Wet-electrospun PCL/PLLA Blend Scaffolds: Effects of Versatile Coagulation Baths on Physicochemical and Biological Properties of the Scaffolds

Majid Salehi [a], Mahdi Naseri-Nosar [b], Sadegh Ghorbani [c], Saeed Farzamfar [d], Mahmoud Azami [b]*

[a] Department of Tissue Engineering, School of Medicine, Shahroud University of Medical Sciences, Shahroud 3613773955, Iran; [b] Department of Tissue Engineering, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran 1417755469, Iran; [c] Department of Anatomical Sciences, School of Medical Sciences, Tarbiat Modares University, Tehran 14115111, Iran; [d] Department of Medical nanotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran 1417755469, Iran

Corresponding author:** Mahmoud Azami, Department of Tissue Engineering, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran 1417755469, Iran. ***E-mail: azamimahmoud@gmail.com; ***Tel***: +98 2143052112

Submitted: 2016-09-05; **Accepted:** 2016-12-14

Introduction: High surface/volume ratio and 3-dimensionality of nanofibers increases cell-scaffold interactions and promote migration and proliferation of cells. Wet electrospinning is a variant of electrospinning technology that is utilized to produce nanofibrous scaffolds. Altering the parameters governing the wet electrospinning process such as applied voltage, polymer concentration, composition and depth of the coagulation bath, and tip to bath distance can affect the morphology of the produced scaffolds. In this study, the influence of various coagulation baths on the physicochemical properties of the wet-electrospun nanofibers was investigated. **Materials and Methods**: Poly (ε-caprolactone)/Poly (L-lactic) acid 15% (w/v) blends under an applied voltage of 15 kV, and a tip-to-bath distance of 10 cm. were used to prepare fibrous scaffolds via wet-electrospinning technique into aqueous solution of sodium hydroxide (NaOH) (pH~13), distilled water, ethanol, water/ethanol (3:7) (v/v) and water/ethanol/methanol (6:2:2) (v/v). The final products were characterized by scanning electron microscopy (SEM), liquid displacement technique, contact angle measurement, compressive and tensile tests. As well as, cell adhesion and cell viability through human adipose-derived stem cells (hADSCs) cell culture. **Results:** Wet-electrospun fibers, except in the almost fully beaded structure of water/ethanol (3:7) (v/v) specimen exhibited random, dispersive and non-woven morphology under SEM observation. The coagulation bath composition significantly influenced on porosity, wettability, mechanical properties and biocompatibility of the scaffolds. The porosity measurement via liquid displacement method showed that except for the specimen in which the blend was spun into NaOH, other scaffolds could not meet the accepted ideal porosity percentage of above 80%. According to the contact angle measurement data, it was expected that all scaffolds experience low cellular attachment and proliferation. Conversely, in vitro hADSCs culture demonstrated that the scaffolds presented a non-toxic environment and enhanced cell proliferation and attachment. **Conclusion:** The data indicated that the scaffold spun into NaOH was the best candidate among other specimens to culture hADSCs.

Keywords: Human adipose-derived stem cells; Poly (ε-caprolactone)/Poly (L-lactic) acid; Scaffold; Wet-electrospinning.

Introduction

Nanofibrous scaffolds, due to their structural similarity to extracellular matrix (ECM) have gained significant attention for tissue engineering applications(1). High surface/volume ratio and 3-dimensionality of nanofibers increases cell-scaffold interactions and promote migration and proliferation of cells(2). Electrospinning technology has been widely used to produce fibers with characteristic dimensions ranging from nanometer to micrometer that could partially mimic the natural ECM (3). Wet electrospinning is a variant of electrospinning technology that utilizes a liquid reservoir collector instead of metallic ones which are used in conventional electrospinning method (4). To provide a better tissue growth environment, three-dimensional (3D) fibrous structures can improve cellular attachment, proliferation and differentiation (5). The production of bulky nanofibrous scaffolds using common electrospinning technique requires special collectors or blowing agents in between nanofibers. In contrast, wet electrospinning is a relatively simple and efficient method to produce 3D scaffolds without sophisticated procedures or special chemical additives (6). Altering the parameters governing the wet electrospinning process such as applied voltage, polymer concentration, composition and depth of the coagulation bath, and tip to bath distance can affect the morphology of the produced scaffolds (7). Material selection and optimization is an important step to fabricate fibrous scaffolds. In this regard, aliphatic polyesters such as Poly L-lactic acid (PLLA) and Poly ε-caprolactone (PCL) due to their biocompatibility, biodegradability and excellent mechanical properties have been used extensively in various biomedical applications (8). To obtain

