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Effect of size of bioactive glass nanoparticles on mesenchymal stem cell proliferation for dental and orthopedic applications

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ABSTRACT

Bioactive glass nanoparticles (nanostructured bioglass ceramics or nBGs) have been widely employed as a filler material for bone tissue regeneration. The physical properties of nBG particles govern their biological actions. In this study, the impact of the size of nBG particles on mouse mesenchymal stem cell (mMSC) proliferation was investigated. Three different sizes of nBG particles were prepared via the sol–gel method with varying concentrations of the surfactant and polyethylene glycol (PEG), and the particles were characterized. Increased concentrations of PEG decreased the size of nBG particles (nBG-1: 74.7 ± 0.62 nm, nBG-2: 43.25 ± 1.5 nm, and nBG-3: 37.6 ± 0.81 nm). All three nBGs were non-toxic at a concentration of 20 mg/mL. Increased proliferation was observed in mMSCs treated with smaller nBG particles. Differential mRNA expression of cyclin A2, B2, D1, and E1 genes induced by nBG particles was noticed in the mMSCs. nBG-1 and nBG-3 particles promoted cells in the G0/G1 phase to enter the S and G2/M phases. nBG particles activated ERK, but prolonged activation was achieved with nBG-3 particles. Among the prepared nBG particles, nBG-3 particles showed enhanced mMSC proliferation via the sustained activation of ERKs, upregulation of cyclin gene(s) expression, and promotion of cell transition from the G0/G1 phase to the S and G2/M phases. Thus, this study indicates that small nBG particles have clinical applications in dental and bone treatments as fillers or bone-tissue bond forming materials.

1. Introduction

Bioglass ceramics (BGC) particles exhibit promising osteoconductive properties and form surface reactive layers when exposed to body fluids, resulting in the formation of chemical bonding between implant and the host tissue [1–3]. This feature is widely explored in periodontal repair and bone regeneration [4]. BGC particles have been mostly used in restorative dentistry as inorganic fillers. The dissolution of the BGC particles results in subsequent ionic exchange, nucleation, and formation of apatite [5,6]. The rate at which bonds are formed with host bone can be controlled, making BGC particles unique compared with other biomaterials [7]. Porous scaffolds that are freeze-casted with bioactive glass particles and mesoporous bioactive glass particles displayed enhanced bioactivity in simulated body fluids [8,9]. Ionic products such as calcium, silicon, and phosphorus released from BGC particles are mainly involved in governing the biological actions in cells.

Bioactive glass nanoparticles (nanostructured bioglass ceramics or nBGs) with enhanced osteointegrative properties provide promising advantages for dental and orthopedic applications. By controlling the particle size to nanoscale dimensions, properties like osteoconductive, dissolution, and other vital characteristics can be greatly improved [10]. The nanostructured surface improves cell adhesion, enhances osteoblast proliferation, promotes differentiation, and increases biomineralization [11,12]. Even though BGC particles have been used in dental and bone applications, the impact of BGC particles at the nanoscale on bone formation and especially on cell proliferation has not been completely understood. The aim of the current study was to synthesize BGC particles of various sizes by altering the surfactant concentration and to study the influence of these particles on mouse mesenchymal stem cell (mMSC) proliferation. This study provides us with new clues to optimize the design of nBG particles of various sizes with improved biological action.

2. Materials and methods

2.1. Materials

Tetraethyl orthosilicate (TEOS), calcium nitrate, citric acid, diammonium hydrogen orthophosphate, polyethylene glycol (PEG) (MW = 20,000 g/mol), Dulbecco's modified Eagle's medium (DMEM), ammoniated water, and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma Aldrich, USA. Fetal bovine serum (FBS) and cell culture reagents were purchased from Invitrogen, USA. Other chemicals used in this study were of analytical grade. Mouse mesenchymal stem cell line C3H10T1/2 was purchased from NCCS,

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Pune, India. Antibodies for ERK/ phospho-ERK were obtained from Santa Cruz Biotechnology, USA.

2.2. Methods

2.2.1. Synthesis of nBG particles

nBG particles (SiO_2:CaO:P_2O_5 = 45:40:15, mol%) at different sizes were synthesized via sol–gel synthesis as described previously [13]. The concentration of PEG, the surfactant, was varied from 0.5% to 1.5% to obtain nBG particles at three different sizes within the nano range (Table 1). Briefly, solution A was prepared by dispersing calcium nitrate and TEOS in a mixture of ethanol and water (1:2) at room temperature. The pH of this solution was maintained at around 1 to 2 by adding 1 M citric acid. The reaction mixture was continuously stirred to obtain a homogeneous and clear solution. Solution B was prepared by mixing diammonium hydrogen orthophosphate and PEG at concentrations of 0.5%, 1%, and 1.5% in 1 L of distilled water to obtain nBG-1, nBG-2, and nBG-3, respectively. Ammoniated water was added to the solution to adjust the pH to 10–11. Under vigorous stirring, solution A was slowly dropped into solution B and the total reaction volume was made up to 1.5 L. Following 48 h of stirring and 24 h of aging at room temperature, the precipitate was isolated by centrifugation at 10,000 rpm. The precipitate was washed with deionized water and dried at 60 °C for 8 h. The obtained powder was ground and calcinated at 700 °C for 6 h with a temperature increase of 1 °C per minute to obtain white nBG particles.

