

Synthesis, characterization and in vitro cytotoxicity assessment of eggshell-derived β -CaSiO₃ nano biomaterial

ABSTRACT

This study explored the conversion of bio waste into a valued bioceramic material for potential tissue regeneration application. Calcium silicate (β -CaSiO₃) nanomaterial was synthesized from waste egg shells (CaCO₃) and silica (SiO₂) precursors using noninvasive but sustainable and industrially scalable ball mill and sonochemical techniques. The development of the β -CaSiO₃ NPs was monitored with Fourier transform infra-red spectroscopy (FTIR), thermogravimetric analysis (TGA), transmission electron microscope (TEM), field emission scanning electron microscopy/energy dispersive spectroscopy (FESEM-EDS) and X-ray diffraction (XRD). The results revealed the formation of polycrystalline nano- β -CaSiO₃ with 100 % peak at $2\theta^\circ = 30.0$ in the (320) crystal plane and sizes less than 50 nm. In vitro cytotoxicity studies on human osteoblast cell line CRL 11372 suggests biocompatibility and non-interference with cell growth at concentrations ranging from 0.0781-1.250 mg/mL of β -CaSiO₃.

Keywords: Eggshell; calcite; diffraction; bioceramic; biocompatible; crystallite.

1. INTRODUCTION

One common source of natural bio-minerals is the chicken eggshell, which comprises of CaCO₃. In the US, millions of kilograms of eggshells are produced as waste yearly [1]. Though this immensely impacts the environment and the potential of deriving functional materials from the eggshell is great, efforts for conversion of these into valuable biomaterials are limited [2]. This mismatch exists even in developed countries where increase in per capita income lead to proportionate increase in the generation of solid residues [3]. To maintain a balanced ecosystem, waste needs to be effectively and sustainably managed in innovative ways, for example by turning them into resources [4] to supplement the practice of recycling and reuse [3]. Besides the impact on environment and landscape by piles of residue, the repugnant gas (H₂S) emitted by microbes decomposing the eggshell may endanger life [3]. On the other hand, this principal untapped source of CaCO₃ can be useful to manufacture biomaterials. The eggshell contains 95% calcite crystals (CaCO₃) [5] which can be converted to β -CaSiO₃ in a more environmentally friendly way compared to artificial precursors which leave behind toxic wastes. β -CaSiO₃ possesses properties suitable for hard tissue repair, due to its biocompatibility, bioactivity and biodegradability [6], and facilitates damaged tissues in-growth to full integration with normal tissues [7]. Teeth root-end perforation repair, pulp-capping, occlusion of dentine tubules [8] and bonelike apatite formation are possible with β -CaSiO₃ [9]. Also, β -CaSiO₃ has shown promise in industrial uses in materials like plastics, ceramics, metals, paints, paper, friction products and for asbestos replacement [10]. The sol-gel and rock mining techniques for deriving CaSiO₃ are the least less sustainable. The sol-gel processes consume toxic chemical that leave behind excess acid wastes, nitrates and salts, even if bio-derived precursors are involved [11-13]. While acids are corrosive, nitrates cause eutrophication in aquatic environments, leading to the suffocation and bioaccumulation in organisms. Nitrates pollute drinking water and lead to reduced oxygen transport by hemoglobin when consumed. They are also linked to cancer of

the digestive tract, bladder and ovary, birth defects, diabetes mellitus, thyroid hypertrophy, spontaneous abortions and respiratory tract infections [14]. Rock mined CaSiO_3 require further refinement and directly lead to the loss of natural habitats, land, water and air pollutions [15]. Hence, the focus of this work was to synthesize $\beta\text{-CaSiO}_3$ NPs from waste residue as an environmentally friendly alternative to the current methods. Nanomaterials have shown better cytocompatible, mechanical, electrical, optical, catalytic and magnetic properties than their micron sized counterparts [16]. However, in dealing with NPs, their toxicity is a major concern which is as important to determine as their desirable properties. The adverse effects to normal physiology, structure of organs and tissues and to the environment by NPs depends on factors like size, shape, surface charge and chemistry, composition, and NPs stability [17-22]. The increased use of nanomaterials in society has made their toxicity a major concern [19]. Printer toners, varnishes, biomedical, cosmetic and food products are already using nanomaterials [17, 19]. The market size for nanoscale pharmaceutical products is projected to increase by US \$180 billion annually from 2010-2015 [23]. With this upsurge in nanomaterials consumption, their toxicity issues cannot be overlooked. Inhalation of 20 nm of TiO_2 at 10 mg/m³ resulted in lung tumor, while 1.4 nm size of gold spheres lead to necrosis, mitochondrial damage, and oxidative stress on cell lines compared to 15 nm [18]. These observations highlight the potential size-dependent toxicity of NPs. Also, the lifespan of mice were reduced by NPs with sizes outside the range of 8-37 nm [24]. Cytotoxic effects of cadmium-based quantum dots on Caco-2 cells at 2-200 nmol/mL concentrations showed that 200 nmol/mL was toxic [25]. Though most nanomaterials are useful, much is not known about their toxicities [19, 24]. In this study bioactive $\beta\text{-CaSiO}_3$ NPs were synthesized through techniques that avoid toxic solvents [13] by the use of eggshell-based CaCO_3 and SiO_2 as precursors. Their cytotoxic effects were examined *in vitro* using human osteoblast cells.

2. MATERIAL AND METHODS

2.1 Separation of CaCO_3 precursor from eggshells

Egg shells were obtained from American dehydrated food Inc., Atlanta Georgia, USA and processed to separate the CaCO_3 crystals. The eggshell consists of ordered layer of calcified shell precisely arranged on three types of collagen and glycosaminoglycan's. Separation of the CaCO_3 requires the denaturing of the organic platform. Hence the eggshells were boiled at 100°C in an electric cooker (Hamilton Beach 33157) for 6 h and blended in a warring blender at high speed for 10 min. This was thoroughly washed with water in the initial stages and ethanol in the final stage before air-drying at room temperature for 24 h.

2.2 Synthesis of calcium silicate ($\beta\text{-CaSiO}_3$)

Beta- CaSiO_3 was synthesized from combined eggshell-source CaCO_3 and SiO_2 (obtained from Nanostructured & Amorphous materials Inc. NM, USA) by ball milling (Spex Sample Prep 8000D). A 1:1 mixture of CaCO_3 and SiO_2 was ball milled for 100 minutes in two separate canisters, each containing 5 g of the precursors and 8 pieces of 6 mm steel balls. About 8 grams of the mixture was then sintered to 1000°C at a rate of 10°C min⁻¹ and allowed to stay for 3 h before cooling to room temperature in a vacuum tube furnace (GLS-1300X). The product (~5 g) was crushed in a ceramic mortar and ball milled again for 10 h in 10 mL polypropylene glycol medium. It was then washed 4 times using ethanol and centrifuged at 12000 rpm for 10 min in Beckman Coulter (ALLEGRA-64R). This was then dispersed in 50 mL ethanol and ultrasonicated in Sonics vibra cell (WCX 750) at amplitude of 50 %, temperature of 25 °C for 3 h, then centrifuged at 12000 rpm in Beckman Coulter (ALLEGRA-64R) before vacuum drying for 24 h.

