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Synthesis and Characterization of a Biocompatible Bioactive Borate Glass

Sanjit Roy[1], Bhaskarjyaa Chatterjee[2], Shrabani Ghosh[3], Gargi Sardar[4], Bholanath Mondal[5] and S C Sarkar [3*]

[1]Center for Bio-separation and Technology, VIT-Velore, amil Nadu 632014, India

[2]Centre for Biotechnology, Anna University, Guindy, Chennai 600025, India

[3] Center for Rural and Cryogenic Technologies, Jadavpur University, Kolkata -700032, India

[4]Department of Zoology, Baruipur College, Kolkata-743610, W.B., India

[5]Central Scientific Services, IACS, Kolkata -700032, India

*Corresponding: swapansarkar1959@yahoo.in

Abstract:

A typical bioactive borate glass of composition $5Na_2O$-30Ca-$65B_2O_3$(NCBO) was prepared by melt quenching method. The NCBO glass powder and solid glass fibers were found to slowly converted to nanocrystalline hydroxyapatite (or $Ca_{10}(PO_4)_6(OH)_2$) by soaking in (0.25M)K_2HPO_4(KDP) solution (with pH = 9.5 and Ca/P ratio ~1.6 at 37^0C) for about one month. We studied biocompatibility (cells adhesion and proliferation) of the NCBO glass by allowing adhesion and proliferation of human cord blood derived mesenchymal stem cells (CB-hMSCs) directly on the thin glass plate. The cells viability on glass surface was determined by MTT [3-(4,5-di-methylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assay analysis. The NCBO glass exhibiting excellent biocompatibility might be suitable for *in vivo* tissue engineering applications.

Keywords: Biactive glass, Stem cells, Biomaterials, Nano-hydroxyapatite

1. Introduction

Bioactive oxide glasses are well studied implant materials mostly used in the human bodies to repair or replace defective bones [1,2].These are surface reactive glasses and induce bioactivity due to strong bonding with living bone tissues. These glasses interact with the simulated body fluid (SBF) *in vitro* and form hydroxyapatite viz. $Ca_{10}(PO_4)_6(OH)_2$(referred to as HAp) on their surfaces. HAp is also formed by interaction in presence of the body fluid *in vivo* [3,4]. Many SiO_2 containing bioactive oxide glasses were prepared earlier and characterized for tissue engineering (TE) applications [5-7] because of their appropriate biocompatibility and biodegradability. These are mostly high temperature (more than 1200^0C) melting glasseswith different compositions of inorganic oxides like SiO_2, CaO, Na_2O and P_2O_5 etc. having excellent biocompatibility. However, it was also reported that silica particles remaining in the human body, after being implanted for a long time, might deposit around the bone joints and cause health problems [8,9]. So different SiO_2 free borate glasses [10-12] like Na_2O-CaO-B_2O_3, Na_2O–BaO–B_2O_3,CaO-B_2O_3 were also prepared and thoroughly investigated for TE applications. These glasses showed faster and almost complete conversion into HAp in presence of SBF or phosphate solutions with appropriate pH value. Nano-hydroxyapatite synthesized by different chemical and physical methods were used for the fabricating TE scaffolds[13,14] and also for drug delivery [13-15]. Upon immersion of these glasses in aqueous phosphate solutions (with Ca/P ~1.6), Ca and phosphate ions react forming HAp. The HPO_4^{2} and OH^{-1} ions, necessary for HAp formation, are supplied by the phosphate ions present in the SBF or in other phosphate solutions [16-19]. Bioactive glass powders were also used for making porous scaffolds[8] and HAp polymer composite scaffolds [20] for tissue engineering (TE) applications.

Importantly, some high melting SiO_2 free Al_2O_3-P_2O_5-ZnO type oxide glass fibres have also been used as substrate [21] for growing human skeletal muscle cells.

