

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

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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 is mainly CaCO₃. In the US, millions of kilograms of eggshells are produced as waste yearly [1]. Though this immensely impacts the environment, the potential of deriving functional materials from the eggshell is great. Efforts for conversion 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 need 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]. Beside the impact on environment and landscape by piles of residue, the repugnant gas (H₂S) emitted by microbes decomposing the eggshell may endanger life [3]. However, 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-

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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 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 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 CaSiO₃ 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 β -CaSiO₃ 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, and is equally 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 TiO₂ 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 15nm [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 β -CaSiO₃ NPs were synthesized through techniques that avoid toxic solvents [13] by the use of eggshell-based CaCO₃ and SiO₂ as precursors. Their cytotoxic effects were examined *in vitro* using human osteoblast cells.

2. MATERIAL AND METHODS

2.1 Separation of CaCO₃ precursor from eggshells

Egg shells were obtained from American dehydrated food Inc., Atlanta Georgia, USA and processed to separate the CaCO₃ crystals. The eggshell consists of ordered layer of calcified shell precisely arranged on three types of collagen and glycosaminoglycans. Separation of the CaCO₃ 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 (β -CaSiO₃)

Beta-CaSiO₃ was synthesized from combined eggshell-source CaCO₃ and SiO₂ (obtained from Nanostructured & Amorphous materials Inc. NM, USA) by ball milling (Spex Sample Prep 8000D). A 1:1 mixture of CaCO₃ and SiO₂ was ball milled for 100 min in two separate canisters, each containing 5 g of the precursors and 8 pieces of 6 mm steel balls. About 8 g

of the mixture was then sintered from room temperature to 1000°C at a rate of 10°C min⁻¹ and allowed to stay for 3 h before cooling back 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.

2.3 Characterization of the precursors and β -CaSiO₃

2.3.1 Fourier transforms infrared spectroscopy

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

2.3.2 X-Ray Diffraction (XRD) Analysis

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

2.3.4 Thermogravimetric Analysis (TGA)

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

2.3.5 Electron Microscopy-Energy Dispersive Spectroscopy

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

2.4 Cell cytotoxicity studies

2.4.1 Preparation of β -CaSiO₃ stock suspensions

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

2.4.2 Cell culture and attachment

Cells were grown in Dulbecco's modified eagle's media (DMEM) containing 10% fetal bovine serum (FBS) and antibiotic (100 units/mL penicillin-G, and 100 μ g/mL of streptomycin) in 100

mm diameter dishes. These were incubated at 37 °C, in a humidified incubator supplied with 5 % CO₂. Frozen fibroblasts (ATCC, CRL 11372) cells were thawed in warm water and transferred into the dishes containing culturing medium and incubated again under the same conditions for 24 h. The cells were removed from the incubator and 5 mL solution made of 0.25 % trypsin and 0.03 % ethylene diamine tetra acetic acid (EDTA) was added to detach them from the dishes. These were then transferred into a 15 mL tube and centrifuged at 900 rpm for 5 min to pellet the cells. The supernatant was replaced with 1 mL of DMEM and mixed to re-disperse the cells. Cell concentration was determined by loading a 10 µL 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 x 10⁵ cell/mL and used in the experiments.

2.4.3 Treatment of cell with CaSiO₃ Suspension

Approximately 100 µL of the cell solution was pipetted out of the 3 mL (1.0 x 10⁵ 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 µL 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 µL of 10 µM staurosporine and 100 µL 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 µL 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 µL 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 is due to absorbed moisture in the sample.

The second transition occurs at around 500 °C, due to the escape of carbon dioxide gas, thus as CaCO_3 is heated up to 500 °C it decomposes to calcium oxide and carbon dioxide (CaO and CO_2). A transition between 550 °C and 700 °C is as a results of the transformation of the reactants to an amorphous phase of calcium silicate. Between 700 - 800 °C a stable form of CaSiO_3 was formed with no noticeable change in weight as seen on the curve. Report show that optimal stabilization of $\beta\text{-CaSiO}_3$ occurs within this range while crystallization occurs afterwards [26]. The transition at 850 °C is attributed to changes from amorphous to crystalline phase. Based on this information it became apparent that CaSiO_3 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*.

