In-Vitro Evaluation of Novel Polycaprolactone/ Chitosan/ Carbon Nano Tube Scaffold for Tissue Regeneration

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ABSTRACT

Background: Many patients lose their organs or tissues due to disease, trauma, or a variety of genetic disorders. Tissue engineering is a multidisciplinary science to regenerate or restore tissue or organ function and an appropriate scaffold is the first and certainly a crucial step in tissue engineering strategies.

Objective: The purpose of this study is to fabricate and evaluate the in-vitro response of porous nano Polycaprolactone (PCL)/ chitosan/ multi-wall carbon nanotube (MWCNTs) scaffold for tissue regeneration.

Material and Methods: In this experimental research, a novel scaffold containing MWCNTs in polycaprolactone/chitosan nanofibrous scaffold was synthesized by electrospinning technique.

Results: According to scanning electron microscopy SEM images, by increasing the number of MWCNT in the scaffold by 2%, the average diameter decreased significantly for fabricated scaffolds with 5% MWCNTs. Based on the results, the scaffolds plunged from submicron to nanoscale fibers at about 80 nm. In addition, by adding more MWCNT to the nanofibrous scaffold, the biodegradation rate was decreased by 32%. However, mechanical characterization demonstrates that the higher level of MWCNT increases young modulus by 96%, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay illustrated that MWCNTs could enhance bioactivity and cell- scaffold relationship in addition to alkaline phosphatase (ALP).

Conclusion: MWCNT significantly improves the physical and mechanical properties of fabricated scaffolds and in-vitro assessment demonstrated that the prepared nanofibrous scaffold containing 4% MWCNT could be a very useful biocompatible material for tissue engineering.

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Keywords

Multi-Wall Carbon Nano Tube; Electrospun Scaffold; Nanofibers; Chitosan; Polycaprolactone; Tissue Engineering

Introduction

Regenerative medicine and tissue engineering as a multi-specialty topic aim to heal or regenerate cells and tissue or organs [1-3]. Tissue regeneration requires sufficient cell-scaffold interaction to control cell fate and regeneration. In addition, vascularization ability, proper scaffold degradation rate, growth factor incorporation, and mineralization should be investigated [4-6]. The scaffolds, with a vital role as a bioactive matrix inducing a desired cellular behavior, and biomaterials are two major components in regenerative medicine and tissue engineering to control

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cell fate in the tissue regeneration process. The cell-scaffold process relation, which is highly important in tissue regeneration, depends on different critical factors such as chemical conformation, physical construction, and mechanical assets [7-10]. A variety of biomaterial is available as a scaffold component for tissue engineering applications, including natural or synthetic biomaterial usually modified for extracellular matrix environments, self-assembling systems, or hydrogels. Each biomaterial offers a unique chemical composition, physical and mechanical properties, structure, and degradation rate [9, 11].

Recently, many reports emphasized the extraordinary properties of carbon nanotubes (CNT) in tissue engineering, due to attractive physical characteristics such as thermal, electrical, and mechanical properties studied enormously for many medical applications and various types of tissue engineering [12, 13]. A carbon nanotube is a material with useful biomedical application and tissue engineering for cell tracking or sensing microenvironments as a biosensor, while it is also used in delivering biological and pharmacological agents or in scaffold structure to incorporate with the extracellular matrix (ECM). CNT can also have single-wall carbon nanotubes (SWCNTs) structure or multi-wall carbon nanotubes (MWCNTs) [12]. Fraczek et al. reported that polymeric matrices in combination with carbon nanotubes have a greater biocompatibility in-vivo and in-vitro than pure polymers [14, 15].

Polycaprolactone (PCL) is a synthetic biopolymer because of its chemical, physical, and mechanical properties and its biocompatibility. Disadvantages of PCL are the lack of cell recognition sites and slower degradation speed compared to other products. Moreover, the scaffolds should deliver suitable cell proliferation and adhesion [16, 17]. Chitosan, a natural biocompatible and biodegradable polymer establishes osteoinductivity and helps tissue healing and its combination with PCL can enhance PCL's bioactivity [18, 19]. Furthermore, MWCNT could be used as a reinforcement element polymer-ceramic composite scaffold due to its greater mechanical profile [20-22].

Scaffolds act like an artificial ECM, which is a temporal model for tissue regeneration with some specified characteristics such as biodegradability, proper biocompatibility, promoting cellular connections and tissue growth, and possessing sufficient mechanical assets. Bio-scaffolds with a various range of nano-fiber width imitates ECM structure affecting cell binding and proliferation [5, 19].

