Original Article

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Electrospinning of Polycaprolactone Nanofibrous Scaffolds Containing Folic Acid for Nerve Tissue Engineering

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Abstract:

Objective: The aim of the study was to employ electrospinning technology to fabricate aligned nanofibrous scaffolds of polycaprolactone (PCL) containing folic acid (FA) for nerve tissue engineering.

Material and Methods: Scanning electron microscopy (SEM) was used to assess the diameter distribution and degree of alignment of the nanofibers. Fourier transform infrared spectroscopy (FTIR) and powder X-ray diffraction (PXRD) were used to analyze the chemical and crystalline structures of the scaffold. Additionally, the content and release behavior of FA in the PCL fibrous scaffolds were examined. Finally, the biocompatibility of the scaffolds was evaluated using rat Schwann cells, assessing cell proliferation, alignment, and morphology.

Results: The study revealed that the nanofiber diameters ranged from 210.07 to 227.36 nm, and the scaffolds maintained an amorphous form with no effects on their chemical structure following the electrospinning process. The investigation demonstrated that PCL fibers could accommodate FA loading within a range of 99.25-102.49% w/w and that the release profile of FA followed Higuchi model. Moreover, the FA-containing PCL nanofibrous scaffolds significantly enhanced rat Schwann cell proliferation during the initial two days of culture when compared to a normal PCL nanofiber scaffold. The hydrophilic properties of folic acid are thought to have facilitated directional growth along the electrospun nanofibers, contributing to the observed results.

Conclusion: Finally, PCL-containing FA nanofibrous scaffolds may be applicable to nerve tissue engineering.

Keywords: electrospinning, folic acid, nerve tissue engineering, polycaprolactone

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Introduction

Peripheral nerve injury, which mainly occurs due to road accidents, is a significant problem affecting more than one million people worldwide[1]. Approximately 5% of injuries to the arm or leg include peripheral nerve injury[2]. Normally, nerves can heal themselves at a rate of 1–3 millimeters (mm) per day, which takes about 2–3 weeks to heal a nerve that has been severed by approximately 1 centimeter (cm)[3]. If the nerve is severed more than 2 centimeters, it cannot heal on its own. Currently, there are three methods to treat completely severed peripheral nerves: direct nerve repair by suturing, autograft transplantation using a sensory nerve from the patient's own skin, and allograft transplantation using nerve tissue from a deceased donor. Direct nerve repair has limitations and can only be performed when the gap between the two nerve ends is not more than 5 millimeters. Autograft transplantation is the primary treatment method, which yields a success rate of 40–50%, but it can only restore sensory nerves and not motor nerves. It also requires up to 18 months of immune-suppressing medication and can result in the failure of the nerve to regenerate if the diameter of the nerve is too large[3]. The final method involves stitching through a nerve guidance conduit (NGC) using a principle similar to autologous nerve transplantation, but with synthesized nerve conduits instead. This method has the advantage of promoting axon growth in the same direction, reducing scar formation from surgery, and accumulating growth factors secreted by nerve cells that are torn. Growth factors have a role in promoting nerve cell growth. There are several methods of making nerve conduits, including freeze-drying, porogen leaching, thermally induced phase separation (TIPS), non-solvent induced phase separation (NIPS), rapid prototyping (Rp), and electrospinning[3–5]. However, the Food and Drug Administration (FDA) of the United States does not recommend using NGC if the nerve gap is larger than 3 centimeters and autograft transplantation may be more successful[6].

