Electronic Supplementary Material (ESI) for Materials Advances.

Synthesis and characterization of β-cyclodextrin/carboxymethyl chitosan/ hydroxyapatite fused with date seeds extract nanocomposite scaffolds for regenerative Bone Tissue Engineering

Reshma Jolly[a], Mohammad Furkan[b], Aijaz Ahmed Khan[c], Syed Sayeed Ahmed[d], Sharique Alam[e], Mohd Ahmadullah Farooqi[f], Rizwan Hasan Khan[b], Mohammad Shakir[a]*

[a]Inorganic Chemistry Laboratory, Department of Chemistry, AMU, Aligarh 202002, India.

[b]Interdisciplinary Biotechnology Unit, AMU, Aligarh 202002, India.

[c]Neuroanatomy Laboratory, Department of Anatomy, J. N. Medical College, AMU, Aligarh, 202002, India.

[d]Department of Oral and Maxillofacial Surgery, Dr. Ziauddin Ahmad Dental College, AMU, Aligarh, 202002,India.

[e]Department of Conservative Dentistry & Endodontics, Dr. Ziauddin Ahmad Dental College, AMU, Aligarh, 202002, India

[f]Department of Building Engineering, College of Architecture and Planning, Imam Abdulrahman Bin Faisal Universtiy, Dammam, 34221, Saudi Arabia.

Author for Correspondence: shakir078@yahoo.com

Phone No: +91-9837430035

2. Materials and Method

S.2.1. Materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide) (MTT), Carboxymethyl chitosan(CMC), β-Cyclodextrin (β-CD), Phosphate Buffer Saline (PBS), Dulbecco's modified Eagle's medium (DMEM) and Triton lysate were purchased from Sigma-Aldrich (USA) and Invitrogen, USA, respectively. Ajwa date fruits (Tamr stage) were harvested from local markets of Saudi Arabia. $[Ca(NO_3)_2.4H_2O]$ (99%), $(NH_4)_2HPO_4$ (DAHP) (99%), NaOH(>97%), CH_3COOH (99.8%), tri-(hydroxylmethyl) aminomethane [TRIS], DMSO, $NaHCO_3$, KCl, $K_2HPO_4.3H_2O$, $MgCl_2.6H_2O$, Na_2SO_4,NaCl, $CaCl_2$, sodium dodecyl sulfate, p-nitrophenyl phosphate, Diethanolamine, EDTA, Glutaraldehyde, Ethanol, HCl, and ammonia solution (25%) have been obtained from Merck, Mumbai, India. All chemicals were of reagent grade and were used as received. All aqueous solutions were prepared with deionized water (DH_2O).

S.2.2. Characterizations

S.2.2.1. Morphological and Chemical Characterizations

To elucidate the surface chemical functionality, Fourier transform infrared (FTIR) spectra (4000-400 cm^{-1}) were recorded on Interspec 2020, spectrolab, UK. X-ray diffractometer (XRD; Philips PW1710 diffractometer at 40 kV, 60 mA) was to investigate the crystallinity and the mineral phase of the samples with Cu Kα radiation (λ=1.540 Å) in the 10^o- 80^o range. The evaluation compressive strength of the nanocomposite scaffolds was done at room temperature using a universal mechanical testing machine (Instron 5967, USA) to analyze the mechanical parameters. The load was applied until the samples were fractured and crosshead speed owas maintained as 1 mm/min. The compressive strength S (in MPa) is calculated by following formula:

$$S = F/A$$

where, F is the maximum compressive load (in Newton), and A is the surface area (in mm^2) of the nanocomposite scaffolds perpendicular to the load axis (in square millimeters). The modulus was calculated from the stress–strain curve slope [1].

Transmission electron microscopy (TEM, Hitachi H-7500 Japan) was used to study the size of nanoparticles (voltage 120 kV) and the average size of nanoparticles was obtained from more than 100 nanoparticles in TEM images through Image J (NIH, USA). Scanning electron microscopy (SEM) and X-ray spectrometry (EDX) (JEOL-JAPAN) was used to study the surface morphology of the synthesized nanocomposite scaffolds. The hydrophilic nature of the nanocomposite scaffolds was determined by the static water contact angle measurement using goniometer equipped with a video capture (DSA-25; Kruss Gmbh, Germany).

S.2.2.2. *in–vitro* calcification assay

The *in-vitro* bioactivity of CH, BCHD1, BCHD2 and BCHD3 nanocomposite scaffolds was evaluated in the simulated body fluid (SBF) solution. In brief, the samples were immersed in SBF 37 ^oC to allow the soaking of SBF solution [2]. After the designated period of 4 and 8 weeks, the nanocomposite scaffolds were withdrawn from SBF, washed with DH_2O, dried, and after sputter-coated with gold, examined under SEM.