For testing biocompatibility of the glass samples, adhesion of living cells on the glass surfaces is a primary requirement to subsequent cell proliferation, protein synthesis and formation of mineral deposits. *In vivo* biocompatibility of glass or polymer based scaffolds were studied mostly with human bone marrow (BM) derived mesenchymal stem cells or other cells procured from animal sources [22]. To the best our knowledge, *in vitro* study of biocompatibility with bioactive oxide glasses using human umbilical cord blood (UCB) derived mesenchymal stem cells (abbreviated as CB-hMSCs) had not been well investigated. It is worthwhile to mention that UCB is an easily available cost-effective source of multipotent stem cells which are potential to differentiate into different lineages like osteogenic, myogenic, adipogenic and chondrogenic lineages [23,24]. So the study of biocompatibility of suitable bioactive glasses using CB-hMSCs is encouraging with regard to TE and CB-hMSCs biomedical applications.

In the present work, our aim is to prepare a relatively low melting point suitable silica free bioactive borate glass of composition $5Na_2O$-30Ca-$65B_2O_3$ (NCBO) and to study bioactivity and biocompatibility of this glass using human cord blood derived stem cells, CB-hMSCs. Bioactivity was studied by soaking the NCBO glass powder and glass fibers in phosphate (0.25M)K_2HPO_4 (hereafter referred to as KDP) solution (with pH = 9.5 and Ca/P~1.6 at 37^0C) and investigating the conversion of NCBO glass powder and glass fibers into HAp. The glass converted HAp powder was characterized by X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), high resolution tunnelling electron microscope (HRTEM) and field emission scanning electron microscope (FESEM) studies. Biocompatibility of the NCBO glass plate was assessed by the MTT[3-(4, 5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

method using CB-hMSCs. This study was intended to establish the importance of multipotent CB-hMSCs and silicon free biocompatible NCBO type borate glasses or glass powders exploring the possibility of their future TE and biomedical applications.

2. Material and Method

2.1. Materials

Analytical grade $CaCO_3$, H_3BO_4, Na_2CO_3, and K_2HPO_4 all of purity 99% or better were purchased from E Merck, India. Dulbecco's modified eagle medium (DMEM), antibiotic solution, foetal bovine serum, phosphate buffer saline (PBS) and foetal bovine serum (FBS) were purchased from (Gibco, USA). Ficoll Hypaque (Histopaque-1077) was obtained from (Sigma, MO, USA). Basic fibroblast growth factor bFGF used for the present work was collected from (Invitrogen, USA). MTT [3-(4, 5-di-methylthiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide] assay for cell viability study was procured from (Sigma Aldrich) and used without further purification.

2.2. NCBO Glass Preparation

The bioactive NCBO glass viz. $5Na_2O$-$30Ca$-$65B_2O_3$ (NCBO) (wt %) was prepared by using $CaCO_3$, H_3BO_4, and Na_2CO_3. Appropriate amounts of these oxides were well mixed and pre-heated first at 300^0C for two hrs and the pre-heated powder after well grinding was melted in a platinum crucible around 1150^oC for one and a half hr. NCBO glass plates were obtained by quickly quenching the melt between two copper blocks kept at room temperature. Thin transparent glass plates of 1-2 mm thickness thus obtained were used for our present studies. NCBO solid glass solid fibres (0.05-0.1 mm diameter) were drawn from the glass melt. Fine glass powder (250-300μm grain size) for soaking inphosphate solution was prepared by grinding the glass pieces in an Agate mortar.

2.3. Conversion of Glass into HAp

The NCBO glass powder (~2.0g) and a few pieces of solid glass fibres (mean diameter ~0.05mm) were immersed in KDPsolution (with pH =9.5 and Ca/P~1.6) in two 200 ml different glass beakersand

kept in the oven at 37^{o}C for 30 days. Glass powders slowly converted to HAP nanoparticles mixed with KDP solution. Here the calcium ions present in the glass react with the phosphate ions producing HAp as reported earlier [16-18]. Once in a week, the solution in the beaker with glass powder was also stirred for better contact of the glass particle surfaces with the KDP solution. The initial pH value of the solution was maintained constant throughout the immersion process of the powdered glass sample by adding required amount of alkaline solution. After soaking for 30 days, the glass powder in the beaker was almost converted to fine white powder mixed with the soaked solutions which was carefully transferred from the beaker. The white powder was separated from the soaked solution by filtration. To remove potassium and other ions, the obtained white powder was washed several times with deionized water until pH of the filtrate reduced to 7.4. Final nanohydroxiapatite powder was dried in a vacuum oven to remove moisture. Similar white HAp powder was also found to form and adhered on the glass fiber surface which were carefully washed with distilled water and dried in vacuum oven.