The key purpose of this research is to fabricate and study the in-vitro response of porous nano PCL/chitosan/ MWCNTs scaffold for further tissue regeneration. The scaffolds were categorized using biodegradation, scanning electron microscope (SEM), and mechanical characterization. Apatite formation capability of the scaffolds was evaluated by SEM analysis after absorbing samples in the solution of simulated body fluid (SBF). Finally, the in-vitro behavior of prepared electrospun scaffolds was tested to understand cell-scaffold interaction.

Material and Methods

In this experimental study, Chitosan (middle molecular weight) and PCL (MW=80,000) were attained from Sigma-Aldrich Chemicals (St. Louis, MO). The main solvent was formic and acetic acid, purchased from Merck (Darmstadt, Germany) without any changes. MWCNT particles were purchased from the Iranian Chemistry Engineering Institute. Cell culture media and fetal bovine serum (FBS), materials like DMEM (Dulbecco Modified Eagle's Medium), trypsin–EDTA, and phosphate-buffered saline (PBS) were also purchased from Biowest Company. Finally, other chemical substances were used in minimal grades.

Nanofibrous scaffolds were constructed by using the electrospinning technique to provide a proper substrate to control cellular behavior for tissue regeneration and various dosages of MWCNT (2, 3, 4, and 5 w/w% of MWCNT) dissolved in the acidic solution to prepare electrospinning solution. To improve the chemical compatibility of MWCNTs in the polymeric matrix of a nanofibrous scaffold, acidic surface modification was used. Therefore, MWCNTs

were double washed in an ultrasonic bath in 5 M nitric acid solution for 120 mins and soaked in 5 M sulfuric acid for another 120 mins to create a carboxyl group on the surface, respectively [23, 24]. Subsequently, the electrospinning solution was prepared by dissolving 13% w/v PCL/ chitosan/ MWCNTs in acetic/ formic acid as a solvent system. PCL/ chitosan with a weight ratio of 3:1 was liquefied in a 50 mL of 1:1 formic/ acetic acid solution system on a mechanical mixer for an hour to build a uniform mixture. Afterward, various amounts of MWCNTs (2, 3, 4, and 5 w/w%) gently were added to the PCL/ chitosan solution, on the magnetic stirrer and the arranged solution was used for electrospinning with 0.2 ml/h federate and a high voltage power source of 15-19 kV potential between the grounded collector and the syringe tip. The electro-spun nano-fiber placed on a plate drum was in various distances between 8 to 12 centimeters from the syringe tip, while the electrospinning procedure was completed in ambient heat.

The surface microstructure and morphology of the scaffolds were studied by an SEM. In advance, each sample was sputter-coated by a gold layer to prepare a conductive layer for SEM observation, analyzed to measure fiber diameter distribution [25].

To evaluate apatite-forming capability and bioactivity of the surface of nano-composite scaffolds, every sample was absorbed in simulated body fluid (SBF) at 37 °C for 7 days, while SBF solutions were synthesized based on Kokubo et al. procedure at pH of 7.40 [26]. Then, after the recommended soaking time, samples were washed with deionized water and left to dry at room temperature during the night. The solution of SBF was made by liquefying reagent-grade $NaHCO_3$, KCl, NaCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2$, and K_2HPO_4 into deionized water in a plastic beaker and then the waters were buffered to pH=7.4 with $(HOCH_2)_3CNH_2$ (Tris) and HCl solutions (1 mol L^{-1}) at 37 °C [26]. The concentrations of ions in the prepared SBF were the same compared to the blood plasma of humans. The morphology of the hydroxyapatite layer on the scaffold surface was assessed with SEM microscopy.

Tensile tests were used to evaluate Young's modulus and mechanical properties. Hence, samples were cut in circular and rectangular shapes and fixed in an Instron machine to starch at a strain rate of 2 mm/min. The Young modulus of each sample was measured based on obtained stress-strain curve [20, 27].

To assess the biodegradation properties of prepared electrospun scaffold samples in various periods (including 14, 21, 28, 35 days) they were incubated in saline phosphate buffer containing 1 mg/ml enzymes of lysosome in a 5% CO_2 incubator. Afterward, each interval-scaffold sample was dried and weighed and the amount lost was calculated as weight loss lost per original weight in percentage.

Scaffold samples with different MWCNTs substances were cultured by differentiated odontoblast cells. To evaluate in-vitro response, samples were assessed using MTT and alkaline phosphatase test. The cellules, excluding the control sample, were cultured in a leaching solution obtained from various scaffolds for 72 h incubation time in 5% CO_2 at 37 °C. The leaching solutions were achieved by the immersion of scaffolds in the medium for 7 days. After specific intervals, the absorbance was evaluated by spectrophotometer (ELX800, USA) at 490 nm while repeating 6 times each test.

