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Cytocompatibility studies of titania-doped calcium borosilicate bioactive glasses *in-vitro*

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ABSTRACT

The present study aims to elucidate the applications of Titania (TiO_2) doped calcium borosilicate glass as a biocompatible material in regenerative orthopedic applications. In this context, we have examined the bioactivity of various concentrations of TiO_2 doped glasses with the help of simulated body fluid (SBF). Cytocompatibility, cell proliferation, and protein expression studies revealed the potential candidature of TiO_2 doped glasses on osteoblast cell lines (MG-63). We hypothesized that TiO_2 doped calcium borosilicate glasses are most cytocompatible material for bone implants. Glasses with composition $31B_2O_3$-$20SiO_2$-$24.5Na_2O$-$(24.5 - x)$ CaO- x TiO_2 ($x = 0,0.5,1,2$) have been prepared by the conventional melt-quenching technique. After immersion of glasses in the SBF, formation of hydroxyapatite layer on the surface was confirmed by X-ray Diffractometer (XRD), Fourier Transform Infrared Spectroscopy (FT-IR) and Scanning Electron Microscopy-Energy Dispersive Spectroscopy (SEM-EDS) analysis. Significant change in the pH of the body fluid was observed with the addition of titania. Degradation test was performed as per the ISO 10993. The results showed that partial substitution of TiO_2 with CaO negatively influenced bioactivity; it decreased with increase in concentration of TiO_2. Vickers hardness tester was used to measure the microhardness values of the prepared glasses. With the increasing of TiO_2 content, the microhardness of the glass samples was increased from 545 Hv to 576 Hv. Cytocompatibility has been evaluated with MG-63 cells by using MTT assay. Further, we observed that there was no change in expressions of cyclin levels even after the incorporation of titania. The antibacterial properties were examined against *E. coli* and *S. aureus*. Strong antibacterial efficacy was observed for 2% TiO_2 in the system. Hence it can be concluded that titania-doped borosilicate glasses may be used as potential materials in bone tissue engineering.

1. Introduction

'Bioceramics' are biocompatible materials, which find use in several clinical applications. Bioceramics can be produced in crystalline and amorphous forms and they are generally classified into two groups based on their chemical composition; calcium phosphates and others, including yttria-stabilized tetragonal zirconia, alumina ceramics, silicate and phosphate families of glasses and glass-ceramics [1]. Bioactive glasses are considered as potential materials for bone substitution, as they can form a direct bond with the living bone without formation of bond with surrounding fibrous tissue. One of the essential conditions for the bioactive material is that it should form a biologically active apatite called hydroxylapatite layer (HAp) on its surface through which the material could form a bond with both soft tissues as well as hard tissues [2]. 45S5 is the best implant material, not only for dental and orthopedic applications but also, for ossicular prostheses, endosseous ridge maintenance, and other applications [1,3].

Boron plays a vital role in bone formation and depends on its concentration in the composition. The highest concentrations of boron are found in bone, nails, and hair [4]. Day et al. [5–9] have extensively studied the use of borate glasses in biomedical applications. Recent studies have demonstrated that the potential bioactivity of borate glasses comes from their lower chemical durability, faster degradation rate and almost complete conversion to hydroxyapatite (HAp) than the widely studied 45S5 bioglass when placed in SBF. Studies have shown that some borate glasses have the ability to support the growth and differentiation of human mesenchymal stem cells, and to promote bone formation more rapidly than silicate based 45S5 glass [10,11]. S. M. Wiederhorn et al. [10] reported that higher amount of borate content weakens the glass structure. Glass structure was strengthened by incorporation of silica as former and CaO as modifier respectively [12].

Titanium has gained importance recently for its broad range of applications in the biomedical field [13]; it is one of the earliest transition metals to be investigated for antitumor properties; also it is found to elicit favorable cell response and is concluded to be one of the best materials suited for biological requirements [14]. Hence, titanium is widely used as a biomaterial for several dental and orthopedic clinical

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purposes. In the present work, titanium dioxide (TiO_2) was incorporated into borosilicate glass to produce compositions with controlled degradation rate and enhanced biological response which is a suitable material for bone tissue engineering applications. Numerous studies were focused on TiO_2 doped glasses and glass ceramics [15–21]. Devi et al. [22] reported titania-doped phosphate glasses and glass-ceramics are bioactive. Though the 2 mol% of TiO_2 content controlled the solubility and improved the density of glass, however poor bioactivity was reported. Mohini et al. [23] studied the *in-vitro* bioactivity of TiO_2 doped borosilicate glasses (up to 10 mol%) and showed that higher concentration of TiO_2 did not affect the bioactivity of the glasses. Titanium is acting as both active as well as inert material depending on the host composition.

Nowadays, clinical interest is aimed at attaining increased biocompatibility and better mechanical surface properties for the development of the biomaterial for dental as well as orthopedic applications. Hence, there is a great need to modify the biomaterial surfaces to produce novel material which meets the clinical demands. So far a tailor-made composition of standard bioglass as a potential tool for orthopedic and dental applications has not yet been fully elucidated. In this context, the present study aims at producing different TiO_2 doped borosilicate bioglasses and also to investigate their bioactivity, cytocompatibility using cell proliferation assays, protein expression studies.