2.4. CB-hMSCs Seeding and Attachment on NCBO Glass Plate

For the study of cell adherence and hence biocompatibility of the glass plate, cord blood mesenchymal stem cells (CB-hMSCs) were isolated in our laboratory from human umbilical cord blood (UCB) similarly to our previous work [25]. UCB samples were collected from ISPAT General Hospital-Rourkela, Orissa, India (with patient's concern and all procedures and formalities had been approved by institutional, NIT-Rourkela, India, Ethical Committee). CB-hMSCs were analyzed by phase contrast microscopy and fluorescence microscopic techniques. For fluorescence microscopic study, cells were fixed with 4% paraformaldehyde (Sigma, USA) and permeabilized with 0.1% Triton X-100 for 5min and blocked with PBS containing 1% Bovine Serum Albumin (BSA) (Invitrogen, USA) for 30min. Nuclei were counterstained using DAPI

(100ng/ml). Thereafter, cells were stained with Phalloidin conjugated to Alexa Fluor and the samples were examined using a Zeiss Axivert 40 CFL florescence microscope.

The CB-hMSCs (primary cells) were seeded on the sterilized (by heat treatment) NCBO glass plate surface along with tissue culture plate taken as positive control for comparison. A thin circular glass plate of 0.5mm thick and 0.8mm diameter was first autoclaved for half an hour at 15 PSI and then normalized by washing three times with PBS solution. Static seeding method was used to seed CB-hMSCs onto the glass plate surface in standard growth medium containing DMEM 90%, FBS 10% and 100X antibiotic-antimycotic solution 1% (approximately). CB-hMSCs ($1x10^3$cells/ml) isolated from fresh cord blood were seeded directly onto both sterile bioactive glass surface and control and kept in a CO_2 incubator at 37^{o}C and 5% CO_2. After 4 days of culture, sample glass plates seeded with cells were washed with PBS for two times and fixed with 2.5% glutaraldehyde for 4hr and dehydrated through a gradient series of ethanol from 70% to 100%. The samples were dried using standard procedure prior to storing at 4^{o}C before analysis.

In vitro cell viability and proliferation of CB-hMSCs on the NCBO glass sheet were determined by MTT assay. MTT assay analysis was done after 3, 5 and 7 days of culture. Cells were seeded in 96 well culture plates and initial media from each well was replaced with 500µL of fresh media and 50µL MTT (5mg/mL) solution. The samples with all the substrates were then kept for incubation at 37^{o}C in 5% CO_2 for 4 hours and the MTT solution was carefully replaced by 300µl of DMSO to each well. Optical density (OD) values were measured using a spectrophotometer (Varioskan Flash, Thermo Scientific) at an absorbance wavelength of 595 nm and a higher OD value indicated stronger cell activity. Isolation of CB-hMSCs from cord blood and their proliferation on NCBO bioactive glass surface were shown schematically in figure 1. Data were

analysed by means of Graph pad Prism software and results were expressed as mean+SD.

