Preparation and investigation of nano-carbonated hydroxyapatite/polymer composite spongy scaffolds for biomedical applications

S.A. Siddiqi[1], F. Manzoor[1], U. Azhar[1], A. Jamal[1], M. Tariq[2], M.S. Anwar[3], M. Saleem[3], A.A. Chaudhry[1], I.U. Rehman[1,4]

1. Interdisciplinary Research Centre in Biomedical Materials (IRCBM), COMSATS IIT. Defence Road, Off Raiwind Road, Lahore
2. Department of Biology, Syed Babar Ali School of Science and Engineering, LUMS, DHA, Lahore 54792, Pakistan
3. Department of Physics, Syed Babar Ali School of Science and Engineering, LUMS, DHA, Lahore 54792, Pakistan
4. Department of Material Science and Engineering, The Kroto Research Institute, University of Sheffield, Broad Lane, S3 7HQ, Sheffield, UK

ABSTRACT

Spongy scaffolds containing nano-carbonated hydroxyapatite (nCHA), with composition $Ca_{10}(PO4)_{6-y}(CO3)_y(OH)_2$,with y=2,4, were prepared by the lyophilization method. Different polymers, natural gelatin (G), chitosan (CS) and polyvinyl alcohol (PVA), alone or in combination, were used to make their composites with nCHA. All the samples were characterized by (XRD), (SEM), (FT-IR) techniques. Particle size and surface area were determined by BET surface area analysis. The nCHA containing scaffolds mediated cytotoxicity (MTT assay) and cell attachment studies (SEM) were carried out on rat bone marrow derived mesenchymal stem cell (MSC). SEM studies revealed varying microstructures for pore sizes, shapes and pore interconnectivity. All the scaffolds were found to be highly porous, porosities beyond 70%. It is observed that the scaffold morphology changes significantly for one nCHA/polymer combination to the other. Furthermore we investigated the biocompatibility of these scaffolds by performing cell viability and cell attachment tests. Our results indicated evident cell attachment on all the materials and also no significant cytotoxicity was observed in cells grown with these materials compared with control. The preparation of spongy scaffolds and their biocompatibility augment their potential use in tissue engineering and biomedical applications.

***Keywords**: carbonated hydroxyapatite, cell attachment, freeze drying, spongy scaffolds, polymer scaffolds, lyophilization*

1 INTRODUCTION

Human bone contains up to 8wt% carbonate ions that occupy phosphate and hydroxide positions in the apatite lattice. It has been shown that the presence of carbonate in synthetic hydroxyapatite (HA) leads to structural disorder and a higher solubility. However, this disorder is useful because it gives rise to higher HA bonding to surrounding tissue. Carbonate-substituted HA is therefore, considered as an optimized biomaterial, which leads to faster bonding between an inserted implant surface and human bone[1].

In recent past, considerable attention has been given to the fabrication of porous ceramic scaffolds for osseous tissue regeneration by different preparation methods. Freeze casting is a simple technique to produce complex-shaped highly porous ceramic or polymeric scaffolds. An ideal scaffold should have the following characteristics: non-toxicity, biocompatibility, suitable mechanical strength, biodegradability with a rate that can match the rate of tissue regeneration, and should not have negative effects on the surrounding tissues and organs. Meanwhile, it should have high porosity and interconnected porous structure to provide adequate space for the cells' seeding, growth and proliferation. With these requirements in mind,we report here thesynthesis of porous scaffolds of nCHA with different biologically accepted polymers (G, PVA, CS) and explore them for their biomedical applications [1,2].

2 EXPERIMENTAL METHODS

2.1 Preparation of Freeze dried spongy scaffolds

The scaffolds are prepared by the procedure given below

2.1.1 Preparation of Gelatin/ Carbonated-HA scaffold

The analytical grade Gelatin powder (Fluka, Germany), 1g, was dissolved in 40 ml distilled water at 40°C with constant stirring for 6h and 1g each of hydrothermally pre-prepared [3], nano-CHA, $Ca_{10}(PO4)_{6-y}(CO3)_y(OH)_2$with y=2,4, were added to transparent solution with constant stirring to obtain a milky slurry of nCHA and G. The slurry was poured into 24-well plate template and kept at 40°C in a freezer for 6 h. Subsequently, the samples were lyophilized for 24 h.

2.1.2 Preparation of PVA/ Carbonated-HA scaffold

Similarly PVA (Merck M_w=72,000) powder, 1g, was dissolved in 40ml distilled water at 90°C with constant stirring for 1h. Pre-prepared nano-CHA, $Ca_{10}(PO4)_{6-y}(CO3)_y(OH)_2$ with y=2,4, 1g each, were added, one by one, to PVA transparent solution with constant stirring to obtain a milky slurry of nCHA and PVA. The slurry was poured into 24-well plate template and kept at -40°C for 6 h in a freezer. Subsequently, the samples were lyophilized for 24 h.