Results

The result of this study showed PCL/ chitosan/ MWCNT fibers can produce submicron fibers and the average diameters decreased significantly by increasing the amount of MWCNTs. Electrospun scaffold with 4 and 5 percentages of MWCNTs exhibited nanofibers mat as seen in Figures 1 (C and D).

The construction of a new level on the exterior of the scaffolds was studied to evaluate the bioactivity of scaffolds, covered completely by a mineralized apatite layer (Figure 2).

The results of this study showed the significant effect of MWCNTs content on nanocomposite scaffolds which is demonstrated in Figure 3. Weight loss is aimed to assess biodegradation behavior. Obtained results are summarized in Figure 4. Furthermore, Alkaline phosphatase results reported in Figure 5 illustrated that MW-

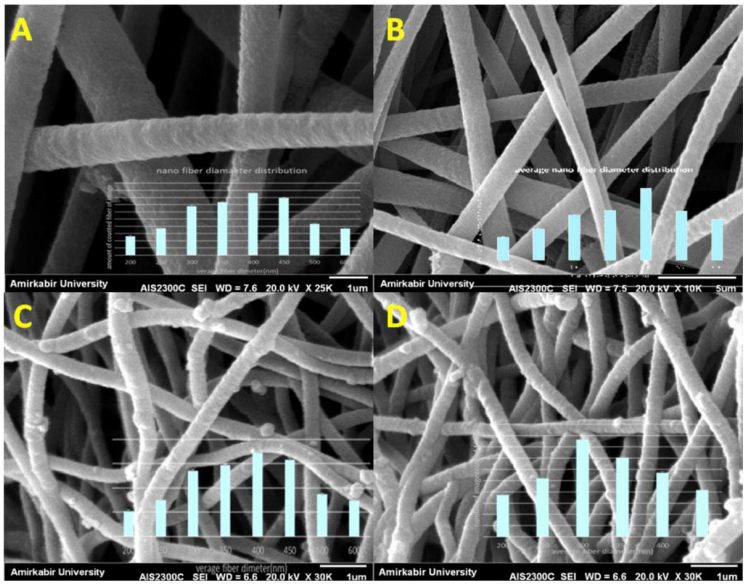

Figure 1: SEM (Scanning electron microscope) image of prepared scaffolds samples with different (multi-wall carbon nanotube) MWCNTs content: A) 2% MWCNT, B) 3% MWCNT, C) 4% MWCNT, and D) 5% MWCNT.

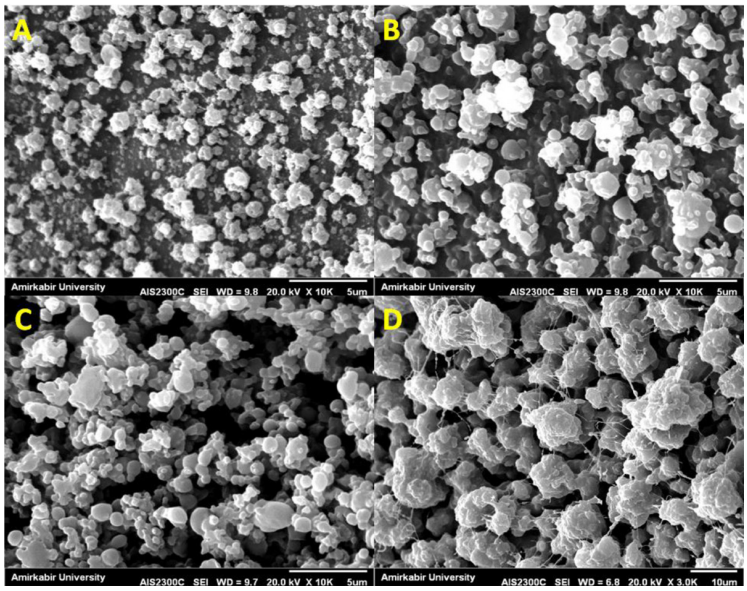

Figure 2: Apatite layer formation after 7 days on a nano-fibrous scaffold: A) 2% MWCNT (multi-wall carbon nano-tube), B) 3% MWCNT, C) 4% MWCNT, and D) 5% MWCNT

CNT content had positively affected the odontoblast cells' behavior action. According to Figure 6, it was shown that the amount of MWCNT increases cell proliferation in against control sample.