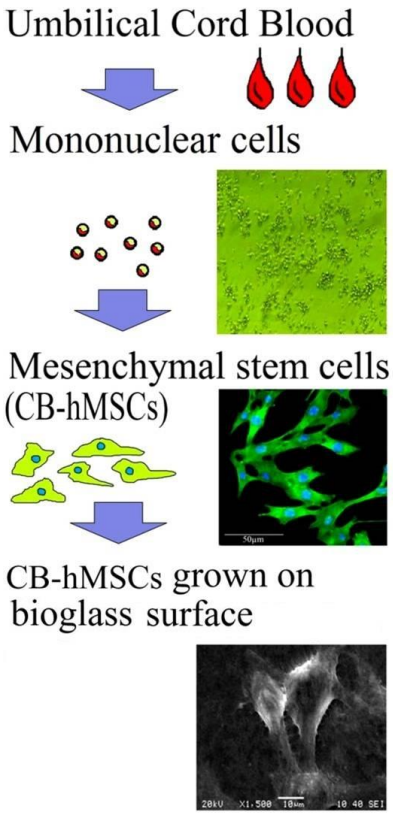

Figure1. A schematic representation of cell culture commencing from isolation of human stem cells CB-hMSCs from cord blood and proliferations of CB-hMSCs on NCBO glass plate.

2.5. Characterization

Glass samples and the white powder obtained after soaking in KDP solution were characterized by XDR (Bruker, Model no. D8 Germany), FTIR (Perkin–Elmer spectrum 100 FTIR spectrometer with a 4 cm^{-1} resolution) and differential thermal analysis (DTA, Instruments: SDQ600). Room temperature electrical conductivity and dielectric constant measurements were carried out with the help of an impedance analyzer (HP 4192A) similarly to our earlier work [26-28]. The glass surface physicochemical property was characterized by water contact angle (CA) measurements against distilled water using

sessile drop method (DAS100S: KRUSS GMbH, Germany). Both the wetting (CA_w) and dewetting (CA_{dw}) contact angles were measured at room temperature at more than ten measurements on drops at two different locations. Glass surface morphology and cell scaffold adherence were studied by field emission scanning electron microscope (FESEM:JEOL JSM-6700F) and scanning electron microscopy (SEM) analysis. Prior to SEM analysis, cell-seeded scaffolds were rinsed in Dulbecco's Phosphate Buffered saline (DPBS) buffer, and fixed with 2.5% gutaraldehyde in DPBS overnight at 4^{o}C. The construct were dehydrated by exposure to a gradient series of alcohol followed by aseptic critical point drying, and coated with platinum before observing under JEOL JSM-840A scanning electron microscope. Live cell assay verified cell viability of CB-hMSCs on the NCBO glass substrate [27,28].

3. Results and Discussion

Figure 2(a) showed the as quenched transparent NCBO glass piece prepared by fast quenching the melt from 1150^{0}C to room temperature. The glass showed highly insulating behavior with room temperature conductivity ~ 2.5x10^{7}S/m and dielectric constant ~15. The SEM microgram of a typical glass particle surface after soaking in KDP solution for one week was shown in Figure 2(b) indicating surface morphology of a glass particle after twenty days soaked in PBS solution.