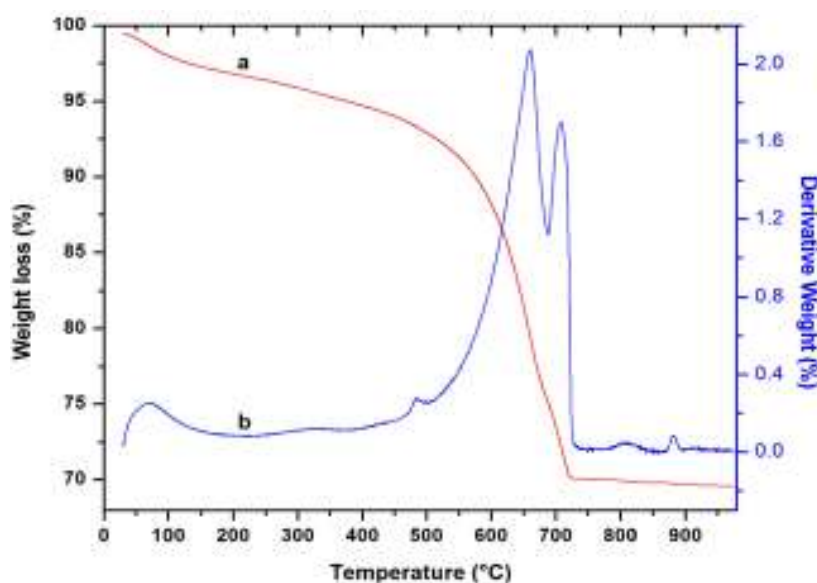


Fig. 1. TGA curves depicting the formation of $\beta\text{-CaSiO}_3$: a) weight loss vs temperature, b) derivative weight loss

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 formation of a stable product. Figure 2 shows FTIR spectra obtained from the mixed precursors and the residue samples. Characteristic stretching bands associated with bonds in $\beta\text{-CaSiO}_3$ were identified between wavenumbers $850\text{-}1000\text{ cm}^{-1}$. These stretching bands observed on the sintered sample (a) are 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.

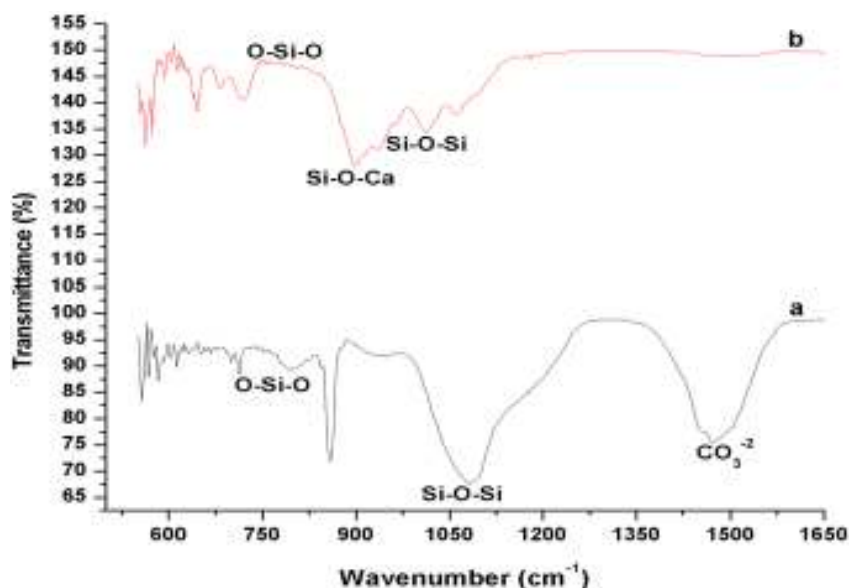


Fig. 2. FTIR Spectra: (a) CaCO_3 and SiO_2 mixture, (b) residue after TGA analysis

