The effect of electrospun poly(lactic acid) and nanohydroxyapatite nanofibers' diameter on proliferation and differentiation of mesenchymal stem cells

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ABSTRACT

Objective(s): Electrospun nanofibrous mats of poly(lactic acid) (PLA) and nanohydroxyapatite (nano-HA) were prepared and proliferation and differentiation of mesenchymal stem cells on the prepared nanofibers were investigated in this study.

Materials and Methods: PLA/nano-HA nanofibers were prepared by electrospinning. The effects of process parameters, such as nano-HA concentration, distance, applied voltage, and flow rate on the mean diameter of electrospun nanofibers were investigated. Scanning electron microscopy (SEM) was used to determine the mean fiber diameter of produced nanofibers. Mechanical propertes of nanofibrous mats were evaluated using a universal testing machine. Response surface methodology was used to model the fiber diameter of electrospun PLA/nano-HA nanofibers.

Results: The average fiber diameter for optimized nanofibers was 125 ± 11 nm. MTT and ALP results showed that optimization of fiber diameter increased the osteogenic differentiation of stem cells.

Conclusion: It could be concluded that optimization of fiber diameter has beneficial effect on cell proliferation and differentiation. Optimized nanofibers of PLA/nano-HA could be good candidates for bone tissue engineering.

Keywords: *Mesenchymal stem cells, Nanofiber, Optimization*

INTRODUCTION

The rapidly growing research in the bone tissue engineering area thus provides a new and promising approach for bone repair and regeneration. Scaffolds for hard tissue engineering applications could be fabricated from a wide variety of classes of materials. Many bone tissue engineering scaffolds have been made as composites, either by the introduction of nanohydroxyapatite (nano-HA) within polymeric matrices or by the mineralization of nano-HA on the surface of polymeric substrates [1,2]. The biologically beneficial characteristics of nano-HA, such as that it is the major inorganic component of the bone matrix, its specific affinity toward many adhesive proteins,

and its direct involvement in the bone cell differentiation and mineralization processes, make nano-HA especially attractive for applications in the bone regeneration field [3]. Mesenchymal stem cells (MSCs) have been widely used in regenerative medicine and tissue engineering studies, and proven to have significant potential in clinical application because of their convenient isolation, lack of significant immunogenicity, high capacity of expansion, and potential to differentiate into tissue-specific cell types [4-10]. Electrospinning is a well-known and ubiquitous technique to produce nanofibers and has been used by many researchers to make nanofibrous matrix for tissue engineering applications [11-15]. Scaffolds for hard tissue engineering applications could be fabricated from a wide variety of classes of materials [16]. Although

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the polymers used are similar to the polymers applied in soft tissue engineering, reinforcement of scaffolds is frequently necessary for hard tissue engineering due to their low mechanical properties. By combining two or more classes of materials into composites, such as a crystalline ceramic (e.g. hydroxyapatite) and a synthetic polymer, scaffolds with improved mechanical properties can be expected [17]. It was previously shown that both the strength and the conductivity of the film/mat of fibers produced by electrospinning are sensitive to fiber diameter [18]. Moreover, size of the fibers along with morphology influences the hydrophobic behavior of polymers [19]. Therefore, controlling the mean fiber diameter which is a function of process parameters is crucial. Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for the statistical modeling and analysis of problems in which a response of interest affected by several variables, with the objective being to optimize this response. In this study, poly(lactic acid) (PLA) polymer solutions with different amounts of nanohydr-oxyapatite were used to produce nanofiber mats by electrospinning.

The experiment parameters were material related variables (i.e., nano-HA concentration in solution) and process-related variables (i.e., applied voltage, distance and flow rate).

The result of the experiment was average fiber diameter. The effect of fiber diameter on proliferation and differentiation of MSCs on the produced nanofibers was investigated.

MATERIALS AND METHODS
Materials

PLA with molecular weight of 75 kD and nanohydroxyapatite (nano-HA) (d" 200 nm) were obtained from Sigma–Aldrich and used without further puriûcation. Chloroform was purchased from Merck.

Solution preparation

Nano-HA powder was dispersed in PLA solution with a two-step method. For this purpose, nano-HA was added in chloroform and sonicated at room temperature for 40 min. In the second step, 1.0 g of PLA pellets was added to the nHA/DMF/Chloroform dispersion.

