



In Vitro Assessment of Synthetic Nano Engineered Graft Designed for Further Clinical Study in Nerve Regeneration

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Abstract

Background: Electrospun nanofibrous scaffolds are considered as promising candidates in neural tissue regeneration due to their ability to support neural cell attachment, spreading and proliferation.

Methods: In this paper, various type of nanofibers scaffold based on polycaprolactone (PCL) were fabricated using electrospinning. The main drawback of PCL scaffolds is their low bioactivity of scaffold surface. To overcome this surface and composition modification was used to enhanced hydrophilicity and bioactivity of scaffold.

Results: The scanning electron microscopy (SEM) results indicate that fiber diameter entirely depends on the solvent system and added component of gelatin and chitosan which by adding gelatin and chitosan fiber diameter decreased. In vitro studies using PC12 cells revealed that the plasma surface modified and blended scaffold with chitosan and gelatin nanofibrous scaffold supports cell attachment, spreading and indicate a significant increase in proliferation of PC12 in the presence of chitosan. The results demonstrated that gelatin and chitosan caused a significant enhancement in the bioactivity of the scaffold, which confirmed by MTT assay and improved the cell spreading and proliferation of neural cell on the scaffolds.

Conclusion: Based on the experimental results, the PCL/chitosan/PPy conductive substrate could be used as a potential scaffold for clinical research in the field of neural regeneration and healing.

Keyword: Electrospinning; Nerve graft; Neural tissue engineering; Nanofibrous; Surface modification.

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Introduction

Nerve regeneration is a difficult biological circumstance, not only in central nervous system but also in the peripheral nervous system. Neural tissue engineering is hopeful advanced approaches to regenerate and heal damaged nervous system.¹⁻⁶

Neural tissue engineering involves three vital elements including scaffolds, cells, and signals. One of the significant challenges in neural tissue engineering is to produce a bioactive scaffold which puissant to support neurite outgrowth. Neural tissue scaffold should provide an ideal environment for physical and chemical signaling.^{7,8} It has demonstrated that morphology and topography of scaffold an influence cell fate and regeneration procedure.

Electrospun nanofibrous scaffolds could mimic the

extracellular matrix (ECM) topography of tissue by preparing fibers with the range of nano to micro scale. Nanofibrous scaffolds represent a promising structure for the regeneration of nerve tissue due to their biomimicking architecture.⁹⁻¹⁴ Yang et al demonstrated that nano-structured porous scaffold could serve as a potential substrate for cell adhesion and differentiation in neural tissue engineering.¹⁵ Nanofiber scaffolds (average diameters less than 200 nm) significantly enhance NSC differentiation and increase neurite length compared to microfibers.¹⁶

Among the various type of synthetic polymers, polycaprolactone (PCL) is an attractive polymer for biomedical applications because of its physicochemical and mechanical properties, biocompatibility and non-

toxic degradation products.¹⁷ However, the poor bioactivity and lack of cell recognition sites, low hydrophobicity and degradation rate of PCL hinder its application. When PCL blended with the natural hydrophilic polymer, the hydrophilic properties of the natural polymer enhances the wettability of composite, with a result in incensement in bioactivity of the scaffold and hydrolytic degradation of scaffold while PCL to reduce the swelling ratio and improve the mechanical properties of chitosan scaffolds.¹⁸

Gelatin is a natural biomimetic polymer derived from the denaturation of collagen. Gelatin is biocompatible, biodegradable and bioactive polymer while in comparison to collagen it does not represent immune rejection reactions. However, gelatin demonstrates a rapid hydrolytic degradation rate which can be the limiting factor of using gelatin in a long-term application.^{19,20} Chitosan is also a natural polysaccharide polymer obtained from deacetylation of chitin is an excellent candidate for tissue engineering applications due to its remarkable properties such as high biocompatibility, hydrophilicity, and antimicrobial activity. It reported that chitosan induces adequate adhesion and proliferation of Schwann cell. Therefore chitosan fibers are considered as a promising material for peripheral nerve regeneration.^{21,22}

However, low mechanical property and high biodegradation rate of the natural polymer are significant drawbacks for their application in tissue engineering. Therefore, preparing composite scaffolds of a natural polymer and a second synthetic polymer with high mechanical properties could be promising candidates because they combine the ideal chemical properties of natural polymer with robust mechanical properties of synthetic polymers.^{23,24}