In curve (b), the stretching band between $1410\text{--}1490\text{ cm}^{-1}$ (asymmetric stretching vibration) and at 856 cm^{-1} (out-of-plane bending vibration) has been reported to be due to vibration of carbonate ion (CO_3^{2-}) [10, 26]. This affirmed that the precursors still existed as a mixture. The broad stretch between $850\text{--}1000\text{ cm}^{-1}$ is due to Si-O-Ca vibration, an indication that $\beta\text{-CaSiO}_3$ was formed. Similar result has been reported in a sol-gel derived $\beta\text{-CaSiO}_3$ from calcium nitrate and tetraethoxysilane, 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 CaCO_3 (egg shell) and SiO_2 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 % CaCO_3 (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 characterized using XRD. Figure 3a shows an X-ray diffraction pattern for the eggshell particles (micron size) with a Bragg's angle of the 100 % peak at $2\theta = 29.4$ in the (104) crystal plane. This matched CaCO_3 (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) precursor was characterized as received. Figure 3b shows the broad characteristic amorphous SiO_2 peak. This peak has an equivalent Bragg's angle of $2\theta = 21.8^\circ$ and conforms to previous reports where 15-30 nm amorphous silica was precipitated [29]. Crystalline CaCO_3 is heavier than the amorphous SiO_2 . 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 onto each other. Hence, the mixture of CaCO_3 and amorphous SiO_2 were ball-milled for 100 min to form a uniform blend for the solid state reaction.

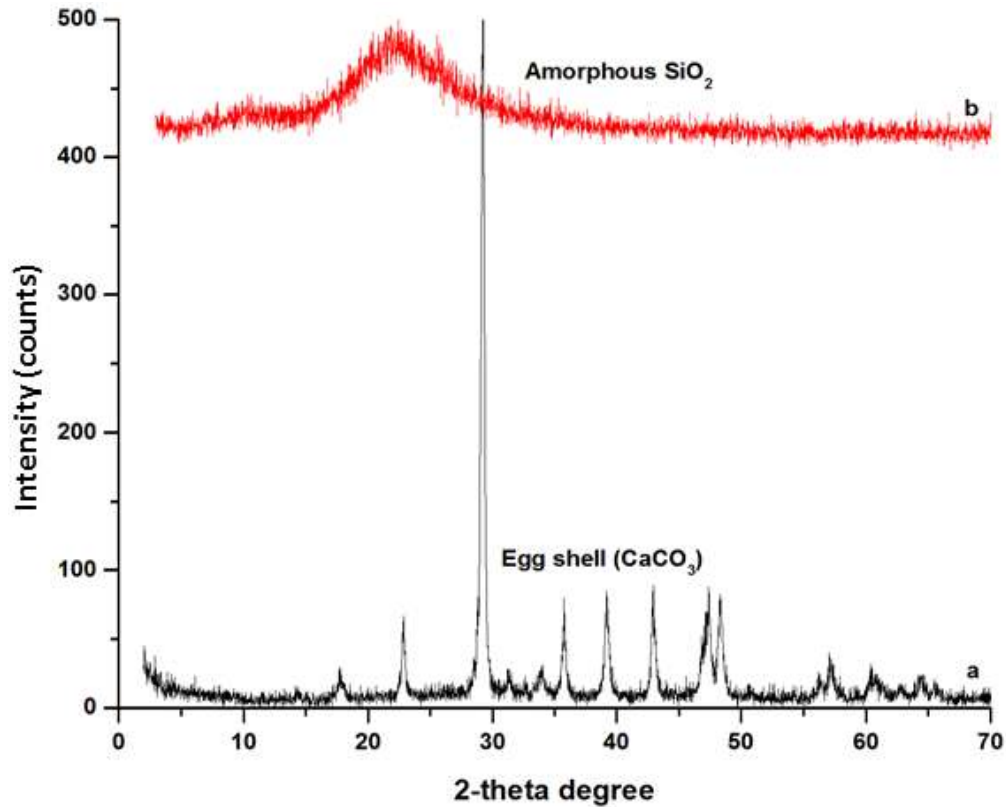


Fig. 3. X-ray diffraction patterns of the precursors: (a) eggshell, (b) silica

Figure 4 presents the X-ray diffraction patterns used for 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]. However, the use of different precursors, differences in the energy of the equipment and powder to ball ratio could be the reason for the different outcome.