This was followed by magnetic stirring until polymer dissolved completely with subsequent sonication for 40 min to obtain a uniform dispersion of the nano-HA in the PLA solution.

Fabrication of nanofibrous scaffold

An electrospinning machine was used to prepare nano- HA/PLA nanofibrous scaffolds. Prepared solutions with different amounts of nano-HA, were placed on a syringe pump used to dispense the solution at a controlled rate.

The collector was a rotating cylindrical drum which was placed at different distances from the needle. A high voltage electric field (Nano spinner TM, Iran) was applied to draw the ultra-fine fibers from the spinneret. The humidity during the experiments was kept the same (65%). To remove any residual solvent present in the fibers, the electrospun nanofibers were subsequently vacuum dried.

Characterization of nanofibers morphology

Scanning electron microscopy (SEM) (SEM; Vega II XMU instrument Tescan, Czech Republic) was used to characterize the morphology of electrospun nanofibers.

The samples were sputtered with a thin layer of gold prior to SEM analysis. An accelerating voltage of 20 kV was applied. Based on SEM images, the average diameter of yarns and electrospun fibers were determined by using image analysis software. The results are reported as average values of 100 measurements.

Design of experiments and response surface method

The influence of the electrospinning process parameters on the PLA/nano-HA fiber diameter was investigated using the Response Surface Method (RSM). In this study, the four operating variables (concentration, collector distance, applied voltage and flow rate) were developed by applying central composite design and the RSM. The independent variables X_1, X_2, X_3 and X4 were as follows (low/high value): nano-HA/PLA concentration ((w/w)%) 0/30; distance (cm) 5/35; voltage (kV) 9/21, and flow rate (ml/h) 0.3/1.5, respectively. The experimental range and coded levels of independent variables are presented in Table 1. Design Expert-7 Software (trial version, Stat-Ease Inc., USA) was used for the statistical design of experiments and data analysis. The obtained results are shown in Table 2 based on the experimental tests.

Table 1. Selected factors and their levels

Variable	Low axial (-2)	Low factorial (-1)	Center (0)	High factorial (+1)	High axial (+2)
X_1 (A)	0.0	7.5	15.0	22.5	30.0
X_2 (B)	5.0	12.50	20.0	27.5	35.0
X_3 (C)	9.0	12.0	15.0	18.0	21.0
X_4 (D)	0.3	0.6	0.9	1.2	1.5

X_1 (A) = Concentration (w/w) %; X_2 (B) = Distance (cm)
X_3 (C) = Voltage (kV); X_4 (D) = Flow rate (ml/h)

Table 2. Experimental conditions and their responses

Run No.	Coded value of X_1 (A)	Coded value of X_2 (B)	Coded value of X_3 (C)	Coded value of X_4 (D)	Response MFD (nm)
1	0	0	0	0	196
2	0	0	0	0	174
3	0	-2	0	0	410
4	1	-1	1	-1	332
5	-1	-1	-1	-1	195
6	0	0	-2	0	241
7	-1	-1	1	-1	215
8	-1	1	1	1	152
9	0	0	0	0	209
10	0	0	0	0	232
11	0	0	0	2	217
12	1	1	1	1	246
13	-1	-1	1	1	250
14	1	1	-1	1	298
15	0	0	2	0	275
16	-2	0	0	0	221
17	1	-1	-1	-1	324
18	1	-1	1	1	315
19	-1	1	-1	-1	495
20	-1	1	-1	1	325
21	0	0	0	-2	226
22	2	0	0	0	248
23	0	2	0	0	212
24	1	1	1	-1	217
25	0	0	0	0	192
26	-1	-1	-1	1	430
27	1	1	-1	-1	305
28	-1	1	1	-1	332
29	0	0	0	0	197
30	1	-1	-1	1	287

MFD: mean fiber diameter.

The regression correlation coefficient (R^2) is used in the context of statistical models and provides a measure of how well the observed response values fit the model. This coefficient ranges from 0 to 1. When R^2 approaches 1, the actual data fit the polynomial model properly. Low values of R^2 imply that the dependent variables in the applied model do not correlate well.

