DBGNPs. DNP = SiO_2; DNP-0Co = SiO_2-8CaO-0Co; DNP-25Co = SiO_2-4.5CaO-3.1CoO; DNP-50Co = SiO_2-2.2CaO-4.6CoO (mol%).

Samples	Q^2		Q^3		Q^4		Q^3/Q^4	Dc %	NBO	BO
	δ_{iso} [ppm]	/ [%]	δ_{iso} [ppm]	/ [%]	δ_{iso} [ppm]	/ [%]				
DNP	-92.1	2.0	-101.2	10.5	-111.4	87.5	0.12	96.4	0.07	0.93
DNP-0Co	-91.0	3.6	-101.2	20.7	-111.6	75.7	0.27	93.0	0.13	0.87
DNP-25Co	-93.1	0.5	-102.8	23.8	-111.8	75.7	0.31	93.8	0.12	0.88
DNP-50Co	-92.0	1.1	-103.0	29.5	-111.7	69.4	0.43	92.1	0.15	0.85