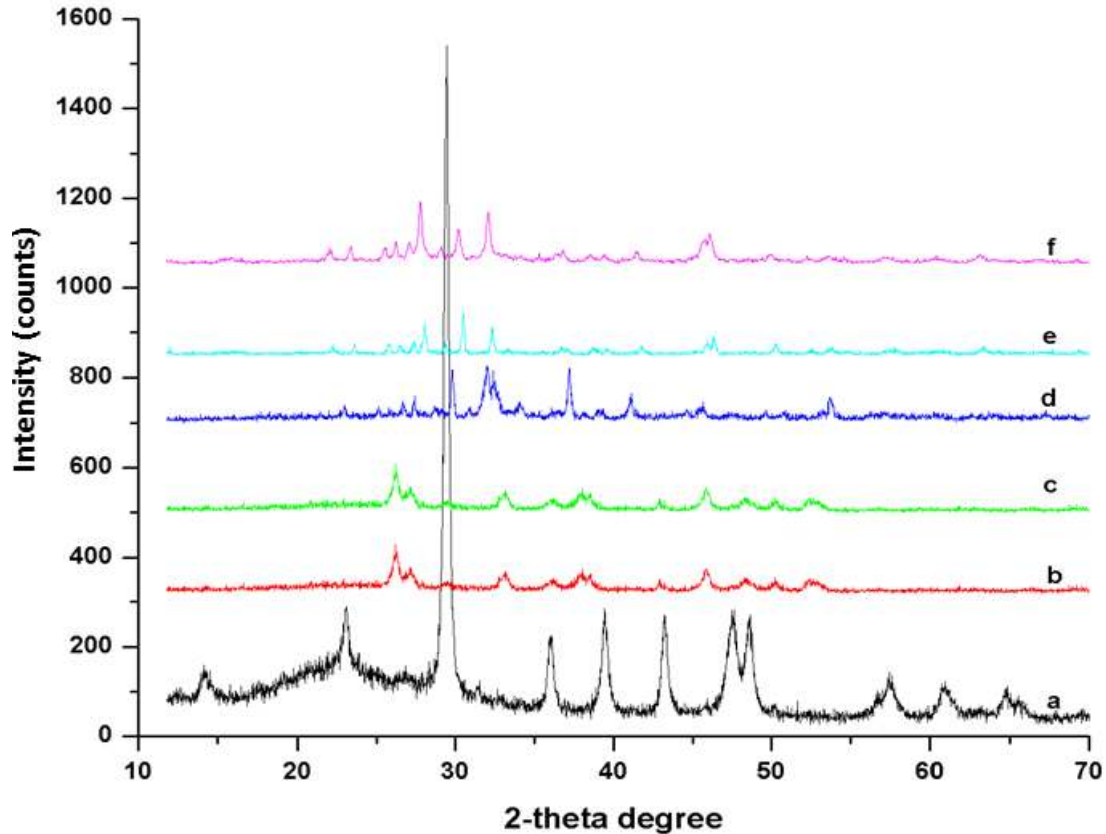


Fig. 4. X-ray diffraction analysis for monitoring the development of β -CaSiO₃ during synthesis: (a) precursors ball milled for 100 min, (b,c) precursors ball milled for 10 and 20 h respectively, (d) Tga residue of the material in a, (e,f) material in a and b sintered at 1000 °C respectively .

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 h 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 h does not yield *Wollastonite*. When the materials in a and b were sintered at 1000 °C for 3 h, the XRD pattern of the resulting materials matched *wollastonite* (β -CaSiO₃) as shown in the curves e and f. These curves have 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 yield the same product. It became apparent after these series of initial experiments that if the required controlled conditions are provided, 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 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.