An ideal scaffold should have sufficient mechanical properties, while the surface should have proper interaction with cells. According to this, another option is to combine nanofibrous properties and enhanced surface characteristic by treating by plasma modification.²⁵⁻²⁷

The primary goal of this study was to design and evaluate nanofibrous electrospun scaffold based on PCL by desirable modification for application in nerve tissue engineering for further clinical assessment. Several formulation variables assessed to optimize fibers essential characteristics such as diameter, degradation rate, and bioactivity. According to this, a series of scaffold were synthesized with different chemical and physical properties to evaluate the effect of these parameters on neural cell behavior.

Finally, morphological and chemical properties of nanofibrous scaffold investigated by using scanning electron microscopy (SEM), atomic force microscopy (AFM) and differential scanning calorimetry (DSC). Cell adhesion, morphology, and proliferation of L929 and PC12 cells on different nanofibrous scaffolds were also studied to understand the effect of solvent, fiber diameter, composition and surface modification incorporation for

further clinical assessment as a promising graft for nerve tissue engineering.

Material and Methods

Materials

PCL, gelatin type A, medium molecular chitosan purchased from Sigma Aldrich. De-ionized water, DMF, chloroform, acetic and formic acid were purchased from Merck and used as received. Fetal bovine serum (FBS), DMEM (Dulbecco Modified Eagle's Medium), phosphate buffered saline (PBS) and trypsin-EDTA acquired from Gibco, Singapore.

Preparation of Electrospun Graft

Different concentrations of electrospinning solution were prepared by dissolving PCL pellets in chloroform, DMF and Acetic acid/formic acid solution as solvent. The prepared solutions magnetically stirred at ambient temperature for 3-6 hours, then for the other 2 samples, chitosan and gelatin were poured gently to the solution with various ratios, to carry out the optimal concentration and percentage finally. Electrospinning process was performed using a commercially available electrospinning setup at solution feed rate of 0.1-1 mL.h⁻¹ and voltage between 10 and 19 kV under a fume hood. Also, O₂ plasma treatment was used to enhance the hydrophilicity of the surface of one of the PCL electrospun nanofibers at 20 W for 15 seconds.

Characterization of the Electrospun Scaffolds

Surface morphology of prepared nanofibrous scaffolds examined by SEM and AFM. Scaffolds (square, 5*5 mm) were placed in the sputtering and coated with a thin gold layer. Also, AFM was taken to study the roughness and fiber diameter range. Furthermore, the thermal properties of the fabricated electrospun scaffolds were discussed using a DSC and pre-calibrated with an indium standard to study the component bonding. Prepared electrospun scaffolds were first quenched to -100°C then gradually heated at a rate of 10°C per minutes to 200°C, respectively.

In Vitro Cell Culture

Prepared scaffold samples were washed with adequate PBS, exposed to UV for 1 hour and incubated in DMEM overnight before cell seeding, respectively. In vitro assessment were done using two different cell line including L929 and PC12 cells (as a neural cell). Besides, to study PC12 cells morphology on the nanofibrous scaffolds, the SEM was employed after seven days.²⁸

Results and Discussion

Chemical Characterization

To understand the interaction between different polymers DSC analysis used. Changes in the melting temperature can discuss phase separation between two or more blended polymers.

Soluble polymers indicate an individual phase blend so that DSC will indicate a unique glass transition, crystallization and melting transition. While Incompatible polymers represent distinct endotherms because of the different glass transition and melting endotherms according to the pure components or with some possible changes from them. In Figure 1, the thermogram of PCL fibers and blended scaffolds show an endothermic curve at 56-59°C, which is related to the melting point of the PCL. The characteristic peak of gelatin is at about 90°C while chitosan showed a linear diagram which represents an early degradation before its melting.

Blended PCL with chitosan thermograms were used to compare PCL nanofibers and PCL/chitosan nanofibers which illustrated a sharp endothermic peak of the melting temperature at 57°C. Therefore, the DSC thermogram of PCL/chitosan almost shows the same thermal behaviors of PCL components.