91 **2.3 Characterization of the precursors and β -CaSiO₃**

92 **2.3.1 Fourier transforms infrared spectroscopy**

93 Fourier transform infrared spectroscopy (FTIR) for functional groups analysis of the
94 synthesized CaSiO₃ was done using Shimadzu (FTIR-8400S) equipped with IR Solutions
95 software.

97 **2.3.2 X-Ray Diffraction (XRD) Analysis**

98
99 X-Ray diffraction pattern of the precursors and the synthesized material and intermediates
100 were determined using a Rigaku (D-max 2100) equipped with monochromatic CuK α
101 radiation (λ = 0.15406 nm). These tests were carried out at 40 KV and 30 mA, 5 °C min⁻¹
102 sampling rate, 0.020 sampling width from 3 °C to 80 ° of 2 θ degrees.

104 **2.3.4 Thermogravimetric Analysis (TGA)**

105 Thermogravimetric analysis of the ball milled mixture was carried out to understand the
106 transformations of the precursors to β -CaSiO₃, using TA Q500 instrument at 10 °C/min up to
107 980 °C under N₂ gas at a flow rate of 60 mL/min.

109 **2.3.5 Electron Microscopy-Energy Dispersive Spectroscopy**

110 Transmission electron microscopy (TEM) was carried using JOEL-2010 microscope. The
111 sample was prepared by dispersing 1 mg powder in 5 mL of ethanol for 10 min in an
112 ultrasonic bath. A drop was placed on a copper grid (200 meshes) for analysis. The
113 microstructures and elemental composition of the β -CaSiO₃ NPs were determined using field
114 emission scanning electron microscope/energy dispersive spectroscopy (FESEM/EDS)
115 JEOL-7000 F operated at 10 kV.

117 **2.4 Cell cytotoxicity studies**

118 **2.4.1 Preparation of β -CaSiO₃ stock suspensions**

119 A stock suspension of β -CaSiO₃ NPs was prepared as 10 mg/mL of phosphate buffered
120 saline (PBS). The stock was serially diluted into the desired concentrations using 9
121 autoclaved vials. About 608 μ L of media was put into the first vial while 320 μ L was put in
122 the remaining eight. Then 32 μ L of the stock was added to the first vial to make a total of 640
123 μ L. This was vortexed and 320 μ L pipetted and serially transferred from the second to the
124 ninth vial.

126 **2.4.2 Cell culture and attachment**

127 Cells were grown in Dulbecco's modified eagle's media (DMEM) containing 10% fetal bovine
128 serum (FBS) and antibiotic (100 units/mL penicillin-G, and 100 μ g/mL of streptomycin) in 100
129 mm diameter dishes. These were incubated at 37 °C, in a humidified incubator supplied with
130 5 % CO₂. Frozen fibroblasts (ATCC, CRL 11372) cells were thawed in warm water and
131 transferred into the dishes containing culturing medium and incubated again under the same
132 conditions for 24 h. The cells were removed from the incubator and 5 mL solution made of
133 0.25 % trypsin and 0.03 % ethylene diamine tetra acetic acid (EDTA) was added to detach
134 the cells from the dishes. These were then transferred into a 15 mL tube and centrifuged at
135 900 rpm for 5 min to pellet the cells. The supernatant was replaced with 1 mL of DMEM and
136 mixed to re-disperse the cells. Cell concentration was determined by loading a 10 μ L aliquot

of the cell suspension onto a counting hamber slide, and TC 10 automated cell counter (Bio-RAD, Hercules, CA) was used to count the cells. A final dilution of cells was made based on the cell count, to make a volume of 3 mL containing 1.0×10^5 cell/mL and used in the experiments.

2.4.3 Treatment of cell with CaSiO₃ Suspension

Approximately 100 μ L of the cell solution was pipetted out of the 3 mL (1.0×10^5 cells/mL) into each of 21 wells of a 96-well plate arranged in triplicates. This was incubated for 24 h to allow cells to attach to the plate surface. The medium was aspirated and 100 μ L of the various serially diluted concentrations of CaSiO₃ NPs were added as treatments in triplicates. The first set of three wells served as negative control (only cells and media) while the second served as positive control (10 μ L of 10 μ M staurosporine and 100 μ L cell each). These were again incubated for 24 h and 48 h in different well plates.

2.4.4 Cell Titer-Glo Viability Assay

Cell viability was determined by Cell Titer-Glo assays (Promega Corp) which measured the luminous intensity of the chemical action of the enzyme luciferase on luciferin substrate. The enzymatic reaction uses Adenosine Triphosphate (ATP) provided only by living cells. This serves as a measure for the viability of treated cells. The degree of enzymatic reaction measured by light emission indicates the degree of cell viability in a well. This reaction and light generation were measured using a micro plate reader (BioTek, Winooski, VT). In each well, 100 μ L of Cell Titer-Glo reagent was added and agitated in an incubator for 2 min at 30 rpm. This was removed and left at room temperature for 10 min before measuring the luminosities.

2.4.5 Optical Microscopy

Optical micrographs of the control and cells treated with 100 μ L of each concentration of β -CaSiO₃ used in the cytotoxicity assays were taken with Olympus IX -71 inverted microscope (model IX2-ILL100) equipped with Olympus SC30 camera and CellSens imaging software. The cells and treatments were incubated in sterile 60 x 15 mm well plates for 5 days. Micrographs were taken at 24 h intervals up to the fifth day to assess the cell interactions with the CaSiO₃ and compare with the viability results.

3. RESULTS AND DISCUSSION

3.1 Monitoring of CaSiO₃ development from the precursors

Thermogravimetric analysis (TGA) was used to monitor thermal transitions related to the reaction between the mixture of CaCO₃ and SiO₂ (1:1) during decomposition and subsequent formation of β -CaSiO₃ from egg shell precursor and to establish the temperature range within which the reaction will spontaneously proceed. Figure 1 shows TGA thermograms obtained during such transition with (a) percent weight lost and its (b) derivative curve. The various transitions can be seen in the derivative weight loss curve in Figure 1b. It shows the first transition at about 100 °C which was attributed to absorbed moisture by the sample.

The second transition occurred around 500 °C due to the escape of carbon dioxide gas, thus as CaCO₃ is heated up to 500 °C it decomposes to calcium oxide and carbon dioxide (CaO and CO₂). A transition between 550 °C and 700 °C was attributed to phase changes in the reactants to an amorphous phase of calcium silicate. Between 700 - 800 °C a stable form of CaSiO₃ was formed with no noticeable change in weight as seen on the curve. Report show

that optimal stabilization of β -CaSiO₃ occurs within this range while crystallization occurs afterwards [26]. The transition at 850 °C was attributed to changes from amorphous to crystalline phases. Based on this information it became apparent that CaSiO₃ was obtainable if the reactants are subjected to controlled temperatures up to 1000 °C. X-ray diffraction performed on the residue after TGA analysis revealed polycrystalline diffracted peaks which matched wollastonite.