a desirable mechanical property of the scaffold the use of blends and copolymers has been proposed for tissue engineering studies. It has been shown that PLLA and PCL blend demonstrate scaffold properties which could not be attained by only one polymer (9). In the current study, we aimed to develop 3D scaffolds using PCL/PLLA blend via a wet electrospinning technique. Based on numerous pre-experiments the electrospinning parameters including the depth of the bath, and tip-to-bath distance were fixed, and the composition of coagulation bath was the only variable. The morphological and biological properties of produced scaffolds were investigated.

Materials and Methods

Materials

PCL with molecular weight of M_w=48,000-90,000 g/mol, PLLA with molecular weight of M_w=60000 g/mol and all other reagents and solvents were purchased from Sigma-Aldrich (Germany). All the chemicals were of analytical grade and used as received without further purification.

Human adipose-derived stem cells (*h*ADSCs) were purchased from Stem Cell Technology Research Center (Tehran, Iran). Dulbecco's modified Eagle's medium/Nutrient F-12 Ham (DMEM/F12) and fetal bovine serum (FBS) were purchased from Gibco, Grand Island (USA). Penicillin and streptomycin were purchased from Sigma-Aldrich (USA) and MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) and DMSO from Carl Roth (Germany).

Sample preparation via wet-electrospinning

PLLA and PCL with ratio of (1:1) with total concentrations of 15% (w/v) in chloroform were spun into aqueous solution of sodium hydroxide (NaOH) (pH~13) (CLWN), distilled water (CLW), ethanol (CLE), distilled water/ethanol (3:7) (v/v) (CLW3E7) and distilled water/ethanol/methanol (6:2:2) (v/v) (CLW6E2M2). The electrospinning device consisted of a 10 ml syringe ended to an 18 gauge metal needle connecting to a positive high voltage source (HV100P OV, Fanavaran Nano-Meghyas, Iran), set to 15 kV. The syringe was placed into a feeding pump (SP1000, Fanavaran Nano-Meghyas, Iran) with 5 ml/h feeding rate. The extruded solution was collected at room temperature on an aluminum-foil grounded electrode connected to the high voltage supply and fixed on the bottom of a polystyrene coagulation bath. The bath had 2 cm depth and was 10 cm beneath the needle tip (Figure 1). After electrospinning, to preserve the lose structure of specimens, they were removed from coagulation bath and immediately transferred to a -20 °C freezer for 2 h and then freeze dried at -77 °C for 24 h (121550PMMA, Christ, Spain).

Scaffold characterization

The morphology of the scaffolds was observed by scanning electron microscope (SEM, AIS2100, Seron Technology, South Korea) after sputter coating with gold for 180 s using a sputter coater (SC7620, Emitech, England) at an accelerating voltage of 20 kV. The fiber average diameter was statistically calculated using a computed image analyzer (ImageJ) by measuring 20 fibers at random, and the average value was reported with standard deviations.

A liquid displacement technique was exploited to determine the porosity of the scaffolds using the following equation (10). Where V_1 is initial volume of 96% ethanol, V_2 is its volume after scaffold soaking (and ethanol filled the pores) and V_3 is volume of the ethanol after the scaffold removal.

$$\text{Porosity (\%)} = \frac{V_1 - V_3}{V_2 - V_3} \times 100$$

Tensile test was performed on dry rectangular specimens (20 mm×10 mm) by an Instron 5566 universal testing machine (Instron, MA) at a strain rate of 10 mm/min. Compression test was performed on cuboid dry specimens (4 mm×4 mm×15 mm) using a dynamic testing machine (HCT400/25, Zwick/Roell, Germany) equipped with a 1 kN load cell and at a crosshead speed of 0.5 mm/min.