Discussion

According to the findings of this study, the electrospun scaffold can have physical properties of the Extracellular matrix (ECM) by producing fine nanofiber, branches, and proper morphology which are the most prominent aspects of a scaffold. As represented in Figure 1, PCL/ chitosan/ MWCNT fibers can produce submicron fibers and the average diameter decreased significantly by increasing the amount of MW-

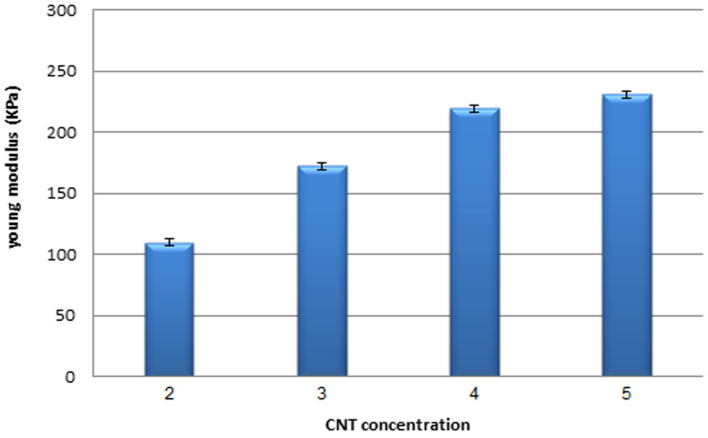

Figure 3: Young modulus of scaffolds with different MWCNT (multi-wall carbon nano-tube) content

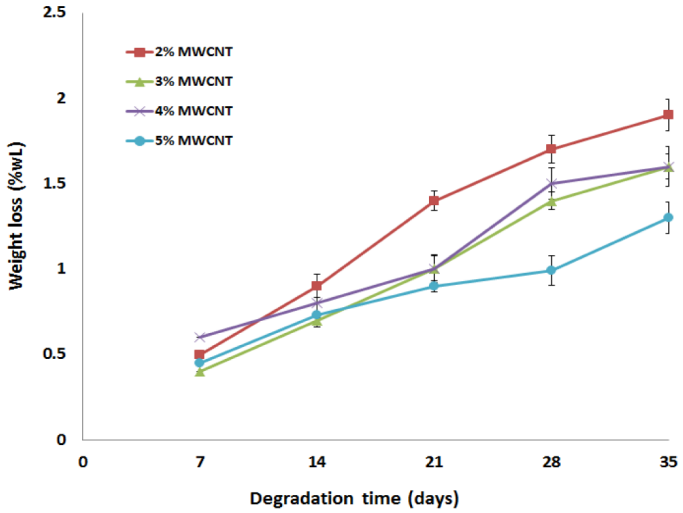

Figure 4: Biodegradation profile of MWCNT (multi-wall carbon nano-tube) contained scaffolds

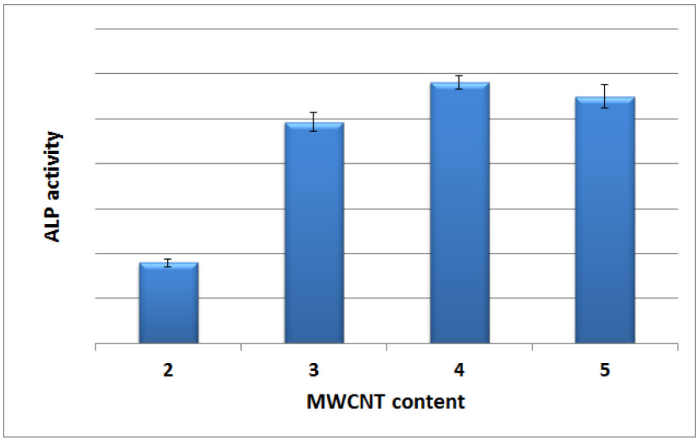

Figure 5: Alkaline phosphatase activity of scaffold samples with different MWCNT (multi-wall carbon nano-tube) content

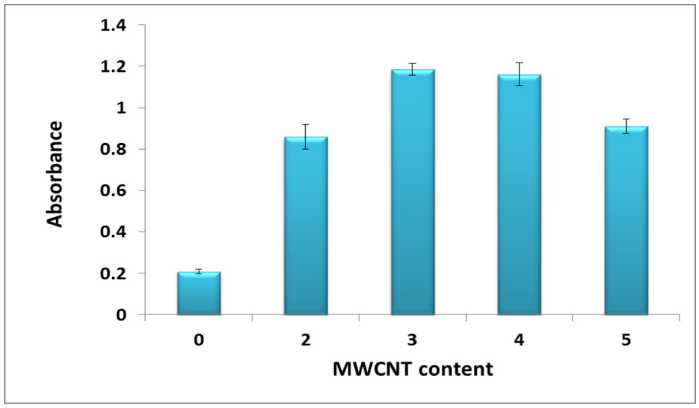

Figure 6: MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay spectrum absorbance

CNT.

