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Original Research

Aligned and random nanofibrous nanocomposite scaffolds for bone tissue engineering

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Abstract

Objective(s): Biological effects of MSCs on aligned and random nanofibrous scaffolds of PCL/PVA/nHA were investigated in this study.
Materials and Methods: Aligned and random nanocomposite nanofibrous scaffolds were electrospun from polycaprolactone (PCL), poly (vinyl alcohol) (PVA) and hydroxyapatite nanoparticles (nHA). The morphology and mechanical characteristics of the nanofibers were evaluated using scanning electron microscopy and tensile testing, respectively.
Results: Scanning electron microscopy revealed fibers with an average diameter of 123 ± 32 nm and 339 ± 107 nm for aligned and random nanofibers, respectively. The mechanical data indicated the higher tensile strength and elastic modulus of aligned nanofibers. The *in vitro* biocompatibility of aligned and random nanofibrous scaffolds was also assessed by growing mesenchymal stem cells (MSCs), and investigating the proliferation and alkaline phosphatase activity (ALP) on different nanofibrous scaffolds.
Conclusion: Our findings showed that the alignment orientation of nanofibers enhanced the osteogenic differentiation of stem cells. The *in vitro* results showed that the aligned biocomposite nanofibrous scaffolds of PCL/nHA/PVA could be a potential substrate for tissue engineering applications, especially in the field of artificial bone implant.

Keywords: Bone tissue engineering, Electrospinnig, Nanofibers

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Introduction

Tissue engineering is a multidisciplinary field that combines both the principles of engineering and life sciences for the development of biological substitutes for the restoration, maintenance or improvement of tissue function. For engineering living tissues, biodegradable scaffold is generally considered as an indispensable element as they are used as temporary templates for cell seeding, invasion, proliferation, and differentiation prior to the regeneration of biologically functional tissue or natural extracellular matrix (ECM). Bone is a highly complex tissue which provides mechanical support, acts as mineral reservoir, supports muscular contraction resulting in motion withstands load bearing and protects internal organs (1, 2). An ideal scaffold to be used for bone tissue engineering should possess characteristics of excellent biocompatibility, adequate pore size, controllable biodegradability and suitable mechanical properties (3, 4).

Of the various techniques available for scaffold fabrication, electrospinning is simple and produces scaffolds with high porosity having interconnected pores and nanoscale matrix features (5, 6). In this process, a polymer solution in a capillary is subjected to an electric field generated by high voltage. A polymer jet is ejected from the capillary when the electric field overcomes the surface tension. As the jet travels toward a grounded collector, it undergoes an instability that stretches the jet and solid fibers are deposited on the collector in the form of a nonwoven fabric (7, 8).

Several reports have shown that the electrospun scaffolds serve as a better environment for cell attachment and proliferation, since they resemble the ECM (9-14). Since extracellular matrix (ECM) has its own specific architecture in each tissue, the biomimetic approach of the scaffold fabrication can be improved via considering the topographies similar to that seen in native ECM (15, 16). This special architecture can affect the tissue-specific cell morphology, function and mechanical properties (16). Mimicking the nanoscale structure of ECM is an effective strategy to design and develop tissue-engineered scaffolds (17, 18).

A more physiologically accurate approach for tissue engineering is to use primary marrow stromal cells (MSCs) to study the cellular response to a synthetic tissue scaffold. A scaffold containing bone MSC extracts has demonstrated accelerated and enhanced bone formation within osseous defects when compared with an unpopulated matrix (19, 20). MSCs serve as a readily available source of undifferentiated cells that are capable of giving rise to diverse tissues, including bone, cartilage, muscle and other tissues of mesenchymal origins. Moreover, MSCs do not appear to be rejected by the immune system, allowing for large-scale production and appropriate characterization, and the subsequent ready availability of allogenic tissue repair enhancing cellular therapeutics (21).

Numerous natural and synthetic polymers have been investigated for the fabrication of nanofibrous scaffolds for bone tissue regeneration (22-25). Among the synthetic polymers, polycaprolactone (PCL) has been intensively studied because it is hydrolyzable in natural environments, as well as in human body (26-29). PCL has good mechanical properties, but the use of electrospun mats of PCL as biomaterial was limited by its hydrophobic nature, which leads to a low adhesion of cells to the scaffold. Its poor hydrophilicity resulted in low cell loading in the initial step of cell culture and reduction in the ability of cell adhesion, migration, proliferation and differentiation (30). Hence, it is very essential to improve the hydrophilicity of PCL so as to overcome the above difficulties arising out of its hydrophobic nature (31). 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. One reason to choose PVA as a hydrophilic additive to electrospun PCL mats is that the electrospun PVA nanofibers in the mats are not easily dissolved in water or cell culture medium, because of their poor solubility at room or body temperature (32). Since the major constituent of natural bone is the mineral hydroxyapatite (HA), it is considered as an essential component for bone tissue engineering. However, using bioceramic nano-hydroxyapatite (nHA) alone as scaffold material is not possible because of its poor mechanical properties (33). Hence a combination of a synthetic polymer or biopolymer and a bioceramic can take advantages of the mechanical properties, degradation stability and cell affinities of the individual components. Thus incorporation of synthetic HA, in particular nHA, into a nanofibrous polymer matrix not only mimics the natural bone structure but also can enhance the mechanical properties and biological response of the scaffolds (34). To devise a near-perfect scaffold for growing MSCs for bone tissue engineering, hybrid nanofibrous scaffolds from nHA, PCL and PVA were fabricated in this study.