2.1.3 Preparation of Chitosan/ Carbonated-HA scaffold

Similarly chitosan powder, extracted from shrimpshells, 81% Degree of Deacetylation (DD), (M_w=88kDa), was dissolved in distilled water at 50°C with constant stirring for 6h. A few drops of acetic acid were added to enhance CS dissolution. Similarly, pre-prepared nano-CHA, with y=2,4, 1g each, were added to CS solution with constant stirring to obtain milky slurries of nCHA and CS. The slurries were poured into 24-well plate template and kept at -40°C for 6 h. Subsequently, the samples were lyophilized for 24 h.

2.1.4 Preparation of PVA/Chitosan/ Carbonated-HA scaffold

Pre-prepared 20ml distilled water solutions, with each of 0.5g PVA solution, at 90°C, and CS, at 40°C, were mixed together at room temperature with constant stirring for 6h. Pre-prepared 1g nano-CHA, powders, with y=2,4, were added to PVA and CS solutions with constant stirring to obtain a milky slurry of nCHA, PVA and CS. The slurries were poured into 24-well plate template and freezed at 40°C for 6 h. Subsequently, the samples were lyophilized for 24 h [4].

2.2 In vitro culture of rat mesenchymal stem cells (MSC)

Rat mesenchymal stem cells (rMSC) were isolated from femur of 4-5 weeks rats using direct adherence method. The femur was isolated under sterile conditions. A disposable aseptic syringe was used to draw antibiotic supplemented L-DMEM medium and to repeatedly fill bone marrow cavity to collect cells in a sterile petri dish. The obtained cell suspension was centrifuged at 250xg for 5 minutes. The cell pellet was resuspended in DMEM (GIbco) containing 10% FBS (Gibco) and 0.1% penicillin and streptomycin (GIbco) and transferred to T25 tissue culture flask. The flasks were incubated at 37 deg. C in a 5 % CO2 incubator. Cells isolated from one rat were cultured in one flask. The first medium was changed after 4 days. Later on the medium was changed on alternative days until the cells become 70–80 % confluent. MSC were sub-cultured at 70–80 % confluence. The cells were trypsinized, counted (dead cells excluded by trypan blue assay) and passaged in T-75 flasks. Second- or third-passage MSC were used for cytotoxicity and SEM analysis.

2.3 Cytotoxicity Assay

Cellular toxicity was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) assay. Prior to cell culture, all the scaffolds were sterilized under UV light for 6 hrs/autoclave. Immediately before cell seeding the scaffolds were washed 2-3 times with PBS and pre-conditioned in DMEM medium for an hour. MSC were seeded in 24-well cell culture plate with 5*104 cells per well with or without scaffolds. Cells seeded in 24-plate wells without scaffold were used as positive control. Post day 7 the medium was discarded and cells/scaffolds were washed with 1 ml PBS. 1 ml (0.5 mg/ml) MTT solution was added to each well and the plate was incubated at 37°C for 3 hrs. The MTT solution was discarded and the cells/scaffolds were washed once with 1 ml PBS. To solubilize the formazan crystals 0.5 ml dimethyl sulfoxide (DMSO) was added to each well and the plate was kept under shaking for 15-20 minutes. The optical density (OD) of the dissolved crystals was measured by using microplate reader at 590 nm. The assay was set up in triplicate with MSC derived from 3 different rats for each sample. % Viability is represented as mean ± SD of 3 independent experiments.

X-ray diffraction (XRD) was performed on XPERT-PRO diffractometer, operated at 40 kV and 40 mA using Cu Karadiation. The detector was scanned over a range of 2θangles from 20°to 80°at a step size of 0.02°. FT-IR (Thermo-Nicolet 6700 P Spectrometer (USA)) was taken in the wavenumber range of 500 cm^{-1} to 4000 cm^{-1} in the photo-acoustic mode in the resolution of 8 cm^{-1} with 256 numbers of consecutive scans.Scanning electron microscopy was performed on Nova NanoSEM-450. SEM was operated on low vacuum mode at 50 Pa. Low vacuum detector was attached with its cone under pole piece. All other parameters are mentioned on bar of the images. The pore size and surface was determined by BET (Tristar II 3020analyzer) using liquid nitrogen.