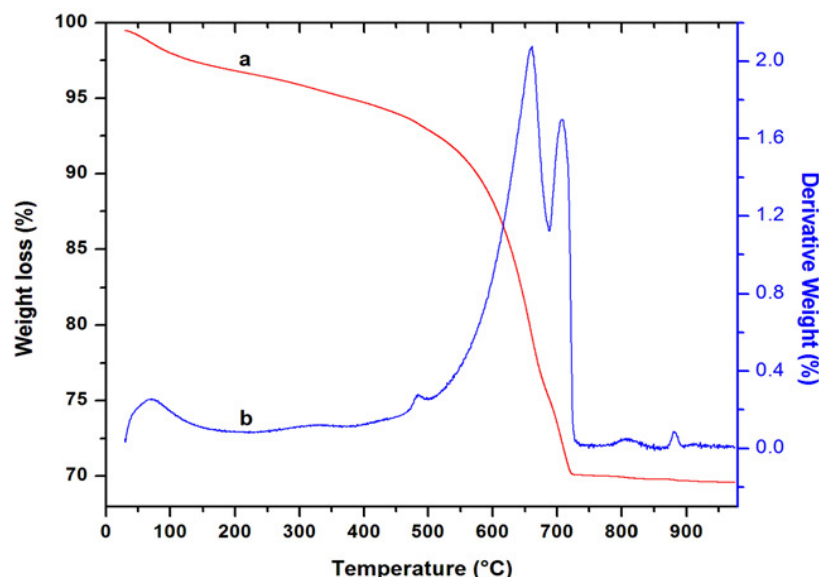


Fig.1. TGA thermograms depicting the formation of β -CaSiO₃: a) weight loss vs temperature, b) derivative weight loss

FTIR analysis was also performed on both the mixture and the TGA residue. This was done to determine the functional groups present in the precursors prior to the heating and those in the residue after the heating. Figure 2 shows FTIR spectra obtained from the mixed precursors and the residue samples. Characteristic stretching bands associated with bonds in β -CaSiO₃ were identified between wavenumbers 850-1000 cm⁻¹. The stretching bands observed on the sintered sample curve (a) was absent in the mixture of reactants in curve (b) prior to TGA analysis. The absence of these bands is an indication that no reaction occurs at this stage between the reactants during the ball milling process.

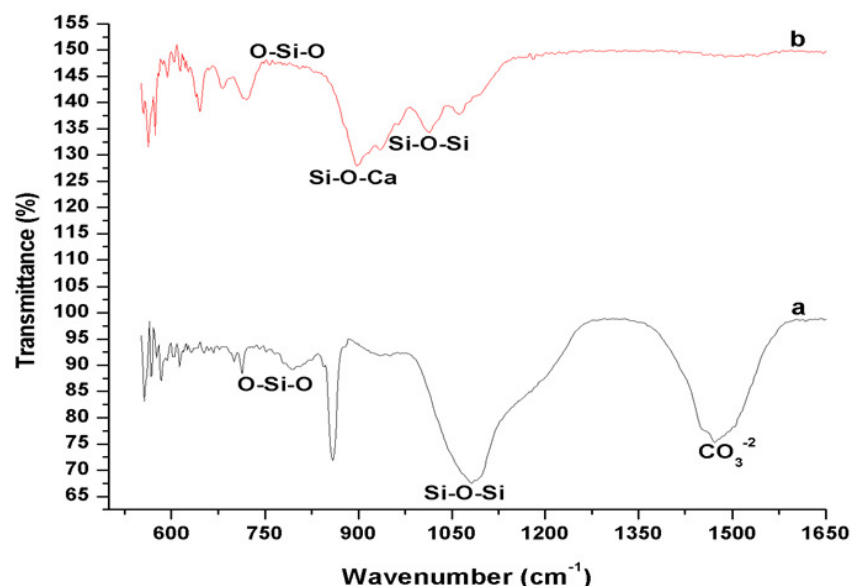


Fig. 2. FTIR Spectra: (a) CaCO₃ and SiO₂ mixture, (b) residue after Tga analysis

In curve (b), the stretching band between 1410-1490 cm⁻¹ (asymmetric stretch vibration) and at 856 cm⁻¹ (out-of-plane bend vibration) has been reported to be due to vibration of carbonate ion (CO₃⁻²) [10, 26]. This affirmed that the precursors still existed as a mixture. This broad stretch between 850 -1000 cm⁻¹ is due to Si-O-Ca vibration, an indication that β-CaSiO₃ was formed. Similar result has been reported in a sol-gel derived β-CaSiO₃ from calcium nitrate and tetraetoxysilane, using water, acetic acid, ammonia and nitric acids as catalysts [27]. Symmetric stretching vibrations of Si-O-Si bonds are also observed at 1080 cm⁻¹ while the stretching mode of O-Si-O featured at 800 cm⁻¹ [28].

3.2 X-ray Characterization of CaCO₃ (egg shell) and SiO₂ precursors

The membrane of an eggshell consists of three layers which include inner membrane, limiting membrane surrounding the egg white shell and the outer shell. These shells are formed by ordered layers made up of 95 % CaCO₃ (calcite) deposited on ~ 3.5% organic matrix (mainly fibrillar proteins with disulphides) cross-linked to three types of collagen (I, V and X) and glycosaminoglycans [5]. Hence, the eggshells were processed to separate the crystalline phase from these organic templates and characterize using XRD. Figure 3a shows an X-ray diffraction pattern for these powdered particles (micron size) with a Bragg's angle of the 100 % peak at 2θ = 29.4 in the (104) crystal plane. This matched CaCO₃ (calcite) with JCPDS-Pdf file # 47-1743 in the data base and is also consistent with past findings on this inorganic mineral [5, 13].

The amorphous silicon dioxide (15 nm sizes) precursor was characterized as received. Figure 3b shows the broad characteristic amorphous SiO₂ peak. This peak has an equivalent Bragg's angle of 2θ = 21.8° and conforms to previous reports where 15-30 nm amorphous silica was precipitated [29]. Crystalline CaCO₃ is heavier than the amorphous SiO₂. In order to uniformly mix the two for reaction in a solid state without phase separations, the mixing has to be such that they can stick to each other. Hence, the mixture of CaCO₃ and amorphous SiO₂ were ball-milled for 100 minutes to form a uniform blend for the solid state reaction.