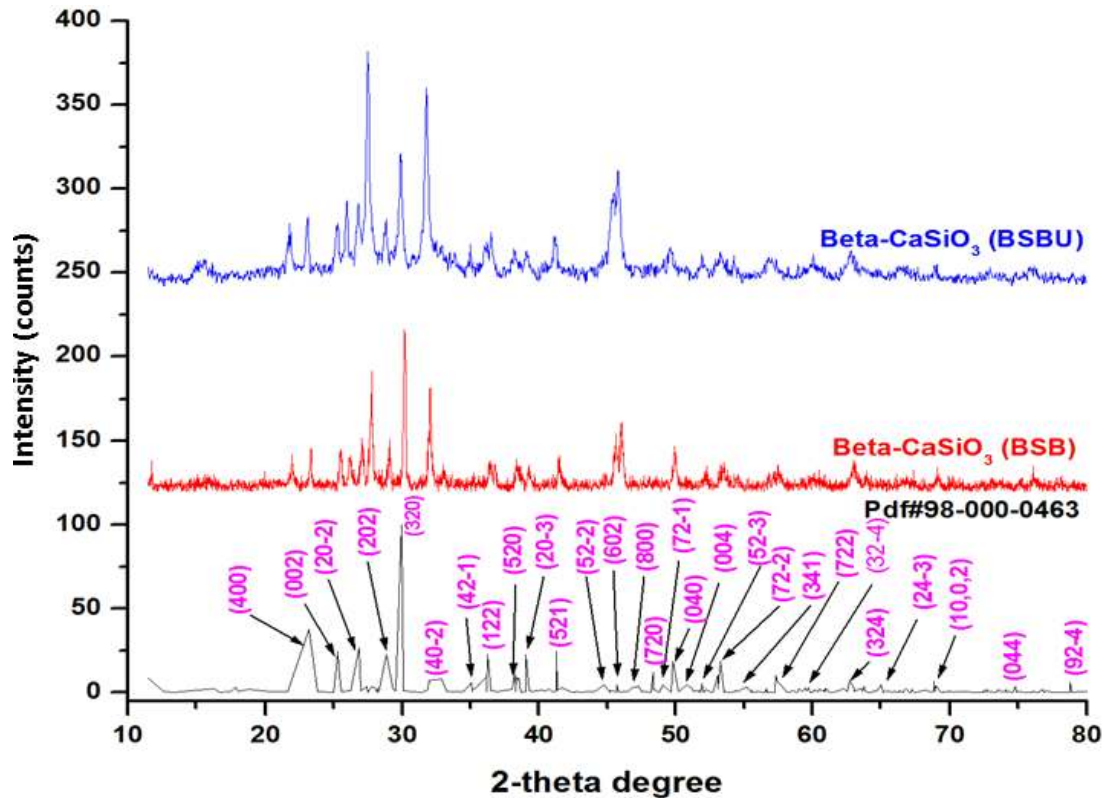


Fig. 5. X-ray diffraction patterns of β -CaSiO₃ subjected to two different treatments

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

The diffraction pattern of BSBU suggest that the particle sizes are smaller in this material than in BSB. This is because the peaks are broader than those of BSB. The broad nature of these peaks signifies the presence of NPs but can also be due to strain or defects in the crystalline material [17]. The narrow peaks in BSB could mean large individual particles or several agglomerates of small particles existed in the material. Particle size reduction can be associated with the random collision of the balls against the particles and the walls of the canisters several times in a second during the ball milling. Singh and Karmakar (2011) [30] reported the synthesis of CaSiO₃ through a solid state room temperature ball milling using CaCO₃ and SiO₂ in a sequential step mechanism they termed: comminution of raw

materials, recombination of comminuted raw material to final product, and comminution of the final product to smaller sizes. Other reports have shown the mechanochemical synthesis of calcium silicate hydrates (CSH) and Sol-gel aging process [31, 32].