3 RESULTS AND DISCUSSION

3.1 XRD of Carbonated Hydroxyapatite

XRD patterns of nCHA, y=2, with gelatin (G), polyvinyl alcohol (PVA) and chitosan (CS) are shown in Fig 1a.Hydroxyapatite peaks are appeared when 2θ is 26.1°, 31.8°, 46.2° and 51.3°. These patterns resemble the JCPDScard number 9-0432 which is the evidence of hydroxyapatite. However, these spectra are different somewhat which is due to the presence of the polymers in CHA. In Fig. 2 XRD pattern of CHA y=4 with polymers has been shown. The spectra clearly show the presence of CHA.

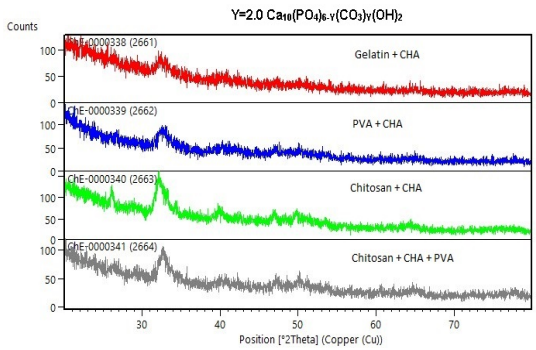


Figure 1 XRD pattern of spongy scaffolds y=2

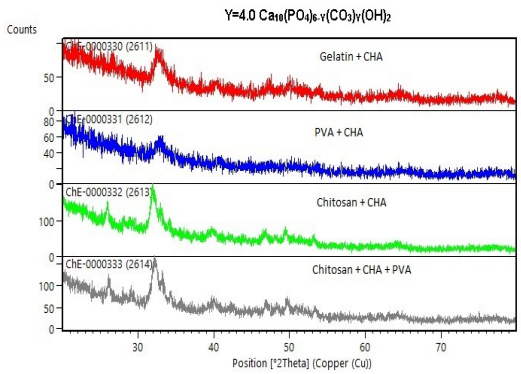


Figure 2 XRD pattern of spongy scaffolds y=4.

3.2 Fourier Transform InfraredSpectroscopy (FT-IR)

The FT-IR spectra of CHA with polymers have shown in the Fig. 3. These are the spectra of CHA y=2 with different polymers. The PO_4^{-3} appeared in the spectra at the peaks 562cm^{-1} and 1014cm^{-1} while CO_3^{-2}peaks appeared in the range of 745cm^{-1} to 755cm^{-1} which is due to the presence of carbonate in the CHA. OH^{-1} stretch can be seen between 3480 cm^{-1} and 3560 cm^{-1}.

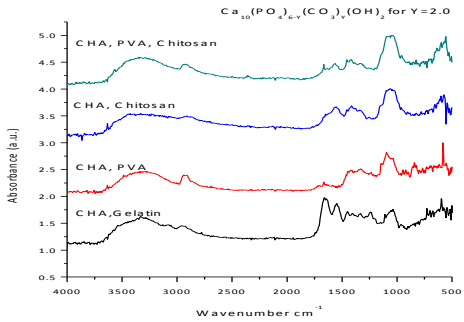


Figure 3 FT-IR of nCHA/polymer scaffolds y=2

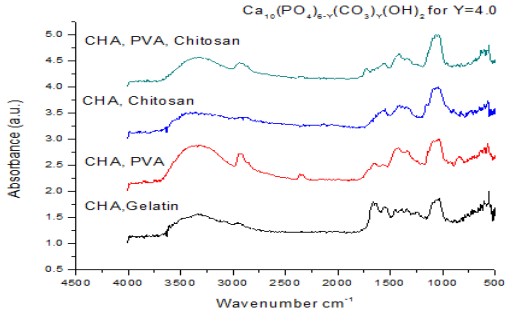


Figure 4 FT-IR of nCHA/polymer spongy scaffolds y=4

3.3 Scanning Electron Microscopy (SEM)

To study the surface morphology of the freeze dryer and MSC cell attached samples, electron microscopy was performed on all the samples and their micrographs has shown below in the fig. SEM images show that the polymer matrix has uniformly dispersed in the nCHA matrix. The interconnected porousstructure can also be seen in the micrographs. It has noted that the pore size and porosity increased from y=2 to y=4

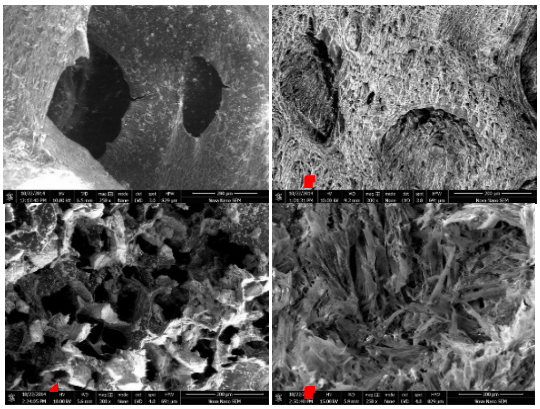


Figure 5 Freeze dried scaffolds of CHA y=4 with A) Gelatin, B) PVA, C) Chitosan, D) PVA and Chitosan

