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Short communication

Fabrication of 3D dual-layered nanofibrous graft loaded with layered double hydroxides and their effects in osteoblastic behavior for bone tissue engineering

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ABSTRACT

Nanofibers coated onto the bone implant as a dual-layered material has supported the regeneration of bone cells suitable for bone tissue engineering. Herein, we report the electrospinning of Poly(3-Hydroxybutyric acid)-Poly (N-vinylpyrrolidone) NanoFibrous Matrix (NFM) loaded with Layered Double Hydroxides (LDH) was coated over the Hydroxyapatite (HAP) pellet as a dual-layered nanofibrous bone graft for bone tissue engineering. The morphology of the fabricated dual-layered nanofibrous bone graft was characterized using scanning electron microscopy. Further, the existence of the LDH and HAP over the dual layer were analyzed with EDX spectrum. The *in vitro* biocompatibility and fluorescence activity of the dual −layered nanofibrous bone graft (NFM-LDH: HAP) against MG63 cell lines was studied over 1, 3, 7 and 14 days. The osteoblast like MG63 cells assisted excellent cell adhesion and proliferation over both top and bottom layer of the dual-layered nanofibrous bone graft. In conclusion, the fabricated bone graft proved to be promising bone implant in tissue engineering application.

1. Introduction

The development of bone graft as a suitable substitute to autograft and allograft types still remains as the one of the most interesting technique in bone tissue engineering [1]. The advances in the bone tissue engineering have led to the development of 3D bone graft coated with nanofibrous matrix to support easy cell adhesion, proliferation and mineralization [2]. Nowadays, nanofibers in bone tissue engineering have attracted significant interest in development of dual layered bone graft through electrospinning technique. The high surface to volume ratio and porosity has promoted cell adhesion and proliferation in nanofibrous scaffolds [3]. Poly (3-hydroxybutyric acid) is a hydrophobic polymer and which is a common metabolite in all higher living organism and further has potential biomedical application [4]. Poly (N −vinylpyrrolidone) is a biocompatible hydrophilic polymer and possesses potential bioadhesive properties to cells and tissues. These properties make it a suitable scaffold for tissue engineering applications [5]. The Hydroxyapatite (HAP) is mainly present in human bone as the major composition along with collagen. However, the unique architecture of HAP in bone along with its biocompatible and osteoconductive ability serves as the promising material for fabricating bone graft [6]. Layered double hydroxides (LDHs), a class of naturally occurring materials generally expressed by the formula $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2](A^{n-})_{x/n}{\cdot}mH_2O$, have attracted increasing attention due to their unique 2D structure with formation of interlayer anions. Synthetically, these materials can be easily prepared by simple co-precipitation method [7], template synthesis using some organic polymers (soft templates) which acquire control over the particle morphology with uniform size distribution [8–10]. The LDH was unique in the cellular and drug delivery application due to its low toxicity, stability and biocompatibility [11]. Furthermore, the LDH materials based on the organic-inorganic hybrid materials has received much scientific attention due to their cheap, ecocompatible and easy in nanocomposites fabrication [12–15]. Romeo et al., showed that the presence of Mg-Al LDH powered improves the electrospinnability of polycaprolactone (PCL) electrospun nanofibers [16]. In this study, a simple method combined with electrospinning techniques was employed for the fabrication of dual-layered nanofibrous bone graft. The morphology and *in vitro* biocompatibility of the

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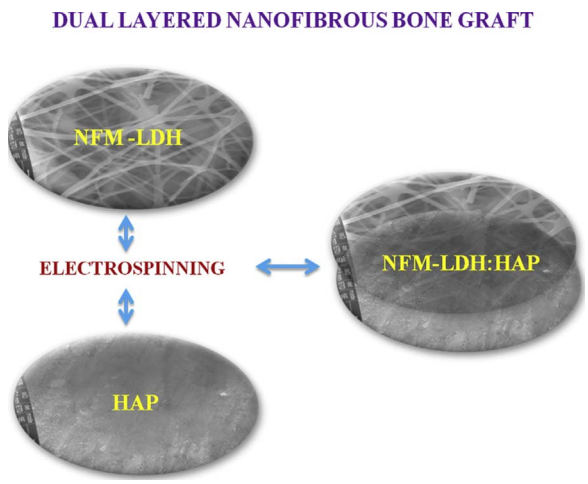

Fig. 1. (a) Schematic representation of fabricated dual layered nanofibrous bone graft.

nanofibrous bone graft was done by investigating MG63 cells over the fabricated bone graft.

2. Materials and methods

Magnesium nitrate hexahydrate ($Mg(NO_3)_2{\cdot}6H_2O$), Aluminium nitrate nonahydrate ($Al(NO_3)_3{\cdot}9H_2O$), Sodium carbonate (Na_2CO_3), Sodium hydroxide (NaOH, 97%), Poly(3-Hydroxybutyric acid) (PHB), Poly(N-vinylpyrrolidone) (PVP), Hydroxyapatite, 1,1,1,3,3,3-hexafuoro-2-propanol (HFIP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Calcein AM, Dulbecco's Modified Eagle's Medium (DMEM), Fetal Calf Serum (FCS), and supplementary antibiotics for tissue culture were purchased from Sigma Aldrich. The MG63 cell lines were obtained from the National Centre for Cell Science (NCCS), Pune India. Other chemicals and culture wares were purchased from Sigma Aldrich, unless specified otherwise were prepared.

