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Precipitation of nanohydroxyapatite on PLLA/PBLG/Collagen nanofibrous structures for the differentiation of adipose derived stem cells to osteogenic lineage

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ABSTRACT

Tissue engineering and nanotechnology have enabled engineering of nanostructured materials to meet the current challenges in bone treatment owing to rising occurrence of bone diseases, accidental damages and defects. Poly(L-lactic acid)/Poly-benzyl-L-glutamate/Collagen (PLLA/PBLG/Col) scaffolds were fabricated by electrospinning and nanohydroxyapatite (n-HA) was deposited by calcium-phosphate dipping method for bone tissue engineering (BTE). The abundance and accessibility of adipose derived stem cells (ADSC) may prove to be novel cell therapeutics for bone repair and regeneration. ADSCs were cultured on these scaffolds and were induced to undergo osteogenic differentiation in the presence of PBLG/n-HA for BTE. The cell-biomaterial interactions were analyzed using cell proliferation, SEM and CMFDA dye extraction techniques. Osteogenic differentiation of ADSC was confirmed using alkaline phosphatase activity (ALP), mineralization (ARS) and dual immunofluorescent staining using both ADSC marker protein and Osteocalcin, which is a bone specific protein. The utmost significance of this study is the bioactive PBLG/n-HA biomolecule introduced on the polymeric nanofibers to regulate and improve specific biological functions like adhesion, proliferation and differentiation of ADSC into osteogenic lineage. This was evident from the immunostaining and CMFDA images of ADSCs showing cuboidal morphology, characteristic of osteogenic lineage. The observed results proved that the PLLA/PBLG/Col/n-HA scaffolds promoted greater osteogenic differentiation of ADSC as evident from the enzyme activity and mineralization profiles for bone tissue engineering.

1. Introduction

Bone is an active organ persistently undergoing remodeling to adapt to mechanical stress, to remain healthy and to repair injuries. The current 'gold standard' for surgical repair of bone loss involves the use of an autograft [1,2]. However, autologous grafts present several limitations such as (i) they are not readily available, (ii) difficult to shape anatomically to match the defect area, and (iii) morbidity at the donor sites [3–5]. Hence, tissue engineering strategies have been adopted to produce scaffolds which can be employed to treat bone defects *in vivo*. Adipose tissue provides an abundant source of multipotent stem cells which are capable of undergoing osteogenic differentiation *in vitro* [6,7] Studies have shown that adipose-derived stem cells (ADSC) have similar immunophenotype, multilineage potential, and transcriptome compared to bone marrow derived MSC (BMSC). Moreover, ADSC have several advantages like (i) they are abundant, (ii) more accessible and (iii) have lower donor morbidity, which in combination make ADSC a better alternative to BMSC, especially from a clinical perspective where large cell numbers are required for regeneration, it appears that ADSC are most useful cell type.

Biomaterial scaffolds that are employed for bone tissue engineering should provide temporary structural and functional support within a bone defect. Besides, the scaffold should also be non-cytotoxic and biodegradable to non-toxic products [8]. Most of these material requirements are satisfied by poly(α-hydroxyl acids) such as poly(L-lactic acid) (PLLA) [9]. Despite good mechanical properties and predictable biodegradation kinetics, PLLA does not provide a favorable surface for cell adhesion and proliferation due to lack of specific cell-recognition moieties [10]. Most mammalian