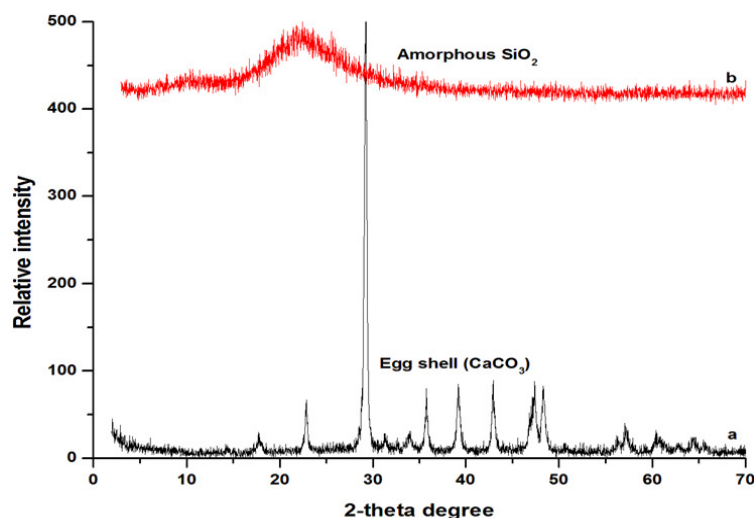


Fig.3. X-ray diffraction patterns of the precursors: (a) eggshell, (b) silica

Figure 4 presents X-ray diffraction patterns used in monitoring the development of β -CaSiO₃ during the synthesis process. In this figure, diffraction pattern (a) denotes SiO₂ + CaCO₃ ball milled for 100 minutes. This pattern is identical to that of calcite, but also shows the amorphous portion due to SiO₂ (extended region between $2\theta = 15-25^\circ$ with one crystal plane of CaCO₃ merging with it as shown in Figure 4a). This indicates that no new material was formed at this stage of the synthesis process contrasting some past report [30]. This is probably due to the use of precursors from different sources, differences in the energy of the equipment and powder to ball ratio.

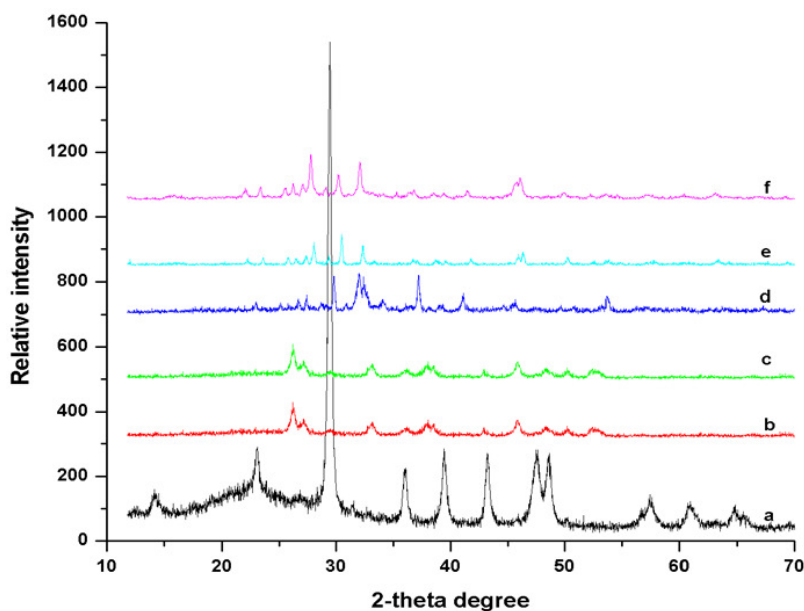


Fig. 4. X-ray diffraction analysis for monitoring the development of β -CaSiO₃ during synthesis: (a) precursors ball milled for 100 minutes, (b,c) precursors ball milled for 10 and 20 h respectively, (d) Tga residue of the material in a, (e,f) material in a and b in sintered at 1000 °C respectively.

The TGA residue of the material in (a) is the one in (d) which reveals a pattern analogous to that of β -CaSiO₃ with some imperfections. The patterns denoted b and c are for SiO₂ and CaCO₃ subjected to ball milling for 10 and 20 hrs respectively. These patterns are unique from that in (a), indicating the formation of a new material. However this could not be identified by the JCPDS database, though past reports have shown similar patterns as calcium silicate hydrates (CSH), obtained by grinding mixtures of calcium hydroxide and SiO₂ in different amounts of water [31] and another after mixing different ratios of calcium oxide and amorphous SiO₂ and aging in water for 7 days [32]. This means that our approach of ball milling for a maximum of 20 hrs does not yield *Wollastonite*. When the materials in a and b were sintered at 1000 °C for 3 hrs, the XRD pattern of the resulting materials matched **wollastonite** (β -CaSiO₃) as shown in the curves e and f. This curves has similar diffraction pattern as the one in d confirming that the transitions observed in the preliminary monitoring of the reaction by TGA can be mimicked in a furnace to generate the same product. It became apparent after these series of initial experiments that if the required controlled conditions can be provided then nanoscale β -CaSiO₃ can be synthesized using the eggshell (bio sourced CaCO₃) and amorphous SiO₂ precursors through these techniques. Therefore a combination of ball milling, sintering and sonochemical techniques were used to synthesis β -CaSiO₃ NPs. Sonochemical technique was used as an additional step to observe the effect of ultrasonic irradiation on the particle sizes and agglomerations.

3.3 Characterization of the β -CaSiO₃ nanomaterial

Sintering causes particles of the β -CaSiO₃ to stick together. Hence ball milling became a necessity after sintering. β -CaSiO₃ NPs synthesized through a cycle of ball milling, sintering and ball milled again, is designated as BSB and those synthesized by ball milling, sintering, ball milled again followed by ultra sonication is designated as BSBU.

The sonochemical technique is imperative in the synthesis of NPs because of its success of in minimizing particle-to-particle affinity, size reduction and morphology modification. The results of both methods are shown in the X-ray diffraction pattern in Figure 5.

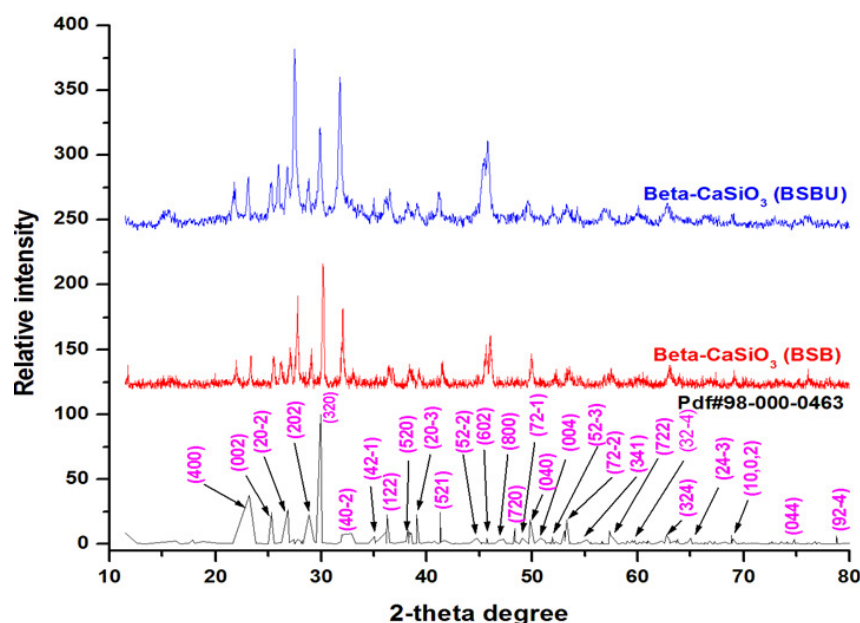


Fig. 5. X-ray diffraction patterns of β -CaSiO₃ treated with two different techniques

293 These patterns reveal prominent peak intensities at Bragg's angles, $2\theta^\circ = 23.2, 25.3, 26.8,$
294 $28.9, 30.0, 32.9, 35.4, 36.2, 38.2, 39.1, 41.3, 44.7, 45.7, 47.3, 48.3, 49.1, 49.7, 51.9, 53.1,$
295 $53.3, 55.2, 57.3, 59.5, 62.7, 65.0, 68.9, 74.8$ and 78.8 . These angles correspond to the
296 crystal planes of standard β -CaSiO₃ shown in Figure 5 in an ascending order and matched
297 JCPDS Pdf file #98-000-0463.

