Regeneration, Reconstruction & Restoration **Regeneration**

Characterization of Wet-electrospun Poly (ε-caprolactone)/Poly (L-lactic) Acid with Calcium Phosphates Coated with Chitosan for Bone Engineering

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Submitted: 2015-08-20**; Accepted:** 2015-12-05; **DOI:** *10.7508/rrr.2016.02.002*

Introduction: Remarkable advances have been made in the development of scaffolds with favorable characteristics for bone tissue engineering using different techniques. Recently, electrospinning process for fabrication of fibrous matrices have gained popularity, mainly because of structural similarity to the extracellular matrix. In this study, the influence of in situ formation of calcium phosphates (CP) stabled with chitosan (CT) layer on the physicochemical properties of the wet-electrospun poly (ε-caprolactone)/poly (L-lactic) acid (PCL/PLLA) scaffolds was evaluated. **Materials and Methods:** To prepare three-dimensional (3D) fibrous scaffold, PCL/PLLA 10% (w/v) blend was fabricated via wet-electrospinning technique. The fabricated scaffolds were characterized regarding morphology, porosity, hydrophilicity and mechanical strength using scanning electron microscopy (SEM), liquid displacement technique, contact angle measurement and mechanical tests, respectively. Moreover, cell adhesion and viability of human adipose-derived stem cells (hASCs) seeded on the scaffolds were investigated using SEM, MTT assay and DAPI staining. **Results:** Wet-electrospun fibers displayed random, dispersive and non-woven morphology. Porosity of the fabricated scaffolds was 80% and CT coating improved the water contact angle value. In vitro seeding of ADSCs on PCL/PLLA/CP+CT demonstrated enhanced cell proliferation and attachment compared to the PCL/PLLA blend. Porosity, wettability, mechanical properties and biocompatibility of the PCL/PLLA scaffolds have significantly influenced by both in situ formation of CP and CT coating. **Conclusion:** The results indicated that the PCL/PLLA scaffold spun into water/ethanol modified with NaOH (pH~10) coagulation bath with in situ surface formation of CP and CT coating can be a candidate scaffold for bone tissue engineering.

Keywords: Wet-electrospinning; Poly (ε-caprolactone); Poly (L-lactic) acid; Human adipose-derived stem cells; Bone tissue engineering

Introduction

Regeneration of bone defects has been one of the arguable issues in regenerative medicine. Three-dimensional (3D) interconnected porous scaffolds have been showed structural similarity to the extracellular matrix (ECM), and enhanced cell adhesion, proliferation and differentiation(1). Moreover, interconnected pores are essential for not only cell in-growth and vascularization but also transportation of nutrients, oxygen, growth factors, and metabolites (2). Biocompatibility, bioactivity and osteoconductivity are important properties of appropriate materials for fabrication of the scaffolds. Preferably, biodegradation rate of the scaffold should be equal to the rate of new bone formation to restore the mechanical stability. To illustrate, complete degradation of scaffold ensure filling of the initial bone defect with newly formed bone tissue (3).

In recent decades, remarkable advances have been made in the development of scaffolds with favorable characteristics for bone tissue engineering using different techniques. Recently, electrospinning process for fabrication of fibrous matrices have gained popularity, mainly because of structural similarity to the ECM, usability to process a wide range of materials, as well as both simple and low cost establishment (4, 5); nonetheless, electrospun nanofibers have been collected on a metal collecting plate and formed in a two-dimensional (2D) sheet. Wet-electrospinning method is a variant of the electrospinning process in which a coagulation bath has been used instead of a solid, mostly metallic one (6); to illustrate, this method has been developed to simple and effective fabrication of 3D high porous scaffolds (7). In addition to applied electric voltage and feeding rate, the parameters governed by electrospinning process, the parameters of coagulation bath should be considered such as liquid composition, depth of the bath and the tip-to-bath distance (8).

