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ORIGINAL RESEARCH PAPER

Preparation and investigation of polylactic acid, calcium carbonate and polyvinylalcohol nanofibrous scaffolds for osteogenic differentiation of mesenchymal stem cells

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ABSTRACT

Objective(s): In this study, the effect of electrospun fiber orientation on proliferation and differentiation of mesenchymal stem cells (MSCs) was evaluated.

Materials and Methods: Aligned and random nanocomposite nanofibrous scaffolds were electrospun from polylactic acid (PLA), poly (vinyl alcohol) (PVA) and calcium carbonate nanoparticles (nCaP). The surface morphology of prepared nanofibrous scaffolds with and without cell was examined using scanning electron microscopy. Mechanical properties of electrospun nanofibrous scaffolds were determined with a universal testing machine. The *in vitro* properties of fabricated scaffolds was also investigated by the MTT assay and alkaline phosphatase activity (ALP).

Results: The average fiber diameter for aligned and random nanofibers were 82 ± 12 nm and 124 ± 25 nm, respectively. The mechanical testing indicated the higher tensile strength and elastic modulus of aligned nanofibers. MTT and ALP results showed that alignment of nanofiber increased the osteogenic differentiation of stem cells.

Conclusion: Aligned nanofibrous nanocomposite scaffolds of PLA/nCaP/PVA could be an excellent substrate for MSCs and represents a potential bone-filling material.

Keywords: *Electrospinnng, Mesenchymal stem cells, Nanofbrous scaffold*

INTRODUCTION

Tissue engineering has enormous potential to provide functional substitutes to guide the regenerative process of the damaged tissue. The main element of tissue engineering is to prepare substitutes. Providing biological and structural cues that mimic the complex properties of the native tissue has become an essential component in the design of tissue-engineering scaffolds. Electrospinning has emerged as a mainstay in tissue engineering due to its versatility in fabricating randomly oriented or aligned fibers that are characteristic of the extracellular matrix [1]. Electrospinning is a versatile and facile technique which can be used to prepare fibers with dimensions of nanometers to micrometers from natural and synthetic polymers. It has been drawn much attention as a potential method to generate various fibers for the biomedical applications [2-6].

Due to the high surface-area-to-volume ratio of the electrospun fibers and the high porosity on the submicrometer length scale of the obtained nonwoven mat, they can be used in many biomedical applications, including drug delivery, wound healing, and scaffolding for tissue engineering [7-10]. Many polymers, including synthetic or natural ones, have been electrospun into NFs with diameters ranging from tens of nanometers to a few micrometers [11-12]. The most commonly used synthetic polymers are polylactide (PLA), polyglycolide (PGA), and polycaprolactone (PCL), along with their corresponding copolymers, due to their biodegradability and biocompatibility. PLA, due to its slow degradation rate, is a good candidate to be used in bone scaffolding applications. In addition, many

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researchers have reported that incorporation of calcium carbonate ($CaCO_3$) or a type of calcium phosphate such as hydroxyapatite (HA) helped to enhance mechanical properties and improve osteoblast proliferation and differentiation [13-16]. Poly (vinyl alcohol) (PVA), recognized as one of the hydrophilic polymers, is also susceptible to ultimate biodegradation. It is a water-soluble, nontoxic polymer, with good biocompatibility. Addition of PVA as a hydrophilic material to PLA/$CaCO_3$ nanofibrous mat could increase cell attachment. In-vitro studies have demonstrated the influence of alignment on the osteogenic and neurogenic differentiation of stem cells cultured on nanofibrous scaffolds [17-18]. Mesenchymal stem cells (MSCs) have been widely used in regenerative medicine and tissue engineering studies, and proven to have signiûcant potential in clinical application because of their convenient isolation, lack of signiûcant immunogenicity, high capacity of expansion, and potential to differentiate into tissue-speciûc cell types [19-25]. To the best of our knowledge, there are no reports about the performance of MSCs on PLA/$CaCO_3$/PVA combination. In the present contribution, aligned and random electrospun nanofibrous scaffolds were produced as potential substrates for MSCs growth, aiming at comparing the effect of fiber orientation and surface morphology on proliferation of MSCs.