298 The diffraction pattern labelled BSBU suggest the particle sizes are smaller in this material
299 than that of BSB. This is because the peaks are broader than those of BSB. The broad
300 nature of these peaks signifies the presence of NPs but can also be due to strain or defects
301 in the crystalline material [17]. The narrow peaks in BSB could mean large individual
302 particles of several agglomerates of small particles existed in the material. Particle size
303 reduction can be associated with the random collision of the balls against the particles and
304 the walls of the canisters several times in a second during the ball milling. Singh and
305 Karmakar (2011) [30] reported the synthesis of CaSiO₃ through a solid state room
306 temperature ball milling using CaCO₃ and SiO₂ in a sequential step mechanism they termed:
307 comminution of raw materials, recombination of comminuted raw material to final product,
308 and comminution of the final product to smaller sizes. Other reports have shown the
309 mechanochemical synthesis of calcium silicate hydrates (CSH) and Sol-gel aging process
310 [31, 32].

311 The relevance of the sonochemical technique has been phenomenal, especially in the
312 modification of inorganic materials [33]. Highly intense ultra sound irradiated liquids produce
313 acoustic cavitation which drives bubble formation and collapse. This leads to the generation
314 of intense and confined heating, high pressures and extreme short lifetimes capable of
315 inducing high energy reactions [34]. The hot spots can rise to 5000°C with about 1000
316 atmospheres with heating and cooling rates more than 10×10^{11} K/s. Cavity collapse close
317 to extended solid surface trigger very fast moving jets of liquid into the surface, and creates
318 shock wave damage to the surface [35]. The physical process responsible for the
319 sonochemical modifications of inorganic materials are; enhancements in mass transport
320 from chaotic mixing and acoustic streaming, generation of damages at the solid-liquid
321 interfaces by shock wave and streams of micro jets, development of high-speed inter-
322 particle collisions in slurries and fragmentation of brittle solid to increase surface areas
323 [35]. Hence, further reduction in particle sizes as shown by the XRD pattern of BSBU sample
324 was due to ultrasonic effects. Particle size of β -CaSiO₃ was confirmed with TEM results
325 shown in Figure 6a-c. They reveal the presence of irregular shaped polycrystalline β -CaSiO₃
326 with diameters less than 50 nm, supporting the XRD results.

327 Ball milling is simple, cheap, applicable to many types of materials and easily scalable to
328 large quantity production. This technique subject's materials severe plastic deformation with
329 compressive loads through continues impacts of the balls on a material. This can produce
330 nanosized materials with superior properties [36]. Also, the sonochemical approach is highly
331 effective in inducing particle break down to nanoscale, dispersion of agglomerates and the
332 production of large quantities of materials [37]. The combination of these two techniques
333 therefore constitutes an effective method for the synthesis of β -CaSiO₃ NPs. The TEM
334 results showed particles in the range of 15 - 30 nm, indicating the effectiveness of the
335 approach. This also conforms to 21 nm size particles produced in ball milling reported
336 elsewhere [30].

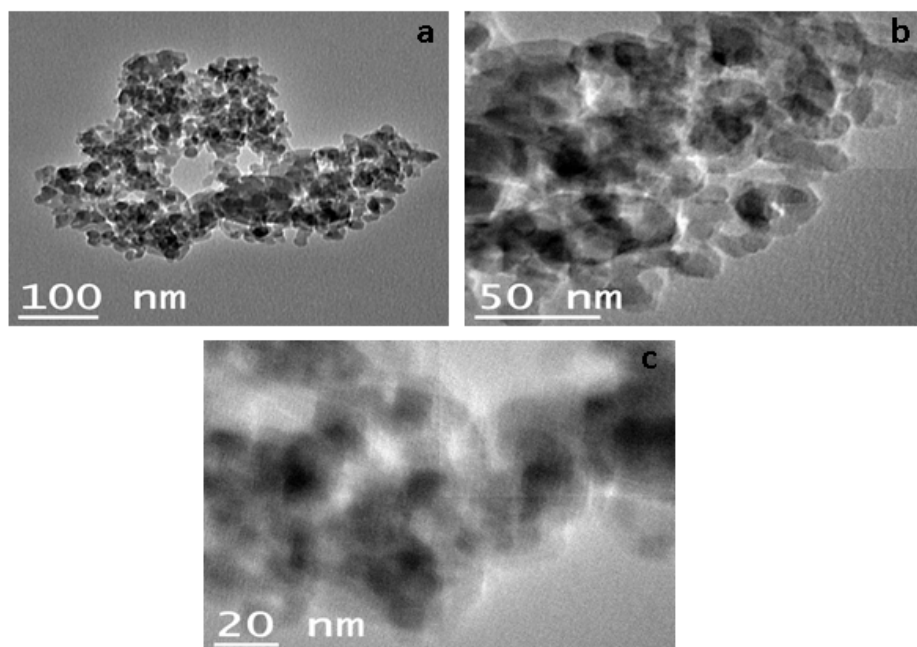


Fig. 6. TEM micrographs of the β -CaSiO₃ NPs; (a) low, (b) medium, (c) high magnification

The FESEM micrograph in Figure 7 further supports the XRD and TEM results. In that, the β -CaSiO₃ synthesized was in the nanometer range as very small particles are evident on the micrograph in Figure 7a. The results for elemental analysis of the rectangular region in Figure 7a by energy dispersive spectroscopy (EDS) revealed the presence of only Ca, Si and O in Figure 7b. This strongly suggests that β -CaSiO₃ was the main material without any detectable impurities in this region.

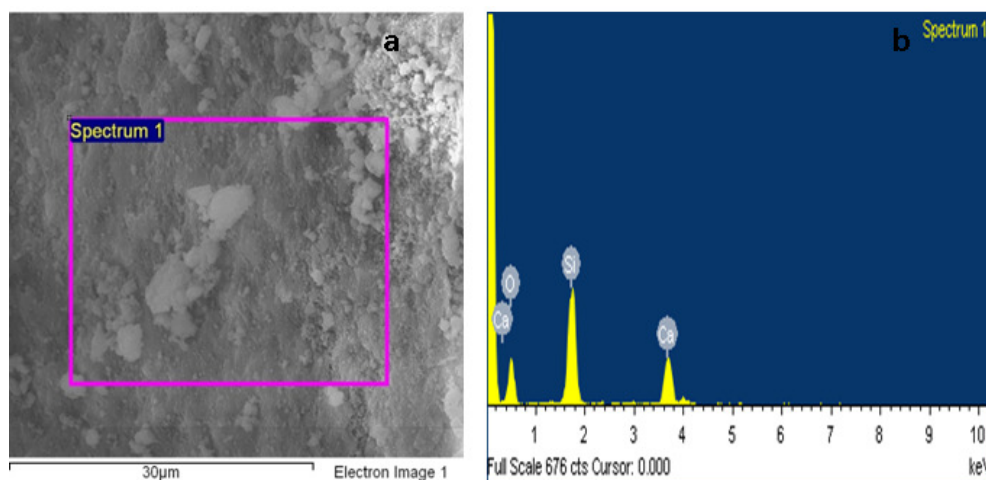


Fig. 7. Scanning electron microscope (SEM) results: (a) micrograph of β -CaSiO₃ NPs, (b) EDS of β -CaSiO₃ NPs.