The electrospinning technique has been widely investigated in medical applications such as tissue engineering scaffolds, wound dressings, and drug delivery[7–9]. This technique is a simple process that can produce nanoscale or microscale fibers with a similar extracellular matrix (ECM) structure[10]. Several polymers such as silicone[11], polytetrafluoroethylene (PTFE)[12], polyglycolic acid (PGA)[13], poly (lactic co-glycolic acid) (PLGA), and collagen[14] have been used in nerve conduit fabrication via electrospinning in various studies. However, these polymers still have limitations in terms of their efficiency and cell growth capability. Polycaprolactone (PCL) is a well-known synthetic polymer used as a scaffold for engineering various tissues such as bones, nerves, muscles, and skin[15]. However, PCL is a highly hydrophobic polymer, which limits cell growth and proliferation. Therefore, other hydrophilic polymers such as gelatin[16], chitosan[15], and polylactic acid[17] have been blended with PCL to prepare electrospun nanofibrous scaffolds. Additionally, research reports have suggested the incorporation of various growth factors and biomaterials such as hydroxyapatite, collagen, and hyaluronic acid to promote cell growth and proliferation in electrospun scaffold structures[9,10]. Folic acid (FA), a type of B vitamin synthesized by humans, undergoes metabolism in the body to become folate. Folate plays a crucial role in deoxyribonucleic acid (DNA) synthesis and repair, which affects cell division and human growth[18,19]. Some studies have reported that folic acid can enhance the growth of nerve cells[20,21], but investigating its incorporation into electrospun polycaprolactone fibers for use as scaffolds in neural tissue engineering is currently lacking.

In this research work, the researchers developed electrospun PCL nanofibrous scaffolds containing FA for application in nerve tissue engineering. The physical and

chemical properties of the electrospun fibrous scaffolds were studied, including the morphology of the fibers using a scanning electron microscope (SEM), chemical properties using Fourier transform infrared spectroscopy (FT-IR), crystallinity of the fibrous scaffolds using X-ray diffractometer, mechanical properties using a texture analyzer, analysis of droplet shape of the fluid on the fibrous scaffold using a drop shape analyzer, and quantification of the release of FA from the nanofibrous scaffolds using a UV-vis spectrophotometer. Finally, the cellular characteristics of the nanofibrous scaffolds were evaluated, including cell growth by Alamar blue assay against rat Schwann cells and cellular morphology on the nanofibrous scaffolds using an SEM.

Material and Methods

PCL (MW 300,000) and FA were purchased from Sigma Aldrich. Rat Schwann cells (*Rattus norvegicus*) were purchased from the American Type Culture Collection (ATCC-CRL-2941™). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, and penicillin-streptomycin were purchased from Gibco BRL (Rockville, MD, USA). All reagents were analytical grade.

Electrospinning of the FA-containing PCL fibrous scaffolds

Using the electrospinning technique, nanofibrous scaffolds were created from a 25% w/w (25PCL) solution of PCL dissolved in 2'2'2' trifluoroethanol. To create variations, FA was dissolved in 25PCL solution at concentrations of 0.5% and 1.0% w/w (25PCL0.5FA and 25PCL1.0FA). The 25PCL, 25PCL0.5FA, and 25PCL1.0FA solutions were dispensed through a 20-gauge stainless steel needle attached to a 5 mL plastic syringe and applied to the emitting electrode of a Gamma High Voltage Research device with a positive polarity and an electric potential of 20 kilovolt (kV). The electrospinning was performed at room temperature with a fixed collection distance of 20 cm and a feed rate of 1.0 milliliter/hour (mL/h) for 4 h, resulting in the collection of nanofibers on a rotating collector at 1,200 rpm for alignment, with the nanofibers having a thickness of approximately 20-30 µm.

Characterization of the FA-containing PCL fibrous scaffolds

The morphology and size distribution of the fibrous scaffolds were observed by scanning electron microscope (SEM LEO 1,450VP, EDAX®, USA). For this process, a small section of the electrospun fibers was sputtered coat with a thin layerof gold prior to SEM observation. The accelerating voltage was fixed at 15 kV. The diameter of the nanofibers was measured using the JmicroVision program. One hundred measurements were made for each sample.

The chemical structure of these fibrous scaffolds was characterized by spectra obtained from a Fourier Transform Infrared Spectrophotometer (FTIR, Nicolet 4,700, USA) in the wave number range of 400-4,000 cm^{-1}.