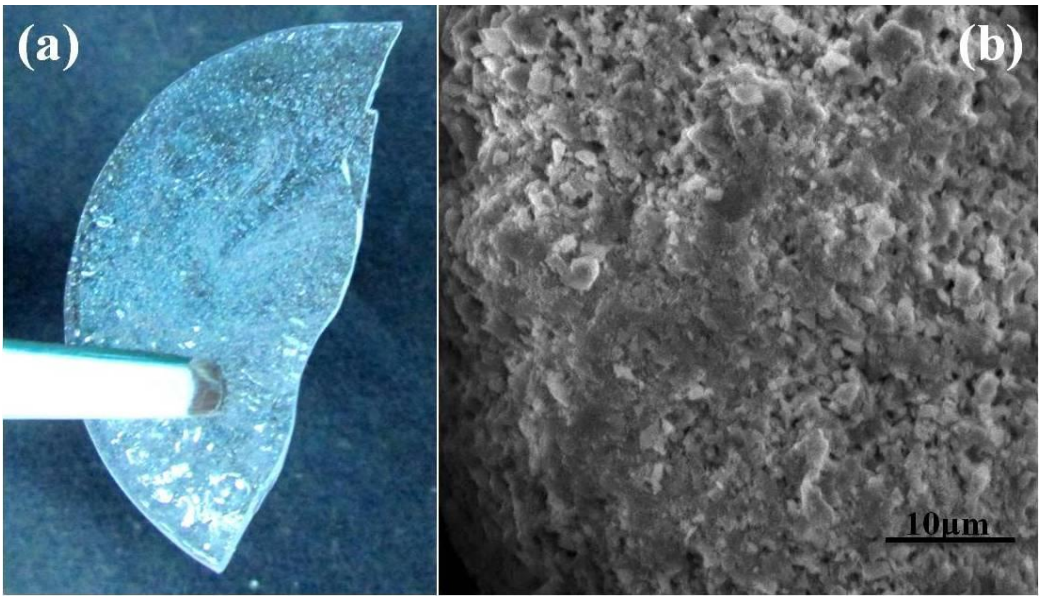

Figure 2. (a) Photograph of a NCBO glass piece and (b) SEM micrograph of the surface of a small NCBO glass particle after soaking for one week in KDP solution.

Figures 3(a) showed the X-Ray diffraction (XRD) pattern of the as quenched glass (before soaking in KDP solution) indicating amorphous character of the NCBO glass.The corresponding XRD pattern of the HAp powders obtained after soaking them in KDP solution was shown in Figure 3(b) representing crystalline structure of the soaked white powder. Most of the XRD peaks were identified (JCPD, 9-0432) with the standard HAp phases. X-ray diffraction pattern showed peak broadening due to ultrafine nature of the powder. Figure 3(c) showed the SEM micrograph of the HAp nanoparticles.

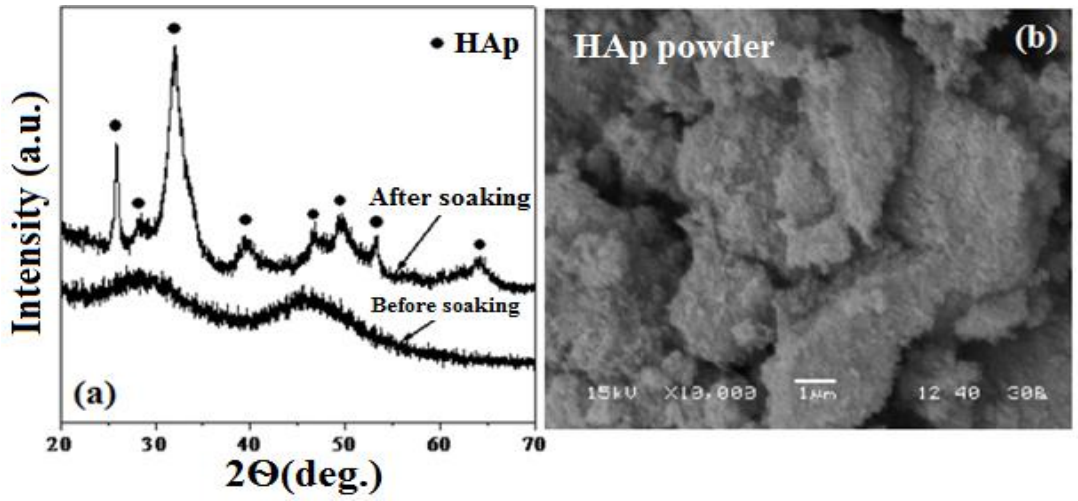

Figure 3. (a) XRD spectra of the as quenched NCBO glass powder (before soaking) and the glass powder after soaking for 30 days in KDP solution. (b) SEM micrograph of the dried soaked glass powder (HAp) after soaking for 30 days in KDP solution.

Figures 4(a and b) depicted interesting bubble like morphology of the surfaces formed by the HAp nanoparticles, respectively, on the thin (<0.05mm diameter) glass fibre and glass piece (~0.5mm thickness) after soaking continuously for thirty days without disturbing the KDP solution in the beaker.