Morphological Observation of Nanofibrous Scaffold

To evaluate the topography and the morphology of electrospun scaffolds SEM micrographs of each specimen shown in Figure 2. The median fiber diameters sizes of all fabricated scaffolds are in the range of 90-1480 nm. Electrospinning parameters could affect the fiber morphology and diameters such as voltage, distance and feed rate, polymers properties, viscosity, and solvent solution. The solvent system for pure PCL, PCL/gelatin and PCL/chitosan prepared by acetic acid and formic acid, which has the high dielectric constant and therefore resulted in the formation of nanoscale fibers.⁵ While for DMF and chloroform because of higher dielectric constant thicker fiber achieved.

According to the SEM images, pure PCL scaffolds had smooth and beaded free fibers while Chitosan addition to the scaffolds composition resulted in the formation of curved and thinner fibers. For the gelatin is also as same as chitosan it made a curvy and thinner fiber with the high degree of water absorption which causes to increase the degradation rate.

Fibers with a thinner diameter resemble natural ECM collagen fibers and provide more surface area for cells compared to thicker fibers. The duration of electrospinning could control fiber density and thickness. The average pore size among fibers was 845.61 ± 34.21 nm, which is smaller than the neural cell size; therefore these nanofibers may be a suitable scaffold for cell seeding.

Also, AFM images of the nanofibrous scaffold were obtained to evaluate the uniformity of nanofibrous scaffold (Figure 3). In contrast, by adding chitosan to the solution, fiber diameter decreased up to 12% and the fibers morphology changed to curvy style.

Furthermore, the length of fiber could be a vital factor in flexibility and strength of the scaffolds. Additionally in tissue regeneration, one of the most fundamental parameters is the ability of vascularization and cell

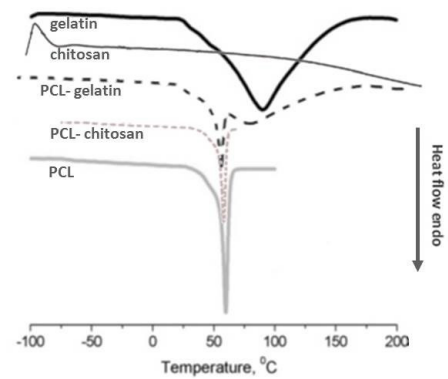


Figure 1. DSC Diagram of the Electrospun Fiber Scaffolds.

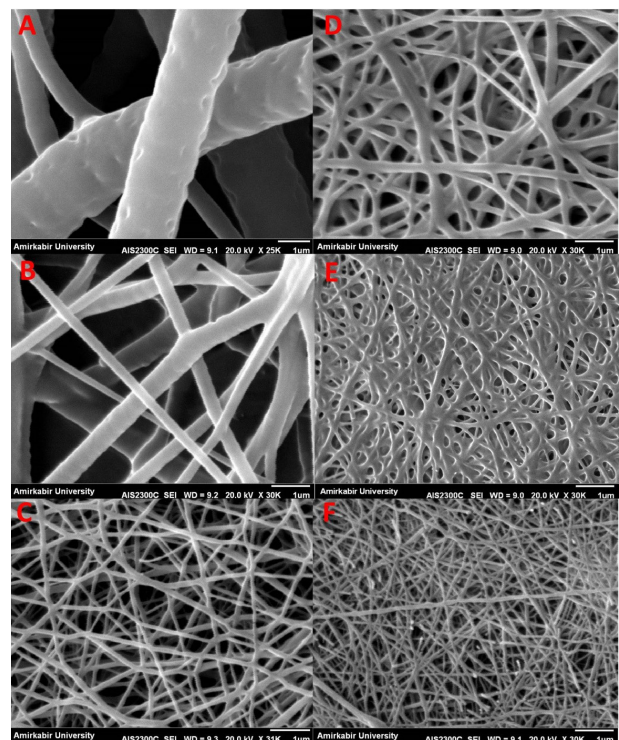


Figure 2. SEM Images of Nanofibrous Scaffold. (A) PCL in chloroform, (B) PCL in DMF, (C) PCL in Acetic/ Formic Acid, (D) Surface Modified PCL With Plasma, (E) PCL/Gelatin, (F) PCL/Chitosan.

migration. In addition to chemical composition, pore size and porosity density (porosity percentage) of fibrous scaffolds play vital function to vascularization and cell growth.^{8,20,29-32} According to this, it observed that by increasing the percentage of Chitosan and gelatin, pore size decreased while the porosity density increased. Also based on SEM images Max pore size could be achieved in PCL scaffold electrospun by chloroform while by using DMF and acetic/formic acid instead of chloroform as a solvent system the mean fiber diameter decreased and smaller pore size were obtained. In contrast, the porosity density increased based on image analysis software.