In other words, by adding to the substance of nanoparticle, structure led to stretching additionally in the fiber and decreasing the fiber width during electrospinning, due to the CNT type that charges would increase its stretching throughout the process in the electric field.

The construction of a new level on the exterior of the scaffolds was studied to evaluate the bioactivity of scaffolds. All scaffold sample surfaces were completely covered by a mineralized apatite layer (Figure 2), showing that carbon nanotube in electrospun nanofiber can successfully boost apatite formation ability, a reliable assessment for scaffold surface bioactivity.

Increasing MWCNTs content causes an increment in the structural strength as shown in Figure 3. Homogeneous distribution of MWCNTs is an important factor for uniform mechanical properties while increasing MWCNT substance by more than 4, 5% may cause heterogeneous distribution as in the Young modulus of a scaffold. Therefore, the capacity of adding MWCNT particles to the scaffold matrix is limited.

The rate of the scaffold biodegradation is one of the chief characteristics in the long-term application which is essential for cell-scaffold integration. The biodegradation rate should be gradually and relevant to cell regeneration to control tissue formation. Chitosan has a faster degradation rate in comparison to PCL and MWCNTs because of hydrolysis degradation of chitosan degradation. In addition, nanocomposite scaffold emphasizes degradation rate of structure because of higher surface area for nanofibers enhancing enzymatic degradation. Weight loss aims to assess biodegradation behavior (Figure 4).

Figure 5 illustrated that MWCNT content had positively affected odontoblast cells' behavior action. Promoting odontoblast cell activity to deliver proper protein synthesis level consists of fibrillar nanostructure that is remarkably like the natural ECM and physicomechanical stimulation of cell fate by MWCNT to differentiate to hard tissue formation and dentinogenesis [28-31]. Based on alkaline phosphatase (ALP) data, increasing the amount of MWCNT to 4% positively affects cell activity, while adding more MWCNT adversely affects cellular behavior related to the MWCNT agglomeration and the increase in surface stiffness.

The amount of the MWCNT increases cell proliferation in against control sample (Figure 6), showing that increasing the content of MWCNT by more than 3% in the scaffold composition leads to a decrease in cell proliferation in contrast to the scaffolding.

On the other hand, MWCNT agglomeration may decrease MWCNT-cell interaction. Moreover, ALP activities represented the same results and confirmed the MTT assay outcomes. Nanofibrous structure of scaffolds and MWCNT adsorb protein of culture media that could enhance cellular behavior, such as cell proliferation and growth.

Conclusion

Various scaffolds based on PCL/ chitosan, including different amounts of the MWCNT, were fabricated by electrospinning procedure to control chemical composition and proper morphological properties for further tissue engineering application. Based on obtained results, adding to the volume of carbon nanotube in PCL/ chitosan/ MWCNT scaffold from 2% to 5% results in decreasing significantly the average diameter from submicron to nanoscale fibers by about 80 nm. According to the biodegradation evaluation, adding the MWCNT to the nanofibrous scaffold reduced the biodegradation rate by 32%. Additionally, mechanical characterizations demonstrated that a higher level of MWCNT increases young modulus by 96%. Finally, the in-vitro behavior of fabricated scaffolds was evaluated by ALP an MTT assay, showing MWCNTs could enhance bioactivity and cell-scaffold interaction. Therefore, the MWCNT caused a significant improvement in physical and mechanical properties of the fabricated scaffold and in-vitro assessment showed the prepared nanofibrous scaffold containing 4% MWCNT could be a great biocompatible substrate for application in tissue engineering.

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Authors' Contribution

R. Fekrazad perceived the idea, visualized and validated the related literature after investigating it. Also R. Fekrazad helped with funding acquisition, project administration and resource management. Lastly, the research was supervised by R. Fekrazad and he was involved with methodology design, manuscript writing, reviewing and editing. F. Tondnevis visualized and investigated the related literature. Conceptualization and Data curation was also done by F. Tondnevis as well as helping with writing, review & editing. MM. Abolhasani helped with visualization, formal analysis alongside with writing the original draft. All the authors read, modified, and approved the final version of the manuscript.

Ethical Approval

The Ethics Committee of Aja University of Medical Sciences approved the protocol of the study (Ethic cod: IR.AJAUMS.REC.1396.118).

Conflict of Interest

None

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