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

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

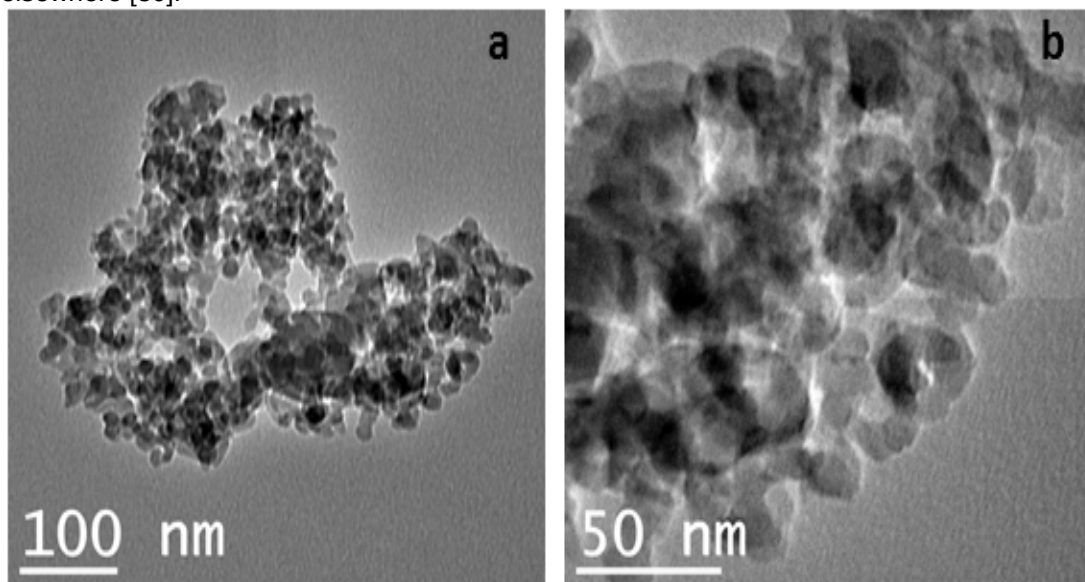
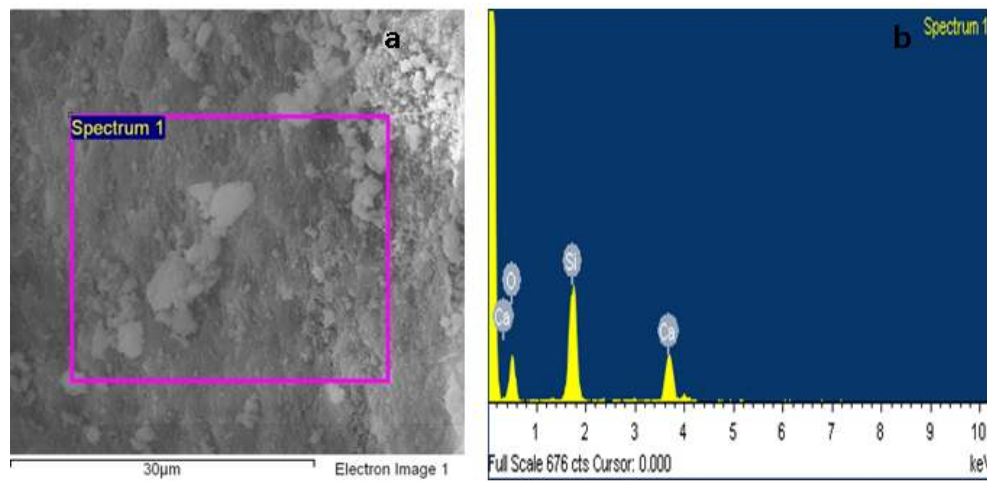


Fig. 6. TEM micrographs of the β -CaSiO₃ NPs; (a) low, (b) medium

The FESEM micrograph in Figure 7 show the results for elemental analysis of the rectangular region in Figure 7a by energy dispersive spectroscopy (EDS) revealed the

348 presence of only Ca, Si and O in Figure 7b. This strongly suggests that β -CaSiO₃ was the
349 main material without any detectable impurities in this region.
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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