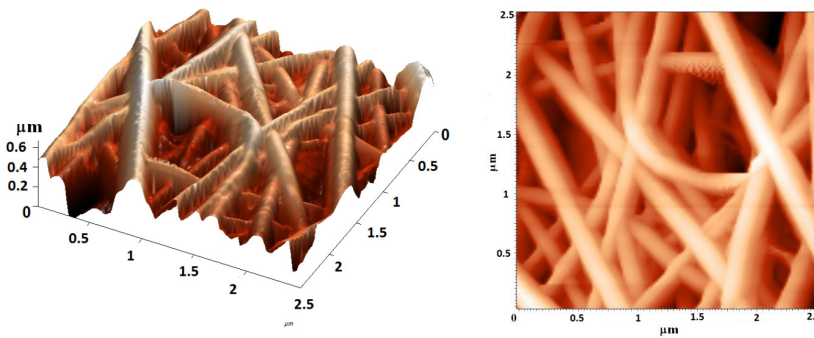


Figure 3. AFM Images of Nanofibrous Scaffold.

In Vitro Assessment

The cell viability of L929 cells was obtained using an SEM after the two days culture. The results demonstrated in Figure 4 and show that cells on the blended fibrous scaffolds prepare the better substrate for cell culturing and cell growth.

To study the behavior of neural cell on the fabricated scaffolds Cell proliferation rate of the PC12 cell were evaluated on the various types of nanofibrous scaffolds (Figure 5). In-vitro results show adding gelatin in the polymer blend improves bioactivity of the scaffolds and cell-scaffold interactions until seven days. However, after that because of the high hydrolysis degradation rate of gelatin and high water absorption its anticipated that gelatin component in blended gelatin scaffold degraded, and after that, the proliferation rate decreased. In comparison to gelatin, chitosan shows slower degradation rate with high bioactivity cause an increase in the proliferation rate in comparison to pure PCL in longer duration.

The proliferation profile of PC12 cells, the cells grown on the pure PCL scaffolds did not show significant

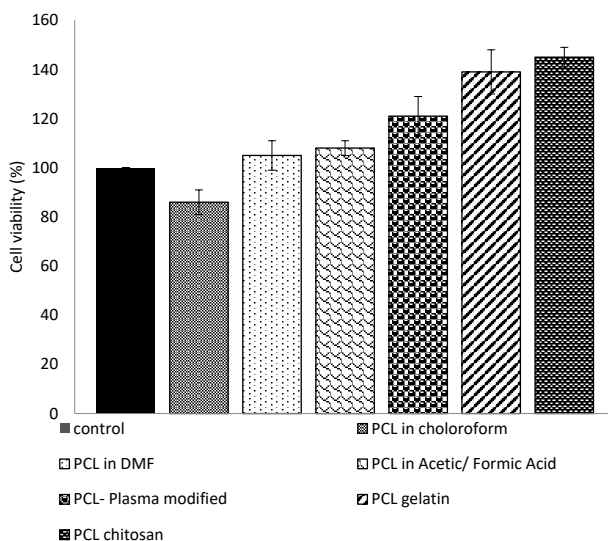


Figure 4. L929 Cell Viability Results on Various Type of Nanofibrous Scaffold.

improvement over the control not in chloroform nor DMF and an acidic solvent. However, the MTT results of the chitosan contained scaffolds shows a dramatic increase of up to 3.72x (as shown in Figure 5). This increased proliferation could be due to two different factors: First increased the hydrophilicity of the surface in chitosan-containing scaffolds; second reduced fiber diameter which also reported in other studies.³³

PC12 cells cultured on the various types of fabricated scaffolds were then visually observed by SEM. The interaction of PC12 cells on the pure PCL, PCL/chitosan, PCL/gelatin, and plasma surface modified nanofibers after seven days shown in Figure 6. In comparison to pure PCL, the cells had a more spreading rate on the surface of PCL/chitosan scaffolds; which was apparently because of reduced fiber diameter and enhanced hydrophilicity. Additionally, the neuronal outgrowth from PC12 cells seeded on the surface of PCL/gelatin scaffold; is clearly visible. Similarly, the plasma surface modified scaffold could also support neuron-like PC-12 cell adhesion and promote the spreading and growth of PC12 cells.