In an effort to closely mimic the ECM, 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. To the best of our knowledge, no report on the performance of electrospun composite fibers of PCL with nHA and PVA for bone tissue scaffolds has been published thus far. In this study, aligned and random composite nanofibrous scaffolds of PCL/nHA/PVA were fabricated and evaluated for the biological behavior and osteogenic differentiation of MSCs in vitro.

Materials and Methods

Materials

PCL with molecular weight of 80 KDa and nHA (≤200 nm) were obtained from Sigma-Aldrich. 99.5% N,N-dimethylformamide (DMF) and chloroform were purchased from Merck (Germany). PVA with molecular weight of 72 KD and 98% degree of hydrolysis was obtained from Merck and used without further purification. 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 chemicals were used as received, without further treatment.

Fabrication of nanofibrous scaffolds

Solutions of PCL/nHA (10.0 %, (w/w)) and PVA with 10 % (w/w) concentration were prepared using the method described previously (35). A hybrid electrospinning was used for the preparation of composite nanofibrous scaffolds (35). Briefly, prepared solutions were fed separately into a blunted needle using a syringe pump with a rate of 0.5 mL/h and 0.3 mL/h for PCL/nHA and PVA solution, respectively. The collector was a rotating cylindrical drum which was placed at a distance of 15 cm from the needles. The rotation speed for the production of random nanofibers was 100 rpm, while it was 3000 rpm for aligned nanofibers.

Characterization of nanofibrous scaffolds

Surface morphology of nanofibrous scaffolds

The surface morphology of electrospun PCL/nHA/PVA aligned and random nanofibrous scaffolds were investigated using scanning electron microscopy (SEM; Vega II XMU instrument Tescan, Czech Republic). Diameters of the fibers were determined from SEM images using image analysis software (Image J, NIH).

Contact angle measurement

The water contact angle of the surface of the scaffolds was 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. The measured contact angle value reflects the hydrophilicity of the scaffolds.

Tensile strength measurement

Tensile properties of nanofibrous mats were determined using a universal testing machine (Galdabini, Italy) at room temperature at a cross-head speed of 50 mm/min. Scaffolds were cut into 10 mm× 60 mm × 0.1 mm specimens for mechanical testing. Five samples were tested for each scaffold.

Cell culture

MSCs were aspirated from the bone marrow of a healthy and adult New Zealand white rabbit which was older than three months and weighted between 2.5-2.8 kg, gradient centrifuged and plated into flasks containing low-glucose Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum and 2% antibiotics (200 μg/mL penicillin and 200 μg/mL streptomycin). MSCs at passage 3 were transferred into culture media containing osteogenic factors (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). Medium was changed every 3 days.

Cell seeding

The electrospun PCL/nHA/PVA scaffolds were sterilized by ethanol (70% v/v) and placed in the osteogenic culture medium for 24 h. MSCs cultured in osteogenic top of the pre-wetted scaffolds (2.0×10^5 medium for one week were seeded onto the cells/scaffold), and the cell/scaffold constructs were placed into the wells of tissue culture plates. The scaffolds were left undisturbed in an incubator for 3 h to allow for the cells to attach to the scaffold. Then, an additional 1 mL of culture medium was added into each well. The cell/scaffold constructs were cultured in a humidified incubator at 37 ˚C with 95% air and 5% CO_2 for 14 days. The medium was changed twice a week.

MTT assay

The proliferation of MSCs on aligned and random scaffolds was determined using the MTT assay. The cell culture medium was removed and 2 mL of MTT solution was added to each well. Following incubation at 37 ˚C for 4 h in a fully humidified atmosphere at 5% CO_2, MTT was taken up by active cells and reduced in the mitochondria to insoluble purple formazan granules. Subsequently, the medium was discarded and the precipitated formazan was dissolved in dimethyl sulfoxide (150 μL/well), and optical density of the solution was evaluated using a microplate spectrophotometer at a wavelength of 570 nm. The analytical assays were performed on a daily basis and at least five wells were randomly taken for examination each time. The same procedure was performed for cultured cells in tissue culture plates (TCPS) as control.