3.4 Cytotoxic effect of β -CaSiO₃

The potential toxicity of NPs has become a major concern as these particles have the ability to interact and disrupt the growth, structure and functions of organs and tissues in living organisms [18]. Toxicity has been extensively investigated and has been found to depend on physiochemical factors such as particle size, shape or morphology, surface charge and chemistry, composition, and subsequent NPs stability [18-32]. High surface area and porosity that characterizes our synthesized calcium silicate nanomaterials make them suitable for tissue regeneration applications. In order to assess the biocompatibility of these newly synthesized materials, we undertook cytotoxicity tests in a controlled experiment based on established cell viability protocols. In this study the effect of β -CaSiO₃ on the viability of human osteoblast cells (ATCC CRL 11372) was investigated. Viability assays are used to assess the overall dose-dependent toxicity of nanoparticles in cultured cells, examining cell survival and proliferation parameters after exposure to the particles [38]. Results of cytotoxicity studies presented in Figure 8 show the status of osteoblast cells during incubation with various concentrations of β -CaSiO₃ NPs for 24 and 48 hrs. The results show that at the tested doses of NPs, including the highest NP concentration of 1.25 mg/mL, the viability of the osteoblast cells was not different from that of control cells (CM). This indicates that β -CaSiO₃ nanoparticles at these concentrations do not adversely affect the physiological activities of the osteoblast cell line. In contrast, cells in the control wells treated with a cytotoxic drug, staurosporine (STS), showed a significant loss in viability, indicating the sensitivity of the cells towards cytotoxic agents.

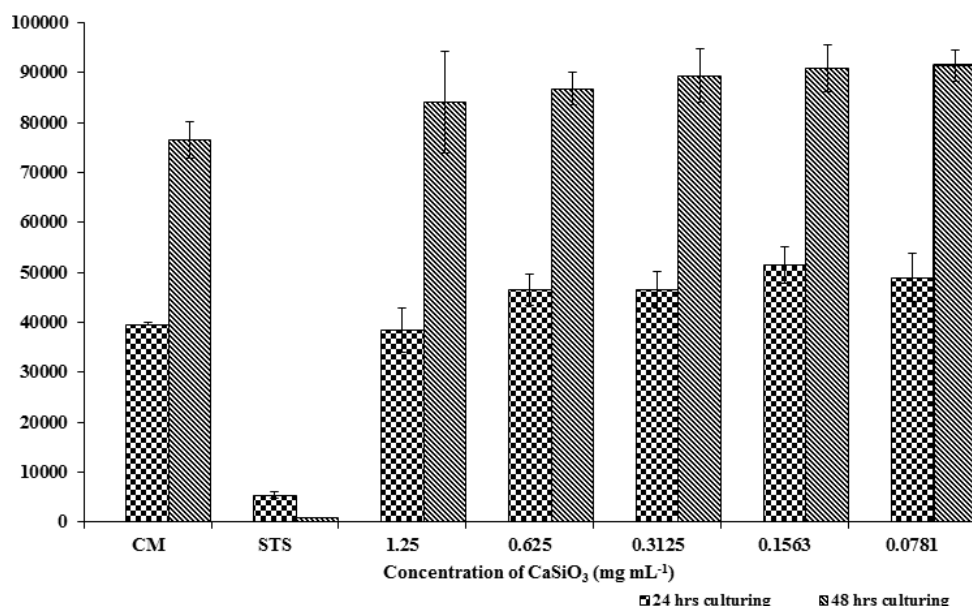


Fig. 8. Luminous intensity in CRL-1137 osteoblast cells after 24 and 48 h incubation with β -CaSiO₃ NPs

If the NPs were toxic a dose dependent reduction of viability would be expected. These results are in agreement with past findings on wollastonite and other silicates in bone-bonding and soft tissue regeneration [39,40]. Also, *in vitro* evaluation of wollastonite and dicalcium silicate coatings seeded with osteoblast cells showed formation of apatite layers on their surfaces [41]. Also, calcium silicate hydraulic cements designed for dentistry was found to be promoting the formation of bone-like apatite, based on the composition of the

390 culturing media and the aging time [9]. These suggest that β -CaSiO₃ is a good candidate for
 391 apical bone healing, particularly when it is doped with alpha-tricalcium phosphate [42]. The
 392 Ca and Si ions in β -CaSiO₃ play key roles in the nucleation and growth of HA and have great
 393 influence on the metabolism of osteoblast cells; this is very relevant in mineralization and
 394 bone-bonding mechanism [43]. Based on our *in vitro* cell line findings, β -CaSiO₃ is a
 395 potential candidate for tissue regenerating applications.
 396