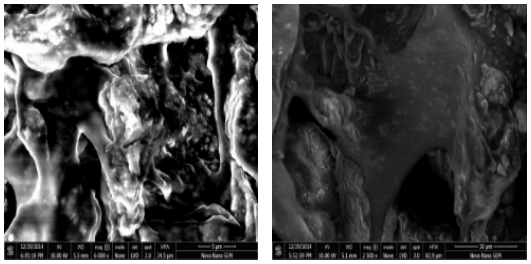


Figure 6 SEm showing MSC cell attachment on the spongy scaffolds

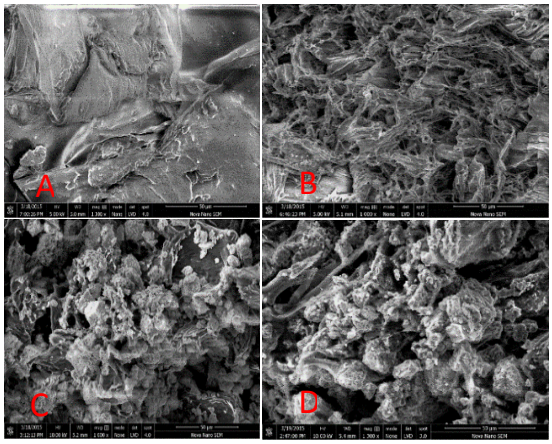


Figure 7 Freeze dried scaffolds of CHA y=2 with A) Gelatin, B) PVA, C) Chitosan, D) PVA and Chitosan

3.4 Surface area and pore size

Surface area plays an important role in the cell attachment. It is shown in the Figs 8,9 that our scaffolds are mesoporous. The pore size is maximum, as shown in the Table 1.In the case of gelatin for nCHAboth for y=2 and 4 that may be due to the dispersion of polymer in the nCHA matrix.

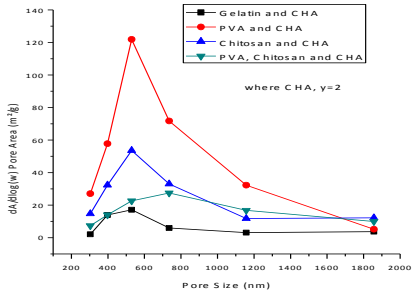


Figure 8 BET of spongy scaffolds for y=2

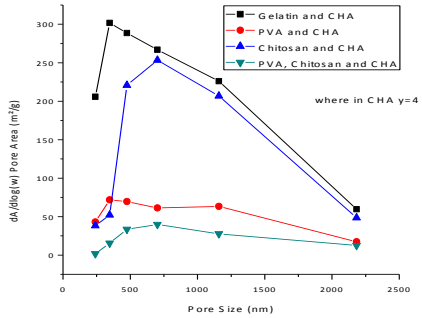


Figure 9 BET of spongy scaffolds for y=4

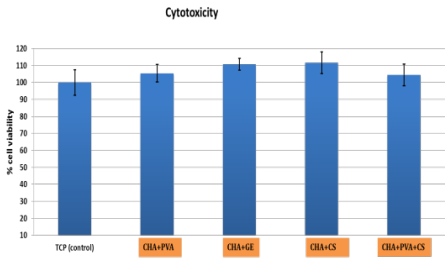
Scaffolds of nCHA with	Y	Surface Area (m^2/g)	Pore size (nm)	Pore volume (cm^3/g)
G	4.0	3.6889	9,59.02	0.884
PVA	4.0	16.4998	57.49	0.237
CS	4.0	27.6406	107.92	0.746
PVA+CS	4.0	18.2690	21.78	0.0994
G	2.0	5.6739	22.66	0.032
PVA	2.0	40.7931	12.04	0.123
CS	2.0	23.5861	19.54	0.115
PVA+CS	2.0	21.0259	13.61	0.071

Table 1 BET data of spongy scaffolds

3.5 Cytotoxicity

Cytotoxicity tests showed that the samples are non-toxic for tissue engineering applications.

Figure 10 Cytotoxicity of spongy scaffolds



4 CONCLUSION

The studies showed that the spongy scaffolds resemble the natural bone and the biological studies suggest that the scaffolds are biocompatible and can be used in bone tissue engineering.

Acknowledgements:

The authors are grateful to HEC, Govt. of Pakistan, for providing funds under NRPU project No. 1835.

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