Cell morphology

Morphological study of the *in vitro* cultured MSCs on aligned and random nanofibrous scaffolds of PCL/nHA/PVA were carried out. After 7 and 14 days of cell proliferation, the cell-cultured scaffolds were processed for SEM studies. The scaffolds were rinsed twice with PBS and fixed in 2.5% glutaraldehyde for 3 h. Thereafter, the scaffolds were rinsed in deionized water and dehydrated through a series of graded ethanol, dried under vacuum, mounted onto aluminum stubs, and sputter coated with gold.

Alkaline phosphatase activity of MSCs

To evaluate alkaline phosphatase (ALP) activity, MSCs were seeded on the aligned and random porous scaffolds, and ALP activity was measured after 3, 7 and 14 days. The adherent cells were removed

from the scaffolds and lysed with PBS, followed by addition of a cell lysis buffer (RIPA buffer). The supernatants were 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 experiments were carried out at least three times and their average expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA). A value of $P \leq 0.05$ was considered to be statistically significant.

Results and Discussion

Morphological characterization of scaffolds

The morphological structure of electrospun nanofibrous scaffolds was observed by SEM. As shown in Figure. 1, though the alignment of every fiber is not perfect in the same orientation, a distinct regularity of the fibers with a specific aligned longitudinal topography is shown on the aligned scaffold. In comparison, the fibers on the random scaffold exhibit a more random orientation. Aligned fibers with diameter in the range of 123 ± 32 nm exhibited a smaller average diameter than random nanofibers with the fiber diameter of 339 ± 107 nm. This is due to the rotation of the cylindrical collector, which exerts a pulling force on the jet, and consequently reduces the size of aligned nanofibers.

Hydrophilic characteristics

Contact angle studies of nanofibrous scaffolds revealed the extent of hydrophibicity of their surface. The contact angles obtained for PCL and PCL/nHA scaffolds were 136±3˚ and 131±2˚, respectively which imply that these scaffolds were highly hydrophobic and non adsorbant for water. The PCL/nHA/PVA nanofibrous scaffolds with water contact angle of zero

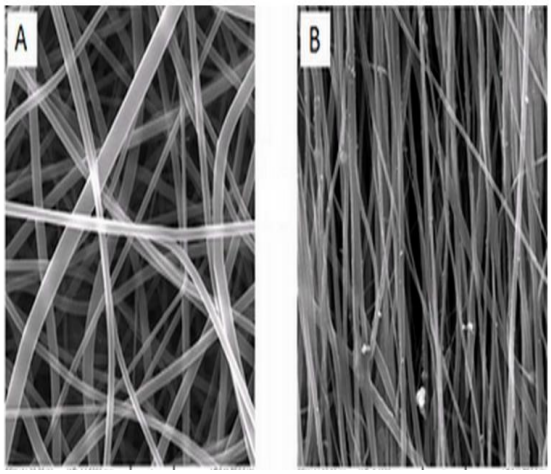

Figure 1. SEM images of nanofibrous scaffolds (a) random, (b) aligned.

were extremely hydrophilic and susceptible to 100% wet-tability by the water droplet due to the presence of hydrophilic groups of PVA on the surface of scaffold.

Mechanical properties of electrospun nanofibers

The tensile strength of aligned and random nanofibers was 2.66 ± 0.12 and 5.82 ± 0.17 MPa, respectively. Elastic modulus, which is a measure of resistance to deformation, was higher for aligned nanofibers (12.2 ± 0.92 MPa) as compared to random nanofibers (5.22 ± 0.23 MPa). The inferior mechanical properties of random nano-fibers, can be attributed to their highly porous structure. Moreover, 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.

Cell proliferation

As shown in Figure 2, both random and aligned scaffolds supported the proliferation of stem cells and there was no significant difference between the rates of cell proliferation on scaffolds. A higher rate of proliferation was observed on aligned scaffolds when compared with

random ones. MTT tests showed that the aligned scaffold was more effective at stimulating cell proliferation than random scaffold at 1, 7 and 14 days. The proliferation of MSCs on aligned PCL/nHA/PVA scaffolds was found to be 8 and 15 % higher after day 7 and 14, respectively compared to random scaffold. The regular alignment of the scaffold may have a positive effect on cell proliferation. One possible reason for the increased cell proliferation is that the mechanical stretching of the aligned nanofibers leads to better cell proliferation. This phenomenon is similar to the mechanical stimulation of cells by matrix stretching studies reported previously (14, 36). 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. This may explain the higher cell density observed on aligned scaffold when compared with random one, especially at longer proliferation period when the population of the cells is high. It can be concluded that the arrangement of cells in controlled architecture has beneficial effects on cell proliferation.