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cells are anchorage-dependent cells and they need a substrate with cell binding moieties for attachment, proliferation and differentiation. Recent studies have demonstrated that cell adhesion and survival could be improved by protein pre-adsorption on the surface [11,12]. Polypeptides like Poly-benzyl-L-glutamate (PBLG) are particularly interesting for this purpose because of their capacity to adopt ordered conformations such as α-helices or β-strands, which may be useful to direct the formation of nanoscale structures and also to present functional ligands by more flexible chains [13]. In the present study PBLG, a polymer of glutamic acid in which the γ-carboxyl groups have been benzoylated, has been electrospun along with PLLA in order to improve cell adhesion and differentiation. Of particular interest in this study is the ability of ligand-functionalized polymeric scaffolds to guide the osteogenic differentiation of progenitor cells, in the absence of any induction medium, for potential use in bone tissue repair. PLLA/PBLG nanofibrous scaffolds have been developed as an ECM analogue to concurrently provide biochemical signaling and nanostructural assembly, thereby creating a biomimetic environment with an instructive capacity for bone regeneration at the molecular level. Moreover, derivatives of glutamic acid like PBLG possess high calcium binding affinity which is essential for differentiation and bone regeneration. Collagen is a natural ECM component of many tissues, such as skin, bone, tendon, ligament, and other connective tissues. Previous studies have shown that electrospun collagen in combination with other synthetic polymeric materials were able to promote bone regeneration [14]. Besides possessing cell binding moieties, the scaffolding materials for bone tissue engineering should also be osteoconductive so that osteoprogenitor cells can adhere and migrate on the scaffolds and subsequently form new bone [9,15]. Studies have shown that calcium phosphate based materials like hydroxyapatite (HA) provide surfaces that are conducive to bone cell attachment and proliferation, termed osteoconductivity. The biological advantages of employing nano-HA (n-HA) are (i) it is the major inorganic component of the bone matrix, (ii) its specific affinity towards many adhesive proteins, and (iii) its direct involvement in bone cell differentiation and mineralization processes, making it an attractive candidate for bone regeneration [16]. However, its brittle nature has limited its applications. This has led to the use of alternative materials like titanium alloy and resorbable polyesters like PLLA to improve its mechanical properties. In the present study, we employed a hybrid strategy to maintain the desired osteoconductivity of n-HA with the formability of polymers like PLLA to create a scaffold material design factor that can enhance osteogenic differentiation of ADSC for bone tissue engineering [17]. The incorporation of n-HA within a degradable polymeric network may provide a more favorable synthetic microenvironment to more closely mimic natural tissue physiology, with the additional properties of higher mechanical strength. For being fully efficient system for BTE, the targeted system should associate simultaneously multiple functionalities like cell binding, calcium binding, differentiation capabilities and osteoconductivity. In the present study, the *in vitro* responses of ADSCs to the surface mineralized PLLA/PBLG/Col/n-HA nanofibrous substrate were investigated, in terms of the initial cell adhesion, proliferation and further osteogenic differentiation and mineralization.

2. Materials and methods

2.1. Fabrication of nanofibrous constructs

The materials used for electrospinning were Type I collagen (Koken Co. Tokyo, Japan), PBLG, PLLA, and 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) were purchased from Sigma Aldrich Chemical Company. PLLA pellets were dissolved in HFP at a w/v ratio of 10%. PLLA/PBLG solution was prepared at a ratio of 90:10 in HFP at the same concentration of 10%. Further PLLA/PBLG/Col solution was also prepared at the ratio of 80:10:10 in HFP. The polymer solution was then loaded into a syringe (Becton Dickinson, BD, N.J, US.) and a high voltage electric field of 12 kV (DC high voltage power supply from Gamma High Voltage Research, Florida, US.) was applied to draw the fibers from the spinneret (27G1/2 needle) onto the collector plate. The spinneret was first grounded to give a flat tip in order to produce continuous and uniform nanofibers. A constant feed rate of 1 mL/h was applied using a syringe pump (KD Scientific Inc., M.A., US.) and a distance of 12 cm was maintained between the tip of the spinneret and the collector plate. The electrospun nanofibers were subsequently vacuum dried so that any residual solvent present could be removed. Biomineralization procedure was then carried out on the PLLA/PBLG/Col samples to precipitate n-HA by calcium-phosphate dipping method. The electrospun PLLA/PBLG/Col nanofibers were initially immersed in 0.5 M $CaCl_2$ solution (Sigma) for 10 min. The samples were then rinsed in DI water for 1 min. The samples were then immersed in 0.3 M of Na_2HPO_4 (Merck & Co. Inc., N.J, US.) for 10 min and rinsed for 1 min in DI water. This entire procedure was considered as 1 cycle. The scaffolds were subjected to 3 cycles of the above treatment. The first cycle was for 10 min and the subsequent cycles were for 5 min in each solution. After biomineralization the scaffolds were freeze dried overnight for characterization studies.