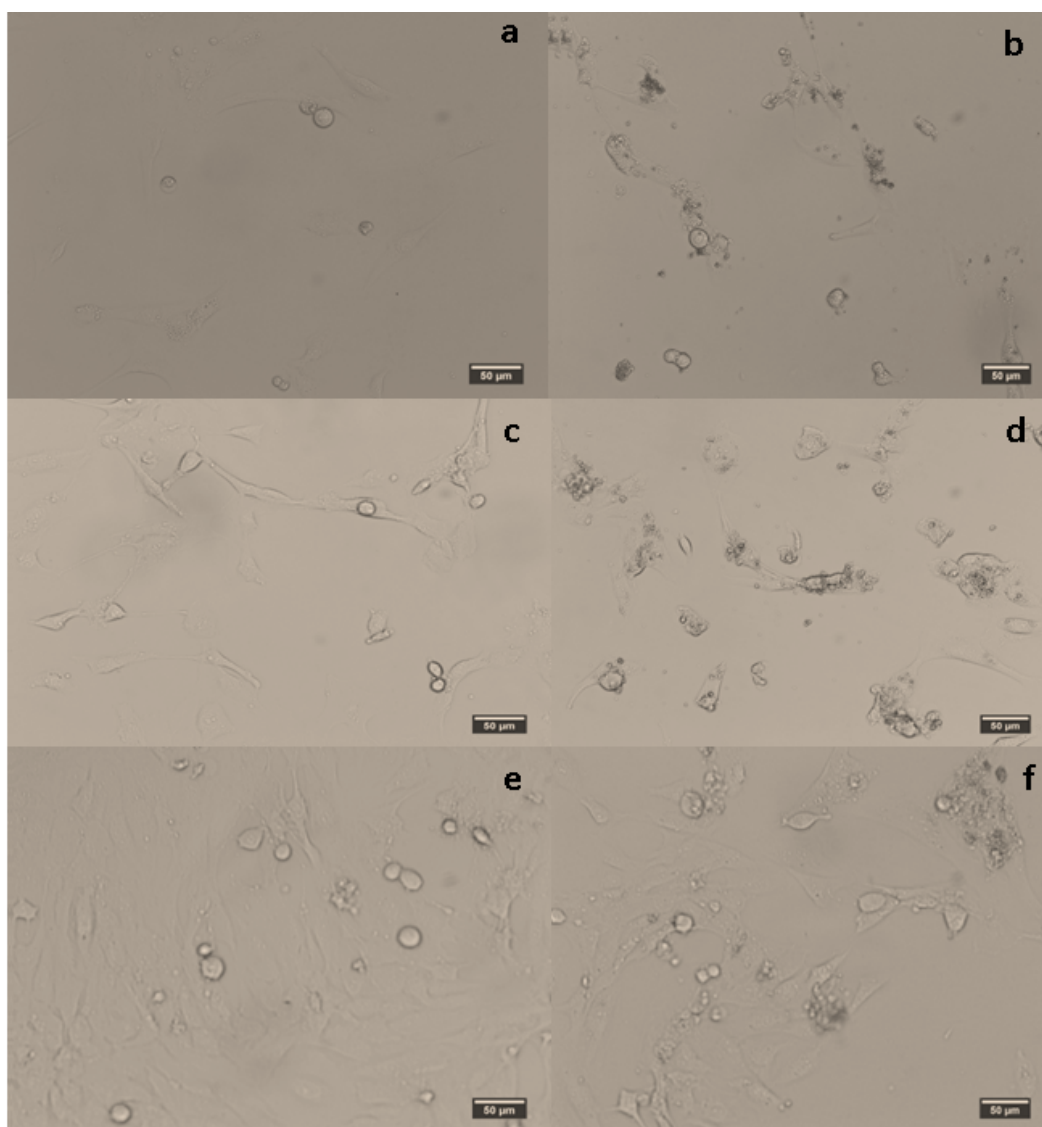


Fig. 9. Optical micrographs:(a,c,e) cultured cell only,(b,d,f) cultured cells with β -CaSiO₃ NPs

Since cytotoxicity may vary with different cell types[20, 24], testing of the NPs *in vivo* are warranted to validate their potential utility for tissue engineering. The optical micrographs in Figure 9 a,c and show the morphology of the untreated cells as they grew from day 1, 3 and 5 respectively. Panels b, d to f depicts the cells incubated with 0.1536 mg/mL concentrations

of β -CaSiO₃ NPs from day 1 (b) to 5 (f). Both isolated and agglomerated particles attached to the cells can be observed on these micrographs (Figure 9 b to f). These micrographs show the absence of osteoblast cell death in the presence of the NPs up to the fifth day, even in the presence of agglomerates. For instance, as depicted in Figure 9, micrograph f taken on day 5 shows more cells per field compared to day 1 (b), suggesting that cell proliferation was not hampered. Also the cells in the first days were visible as singlets, but by the fifth day they had expanded to occupy the plate surface due to continued division. Since this trend is also visible in the control cells (a - e) we conclude that the NPs did not interfere with cell proliferation.

4. CONCLUSION

The conversion of the eggshell to β -CaSiO₃ through a sustainable approach was successful. CaCO₃ separated from eggshells was reacted with SiO₂ at high temperature. The development of β -CaSiO₃ during the synthesis process was monitored using FTIR, XRD TGA. FESEM-EDS used for elemental analysis showed that the materials contain only Ca, Si and O while the TEM micrograph showed the particles in nanometer dimensions. The characterization showed that the use of only mechanical attrition on CaCO₃ and SiO₂ precursors could only produce calcium silicate hydrates (CSH). However, addition steps of sintering (3 hours) re-ball milling and ultra sonication in ethanol resulted into a β -CaSiO₃ phase with particle sizes less than 50 nm. Cytotoxicity tests on human osteoblast cell line ATCC CRL 11372 indicated that the β -CaSiO₃ is biocompatible, an indication that it can be used as a tissue regenerative material and other biomedical engineering applications upon further investigation. This is essentially a conversion of bio waste into a useful bio material. Such findings are promising and suggest that the natural and more environmentally benign eggshells can substitute toxic precursors in the synthesis of multifunctional β -CaSiO₃ and others like calcium silicate hydrates with the use of sustainable techniques, instead of discarding it in the already waste populated landfills.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

- Walton HV, Cotteril OJ, Vandepop JM. Composition of shell waste from egg breaking plants. Poult. Sci. 1973;52(5):1836-41.
- Adams RG, Franklin MR. Vacuum treatment of an input stream without ruining delicate output fractions. US Pat. 7017 277 B1, 2006.
- Felipe-Sese M, Eliche-Quesada D, Corpas-Iglesias FA. The use of solid residues derived from different industrial activities to obtain calcium silicates for use as insulating construction materials. Ceram. Int. 2011;37(8):3019-28.
- Dodson RJ, Hunt JA, Parker LH, Yang Y, Clark HJ. Elemental sustainability: Towards the total recovery of scarce metals. Chem. Eng. Process. 2012;51:69-78.
- Nys Y, Gautron J, Garcia-Ruiz JM, Hincke MT. Avian eggshell mineralization biochemical and functional characterization of matrix proteins. CR Palevol. 2004;3(6-7): 549-56.
- Lin K, Chang J, Liu Z, Zeng Y, Shen R. Fabrication and characterization of 45S5 bioglass reinforced macroporous calcium silicate bioceramics. J. Eur. Ceram. Soc. 2009;29(14):2937-43.