The hydrophilicity of all specimens was determined using static contact angle measurements with sessile drop method using a contact angle measuring system (G10, KRUSS, Germany) (11). Finally, to monitor the pH alteration during the in vitro degradation, the scaffolds were put in normal saline (pH 6.70) at 26 °C for a total period of six weeks. The pH was measured every week by Inolab pH 720 (Manufacturer: WTW, Germany)

Cell culture and seeding

Human adipose-derived stem cells (hADSCs) were cultured in DMEM/F12 supplemented with 10% (v/v) FBS, 100 unit/ml of penicillin and 100mg/ml of streptomycin in a humidified incubator at 37 °C with 5% CO2. For scaffold sterilization, the specimens were exposed to radiation by UV light for 2h and then were immersed in 70% ethanol for 1h and dried under vacuum for 1h. Next, the scaffolds were washed twice with PBS and once with DMEM/F12. Finally, the scaffolds were transferred to 96-well plate and each cultured with 5×103 third passage cells. After 1 h incubation, 0.15 ml cell culture medium with FBS was added to each well. The medium was refreshed every 24 h(12).

Cell proliferation and attachment studies

Cell proliferation was investigated by MTT assay after three days of incubation. The media on cells was removed from each well and 0.2 ml of 5 mg/ml MTT was added. The cells then were incubated at 37 °C for 4 h. Formed purple formazan crystals were dissolved by adding 0.1 ml DMSO. The absorption was read at 570 nm using a microplate reader (Anthos 2020, Biochrom, Germany) (13). The hADSCs in conventional culture were treated identically in the other wells of the plate without scaffolds as negative control. The mean for the triplicate wells for each specimen was reported.

Table 1. Parameters of the wet-electrospun fibrous scaffolds

Samples	Mean Diameter (µm)	Porosity (%)	Contact angle (°)
CLWN	3.95±2.48	78	108.5±2.30 *
CLW	2.94±1.34	72	113.6±0.72 *
CLE	3.33±1.07	70.3	119.7±6.72
CLW3E7	1.01±0.40	29	122.4±1.34
CLW6E2M2	1.34±0.55	42.5	118.1±0.68

Mean contact angle values±SD (n=3 in each group). * indicated there was a statistically significant (P<0.05) between the two specimens compared with CLW3E7

Table 2. The mechanical properties of wet-electrospun scaffolds

Samples	Compressive modulus (MPa)	Tensile strength (Mpa)
CLWN	0.77±0.20	1.33±0.37
CLW	0.69±0.04	1.26±0.71
CLE	0.62±0.03	1.29±0.72
CLW3E7	0.81±0.04	1.52±0.80
CLW6E2M2	0.88±0.06	1.68±0.68

Mean compressive modulus and tensile strength values±SD (n=3 in each group). There is no statistically significant difference in compressive modulus and tensile strength of scaffolds

Table 3. pH values of all scaffolds in the normal saline as a function of the degradation time

Samples Day (s)	0	7	14	21	28	35	42
Control	6.70	6.70	6.70	6.70	6.70	6.70	6.70
CLWN	6.70	6.67	6.65	6.64	6.60	6.58	6.55
CLW	6.70	6.69	6.67	6.63	6.61	6.59	6.54
CLE	6.70	6.67	6.66	6.62	6.60	6.57	6.52
CLW3E7	6.70	6.69	6.66	6.65	6.62	6.60	6.55
CLW6E2M2	6.70	6.68	6.67	6.65	6.61	6.58	6.53

The percentage of attached hADSCs on scaffolds was tested with the aid of MTT assay. The cells were seeded on scaffolds and immediately transferred to incubator and were incubated for 6 h. The constructs were washed twice with PBS for 30 s, and transferred to new wells. The percentage of cell attachment was calculated using the mean absorbance value of construct specimen divided by that of negative control (12).

Statistical Analysis

The results were statistically analyzed by Minitab 17 software (Minitab Inc., State College, USA) using student t test and the data were expressed as mean±SD, n≥3. In all evaluations, P<0.05 was considered as statistically significant.