These results indicated that PC12 growth rate on PCL/

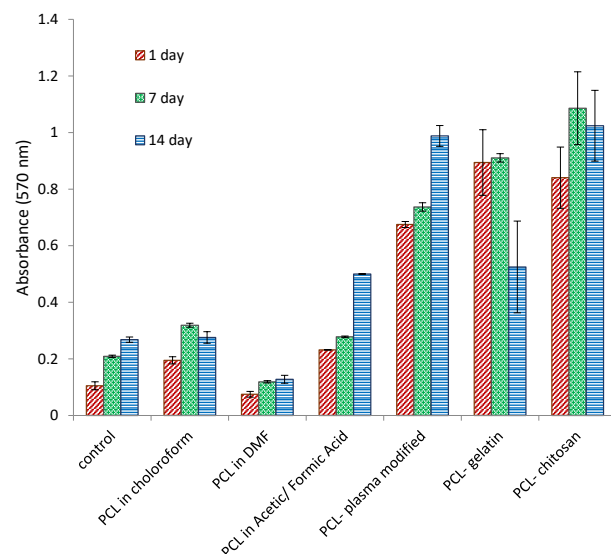


Figure 5. PC12 Cell Proliferation on Various Type of Nanofibrous Scaffold.

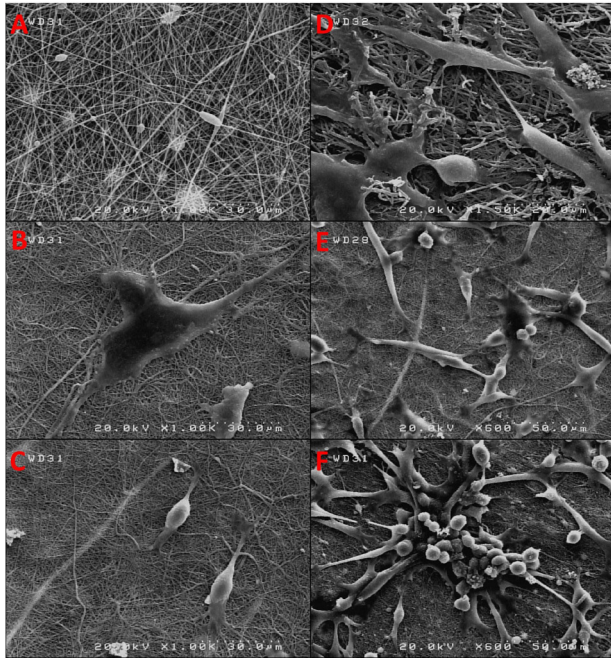


Figure 6. SEM Images of PC12 Seeded on the Nanofibrous Scaffold. (A) PCL in Chloroform, (B) PCL in DMF, (C) PCL in Acetic/Formic Acid, (D) Surface Modified PCL With Plasma, (E) PCL/Gelatin, (F) PCL/Chitosan.

chitosan nanofibers was 372% higher than cell growth on pure PCL nanofibers. Higher hydrophilicity of plasma surface modified and blended scaffold with chitosan and gelatin results in greater cell attachment and proliferation of PC12 cells. These results demonstrated that PCL nanofibrous scaffolds with plasma surface modification or blended with natural polymers such as chitosan and gelatin could support the adhesion, cell growth and proliferation of PC12.

Conclusion

Electrospun scaffolds of PCL in different solvent systems, PCL with gelatin and chitosan and surface modified PCL with plasma fabricated with the average fiber diameters of 90 to 1480 nm. DSC thermogram as a chemical analysis of scaffolds demonstrated the existence of gelatin and chitosan polymer in the electrospun of fibers. The modified surface and blended scaffold together with nanofibrous structure might provide essential topographic cues that favor neural growth, cell attachment, and proliferation. According to the results in fabricated nanofibrous scaffolds, gelatin and chitosan decrease the fiber diameter while cell activity increased which confirmed by cell adhesion and cell proliferation results. These results suggested that PCL nanofibrous scaffolds containing gelatin and chitosan could be a potential graft for nerve tissue engineering.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

Ethical Statement

Not applicable.

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