The poly (lactic acid) (PLA) and poly (caprolactone) (PCL), Food and Drug Administration approved polymers, are interesting biodegradable polyesters from which different shapes can be easily manufactured (9). Kostakova *et al.*, demonstrated dual fibrous structures composed of microfibers and nanofibers uniformly distributed in fabrication of wet electrospun PCL 3D

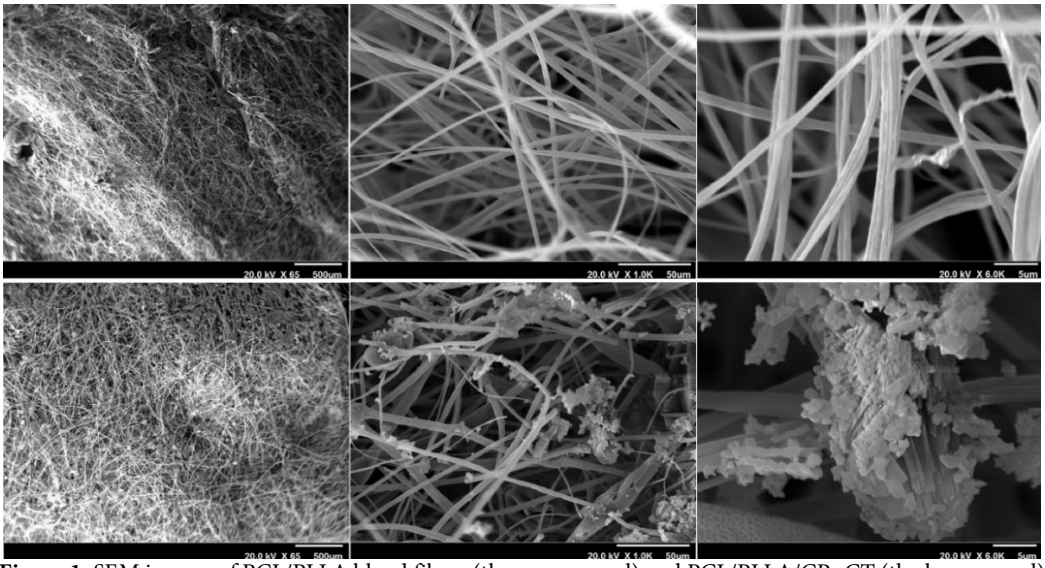


Figure 1. SEM images of PCL/PLLA blend fibers (the upper panel) and PCL/PLLA/CP+CT (the lower panel)

scaffold (10). They have also showed no formation of the Plateau- Rayleigh instability during wet- electrospinning in comparison with the classic electrospinning. Furthermore, in a study conducted by Wan *et al.*, fabrication of a PLA/CT composite scaffold has been demonstrated by wet-electrospinning method (11). They have revealed formation of both microfibers and nanofibers depends on the concentration of polymer solutions and the composition proportions of coagulation solutions.

Since all the essential characteristics of scaffolds for an specific clinical application is not commonly provided by using only one type of polymer, using blends, copolymers and composites have been proposed to enhance the fundamental properties of the tissue engineering scaffolds (12). Polymer blending is both simpler and more effective method compared to copolymerization for achievement of biomaterials with favorable characteristics (13). It is demonstrated that properties of PLLA and PCL blend could not be achieved by using just one polymer (12). For instance, blending of PLLA with PCL could enhance its elasticity (14-16).

Chitosan, a partially N-deacetylated derivative of chitin, is one of the most abundant natural polysaccharide which has been demonstrated as a biodegradable and biocompatible material with anti-microbial activity and cell affinity (17, 18). Moreover, it has been a similarity between the chemical structure of CT and glycosaminoglycans in ECM, and because of having amino groups, CT can act as a weak cationic polyelectrolyte after dissolving into solvents. In fact, CT has ideal properties for a wide range of biomedical applications.