Results

Morphology of wet-electrospun scaffolds

Figure 2 and table 1 show the morphology and mean diameter of fibers of all scaffolds. SEM micrographs illustrated that (in all scaffolds) the fibers were oriented in a random, dispersive manner, forming a non-woven porous structure. In CLWN and CLE scaffolds, the fibers had a similar cylindrical fiber shape which means in these scaffolds, the composition of the coagulation baths did not significantly influenced the morphology of the wet-electrospun fibers (Figure 2a, 2b and 2c respectively). Conversely, the mean diameter of the fibers in these scaffolds was under the influence of bath composition. In the PCL/PLLA in distilled water/ethanol (CLW3E7) scaffold, large circular beads were dominant in the structure and very few fibers were observed (Figure 2d). In the PCL/PLLA in distilled water/ethanol/methanol (CLW6E2M2) scaffold, some beads were observed but their presence was negligible (Figure 2e).

Porosity and mechanical studies

Porosity and mechanical properties are important parameters for tissue scaffolds. Porosity is critical for the transport of oxygen and nutrients into the scaffolds and mechanical properties are essential for the scaffolds to withstand the forces during surgical operation, physiological activities and/or tissue growth. Table 1 shows the very low porosity percentage of the CLW3E7 and CLW6E2M2 scaffolds (29% and 42.5% respectively). The lowest porosity in CLW3E7 scaffold can be

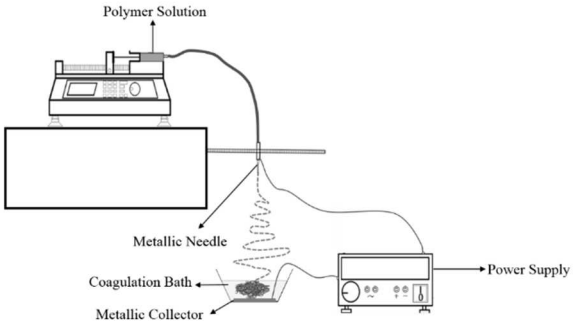


Figure 1. Scheme of the used wet-electrospinning system

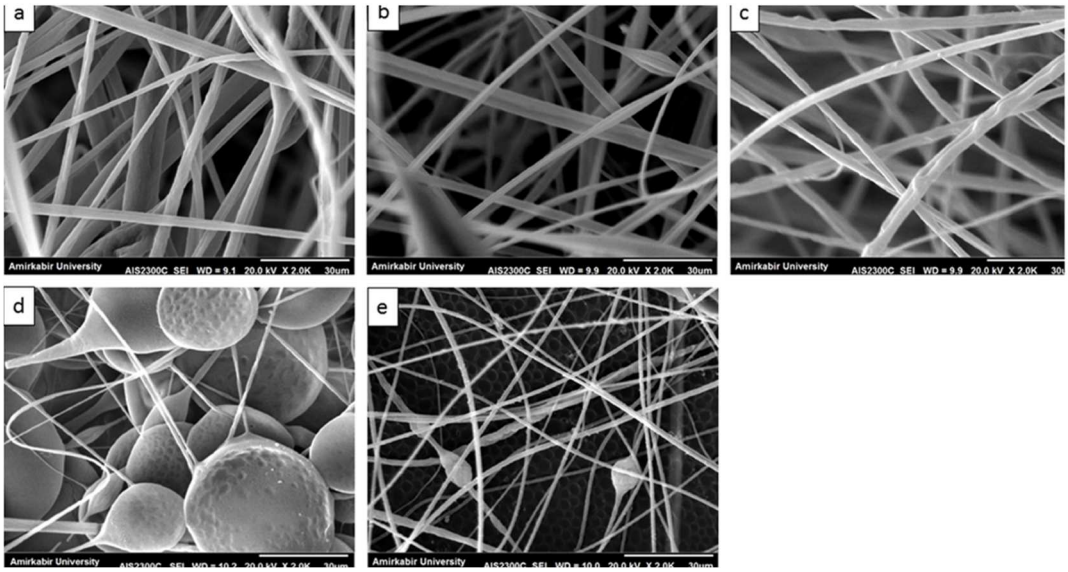


Figure 2. SEM images of the scaffolds (a) CLWN (b) CLW (c) CLE (d) CLW3E7 (e) CLW6E2M2