The crystallinity of the fibrous scaffolds was evaluated by a powder X-ray diffractometer (PXRD, Miniflex II, Rigaku, Japan) with nickel-filtered Cu radiation generated in a sealed tube operated at 30 kV and 15 mA. The diffraction patterns of the nanofiber mats were recorded in the 2θ range of 5–45° at a scanning rate of 4 min^{-1}.

The mechanical properties of the electrospun fibers was assessed using a texture analyzer (TA.XT plus, Stable Micro Systems, UK) with 5 kg load cell equipped with a tensile grips holder. Fiber samples with a size of 5x25 mm and thickness ranging from 20-30 µm were cut and tested for tensile stress, strain, and Young's modulus.

The water wettability test was performed using a drop shape analyzer by measuring the contact angle between water and a fiber sheet. A distilled water droplet was placed on the sample surface, with parameters adjusted to a contact surface area of 0.2 square millimeters, a width of 1 cm, a length of 0.02 mm, and a height of 2 cm. Images

were captured before and after 5-second intervals for a 60-second period and the average angle] between the water droplet and the sample surface were measured.

Determination of the folic acid content and release profile of the PCL fibrous scaffolds containing FA

Each PCL fibrous scaffold was placed in a phosphate buffer solution with pH 7.4 and incubated at 37 °C for 24 h. The amount of phthalic acid in the fibers was determined using a UV-visible spectrophotometer at 281 nm[22]. The actual content of FA in the fibers was calculated and expressed as a percentage using the equation: (1)

$$\% \text{ FA content} = (La/Lt) \times 100 \quad (1)$$

Where La is the amount of FA embedded in the fibrous scaffolds, and Lt is the theoretical amount of FA (obtained from feeding condition) incorporated into the fibrous scaffolds.

Each PCL fibrous scaffold was placed in a pH 7.4 phosphate buffer solution (PBS) at a volume of 10-20 mL and incubated at 37 °C with continuous shaking at 100 rpm in a shake incubator. Sample solutions were collected at various time points (0.5, 1, 2, 4, 6 h, etc.). The collected samples were analyzed to determine the amount of folic acid released.

Cell cultures

Schwann cells from *Rattus norvegicus* (CRL-2941™) obtained from the American Type Culture Collection (ATCC) were cultured in DMEM supplemented with 10% v/v fetal bovine serum (FBS), 1% v/v L-glutamine and 1% v/v penicillin/streptomycin. The cells were maintained in an incubator at 37 °C with 5% CO_2 for studying cell growth and morphology of nerve fibers. Approximately 10,000 cells/mL were seeded onto the nerve fibers in 1 mL of cell culture medium, which was attached to a cell crown.

Cell growth study on PCL fibrous scaffolds containing FA

Cell proliferation was evaluated using the alamarBlue assay at various time points subsequent to the cells being seeded onto nerve fibers, which was done on day 1, day 2, day 4, and day 7. At each time point, the cellular medium was removed, and replaced with 1 mL of fresh medium supplemented with 5 mM alamarBlue dye. The samples were then incubated at 37 °C in an environment containing 5% CO_2 for a duration of 2 h, following which 200 µL of the cell culture medium was transferred into a black 96-well plate for fluorescence measurements at an excitation wavelength of 530 nm and an emission wavelength of 590 nm utilizing a microplate reader (Microplate Reader, FLUOstar Omega, BMG Labtech, NC, USA). The relative fluorescent units were converted to cell numbers using a calibration curve constructed by measuring the fluorescence of known numbers of cells.

Morphological study of cells on PCL fibrous scaffolds containing FA

On day 4, the cellular samples were fixed with 1.5% glutaraldehyde for 30 minutes at 4 °C, followed by a rinse with 0.1M phosphate buffer. The samples were subsequently dehydrated using a series of increasing ethanol concentrations (50%, 70%, 90%, and 100% v/v), and an overnight incubation in hexamethyldisilazane (HMDS). The resulting morphology of the cells adhering to the nanofiber scaffolds was observed using scanning electron microscopy (SEM), after sputtering coat with a thin layer of gold.