Nevertheless, the bioactivity of PLA scaffolds is low because of their hydrophobic nature (19). Surface coating of polyesters with apatite layer can lead to improvement of bioactivity without changing the substantial properties of the material. Since chemical structure of such calcium phosphate coatings is comparable to the mineral phase of natural bone, desirable cell adhesion and proliferation with favorable cell-substrate interactions have been demonstrated by calcium phosphate coatings (20-23). Composites of polymer-ceramic have enhanced both bioactivity and mechanical properties compared to the pure polymer scaffolds (24, 25). Moreover, acidic degradation of polymer by-products which can lead to a local inflammation could be neutralized by the resorption of the apatite layer (26). In this study, we fabricated and characterized a 3D porous PCL/PLLA (poly (L-lactic acid)) blend scaffold with in situ formation of CP stabled by CT coating layer (PCL/PLLA/CP+CT) as a candidate for bone tissue engineering.

Materials and Methods

PCL (M_w = 48,000 - 90,000 g/mol), PLLA (M_w = 60000 g/mol), CT (medium molecular weight), calcium chloride, sodium phosphate dibasic dihydrate and all other reagents and solvents were purchased from Sigma-Aldrich (Germany). Analytical grade of all the chemicals was used without further purification.

Human adipose-derived mesenchymal stem cells (hASCs) were provided 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, streptomycin and DAPI staining solution were purchased from

Sigma-Aldrich (Germany). MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) and DMSO were purchased from Carl Roth (Germany).

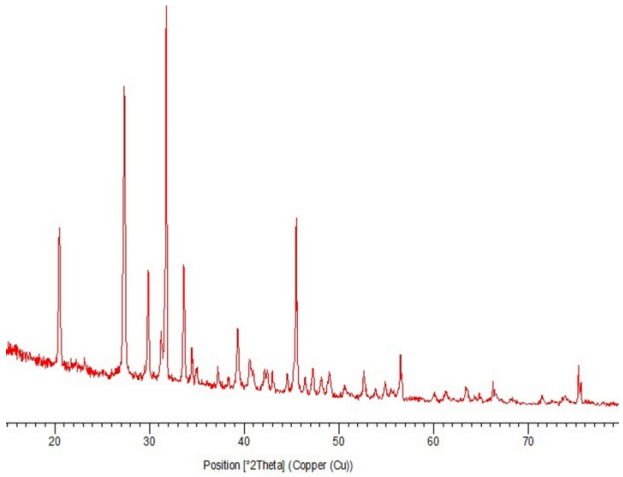
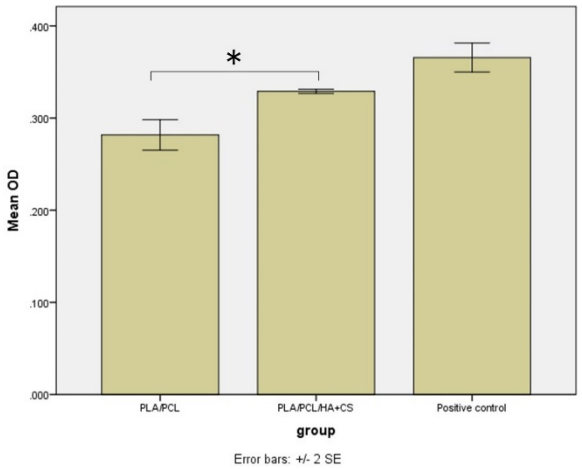


Figure 2. XRD pattern of CP formed on the scaffold



groups

Scaffold fabrication

Wet-electrospinning of PLLA and PCL (1:1) with total concentrations of 10% (w/v) in chloroform were performed into aqueous solution of sodium hydroxide (NaOH) (pH~10) distilled water/ethanol (1:1) (v/v). A 15ml syringe ended to a 20 gauge metal needle connecting to a positive high voltage source (HV100P OV, Fanavaran Nano-Meghyas, Iran), set to 12kV consisted our electrospinning device. A feeding pump (SP1000, Fanavaran Nano-Meghyas, Iran) was used with 1 ml/h feeding rate. The fibers were collected on an aluminum-foil grounded electrode connected to the high voltage supply and fixed on the bottom of a polystyrene coagulation bath at room temperature. The depth of bath was 5cm, and the tip to bath distance was 15cm. After removing the coagulation bath, electrospun fibers immediately transferred to a -80°C freezer for 12h and then freeze dried at -77°C for 24h (121550PMMA, Christ, Spain). The surface of specimens was functionalized by immersing in 0.1M NaOH for 24h. After that, the specimens were washed by distilled water 3 times and dried. The samples were immersed in calcium chloride solution and then placed in sodium phosphate solution for apatite forming. The scaffolds were dried and placed in CT 2% (W/V), then freeze-dried in -20°C for 24h. For crosslinking the CT layer, the scaffolds were immersed in glutaraldehyde 2.5 wt% for 24h. The scaffolds were placed into 100 mM glycine solution for 1h at 37°C for blocking the residual aldehyde groups, and then, washed 3 times with distilled water.