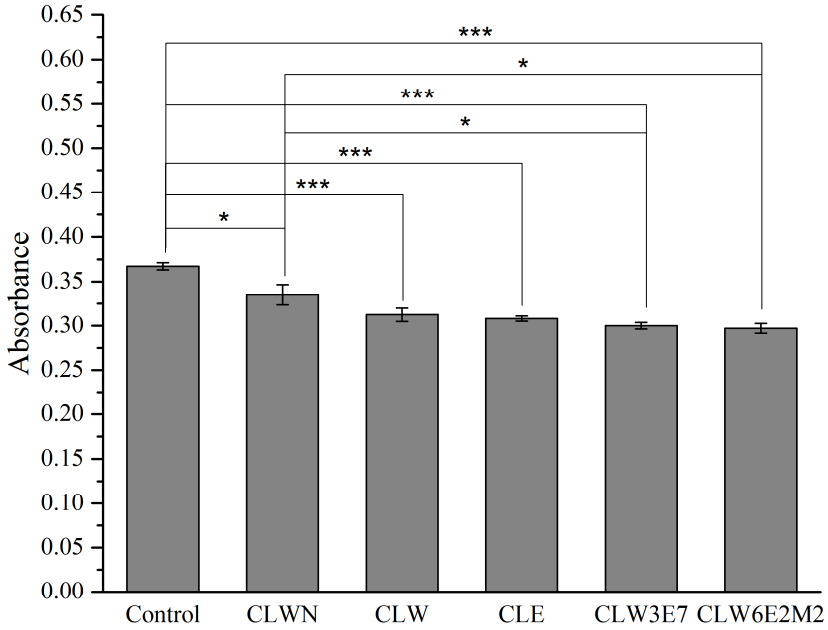
attributed to the almost fully beaded structure of this scaffold. Tensile strength and compressive modulus of all specimens are demonstrated in Table 2.

Hydrophilicity studies

The surface hydrophilicity can be assessed by measuring contact angle. The lower contact angle means the surface is more hydrophile and has higher surface energy(14). The contact angles of all specimens are reported in Table 1. The optimal water contact angle values for maximal cell adhesion have been reported to be in the range of 45-70° or in the region of 30-60°(15). Hence, all the scaffolds in this study for higher interaction with cells need to be modified through addition of hydrophilic polymers or surface modification. The high hydrophobicity of PCL and PLLA was expected due to the presence of five hydrophobic CH_2 and one CH_3 group in their repeating units respectively (15-17). Wan et al.(18) showed that presence of ethanol in the coagulation bath produces fibers with a smooth surface. Thus, CLE, CLW3E7 and CLW6E2M2 scaffolds which were produced from an ethanol contained bath had higher contact angels compared with CLW and CLWN scaffolds due to their lower roughness. Furthermore, NaOH treatment can decrease the contact angle values which explains the lower contact angle value of CLWN scaffold compared with CLW, CLE, CLW3E7 and CLW6E2M2 scaffolds(19). CLW3E7 scaffold had the highest contact angle among PCL/PLLA scaffolds (122.4±1.34°).

pH alteration during in vitro degradation

The pH changes were monitored every seven days for 6 weeks (Table 3). The scaffolds did not measurably degrade over the course of the 6-week experiment. This outcome is favorable because it reduces the inflammatory reactions during the application of the scaffolds(20).



Samples	CLWN	CLW	CLE	CLW3E7	CLW6E2M2
Cell Attachment (%)	87.42 ± 1.78	78.31 ± 1.34	82.08 ± 0.45	85.22 ± 2.22	80.19 ± 3.11

Figure 3. The effect of scaffolds on viability and attachment of hADSCs. Values represent mean±SD, n=3, **P*<0.05, ***; *P*<0.005.

Cell proliferation and attachment studies

MTT assay was carried out to evaluate the proliferation and attachment of *h*ADSCs on wet electrospun scaffolds and the results are shown in Figure 3. It can be seen that all scaffolds provided a favorable environment, as well as, control and showed increased cell proliferation. The control *h*ADSCs, without doubt, had the highest attachment percentage.

After three days of incubation, the CLWN scaffold displayed the highest absorbance compared with control and other scaffolds. It means that the CLWN specimen provided the best support in proliferation of *h*ADSCs in this study. The CLW, CLE, CLW3E7 and CLW6E2M2 scaffolds had no significant difference in proliferation and attachment tests.