2. Materials and methods

2.1. Media and chemicals

All chemicals used in this work were of analytical grade and without further purification. Silica (SiO_2, 99.99%) taken from Umicore thin film coating quality, B_2O_3 (99.9%), CaO (99.9%), TiO_2 (99.9%) have been purchased from Sigma-Aldrich. Na_2CO_3 with 99.9% purity was purchased from Sisco Research Laboratories.

DMEM (Gibco, 11965092), trypan blue (Himedia, RM 100125), MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Himedia, TC191), NP-40 (Sigma, I8896), 1× PBS(pH 7.4) (Gibco, 10010023), FBS (Gibco, 10270106), anti-anti (Gibco, 15240062), sodium pyruvate (Gibco, 11360070), and trypsin-EDTA (Gibco, 25200056) were purchased from Invitrogen. Ponceau stain was from Himedia, RM977 was purchased from Sigma-Aldrich.

Reagent A and Reagent B for protein estimation (Catalog no: 5000113) were purchased from Bio-Rad, PVDF membrane was from GE healthcare (Cat. No. 10600002).

Cyclin A, cyclin B1 antibodies were from Santacruz, β-tubulin antibody was of Sigma-Aldrich, mouse, and rabbit HRP conjugated secondary antibodies were from GE Healthcare, Alexa fluor 588, and DAPI were from Invitrogen. β-Actin was from Cell Signaling Technology, USA.

2.2. Cell line

MG 63 Human osteoblast cells were purchased from National Centre for Cell Science (NCCS), Pune, India. The expression of various features by these cells makes them handy in investigating the osteoblast response on different biomaterials as they are derived from osteosarcoma cells [14,24].

2.3. Preparation of calcium borosilicate glass

The glasses with the composition $31B_2O_3$-$20SiO_2$-$24.5Na_2O$-(24.5 − x) CaO- x TiO_2 (mol%) (x = 0,0.5,1,2) were prepared by melt quenching technique, as described earlier [12]. Appropriate amounts of analytical grade oxide powders with 99.9% purity were heated in a platinum crucible in an electric furnace for 3 h under the temperature range 1000 °C–1200 °C. The melts were cast into pre-heated stainless steel moulds, annealed at 350 °C for 2 h. For convenience, these glass systems are labelled T_0, $T_{0.5}$, T_1 and T_2 according to the TiO_2 content in the glass matrix as shown in Table 1. The resulting glasses were grounded and sieved to obtain a fine glass powder.

2.4. Preparation of SBF

In order to investigate the *in-vitro* bioactivity of a material, SBF is widely used for its mimicking nature with human blood plasma. Simulated body fluid was prepared by using standard procedure developed by Kokubo et al. [25] in polypropylene bottles. The experiment was performed in a static condition. Powder to SBF solution ratio was used as 0.1 g of glass powder in 50 mL at 37 °C [26].

2.5. Characterization

The amorphous nature of glasses was confirmed by X-ray diffraction (XRD) analysis (PANanalytical X'Pert POWDER); The radiation source was CuKα with the scanning angle ranging from 20° and 60°. The measurement was done with the step size of 0.02° with the time per step being 50 s. IR absorption spectra were measured to identify the functional groups in the glass specimens before and after immersion into SBF solution to confirm the formation of apatite layer. The spectra were recorded using KBr pelleted samples with a resolution of 4 cm^{-1} in a wavelength range of 4000–400 cm^{-1} using FTIR transmittance spectrometer (S 100; PerkinElmer). pH measurements were done with Thermo Scientific (ORION pH 7000). The surface morphologies and elemental compositions of the glasses were examined using SEM-EDS (Carl Zeiss EVO 18 and Oxford). Confocal microscopy images of the samples have been obtained with Carl Zeiss, NLO 710 Germany. The hardness of the glass samples were carried out by using Vickers hardness tester (HMV-2000 SHIMADZU). The applied load was 100 g and loading time was 15 s. Each sample was measured ten times, and the mean value of the test results was taken.

2.6. Degradation study

Degradation test was performed as per the ISO 10993 "Biological evaluation of biomedical devices – Part 14: Identification and quantification of degradation products from ceramics". These tests were performed at 37 °C in SBF at pH 7.4, weight loss as a function of immersion time were measured up to 21 days. A ratio of 0.50 g powders to 50.0 mL solution was used, and the SBF was replaced for every three days. Weight changes were measured by separating the powders from SBF, washing with deionized water, and dried at 95 °C.

2.7. Cytotoxicity and cell proliferation assay with MTT

The cytocompatibility of the prepared glasses has been assessed by using MTT Assay [27]. For this, approximately 5×10^3 MG-63 cells were added to each well of 96-well plate. The cell counting was done by trypan blue staining assay using hemocytometer. Cells were treated with each of the four compounds within a range of concentrations from 50 μg/mL to 1000 μg/mL. The work was done in triplicates. After 48 h, 20 μL (5 mg/mL) of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent was added to each well and incubated for 4 h. After that medium with MTT was removed and 100 μL of

Table 1
The composition of glasses with various concentrations of TiO_2 (mol%) and microhardness.

Sample	Composition (mol%)					Microhardness (Hv)
	B_2O_3	SiO_2	Na_2CO_3	CaO	TiO_2	
T_0	31	20	24.5	24.5	0	545 (±20)
$T_{0.5}$	31	20	24.5	24	0.5	558 (±22)
T_1	31	20	24.5	23.5	1	564 (±20)
T_2	31	20	24.5	22.5	2	576 (±21)