2.2.2. Physico-chemical characterization

To assess the size and morphology of nBG particles, they were dispersed in ethanol and visualized using a high resolution transmission electron microscope (JEOL-JEM 2100F, Japan). The elemental composition of nBG particles was determined by EDS analysis using a JEOL JSM 6490 LA, USA. Samples were placed on a stub coated with carbon tape and thereafter subjected to platinum coating with JEOL JFC 1600 for 2 min at 10 mA. Functional group analysis was carried out on the nBG particles using a FT-IR spectrometer (Perkin-Elmer RX1, USA). The powdered samples were mixed with KBr and made into homogenous disks using the KBr press. The spectra were recorded in transmission mode over the mid-infrared range of 400–450 cm^{-1}. The XRD patterns of the nBG particles were acquired at room temperature using a Panalytical XPERTPRO powder diffractometer (CuKα radiation) operating at a voltage of 40 kV. Diffraction patterns were recorded in a 2θ range of 0–100° and scanned at a speed of 2° min^{-1}.

2.2.3. Cell culture studies

Mouse mesenchymal stem cells (mMSCs; C3H10T1/2) were procured from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured to confluence in DMEM supplemented with 10% FBS. The cells were trypsinized and sub-cultured for further experiments.

2.2.4. Cytotoxicity studies

The cytotoxicity of the synthesized nBG particles was assessed by colorimetric detection using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. mMSCs were seeded in a 24-well plate at a density of 10,000 cells/cm^2. nBG particles of each size were individually incubated in DMEM for 24 h at different concentrations (0, 5, 10, 20, 30, 40, and 50 mg/mL) and conditioned media were obtained. The cells were then treated with conditioned media for 24 h and 48 h. Cells without nBG treatment served as a negative control and cells treated with 0.1% Triton X-100 served as a positive control. At the end of the incubation period, 500 μL of a 5 mg/mL solution of MTT in phosphate buffered saline (PBS) was added to each well and incubated for 3 h. The medium was removed and formazan crystals were solubilized by adding 100 μL of dimethyl sulfoxide (DMSO). The optical density was recorded at 570 nm.

2.2.5. Ionic dissolution measurements

nBG particles (20 mg/mL) were incubated in DMEM at 37 °C for 24 h. The solution was centrifuged and the supernatant was filter-sterilized (0.22 μm). The supernatant was then subjected to analysis using an inductively coupled plasma optical emission spectrometer (ICP-OES) (PerkinElmer, Optima 5300 DV, USA) to measure the calcium (Ca), silicon (Si), and phosphorus (P) levels released from the nBG particles.

2.2.6. Cell count, viability, and cell cycle phase analyses

mMSCs were seeded in 6-well plates and serum-starved for 6 h in DMEM containing 0.1% FBS to obtain a synchronous population. Medium (20 mg/mL) conditioned with nBG particles was prepared individually as mentioned previously. The serum-starved cells were incubated with 1 mL of conditioned medium for 24 h, 48 h, and 72 h. Cells without treatment served as a control. After the incubation periods, cells were harvested and subjected to cell count and viability assays according to the manufacturer's protocol (Muse™ Cell Analyzer, Merck-Millipore, Germany). The cells were harvested and washed several times with 1× PBS to remove cellular debris. They were resuspended in 50 μL of ice cold 1× PBS and fixed with ethanol at −20 °C for 3 h. The fixed cell suspension (250 μL) was washed several times with 1× PBS and centrifuged. The cell pellet was finally resuspended in 200 μL of Muse cell cycle reagent and incubated in the dark for 30 min. The suspension was then analyzed for cell cycle phases using the Muse Cell Analyzer.

2.2.7. Real-time RT-PCR analysis

mMSCs were seeded in a 6-well plate at a density of 5×10^5 cells per well and treated with nBG particles for 24 h. Total RNA was isolated from the cells using TRIzol reagent. One microgram of total RNA from each sample was reverse-transcribed and cDNA was used for real-time PCR. The primers used in this study are shown in Table 2. Real-time PCR analysis was performed according to the manufacturer's protocol (BioRad, USA). The threshold cycle (Ct) values were calculated from the amplification plots obtained. GAPDH was used as an internal control. The value of ΔCt for each sample was calculated by subtracting the Ct values of the internal control GAPDH. The ΔΔCt and fold change was then calculated as described earlier [14].

2.2.8. Immunoblotting

mMSCs were serum-starved and treated with conditioned media for 5, 15, and 30 min. Cells without treatment served as a negative control. Cells treated with 10% FBS served as a positive control. After treatment, the medium was removed, the cells were washed with ice-cold 1× PBS, and whole cell protein lysates were prepared. Proteins were separated

Table 1
Composition and various PEG concentrations used in nBG synthesis.

Description	SiO_2 (mol%)	CaO (mol%)	P_2O_5 (mol%)	PEG %	Particle size (nm)
nBG-1	45	40	5	0.5%	74.70 ± 0.62
nBG-2	45	40	5	1%	43.25 ± 1.50
nBG-3	45	40	5	1.5%	37.60 ± 0.81

Table 2
Primer sequences used for real-time reverse transcriptase PCR analysis.

Gene		5′ → 3′ sequence
Cyclin A2	Forward	CCTGCAAACTGCAAAGTTGA
	Reverse	AAAGGCAGCTCCAGCAATAA
Cyclin B1	Forward	GAGATGTACCCTCCAGAA
	Reverse	CCATGTCGTAGTCCAGCA
Cyclin D1	Forward	TGAACTACCTGGACCGCT
	Reverse	GCCTCTGGCATTTTGGAG
Cyclin E1	Forward	GGGAGACCTTTTACTTGGC
	Reverse	GGCAGTCAACATCCAGGAC
GAPDH	Forward	GAGAGACCCCACTTGCTGCCA
	Reverse	CTCACACTGCCCCTCCCTGGT