Discussion

In the current study the effects of different coagulation baths on mechanical and biological properties of yielded scaffolds were investigated. In SEM observations the presence of beads in CLW3E7 and CLW6E2M2 scaffolds were hypothesized to be due to reduction in electrical conductivity of water after mixing with ethanol(21). Since sufficient charge is essential for the jet to draw and stretch the fibers in electrospinning for fiber formation, decreasing the electrical conductivity led to the formation of beads in these structures. Scaffold porosity is an essential requirement for tissue engineering applications. It is generally accepted that the essential porosity for tissue engineering scaffolds is above 80% (22). In this study, except for CLWN scaffold with about 80% porosity, all other specimens could not fulfill this requirement. The highest porosity percentage in CLWN scaffold is attributed to NaOH presence in its coagulation bath composition. It was reported that by the presence of NaOH in the coagulation bath solution, the fibers would undergo a severe contraction and have smaller diameter, resulting in a scaffold with higher porosity(23). Mechanical testing of the CLW3E7 and CLW6E2M2 scaffolds displayed higher mechanical properties compared with other PCL/PLLA scaffolds which may be related to their very low porosity. However, increasing the porosity had different influence on the mechanical properties of CLWN scaffold. Although CLWN had higher porosity compared with CLW and CLE scaffolds, it showed better mechanical properties which may be attributed to the different composition of their coagulation bath. The presence of NaOH increased its porosity as well as its mechanical properties. Surface wettability examinations showed that CLW3E7 scaffold had the highest contact angle among other scaffolds. This high hydrophobicity can be explained by its almost fully beaded structure. The beads in the structure can

decrease the surface energy and consequently make the surface more hydrophobe. Finally, the influence of hydrophobic solvents such as chloroform on the enhanced hydrophobicity of all scaffolds cannot be neglected.

Conclusion

3-D fibrous scaffolds can be produced via wet-electrospinning of PCL/PLLA 15% (w/v) blends under an applied voltage of 15 kV, and a tip-to-bath distance of 10 cm into NaOH, ethanol, water/ethanol (3:7) (v/v) and water/ethanol/methanol (6:2:2) (v/v) as coagulation baths. The morphology and mean diameter of the scaffolds was found to be dependent to the coagulation bath composition. Furthermore, the coagulation bath composition significantly influenced porosity, wettability, mechanical properties and biocompatibility of the scaffolds.

Acknowledgement

This study was funded by Tehran University of Medical Sciences (Grant no. 33495).

Conflict of Interest: 'None declared'.