X-ray diffraction analysis

The apatite formed in situ on the polymeric blend was analyzed by X-ray diffraction analysis (XRD, voltage of 40 kV, current settings of 40 mA, Cu Kα radiation, Philips, X'PERT). The XRD diagram was recorded in the interval of $15^{o} \leq 2\theta \leq 80^{o}$ at scan speed of 2^{o} min^{-1}.

Figure 3. MTT assay of the adhesion viability of hASCs cells seeded onto the scaffolds. Asterisk shows the significant differences between the

Morphology of the scaffold

After sputter coating with gold for 300 s using a sputter coater (SC7620, Emitech, England) at an accelerating voltage of 20 kV, the morphology of the scaffolds was observed by scanning electron microscope (SEM, AIS2100, Seron Technology, South Korea).

Porosity assessment

To determine the porosity of the scaffold, liquid displacement technique was employed using the following equation;

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

Where V_1 is the initial volume of ethanol (96%), V_2 is the volume after immersing the scaffold (and ethanol filled the pores) and V_3 is volume of the ethanol after the scaffold removal.

Tensile strength of the scaffold

Rectangular specimens (80 mm×10mm) were selected for tensile strength analysis performed by an Instron 5566 universal testing machine (Instron, MA) at a strain rate of 10 mm/min.

Contact angle measurement

The hydrophilicity of the scaffold was assessed using static contact angle measurement with sessile drop method using a contact angle measuring system (G10, KRUSS, Germany) (27).

Cell culture and seeding

hASCs were cultured in a culture medium consisted of 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. The scaffolds were sterilized under irradiation of UV light for 1h and then were placed in 70% ethanol for 1h, and dried under vacuum for 2h. Next, the scaffolds were washed with PBS 2 times and with DMEM/F12. Finally, 5×10^{3} third passage cells were seeded on the scaffolds in 96-well plate. After 1h incubation, 0.15ml cell culture medium with FBS was added to each well. The medium was changed every 24h (28).

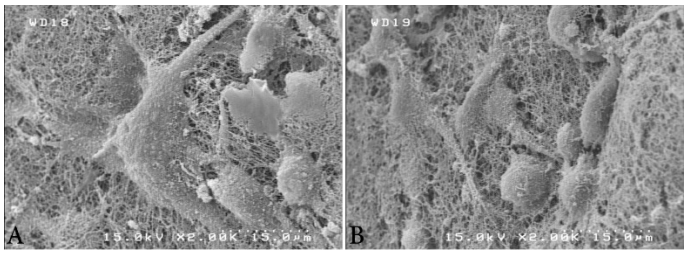


Figure 4. SEM images of cell attachment and interactions with PCL/PLLA blend fibers (left image) and PCL/PLLA/CP+CT (right image)

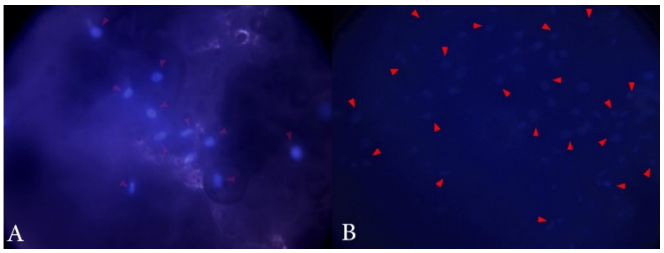


Figure 5. DAPI staining of cells seeded on the PCL/PLLA blend fibers (A) and PCL/PLLA/CP+CT (B)

Cell proliferation on the scaffold

MTT assay was employed to cell proliferation investigation after 72h. The medium was replaced with 5 mg/ml MTT solution, and incubated for 4 h at 37 °C. After adding 0.1 ml DMSO for dissolving the formed purple formazan crystals, the absorption was read at 570 nm using a microplate reader (Anthos 2020, Biochrom, Germany) (29). hASCs were culture without scaffolds as positive control. Triplicate wells for each specimen was assessed.