MATERIALS AND METHODS

Materials

PVA with molecular weight of 72 kD and 98% degree of hydrolysis was obtained from Merck. PLA with molecular weight of 75 kDa was purchased from Sigma- Aldrich. Calcium carbonate nanoparticles (nCaP) were supplied from Sigma-Aldrich. Chloroform was purchased from Merck (Germany). Dulbecco's modified Eagle's Medium (DMEM) was obtained from Sigma; fetal bovine serum (FBS), antibiotics and trypsin-EDTA were purchased from GIBCO invitrogen (Carlsbad, CA, USA). All other reagents and chemicals were used without further purification.

Solution preparation and electrospinning

A weighed amount of PVA powder was dissolved in de-ionized water to obtain a 8 % (w/w) %) solution and gently stirred with a magnetic stirrer at 80 º C for 5 h. Prepared solution, then cooled to room temperature and stirred for 3 h to ensure homogeneity. $CaCO_3$ nanoparticulate powder (10.0 (w/w) %) was dispersed in 10 mL Chloroform to form a suspension.

Then 1.0 g of PLA pellets were added to the suspension. This was followed by magnetic stirring until the polymer dissolved completely. A double-spinneret electrospinning machine (ANSTCO-N/VI, Asian Nanostructures Technology Co., Iran) was used for the preparation of composite nanofibrous scaffolds. The solutions were fed into 5 ml standard syringes using a syringe pump with a rate of 0.7 and 0.2 ml/hr for PLA/ $CaCO_3$ and PVA solution, respectively. The ground collection plate of aluminum foil was located around 15 cm from the needle tip. A high voltage (Nano spinner TM, Iran) was applied to draw the ultra-fine fibers from the spinneret. Aligned nanofibers were formed using a rotating disk setup at 3000 rpm with the same parameters to obtain well-aligned nanofibers.

Characterization of the surface morphology

The fibers' morphology was characterized using a scanning electron microscope (SEM; Vega II XMU instrument Tescan, Czech Republic) after specimens were coated with gold using a sputter coater. The fibers' diameter was determined from SEM images using image analysis software.

Mechanical characterization of nanofibrous scaffolds

Tensile properties of electrospun nanofibrous scaffolds were determined with a universal testing machine (STM-20, SANTAM Design & Manufacturing Co., Iran) using low-force load cell of 10 N capacity. Strip-shaped specimens (60 mm × 10 mm) were tested at a crosshead speed of 50 mm/min. At least six samples were tested for each type of electrospun nanofibrous scaffold. Ultimate strength and Young's modulus, were calculated based on the generated tensile stress–strain curves.

Wettability of nanofibrous scaffolds

The water contact angles of PLA, PVA and PLA/$CaCO_3$/PVA nanofibrous scaffolds were measured by Sessile drop method with a G10 contact angle goniometer (Kruss, Germany) at room temperature. A water droplet was placed on the scaffold surface and the contact angle was measured after 10s.

Culture of mesenchymal stem cells in nanofibrous scaffolds

MSCs were aspirated from iliac crest marrow of New Zealand white rabbits. Aspirated marrow was transferred to sterile tubes to which 20 ml DMEM medium was added.

The mixtures were centrifuged at 1000 rpm for 5min. Following removal of supernatant, cells were resuspended in 10 ml of DMEM containing 10% FBS and 1% antibiotics, and 10^5 cells were then plated and cultured in culture dishes at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Non-adherent cells were removed by changing the culture medium after 5 days of culture. After 2 weeks of primary culture, each dish of cells was passaged into three culture dishes every 7 days and cells from passage 2 were used for cell culture experiments. MSCs were seeded onto NFMs at a density of 10^5 cells/well in 24-well culture plates containing osteogenic differentiation medium (50 µg/l L-ascorbic acid, 10^{-8} mol/l dexamethasone, 10 mmol/l β-glycerophosphate, 10 mmol/l vitamin D3, 100 µg/ml penicillin, 100 µg/ml streptomycin, 0.3 µg/ml amphotericin, 2.2 g/L sodium bicarbonate and 15% fetal bovine serum) and incubated in a humidified CO_2 incubator at 37 °C.

Cell morphology

Cell/scaffold samples were fixed in 3% glutaraldehyde for 2 h at room temperature, dehydrated through a graded series of ethanol soaks, dried and sputter coated with gold. Then, observed under a SEM (SEM; Vega II XMU instrument Tescan, Czech Republic) at an accelerating voltage of 10 kV.