References

1. Duan N, Geng X, Ye L, Zhang A, Feng Z, Guo L, et al. A vascular tissue engineering scaffold with core–shell structured nano-fibers formed by coaxial electrospinning and its biocompatibility evaluation. Biomedical Materials. 2016;11(3):035007.
2. Cui W, Zhou Y, Chang J. Electrospun nanofibrous materials for tissue engineering and drug delivery. Science and Technology of Advanced Materials. 2016.
3. Kumar S, Gandhimathi C, Venugopal J, Tay S, Ramakrishna S. Simultaneous electrospun-electrosprayed biocomposite nanostructured substrates for bone tissue engineering. The FASEB Journal. 2015;29(1 Supplement):876.14.
4. Nosar MN, Salehi M, Ghorbani S, Beiranvand SP, Goodarzi A, Azami M. Characterization of wet-electrospun cellulose acetate based 3-dimensional scaffolds for skin tissue engineering applications: influence of cellulose acetate concentration. Cellulose. 2016;23(5):3239-48.
5. Chen H, Peng Y, Wu S, Tan LP. Electrospun 3D Fibrous Scaffolds for Chronic Wound Repair. Materials. 2016;9(4):272.
6. Kostakova E, Seps M, Pokorny P, Lukas D. Study of polycaprolactone wet electrospinning process. Express Polym Lett. 2014;8:554-64.
7. Yokoyama Y, Hattori S, Yoshikawa C, Yasuda Y, Koyama H, Takato T, et al. Novel wet electrospinning system for fabrication of spongiform nanofiber 3-dimensional fabric. Materials letters. 2009;63(9):754-6.
8. Webb AR, Yang J, Ameer GA. Biodegradable polyester elastomers in tissue engineering. Expert opinion on biological therapy. 2004;4(6):801-12.
9. Khatri Z, Jatoi AW, Ahmed F, Kim I-S. Cell adhesion behavior of poly (ε-caprolactone)/poly (L-lactic acid) nanofibers scaffold. Materials letters. 2016;171:178-81.
10. Ho ST, Hutmacher DW. A comparison of micro CT with other techniques used in the characterization of scaffolds. Biomaterials. 2006;27(8):1362-76.
11. Ponsonnet L, Reybier K, Jaffrezic N, Comte V, Lagneau C, Lissac M, et al. Relationship between surface properties (roughness, wettability) of titanium and titanium alloys and cell behaviour. Materials Science and Engineering: C. 2003;23(4):551-60.
12. Chen L, Bai Y, Liao G, Peng E, Wu B, Wang Y, et al. Electrospun Poly (L-lactide)/Poly (ε-caprolactone) Blend Nanofibrous Scaffold: Characterization and Biocompatibility with Human Adipose-Derived Stem Cells. PLoS ONE. 2013;8(8):e71265.
13. Salehi M, Naseri Nosar M, Amani A, Azami M, Tavakol S, Ghanbari H. Preparation of Pure PLLA, Pure Chitosan, and PLLA/Chitosan Blend Porous Tissue Engineering Scaffolds by Thermally Induced Phase Separation Method and Evaluation of the Corresponding Mechanical and Biological Properties. International Journal of Polymeric Materials and Polymeric Biomaterials. 2015;64(13):675-82.
14. Edlund U, Sauter T, Albertsson AC. Covalent VEGF protein immobilization on resorbable polymeric surfaces. Polymers for Advanced Technologies. 2011;22(1):166-71.
15. Abedalwafa M, Wang F, Wang L, Li C. Biodegradable poly-epsilon-caprolactone (PCL) for tissue engineering applications: a review. Rev Adv Mater Sci. 2013;34:123-40.
16. Savarino L, Baldini N, Greco M, Capitani O, Pinna S, Valentini S, et al. The performance of poly-ε-caprolactone scaffolds in a rabbit femur model with and without autologous stromal cells and BMP4. Biomaterials. 2007;28(20):3101-9.
17. Zhu X, Cui W, Li X, Jin Y. Electrospun Fibrous Mats with High Porosity as Potential Scaffolds for Skin Tissue Engineering. Biomacromolecules. 2008;9(7):1795-801.
18. Wan Y, Cao X, Zhang S, Wang S, Wu Q. Fibrous poly(chitosan-g-dl-lactic acid) scaffolds prepared via electro-wet-spinning. Acta Biomaterialia. 2008;4(4):876-86.
19. Cipitria A, Skelton A, Dargaville T, Dalton P, Hutmacher D. Design, fabrication and characterization of PCL electrospun scaffolds—a review. Journal of Materials Chemistry. 2011;21(26):9419-53.
20. Wang X, Song G, Lou T, Peng W. Fabrication of nano-fibrous PLLA scaffold reinforced with chitosan fibers. J Biomater Sci Polym Ed. 2009;20(14):1995-2002.
21. Personna YR, Slater L, Ntarlagiannis D, Werkema D, Szabo Z. Electrical signatures of ethanol–liquid mixtures: Implications for monitoring biofuels migration in the subsurface. Journal of contaminant hydrology. 2013;144(1):99-107.
22. Yang F, Murugan R, Ramakrishna S, Wang X, Ma Y-X, Wang S. Fabrication of nano-structured porous PLLA scaffold intended for nerve tissue engineering. Biomaterials. 2004;25(10):1891-900.
23. Wan Y, Cao X, Zhang S, Wang S, Wu Q. Fibrous poly (chitosan-g-DL-lactic acid) scaffolds prepared via electro-wet-spinning. Acta biomaterialia. 2008;4(4):876-86.

Please cite this paper as: Salehi M, Naseri-Nosar M, Ghorbani S, Farzamfar S, Azami M. Wet-electrospun PCL/PLLA Blend Scaffolds: Effects of Versatile Coagulation Baths on Physicochemical and Biological Properties of the Scaffolds. Regen Reconstr Restor. 2017;2(1):1-6.