2.1. Synthesis of Mg-Al layered double hydroxides

Aqueous solution of metal salts (Mg and Al nitrates with 1:2) was added drop wise at a rate of 1 mL/min to a flask containing sodium carbonate solution and adjusted to pH 9. The synthesis was performed at room temperature and under constant magnetic stirring to ensure homogeneity of the reaction medium. The solution was stirred continuously for 2 h and the precipitate was aged overnight. The precipitate was then washed several times with distilled water to attain neutral pH and to remove the impurities. The samples was then dried at 50 °C in hot air oven and grounded into a fine powder to yield the final product, hereafter noted LDH [17].

2.2. Fabrication and characterization of the dual layered nanofibrous bone graft

The HAP pellet was made using the manually operated hydraulic press applied with 10 ton force and kept separately. The 6 wt% concentration of hydrophobic polymer PHB and hydrophillic polymer PVP solution were prepared by dissolving in HFIP. The dissolved solutions were blended with 1:1 ratio with 0.2% prepared LDH at constant stirring for 12 h. Further, the uniformly blended PHB-PVP-LDH mixture was electrospun over the prepared HAP pellet placed above the rectangular aluminum substrate at a distance of 10 cm perpendicular to a 24G needle connected to the positive terminal of the high-voltage DC power supply (ZEONICS, Bangalore, India). The polymer solution was extruded at 0.8 mL/h using a computer controlled syringe pump and was subjected to an electric potential of 1.5 kV/cm. The nanofibrous matrix with LDH (NFM-LDH) collected over the HAP pellet as a dual-layered bone graft (NFM-LDH: HAP). The fabricated bone graft was ethylene oxide sterilized and stored at room temperature until further use [5,18]. The schematic representation was depicted in Fig. 1. The prepared LDH and NFM-LDH samples were characterized for their XRD patterns [13]. The surface and cross-sectional morphology of prepared samples (LDH, NFM, NFM-LDH, NFM-LDH: HAP) were analyzed by scanning electron microscopy (JEOL JSM-6460 LV and F E I Quanta FEG 200 − HRSEM) along with EDX analysis for the top and bottom layer separately. The samples were coated with gold to enhance the surface conductivity before scanning [19]. Approximately fifty places were taken and the pore size on the fabricated scaffold was measured using the UTHSCA Image tool software. The porosity of the fabricated material was achieved by the liquid displacement method [14].

2.3. In vitro biocompatibility and cell proliferation

The ethylene oxide sterilized scaffolds were further evaluated for the *in vitro* cell viability using MTT assay. The MG63 cells were seeded over both the side of the bone graft separately placed in 24 well plates (Corning, NY) and maintained in DMEM with 10% fetal calf serum supplemented with penicillin (120 units/mL), streptomycin (75 mg/mL), gentamycin (160 mg/mL), and amphotericin B (3 mg/mL) at 37 °C at a density of 5×10^4 cells/mL and then incubated over a time interval for 1, 3, 7 and 14th day in a humidified atmosphere of 5% CO2. Cells cultured in blank wells were used as control. After 1, 3, 7 and 14th day the culture medium was replaced with a serum-free medium containing 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and incubated at 37 °C for 4 h in a humidified atmosphere of 5%

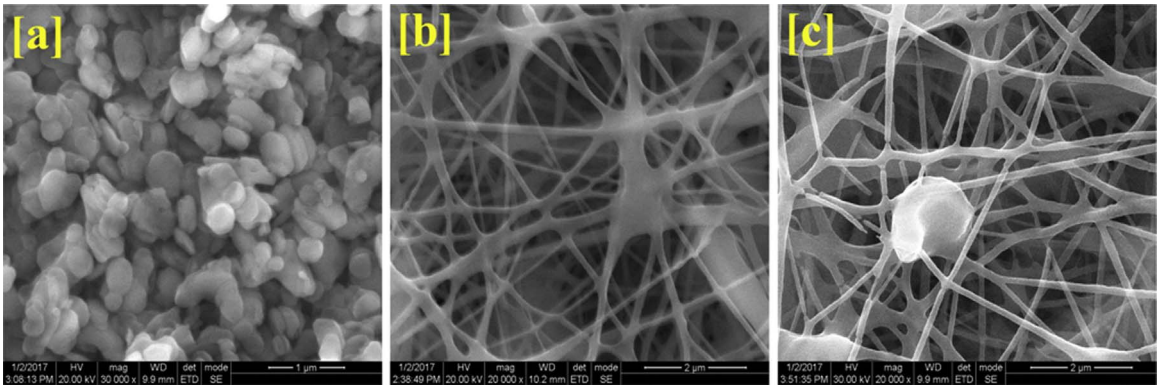

Fig. 2. SEM images of (a) synthesized Layered Double Hydroxides (LDH,) fabricated (b) Nanofibrous matrix (NFM) and (c) Nanofibrous Matrix loaded with Layered Double Hydroxides (NFM-LDH).