Data analysis

In this study, triplicate samples were utilized to collect data, and the resulting values were presented as mean± standard deviation (S.D.). To evaluate statistical significance, Student's t-test was performed, and a significance level of $p\text{-value}<0.05$ was employed.

Results

Characterization of PCL fibrous scaffolds containing FA

Figure 1 shows the SEM images of the 25PCL, 25PCL0.5FA and 25PCL1.0FA nanofibrous scaffolds. The average diameters of the 25PCL, 25PCL0.5FA and 25PCL1.0FA nanofibrous scaffolds were 210.07±55.71, 219.79±61.60 and 224.36±63.76 nm, respectively. When the amount of FA was increased the average diameter of the nanofibers slightly increased. The diameter distribution and degrees of alignment are shown in Figure 1. The fibers exhibited narrow distributions for both diameter and degrees of alignment.

The FTIR spectra of the 25PCL, 25PCL0.5FA, 25PCL1.0FA nanofibrous scaffolds and FA powder are demonstrated in Figure 2A. The spectra of the 25PCL scaffolds displayed C=O stretching at 1731 cm−1, –COO– vibrations at 1,239 cm−1 and C–H stretching at 2,998 cm−1 that found in PCL[16]. The spectra of the FA powder also displayed C=O stretching at 1,731 cm−1 and –COO– vibrations at 1,239 cm−1[23]. The spectra of the 25PCL0.5FA and 25PCL1.0FA scaffolds did not differ from the spectrum of 25PCL. Figure 2B shows the PXRD patterns of the 25PCL, 25PCL0.5FA, 25PCL1.0FA nanofibrous scaffolds and FA powder. The 25PCL nanofibrous scaffold showed diffraction peaks at 21.5 degrees and 23.6 degrees[24]. The FA powder showed a major diffraction peak at 14.2 degrees. When FA was loaded into the 25PCL nanofibrous scaffold, the PXRD pattern did not change.

Table 1 presents the mechanical properties of the nanofibrous scaffolds made from 25PCL, 25PCL0.5FA, and 25PCL1.0FA. Incorporation of FA into the 25PCL scaffold resulted in a significant increase in tensile stress and Young's modulus, from 3.08±0.4 to 15.31±3.26 kilopascal (kPa) and 33.33±2.15 to 246±23.51, respectively. However, the maximum strain of the scaffold slightly decreased from 9.26±1.26 to 6.19±0.92% upon FA loading. The water contact angles for the 25PCL, 25PCL0.5FA, and 25PCL1.0FA nanofibrous scaffolds are presented in Table 1. Incorporation of FA led to a reduction in water contact angle of 25PCL0.5FA nanofibrous scaffolds from 117.61±1.25 to 100.94±2.38 degrees and 25PCL1.0FA nanofibrous scaffolds from 117.61±1.25 to 99.35±1.78 degrees.

Content (%) and release profile of PCL fibrous scaffolds containing FA

Table 1 shows that the 25PCL0.5FA and 25PCL1.0FA nanofibrous scaffolds had content (%) values of 102.49±0.50 and 99.25±0.44, respectively. Figure 3 illustrates the release of FA from the PCL nanofibrous scaffolds in pH 7.4 phosphate buffer. The maximum release of FA was observed to be 30 μg/mL in 24 h for 25PCL0.5FA and 45 μg/mL in 24 h for 25PCL1.0FA.

Table 1 Content (%), contact angle and mechanical properties of the 25PCL, 25PCL0.5FA and 25PCL1.0FA nanofibrous scaffolds. Each value represents the mean±S.D. from three independent experiments.