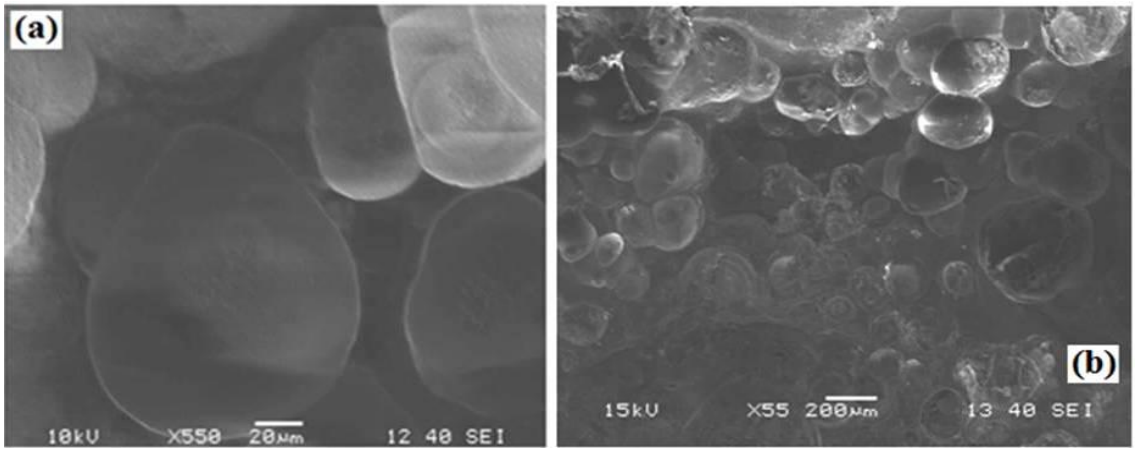

Figure 4. FESEM micrographs of the glass fibres after soaking in KDP solution for thirty days showing bubble like surface morphology formed by nanocrystalline HAp on the (a) thin (<0.01mm diameter) and (b) thick (<0.5mm diameter) glass fibre surfaces.

Figure 5(a) represented the HRTEM image of the HAp particles indicating agglomeration of HAp particles. The major factors causing the agglomeration of nano-powder when drying from aqueous solutions were the capillary pressure between the adjacent particles due to the evaporation of water and also the hydrogen bond originating from the water molecules on the surface of adjacent particles.

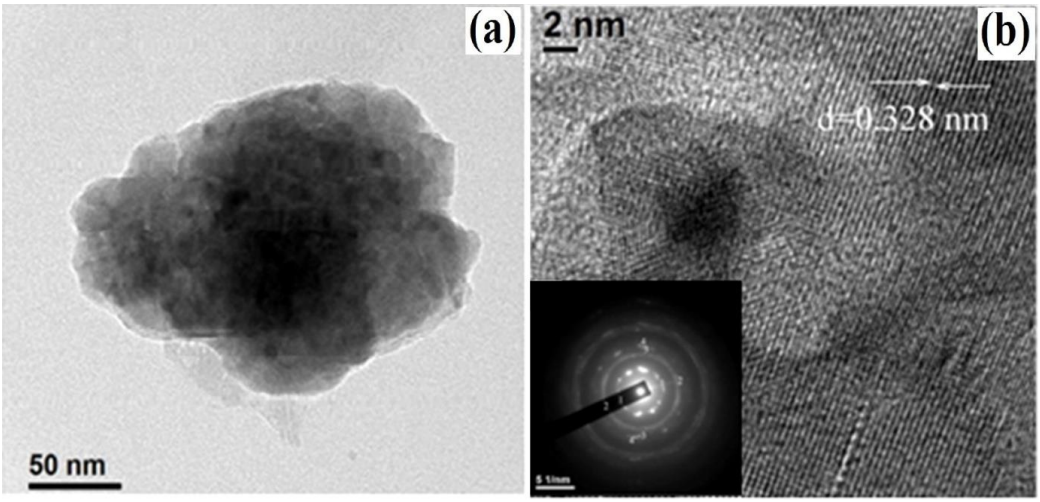

Figure 5. **.** (a) HRTEM image of a HAp nanoparticle and (b) SAED image representing nanocrystalline morphology of the HAp powder obtained after soaking the NCBO glass powder in KDP solution.