- 459 7. Hench LL, Polak JM. Third-generation biomedical materials.Science. 2002;
460 295(5557):1014-17.
- 461 8. Gandolfi MG, Farascioni S, Pashley DH, Gasparotto G,Prati C. Calcium silicate coating
462 derived from portland cement as treatment for hypersensitive dentine. J. Dent.
463 2008;36(8):565-78.
- 464 9. Gandolfi MG, Ciapetti G, Taddei P, Perut F, Tinti A, Cardoso MV, et al. Apatite
465 formation on bioactive calcium-silicate cements for dentistry affects surface topography
466 and human marrow stromal cells proliferation.Dent. Mater. 2010;26(10):974-92.
- 467 10. Thompson SP, Day SJ, ParkerJE, Evans A, Tang CC. Fine-grained amorphous calcium
468 silicate CaSiO_3 from vacuum dried sol-gel-production, characterization and thermal
469 behavior. J. Non-Cryst. Solids. 2012;358(5):885-92.
- 470 11. Kaili L, Wanyin Z, Siyu N, Jiang C, Yi Z, Weijun Q. Study of mechanical property and
471 *in vitro* biocompatibility of CaSiO_3 ceramics. Ceram. Int. 2005;31 (2):323-26.
- 472 12. Siyu N, Jiang C, Lee C, WanyinZ. Comparison of Osteoblast-like cell response to
473 calcium silicate and tricalcium phosphate ceramic in vitro. J. Biomed. Mater. Res. Part
474 B: Appl. Biomater. 2007;80B(1):174-83.
- 475 13. Nuchnapa T, Tunchanoke K, Supawinee K, Ruksapong K, Anuvat S. An innovative
476 CaSiO_3 dielectric material from eggshells by sol-gel process. J. Sol-Gel. Sci. Technol.
477 2011;58(1):33-41.
- 478 14. Julio AC, Alvaro, A. Ecological and toxicological effects of inorganic nitrogen pollution in
479 aquatic ecosystems: A global assessment. Environ. Int. 2006;32 (6):831-49.
- 480 15. Juan CA, Rodney A, Klaus SL, Francis M, Edward R, Juan CS, Katsunori Y, Ron Z.
481 Mineral carbonation and industrial uses of carbon dioxide. In: Bert M, Ogunlade D,
482 Heleen de C, Manuela L, Leo L. (Eds.). Carbon dioxide capture and storage. United
483 Kingdom: Cambridge University Press; 2005.
- 484 16. Zhang L, Webster JT. Nanotechnology and nanomaterials: Promises for improved
485 tissue regeneration. Nano Today. 2009;4(1):66-80.
- 486 17. Li X, Wang L, Fan Y, Feng Q, Cui F-Z. Biocompatibility and toxicity of nanoparticles
487 and nanotubes. J. Nanomater. 2012;19. DOI:10.1155/2012/548389.
- 488 18. Buzea C, Blandino PII,Robbie K. Nanomaterials and nanoparticles: Sources and
489 toxicity. Biointerphases. 2007;2(4):17-172.
- 490 19. Kong B, Seog HJ, Graham LM, Lee BS. Experimental considerations on the cytotoxicity
491 of nanoparticles. Nanomedicine-UK. 2011;6(5):929-41.
- 492 20. Shinde SK, Grampurohit ND, Gaikwad DD, Jadhav L,Gadhav MV,Shelke PK. Toxicity
493 induce by nanoparticles. Asian Pac. J. Trop. Dis. 2012;2(4):331-34.
- 494 21. Ai J, Biazar E, Jafarpour M, Montazeri M, Majdi A, Aminifard S, et al. Nanotoxicology
495 and nanoparticle safety in biomedical designs. Int. J.Nanomed. 2011;6:1117-27.
- 496 22. Sohaebuddin KS, Thevenot TS, Baker D, Eaton WJ, Tang L. Nanomaterial cytotoxicity
497 is composition, size, and cell type dependent. Part. Fibre Toxicol. 2010;7:22. DOI:
498 10.1186/1743-8977-7-22.
- 499 23. Webster JT. Nanomedicine: real commercial potential or just hype? Int. J. Nanomed.
500 2006;1(4):373-374.
- 501 24. Alkilany MA, Catherine J, Murphy JM. Toxicity and cellular uptake of gold
502 nanoparticles: what we have learned so far? J. Nanopart. Res. 2010;12 (7): 2313-33.
- 503 25. Wang L, Nagesha KD, Selvarasah S, Dokmeci RM, Carrier LR. Toxicity of CdSe
504 Nanoparticles in Caco-2 Cell Cultures. J. Nanobiotechnology. 2008; 6:11.
505 DOI:10.1186/1477-3155-6-11.
- 506 26. Meiszterics A, Sinko K. Sol-gel driven calcium silicate ceramics. Colloid Surfaces A.
507 2008;319(1-3):143-48.
- 508 27. Saravanapavan P, Hench LL. Mesoporous calcium silicate glasses I. Synthesis. J.
509 Non-Cryst. Solids. 2003;318 (1): 1-13.
- 510 28. Saito F, Mi G, Hanada M. Mechanochemical synthesis of hydrated calcium silicates by
511 room temperature grinding. Solid State Ionics.1997;101-103(1):37-43.

- 512 29. Musić S, Filipović-Vinceković N, Sekovanić L. Precipitation of amorphous SiO₂ particles
513 and their properties. *Braz. J. Chem. Eng.* 2001;28(4):89 -94.
- 514 30. Singh SP, Karmakar B. Mechanochemical synthesis of nano calcium silicate particles
515 at room temperature. *New J. Glass Ceram.* 2011;1(2):49-52.
- 516 31. Sugiyama D. Chemical alteration of calcium silicate hydrate (C-S-H) in sodium chloride
517 solution. *Cement and Concrete Res.* 2008;38(11):1270-75.
- 518 32. Rangari KV, Mohammad MG, Jeelani S, Butenko VY, Dhanak RV. Synthesis and
519 characterization of diamond-coated CNTs and their reinforcement in nylon-6 single
520 fiber. *ACS. Appl. Mater. Inter.* 2010; 2(7):1829-34.
- 521 33. Suslick KA. Sonochemistry of transition metal compounds. In: King BR. (Eds.),
522 *Encyclopedia of inorganic chemistry*. Vol.7. New York: J. Wiley & Sons;1994.
- 523 34. Suslick KS, Crum LA. Sonochemistry and Sonoluminescence, In: Crocker MJ.(Eds.),
524 *Encyclopedia of Acoustics*. Vol.1. New York:Wiley-Interscience;1997.
- 525 35. Suslick KS, Fang MM, Hyeon T, Mdeleleni MM. Applications of sonochemistry to
526 materials synthesis. In: sonochemistry and sonoluminescence. Crum LA, Mason TJ,
527 Reiss J, Suslick KS. (Eds.). Netherlands; Kluwer Publishers:1999.
- 528 36. Rao BJ, Catherin JG, Murthy NI, Rao VD, Raju NB. Production of nano structured
529 silicon carbide by high energy ball milling. *Int. J. Eng. Sci. Technol.* 2011;3(4):82-88.
- 530 37. Saravanan P, Gopalan R., Chandrasekaran V. Synthesis and characterization of
531 Nanomaterials. *Defence Sci. J.* 2008;58(4):504-16.
- 532 38. Sprio S, Tampieri A, Celotti G, Landi E. Development of hydroxyapatite/calcium silicate
533 composites addressed the design of load-bearing bone scaffolds. *J. Mech. Behav.*
534 *Biomed. Mater.* 2009; 2 (2):147-55.
- 535 39. Fujiwura M, Shiokawa K, Kubota T. Direct encapsulation of proteins into calcium
536 silicate by water/oil/water interfacial reaction method and their responsive release
537 behaviors. *Mater. Sci. Eng. C.* 2012;32(8):2484-90.
- 538 40. Liu X, Ding C, Chu KP. Comparison of in-vitro evaluation of wollastonite and dicalcium
539 silicate coating. *Key Eng. Mater.* 2005;288-289:359-62.
- 540 41. Gandolfi GM, Taddei P, Tinti A, Dorigo SDE, Prati C. Alpha-TCP improves the apatite-
541 formation ability of calcium-silicate hydraulic cement soaked in phosphate solutions.
542 *Mater.Sci. Eng.C.* 2011;31:1412-22.
- 543 42. Wu C, Chang J, Wang J, Ni SE, Zhai W. Preparation and characteristics of a calcium
544 magnesium silicate (bredigite) bioactive ceramic. *Biomaterials.* 2005; 26 (16):2925-31.
- 545 43. Christenson EM, Anseth KS. van den Beucken JJ, Chan CK, Ercan B, Jansen J A, et
546 al. Nanobiomaterial Applications in Orthopedics. *J. Orthopaed. Res.* 2007;25(1):11-22.