Mechanical characterization of nanofibrous scaffolds

The tensile measurements were performed on the nanofibrous webs using a universal testing machine (STM-20, SANTAM Design & Manufacturing Co., Iran). Prepared scaffolds were cut into 10 × 60 mm specimens, and a tensile test was conducted at 50 mm/min crosshead speed at room temperature. At least six samples were tested for each type of electrospun nanofibrous scaffold.

Cell culture

MSCs were seeded onto nanofiber mats 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 ìg/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 proliferation

Proliferation of MSCs on nano-HA/PLA scaffolds in comparison to TCPs (tissue culture plates) was evaluated via MTT assay. Sterilized nanofibrous membranes were placed in a 24-well culture plate, seeded with a cell density of 5×10^3 cells per cm^2, and incubated at 37 °C, 5% CO_2 under basal medium. After 1, 7, and 21 days of cell seeding, 50 µL of MTT solution (5 mg/mL in DMEM) was added to each well followed by incubation at 37 °C for 3 h. For dissolution of the dark-blue intracellular formazan, the supernatant was removed and 1 mL of DMSO (Merck) was added. The optical density was read spectrophotometrically at a wavelength of 570 nm. The same procedure was performed for cultured cells in TCPS as control.

Alkaline phosphatase (ALP) activity

ALP is used to measure ALP activity by a kinetic rate method using 2-amino-2-methyl-1-propanol buffer. In this reaction, ALP catalyzes the hydrolysis of colorless organic phosphate ester substrate, *p*-nitrophenyl phosphate, to a yellow product, *p*-nitrophenol, and phosphate. This reaction occurs at an alkaline pH of 10.3. Nanofibrous scaffolds with MSCs were washed twice with PBS and added with 1 mL of 10 mM Tris buffer to isolate the sample. The sample (1 mL) was again sonicated for 2 min at 4°C. The sonicated sample was centrifuged at 3000 rpm for 5 min at 4°C, then the supernatant was used for the assay of ALP activity.

Statistical Analysis

All experiments were conducted at least for n= 3. Data are expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to compare results. A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION
Experimental model

The experimental conditions and their responses are shown in Table 2. A polynomial model for the average variations of DOX/PLA nanoûbers was chosen and ûtted to the results. Adequate model for prediction of the response variables is given by the following equation:

$$MFD = 147.24 + 54.36A \text{ " } 45.37B + 72.59AD + 84.95BC \text{ " } 28.22BD \text{ " } 48.17ABC$$

All the variables are in coded values. Values of R-square and adjusted R-square for the model are 0.92 and 0.8, respectively. The high value of R^2 indicates that the cubic equation is capable of representing the system under the given experimental domain.

MFD optimization

An optimization technique was used in this research for optimization of PLA/nano-HA nanofibers diameter using design expert software.

The aim of electrospinning process optimization was to find the conditions of producing fibers with minimum diameter. Optimum conditions are as follows: 12.5 (w/w)% concentration, 14.5 kV voltage, 25 cm distance and 0.8 ml/h flow rate. According to the above conditions, the minimum average diameter of PLA/nano-HA nanofibrous mats was about 129 nm. In order to determine the reliability of the fibers produced from the process, a test was conducted to measure the fiber diameter from a given set of process parameters. SEM image of optimum condition is shown

in Fig 1. As can be seen, the MFD was 125 ± 11 nm. Comparing the experimental results with the response provided by the model shows that the difference is less than 5% and they are close to each other.
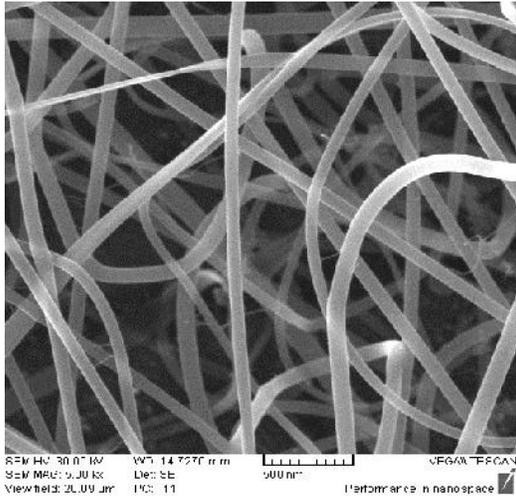