Characterization of cell behaviors cultured in nanofibrous scaffolds

To determine cell proliferation and differentiation, cells/scaffolds were recovered at days 1, 7, and 21 for determination of cell number and alkaline phosphatase (ALP) activity. Tissue culture plates (TCPs) were used as control. The proliferation of MSCs on aligned and random scaffolds was determined using the MTT assay.

The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells. First, each culture medium was aspirated and replaced with 250 ml per well of MTT solution at 0.5 mg/ ml for a 24-well culture plate. Secondly, the plate was incubated for 1 h at 37 °C. The solution was then aspirated and 900 ml per well of dimethylsulfoxide (DMSO) containing 125 ml per well of glycine buffer (pH=10) was added to dissolve the formazan crystals. Finally, after 10 min of rotary agitation, the absorbance of the DMSO solution was measured by using a microplate spectrophotometer.

The analytical assays were performed on a daily basis and at least five wells were randomly taken for examination each time. To evaluate alkaline phosphatase (ALP) activity, the total protein of cells on TCPS and scaffolds was extracted using 200 μL of RIPA buffer. The lysate was then centrifuged at 14000 g at 4 °C for 15 min to sediment cell debris. The supernatant was collected and ALP activity was measured with an ALP assay kit (Parsazmun, Tehran, Iran), using *p*-nitrophenyl phosphate (p-NPP) as substrate and alkaline phosphatase provided in the kit as a standard. The activity of enzyme (IU/L) was normalized against total protein (mg/dl).

Statistical Analysis

All quantitative results were obtained from triplicate samples. Data were expressed as the mean ± standard deviation. Statistical analysis was carried out using the unpaired Student's t-test and one-way analysis of variance. A value of $P < 0.05$ was considered to be statistically signiûcant.

RESULTS AND DISCUSSION

Characterization of nanofibers

Fabricated PLA/nCaP/PVA scaffolds showed porous structure with interconnected pores and uniform smooth nanofibers with an average diameter of 82 ± 12 nm and 124 ± 25 nm for random and aligned nanofibers, respectively. SEM images of prepared scaffolds are shown in Fig 1. As shown, the fibers on the random scaffold exhibit a more random orientation. MFD of aligned nanofibers is smaller than random ones. This could be due to the rotation of the cylindrical collector, which exerts a pulling force on the jet, and consequently reduces the size of aligned nanofibers.

Mechanical properties

Fig. 2 shows the stress-strain curves of aligned and random nanofibers. As shown, the tensile strength of aligned and random nanofibers was 2.86 ± 0.72 and 6.54 ± 0.82 MPa, respectively. Young's modulus for aligned nanofibers was 119.02 ± 31.20 MPa, which is higher compare to random nanofibers (65.27 ± 12.62 MPa). Yin et.al found that the alignment of PLLA nanofibers significantly increased the mechanical properties of prepared nanofiberous scaffolds [26].

During tensile loading, only the fibers oriented along the loading direction experience the stretching force, while the fibers that are oriented perpendicular to the loading direction do not experience any force.