2.2. Material characterization

The surface morphology of electrospun nanofibrous scaffolds was studied under Scanning Electron Microscope (JEOL JSM − 5600LV) at an accelerating voltage of 15 kV, after gold coating (JEOL JFC-1200 fine coater, Japan). For measuring the fiber diameter of electrospun fibers from the SEM images, $n = 10$ fibers were chosen at random on each of the scaffolds. For each scaffold material $n = 5$ samples were chosen for analysis and the average fiber diameter was then calculated along with SD using image analysis software (Image J, National Institutes of Health, USA). Functional groups present in the scaffolds were analyzed using Fourier Transform Infrared (FTIR) spectroscopic analysis on Avatar 380, (Thermo Nicolet, Waltham, MA, USA) over a range of 400–4000 cm^{-1} at a resolution of 8 cm^{-1}. The hydrophobic or hydrophilic nature of the electrospun fibers was measured by sessile drop water contact angle measurement using VCA Optima Surface Analysis system (AST products, Billerica, MA).

2.3. Cell culture

2.3.1. Isolation of adipose derived stem cells (ADSC)

The standardized protocol for ADSC isolation as pioneered by Rodbell and Jones [18] was followed in the present study. The adipose tissue was collected from rabbits in accordance with the NUS ethical guidelines. The tissue were minced finely and washed with sterile phosphate-buffered saline (PBS) to remove red blood cells. It was followed by digestion with 0.1% collagenase for 30 min at 37 °C. Following incubation, the collagenase was neutralized with an equal volume of standard culture media and centrifuged to separate the floating population of mature adipocytes from the pellet containing a heterogeneous population of blood cells, pericytes, endothelial cells, fibroblasts, preadipocytes, and other cells. The final isolation step consists in selecting only the preadipocytes (i.e., the ADSC population), by taking advantage of the adherent property of these cells to plastic after plating. The isolated ADSCs were confirmed using immunofluorescent staining. The cells were fixed in 100% ice cold methanol for 15 min. The samples were then washed with PBS once for 15 min and incubated in 0.5% Triton-X 100 solution for 5 min to permeabilize the cell membrane. Non-specific sites were blocked by incubating the cells in 3% BSA (Sigma) for 1 h. Primary antibody ADSC specific marker protein CD 105 (abcam, USA) was added in the dilution 1:100 for 90 min at room temperature. This was followed by the addition of secondary antibody Alexa Fluor 488 (Invitrogen) in the dilution 1:250 for 60 min at room temperature. The samples were washed with PBS thrice to remove the excess staining and then incubated with DAPI in the dilution 1:5000 for 30 min at room temperature. The samples were then removed and mounted over a glass slide using Vectashield mounting medium and examined under the fluorescent microscope (Olympus FV 1000). Fig. 1a shows the ADSCs stained with the immunofluorescent marker protein CD 105 and Fig. 1b shows an optical microscope image of the isolated ADSCs.

The electrospun fibers collected on round glass cover slips of 15 mm diameter, were placed in a 24-well plate with a stainless steel ring to prevent swelling. The fibers were sterilized under ultraviolet light for 2 h washed thrice with PBS for 15 min each in order to remove any residual solvent, and subsequently immersed in complete medium (DMEM/F12 medium/10% FBS/1% antibiotics) overnight before cell seeding. The isolated ADSC were then seeded on the scaffolds at a cell density of 10,000 cells per well.

2.4. Cell proliferation

Cell proliferation on the nanofibrous substrates was determined using the colorimetric MTS assay (CellTiter 96 Aqueous One solution, Promega, Madison, WI). The reduction of yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4sulfophenyl)-2H tetrazolium] in MTS to form purple formazan crystals by the dehydrogenase enzymes secreted by mitochondria of metabolically active cells formed the basis of this assay. The formazan dye shows the