The percentage of cells attached on the scaffolds was tested using MTT assay. The cells seeded on the scaffolds were incubated for 6h. The constructs then were washed with PBS 2 times for 30s. The percentage of cell attachment was calculated using this equation (28):

$$\text{Cell attachment (\%)} = \frac{ODt}{ODc} \times 100$$

In which ODt and ODc are the mean absorbance value of the scaffolds and the positive control, respectively.

Cell attachment morphology

Cell attachment was evaluated 72h after cell seeding. For cell fixation, scaffolds were immersed in glutaraldehyde 2.5wt% for 2h, and dried in ethanol 30, 70, 80, 90 and 100% each for 10min. The scaffolds loaded with cells were freeze-dried and evaluated using SEM.

DAPI staining

For staining the adherent cells, 50 µl of DAPI (4',6-diamidino-2-phenylindole) staining solution was added into each well 48hafter cell seeding, and incubated for 5min at room temperature. DAPI solution was replaced and washed with PBS 3 times each for 3 min to remove the unbinding DAPI. The cells were observed under a fluorescence microscope with optical filter owning 360nm and 460nm excitation and emission wavelengths.

Statistical Analysis

The outcomes of MTT assay were statistically analyzed by SPSS-21 software using independent sample t-test. In all comparisons, $P<0.05$ was considered as statistically significant. All data were expressed as mean and standard deviation.

Results

In situ formation of CP on the polymer blend surface was observed in SEM images (Figure 1). Also, wet-electrospun PCL/PLLA fibers revealed random, dispersive and non-woven morphology. XRD pattern of the PCL/PLLA/CP+CT scaffold has been shown in Figure 2. CP formed in situ showed characteristic peaks of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$, Joint Committee on Powder Diffraction Standards (JCPDS) #09-0432] and β-tricalcium phosphate [$Ca_3(PO_4)_2$, b-TCP, JCPDS #09-0169].

The porosity percentage of the PCL/PLLA/CP+CT scaffold was 80% compared to PCL/PLLA about 91.5%. Tensile strength of PCL/PLLA blend was reported 1.56 ± 0.37 MPa, but it was reported 1.93 ± 0.46 MPa for PCL/PLLA/CP+CT. The contact angle of PCL/PLLA blend and PCL/PLLA/CP+CT were reported 112±0.59 and 82.3±0.72, respectively.

The results of MTT assay performed to assess the proliferation of hASCs on the wet-electrospun scaffolds are shown in Figure 3. The PCL/PLLA/CP+CT scaffold displayed higher absorbance significantly compared to the PCL/PLLA scaffold (P=0.006). Cell attachment obtained from MTT assessment was reported 72.32% and 83.96% for PCL/PLLA and PCL/PLLA/CP+CT scaffolds, respectively. The cells attached on the PCL/PLLA/CP+CT scaffold can be determined in the SEM images (Figure 4). Furthermore, the attached cells on the both scaffolds were determined via DAPI staining (Figure 5).

Discussion

In the current study we demonstrated in situ CP formation stabled with a CT layer on PCL/PLLA 10% (w/v) blend as a suitable 3D scaffold for bone tissue engineering. Wet-electrospinning was performed under an applied voltage of 12 kV, and a tip-to-bath distance of 15 cm. Water/ethanol (1:1) (v/v) + NaOH was considered as coagulation bath because the presence of ethanol produces fibers with a smooth surface (30).