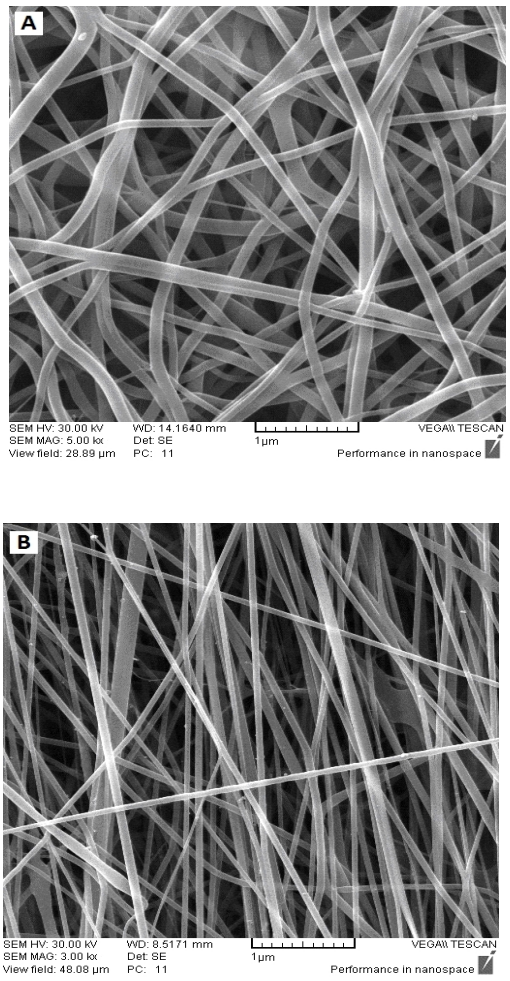

Fig. 1. SEM images of (a) random and (b) aligned nanofibrous scaffold

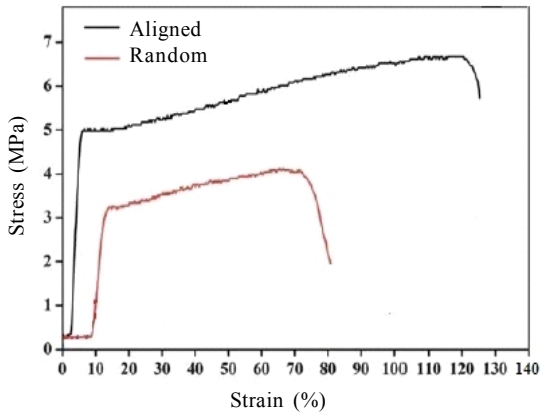

Fig. 2. Stress-Strain curves of random and aligned nanofibers

Hydrophilic properties of nanfibers

The water contact angles for PLA and PLA/nCaP scaffolds were 145 ± 2Ú and 141 ± 3Ú, respectively which imply that these scaffolds were highly hydrophobic. The PLA/nCaP/PVA nanofibrous scaffolds with water contact angle of zero were extremely hydrophilic and susceptible to 100% wettability by the water droplet due to the presence of hydrophilic groups of PVA on the surface of scaffold.

Morphology and Proliferation of MSCs

The morphology of MSCs on random and aligned scaffolds of PLA/CaP/PVA was studied on days 7 and 14 after cell seeding. As shown in Fig. 3, the cell density on the aligned electrospun nanofibers was similar to random scaffold after one week , but the surface of aligned nanofibrous mat was fully covered at days 14.

This result suggests that the aligned nanofibrous scaffolds can provide better environment for cell adhesion, proliferation and distribution compared to the random ones. MSCs on both scaffolds exhibited typical flattened polygonal morphology and spread and attached well on the surface of nanofibers. According to the MTT results (Fig. 4), cell numbers were found to be similar for all types of membranes during the early phase of cell proliferation (day 1). A significant increase in cell number was observed on both random and aligned scaffolds at 7 and 21 days. After 21 days of culture, more cells are found in the aligned NFM than random one. It could be due to the regular alignment of the scaffold, which may have a positive effect on cell proliferation. In addition, the regular alignment of the adherent cells, which allows the cells occupying the space more compactly, and consequently more cells can be packed within the fixed size of the scaffold surface area.

Differentiation of MSCs

ALP activity is a marker of early osteoblastic differentiation and commitment of MSCs toward the osteoblastic phenotype. Fig. 5 shows the ALP activity of prepared nanofibrous scaffolds. As can be seen, ALP activity increased from 7 days to 21 days of culture. However, MSCs in the aligned NFMs showed the highest ALP activity ($p < 0.05$), and the enhancement of osteo-differentiation could be related to the crucial role of fiber alignment in the stimulation of bone cell response and thus its significance in the bone regeneration.