S.2.2.3. Protein adsorption

The CH, BCHD1, BCHD2 and BCHD3 nanocomposite scaffolds were pre-washed with PBS, air dried and incubated with 500 µL fetal bovine serum (FBS-GIBCO) at 37 ^oC for 2 h to evaluate their protein adsorption ability. Thereafter, the samples were rinsed with PBS following the method reported previously [4]. Results were expressed as the means ±SD of three independent measurements.

S.2.2.4. Cell Proliferation assay

The cell proliferation on CH, BCHD1, BCHD2 and BCHD3 nanocomposite scaffolds was performed on human osteoblasts MG-63 cell line to quantitatively measure the cell proliferation rate. Briefly, MG-63 cells at a density of 0.5 x 10^4/well were seeded onto nanocomposite samples at various concentrations (0-64 µg/ml) in 96-well culture plates incubated at 37 ^oC and 5% CO_2 [5]. Then, the samples were removed from the wells after 24 h and the wells were washed with phosphate-buffered saline (PBS, pH = 7.4). The cells adherent to the well walls were found to be viable and incubated with 0.5% MTT solution. The media containing MTT was removed and subsequently 0.1% dimethyl sulfoxide (DMSO) was added. The amount of the blue formazan compound is an indicative of the number of living cells and optical density was read at 570 nm using spectrophotometry.

S.2.2.5. Alkaline phosphatase (ALP) assay

ALP activity of osteoblast cells (isolated from femur bone shaft of the Wistar rat) was assessed using ALP assay. The cell suspensions were seeded on CH, BCHD1, BCHD2 and BCHD3 nanocomposite scaffolds and cultured in polystyrene 6-well dishes following the procedure reported in the previous studies [4]. The absorbance of reaction product was measured at 405 nm using a plate reader (Bio-Rad). The value of ALP activity was normalized by total protein content, and expressed as the total protein content (U/g protein).

S.2.2.6 Lactate dehydrogenase (LDH) assay:

Lactate dehydrogenase (LDH) leakage assay was performed to compute the membrane integrity of cells cultured on CH, BCHD1, BCHD2 and BCHD3 nanocomposite samples. After 24h of cell seeding, the cell culture medium was collected from each well for LDH measurement using a LDH assay Kit (Span Diagnostic kits, Mumbai, India) according to manufacturer's protocol [6]. The absorbance values after colorimetric reaction were recorded at 450 nm using microplate reader. The lysed cells on tissue culture plate (TCP) were used as

control. The LDH leakage was expressed as the percentage of the amount of LDH released from test group to the maximum amount of LDH released from the control.

References

[1] R. Govindan, G. S. Kumar, E. K. Girija, Polymer coated phosphate glass/hydroxyapatite composite scaffolds for bone tissue engineering applications, *RSC Adv*., 2015, **5,** 60188-60198. DOI: 10.1039/c5ra09258b

[2] L. C. Palmer, C. J. Newcomb, S. R. Kaltz, E. D. Spoerke, S. I. Stupp, Biomimetic Systems for Hydroxyapatite Mineralization Inspired By Bone and Enamel, *Chem. Rev.,* 2008, **108,** 4754-4783. DOI:10.1021/cr8004422

[3] H. Liu, H. Yazici, C. Ergun, T. J. Webster , H. Bermek, An in vitro evaluation of the Ca/P ratio for the cytocompatibility of nano-to-micron particulate calcium phosphates for bone regeneration, *Acta Biomater*., 2008, **4,** 1472-1479. DOI:10.1016/j.actbio.2008.02.025

[4] A.K. Jaiswal, H. Chhabra, V.P. Soni, J.R. Bellare, Enhanced mechanical strength and biocompatibility of electrospun polycaprolactone-gelatin scaffold with surface deposited nano-hydroxyapatite, *Mater. Sci. Eng. C*., 2013, **33,** 2376-2385. DOI: http://dx.doi.org/10.1016/j.msec.2013.02.003

[5] D. Gopi, S. Nithiya, E. Shinyjoy, D. Rajeswari, L. Kavitha, Carbon Nanotubes/Carboxymethyl Chitosan/Mineralized Hydroxyapatite Composite Coating on Ti-6Al-4V Alloy for Improved Mechanical and Biological Properties, *Ind. Eng. Chem. Res*., 2014, **53,** 7660-7669. DOI:dx.doi.org/10.1021/ie403903q

[6] H. Xie, P. Wang, J. Wu, Effect of exposure of osteoblast-like cells to low dose silver nanoparticles: uptake, retention and osteogenic activity, Artif. Cells, *Nanomed., Biotechnol*. ,2019, **47(1),** 260-267. DOI: https://doi.org/10.1080/21691401.2018.1552594