Both porosity and mechanical properties are important parameters for scaffolds in tissue engineering. Porosity is essential to transport oxygen and nutrients into the scaffolds,

and mechanical properties are critical to withstand the forces during not only surgical operation but also physiological activities and/or tissue growth (31, 32). The lower porosity of PCL/PLLA /CT+CP scaffold compared to PCL/PLLA can be attributed to the CT coating on this scaffold; nevertheless, its porosity is acceptable for bone tissue engineering (3). In this study, we showed that in situ CP formation increased the tensile strength of the PCL/PLLA blend scaffolds. Also, lower porosity of PCL/PLLA/CP+CT scaffold could lead to higher mechanical properties compared to PCL/PLLA scaffold. In fact, in case of porous scaffolds, combination of mechanical and biological properties of polymers and inorganics has been well-documented for bone tissue engineering (33).

The surface hydrophilicity could be determined using water contact angle measurement. It have been demonstrated that the lower contact angle of the surface the more hydrophilicity and the higher surface energy (34). To reach the most cell adhesion, the optimal contact angle values have been reported to be in the range of 45-70° or in the region of 30-60° (35). Due to the presence of five hydrophobic CH_2 and one CH_3 group in the repeating units of PCL and PLLA, respectively (35), the high hydrophobicity could be expected from these polymers as we observed in our study. Hence, PCL/PLLA blend have needed to be modified through addition of hydrophilic polymers; to illustrate, we modified the surface of the scaffold using CT in this study to reach higher cell adhesion to the scaffold. However, it should be noticed that the lower contact angle value of PCL/PLLA/CP+CT scaffold can be explained by NaOH surface treatment which can decrease the contact angle values (32).

In this study we used mesenchymal stem cells derived from adipose tissue which demonstrated osteogenic differentiation ability in the literature (36). According to the MTT assay outcomes, not only both scaffolds provided a desirable environment for cellular viability, but also cell can proliferate increasingly on the surface of both scaffolds. The positive control of hASCs undoubtedly showed the highest mean of absorbance. Moreover, hASCs showed significant favorable proliferation on PCL/PLLA/CT+CP scaffold compared to the PCL/PLLA blend scaffold. To illustrate, cell compatibility and appropriate hydrophilicity of the PCL/PLLA/CP+CT scaffold and favorable hASCs interactions with these nanofibers have allows the scaffold to act as a desirable matrix like ECM for growing and proliferation of the cells.

DAPI, a kind of fluorescent dye, can bind DNA strands constantly, and be detected by fluorescence microscope. Both live and fixed cells can be stained by DAPI. The largest excitation and emission wavelengths for DAPI are 340 nm and 488 nm, respectively; however, when DAPI binds with double-strand DNA, the largest excitation and emission wavelengths changed to 360 nm and 460 nm, respectively. With regard to DAPI staining outcomes, it can be concluded that the rate of cell attachment and viability on the PCL/PLLA/CP+CT scaffold was more compared to the PCL/PLLA scaffold due to both more hydrophilicity and having functional groups of CT in the fabricated scaffold.

Investigation of the osteogenic differentiation of stem cells seeded on the scaffold was the limitation of our study. Alizarin red staining, test of alkaline phosphatase activity and evaluation of osteogeinc gene expression using reverse transcriptase polymerase chain reaction can be recommended (37) to confirm the bone regeneration potential of the PCL/PLLA/CP+CT scaffold in future studies.

Conclusion

In conclusion, in situ CP formation with a CT layer coating on PCL/PLLA blend has significantly influenced porosity, wettability, mechanical properties and biocompatibility of the scaffolds. Nonetheless, for the application of the scaffold in bone tissue engineering, evaluation of differentiation ability and the expression of osteogenesis factors have been requested in future studies before *in vivo* studies and clinical trials.

Conflict of Interest: 'None declared'.

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Please cite this paper as: Salehi M, Bastami F. Characterization of Wet-electrospun Poly (ε-caprolactone)/Poly (L-lactic) Acid with Calcium Phosphates Coated with Chitosan for Bone Engineering. Regen Reconstr Restor. 2016; 1(2): 69-74. DOI: 10.7508/rrr.2016.02.002.