The selected area electron diffraction (SAED) diagram indicated nanocrystalline feature of the HAp particles. Figure 6 (a) showed the differential thermal analysis (DTA) curve of the NCBO glass sample. The glass transition temperature (T_g) was found to be around $(230\pm5)^0$C. Figures 6 (b and c) indicated, respectively, the FTIR spectra of the as quenched NCBO glass and the HAp obtained from the soaked glass powder.

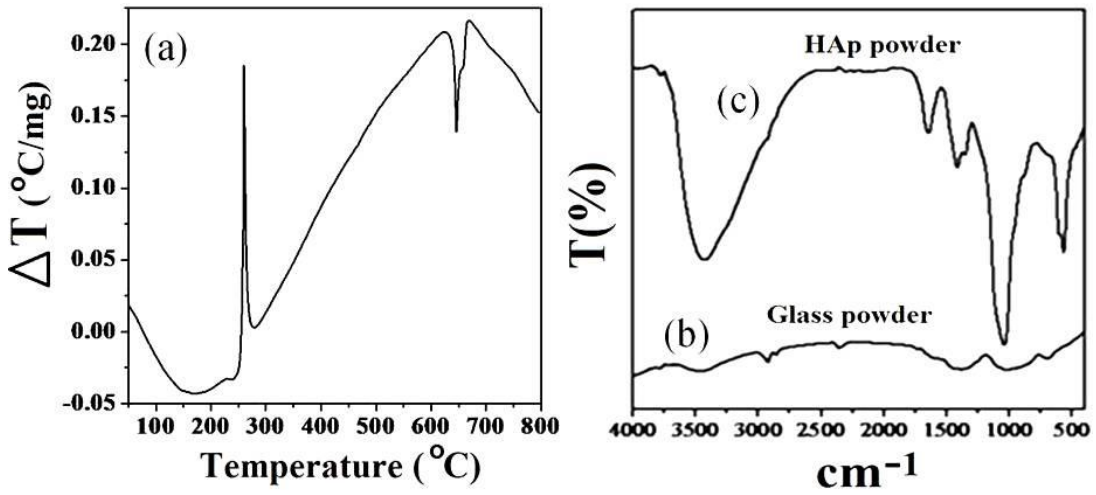

Figure 6 (a) DTA curve of NCBO glass, (b) FTIR spectra of NCBO glass and (c) HAp powder obtained from the KDP solution oaked glass powder.

The bands around 3439 cm^{-1} and 2346 cm^{-1}showed by the FTIR spectra of the as quenched NCBO glass sample represented symmetric bending mode of the O-H functional group and the band around 3479 cm^{-1} might be assigned to the asymmetric stretching C=C−H functional group present in the as quenched glass. The bands at 3430 and 631cm^{-1}showed by the NCBO glass powder (figure 4b) were attributed to the vibrations of the hydroxyl group. The bands around1 037.63 and 963cm^{-1} were the characteristic bands of phosphate stretching vibrations, while the bands at 864 and 565 cm^{1} appeared due to phosphate bending vibrations. All these results revealed that the NCBO glass powder was converted to HAp by soaking in KDP solution.

4. Conclusion

The SiO_2 free NCBO glass was prepared by melt quenching in the form of thin plates and fibres. The NCBO glass powder was found to be completely converted to nanocrystalline HAp by soaking in KDP solution. Our preliminary observation also demonstrated biocompatibility of the bulk NCBO glass plate using human umbilical cord blood derived multipotent CB-hMSCs ,which adhered and proliferated on the glass surface. Glass fibres showing

HAp formation on their surfaces, soaked in KPD solution, also indicated their biocompatibility suitable for cells culture. As CB-hMSCs are easily available from cost effective source of human cord blood, we believe there is immense possibility of differentiation and proliferation of such CB-hMSCs into osteoblasts or other lineages on these types of SiO_2 free moderately low temperature melting borate glass surfaces for future *in vivo* TE and biomedical applications.

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