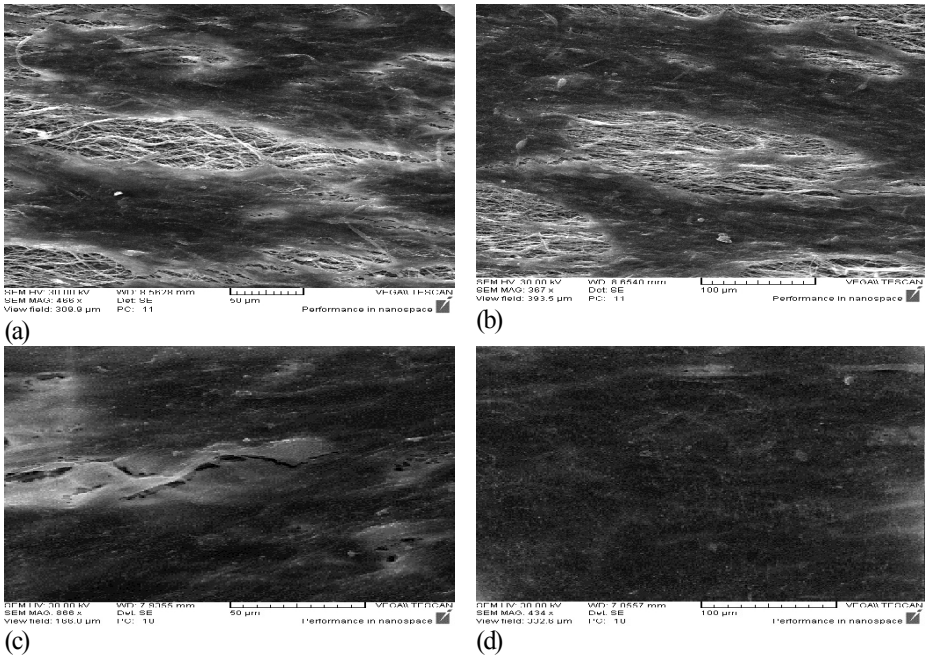

Fig. 3. SEM images of MSCs on (a,b) random and (c,d) aligned scaffold after (a,c) 7 days and (b,d) 14 days

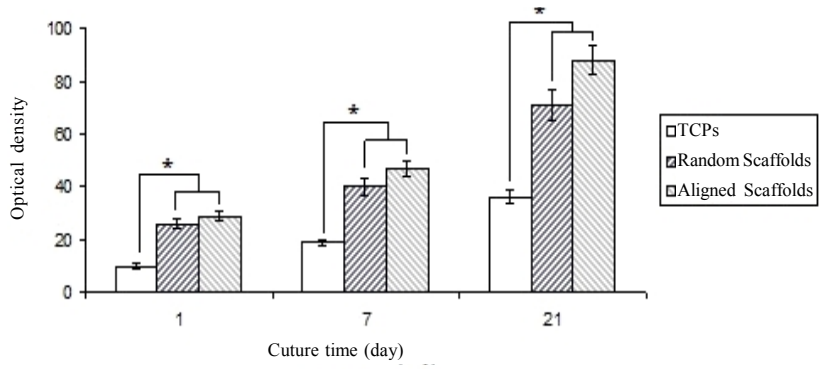

Fig. 4. MTT assay results for scaffolds (random and aligned) and TCPs

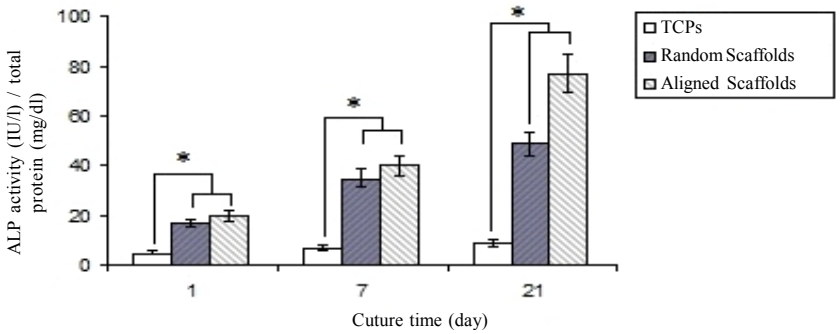

Fig. 5. ALP results for scaffolds (random and aligned) and TCPs

CONCLUSION

In summary, a novel nanofibrous nanocomposites of PLA/nCaP/PVA were prepared by electrospinning to obtain aligned and random scaffolds. The impact of orientation on MSCs proliferation and differentiation was investigated. The results of this study showed that aligned electrospun composite scaffold proved to have significantly improved cell growth and osteoblast responses when compared to random nanofibrous scaffolds. Based on these studies, aligned electrospun PLA/nCaP/PVA composite nanofibrous scaffold is considered as a promising material for bone tissue regeneration.

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