358 The potential toxicity of NPs has become a major concern as these particles have the ability
359 to interact and disrupt the growth, structure and functions of organs and tissues in living
360 organisms [18]. Toxicity has been extensively investigated and has been found to depend on
361 physiochemical factors such as particle size, shape or morphology, surface charge and
362 chemistry, composition, and subsequent NPs stability [18-32]. High surface area and
363 porosity that characterized the synthesized calcium silicate nanomaterial makes it more
364 suitable for tissue regeneration applications. In order to assess the biocompatibility of these
365 newly synthesized materials, cytotoxicity tests in a controlled experiment based on
366 established cell viability protocols were conducted. In this study the effect of β -CaSiO₃ on
367 the viability of human osteoblast cells (ATCC CRL 11372) was investigated. Viability assays
368 are used to assess the overall dose-dependent toxicity of nanoparticles in cultured cells,
369 examining cell survival and proliferation parameters after exposure to the particles [38].
370 Results of cytotoxicity studies presented in Figure 8 show the status of osteoblast cells
371 during incubation with various concentrations of β -CaSiO₃ NPs for 24 and 48 h. The results
372 show that in all the tested doses of NPs, , the viability of the osteoblast cells was not
373 different from that of control cells (CM). This indicates that β -CaSiO₃ NPs at these
374 concentrations do not adversely affect the physiological activities of the osteoblast cell line.
375 In contrast, cells in the control wells treated with a cytotoxic drug, staurosporine (STS),
376 showed a significant loss in viability, indicating the sensitivity of the cells towards cytotoxic
377 agents.
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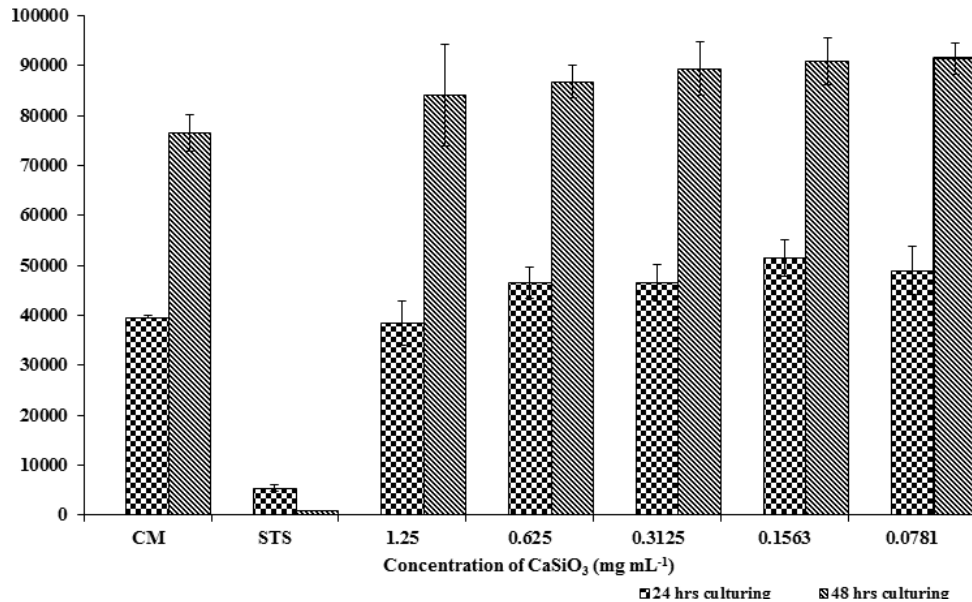


Fig. 8. Luminous intensity in CRL-1137 osteoblast cells after 24 and 48 h incubation with β -CaSiO₃ NPs