Fig. 1. SEM image of optimized nanofibers

Mechanical properties of prepared nanofibers

The mechanical properties of biocomposite nanofibrous scaffolds were evaluated by tensile testing. The tensile strength of PLA/nano-HA nanofibers (Exp. No. 19) was 2.53 ± 0.12 MPa and the elastic modulus was 7.18 ± 0.87 MPa. By optimizing of nanofibers' diameter, the tensile strength increased to 6.12 ± 0.21 MPa with an elastic modulus of 15.3 ± 0.95 MPa. According to Baji et al. [20], the mechanical properties of the nanofibers were affected by mean fiber diameter. Upon increasing the fiber size, both tensile strength and modulus decreased and the larger diameter fibers tend to display bulk-like properties. In fibers with finer diameter, the lamellae and fibrillar structures align themselves along the fiber axis, which play a critical role in enhancing the mechanical properties of the fibers.

Cell proliferation results

The effect of PLA/nano-HA fiber diameter on cell attachment and proliferation has shown in Fig 2. As can be seen, a significant increase in cell number was observed on both optimized and non-optimized scaffolds at 7 and 21 days. There was no significant difference, between the scaffolds at 1 day. The proliferation of MSCs on optimized PLA/nano-HA scaffold was found to be 15 and 40 % higher after day 7 and 21, respectively compared to non-optimized scaffold. One possible reason for the increased cell proliferation is that the mechanical stretching of the optimzed nanofibers leads to better cell proliferation.

This phenomenon is similar to the mechanical stimulation of cells by matrix stretching studies reported previously [21].
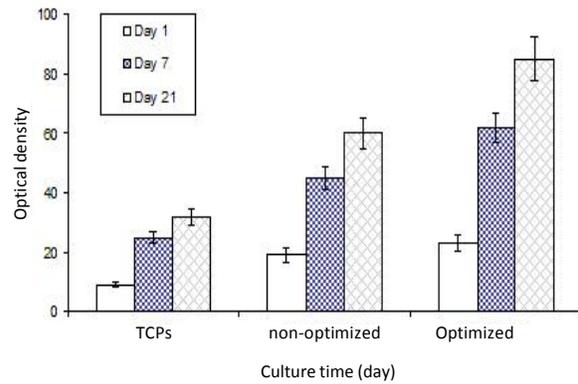

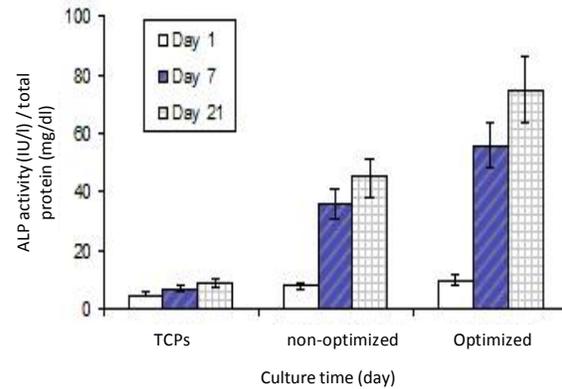Fig. 2: Proliferation results for scaffolds (non-optimized and optimized) and TCPs

Fig. 3. ALP results for scaffolds (non-optimized and optimized) and TCPs

ALP results

Fig 3 shows the ALP results for fabricated scaffolds at optimized and non-optimized conditions. As shown, ALP activity of the cells at days 7 and 21 was significantly higher on the optimized nanocomposite

fiber than on the non-optimized PLA/nano-HA fibers (*P* d" 0.05), confirming the crucial role of fiber size in the stimulation of bone cell response and thus its significance in the bone regeneration.

CONCLUSION

PLA/nano-HA nanofiber mats were successfully fabricated by electrospinning. The overall optimal electrospinning conditions were 12.5 (w/w)% concentration, 14.5 kV voltage, 25 cm distance and 0.8 ml/h flow rate. The favorable attachment and proliferation and differentiation of MSCs on the optimized nanofibers indicated good cytocompatibility compare to non-optimized nanofibers. The PLA/nano-HA fibrous composites prepared by electrospinning by using the optimized parame-ters could lead to the construction of suitable porous scaffolds for bone tissue engineering. It could be concluded that optimization of fiber diameter has beneficial effect on cell proliferation and differentiation.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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