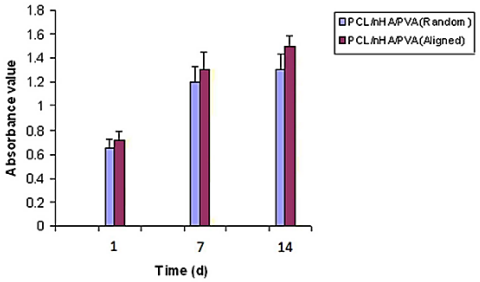

Figure 2. Proliferation of MSCs on scaffolds during a 14-day culture period.

Morphological studies of MSCs

Figure 3 shows the SEM micrographs of the aligned and random PCL/nHA/PVA scaffolds after 7 and 14 days of cell culture. The cell density on the aligned electrospun nanofibers was more random electrospun ones. This result suggests that the aligned nanofibrous scaffold can provide better environment for cell adhesion, proliferation and distribution compared to the random ones. In the present study, the diameters of the aligned electrospun nanofibers were significantly smaller than those of random nanofibers. Therefore, small diameter and uniform structure of aligned nanofibrous scaffold provided a higher surface-to-volume ratio than random ones. It has been reported that highly packed fiber or high-surface-density fiber provided a better environment for cell adhesion and proliferation (13, 37).

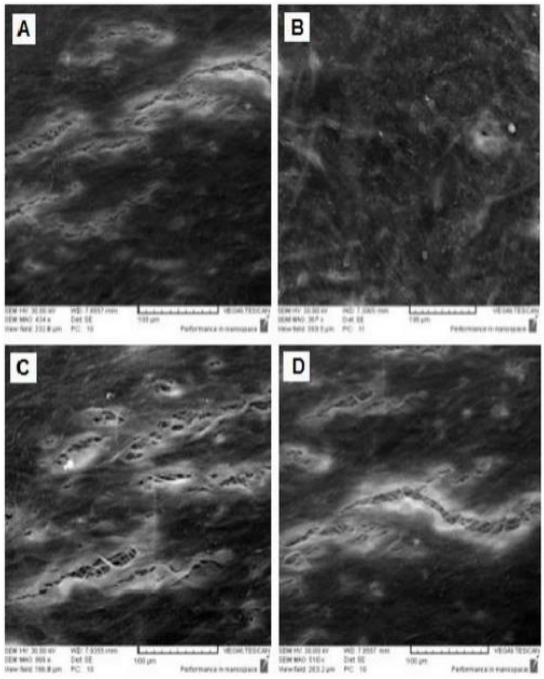

Figure 3. Morphology of MSCs on nanofibrous aligned (a,b) and random (c,d) on days 7 (a,c) and 14 (b,d).

ALP expression

The bone forming potential of the cells was further analyzed by their expression of ALP activity (Figure 4), since ALP is regarded to be an important phenotype of bone-forming cells. Of particular note, the ALP activity of the cells at days 7 and 14 was significantly higher on the aligned nanocomposite fiber than on the random PCL/nHA/PVA fibers ($P \leq 0.05$), confirming the crucial role of fiber alignment in the stimulation of bone cell response and thus its significance in the bone regeneration.

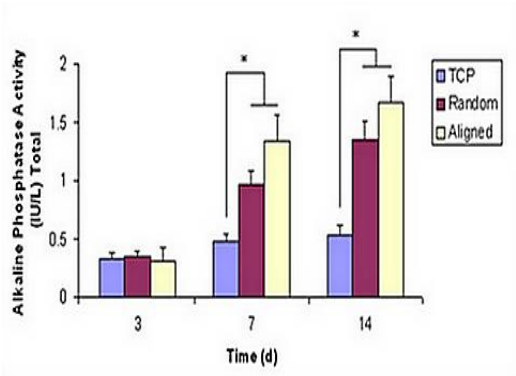

Figure 4. ALP activity of MSCs on scaffolds and TCPS during osteogenic differentiation: asterisk shows significant difference with $p < 0.05$.

Conclusion

In an attempt to devise a near-perfect scaffold for growing MSCs for bone tissue engineering, aligned and random nanocomposite scaffolds of PCL, PVA and nHA were fabricated by electrospinning. Although randomly oriented nanofibrous scaffolds are useful in tissue engineering, but the results of this study showed that aligned nanofibers highly supported the cell proliferation and differentiation process MSCs cultured on aligned composite. Nanofibrous scaffold showed high proliferation rate and a moderate increase in ALP activity. Based on these studies, it can be concluded that aligned electrospun PCL/nHA/PVA nanocomposite scaffold is potentially a promising biomaterial for bone tissue engineering.

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