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

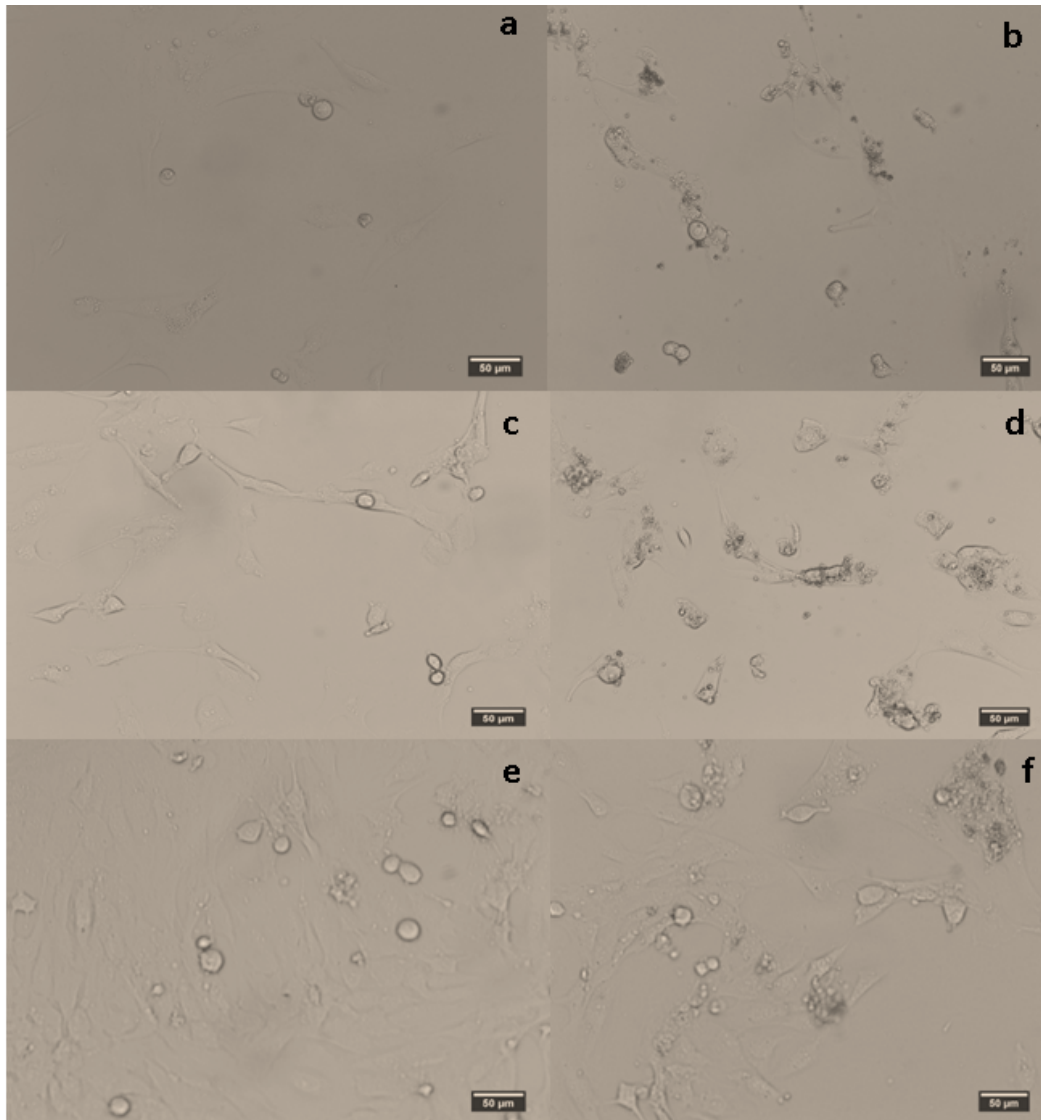


Fig. 9. Optical micrographs: (a, c, e) cultured cell only, (b, d, f) cultured cells with β - CaSiO_3 NPs

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 e 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 - CaSiO_3 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). Themicrographs also show that no cell death occurred in the presence of the NPs by 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, additional steps of sintering (3 h) 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 regeneration 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.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

Authors BT, VR and TS designed the study. BT managed literature search, wrote the protocol and first draft of the manuscript. BT and VA managed the analyses of the study. All authors read and approved the final manuscript.

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