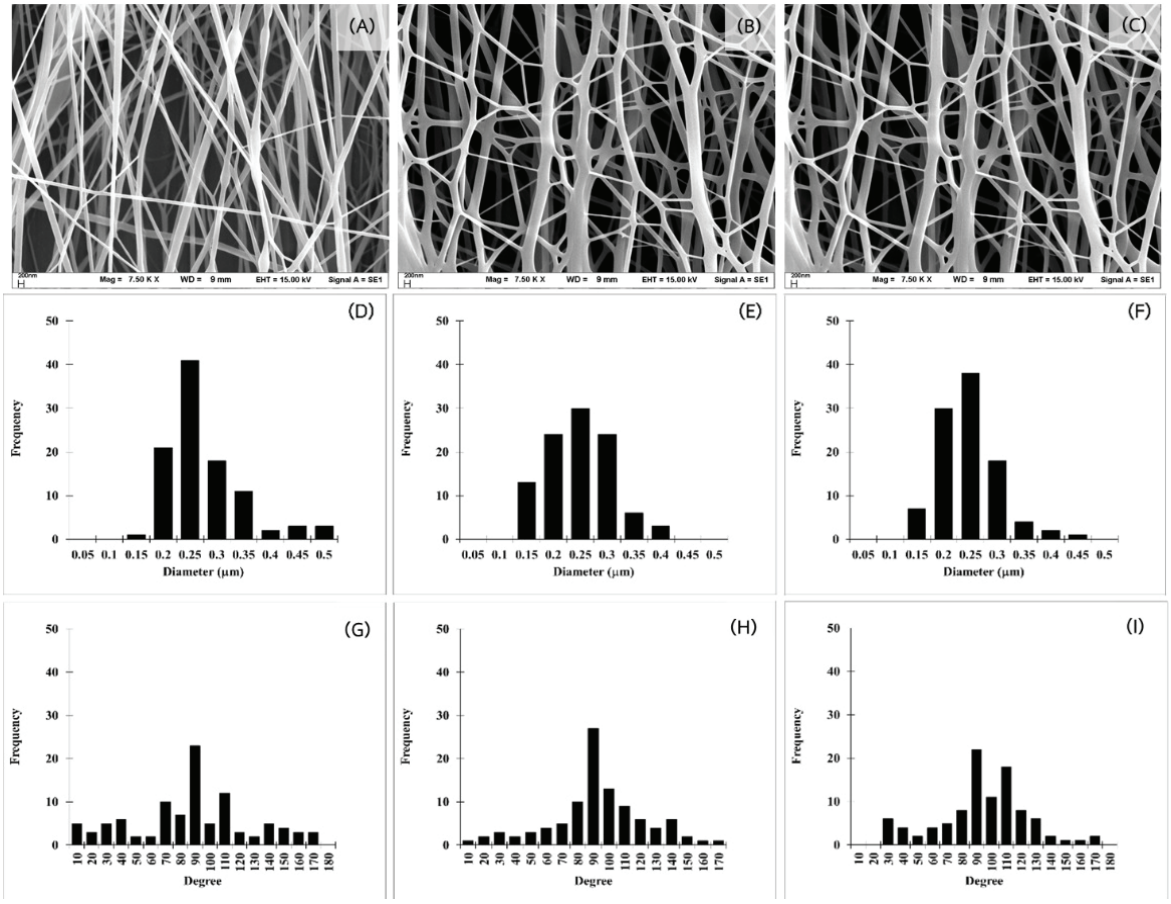
Sample	Content (%)	Contact angle (degree)	Tensile Stress (kPa)	Maximum strain (%)	Young' s modulus (kPa)
25PCL	–	117.61±1.25	3.08±0.40	9.26±1.26	33.33±2.15
25PCL0.5FA	102.49±0.50	100.94±2.35	10.52±1.58	5.63±1.95	194.93±35.76
25PCL1.0FA	99.25±0.44	99.35±1.78	15.31±3.26	6.19±0.92	246.04±23.51

PCL=polycaprolactone, FA=folic acid, kPa=kilopascal, S.D.=standard deviation

Cell study on PCL fibrous scaffolds containing FA

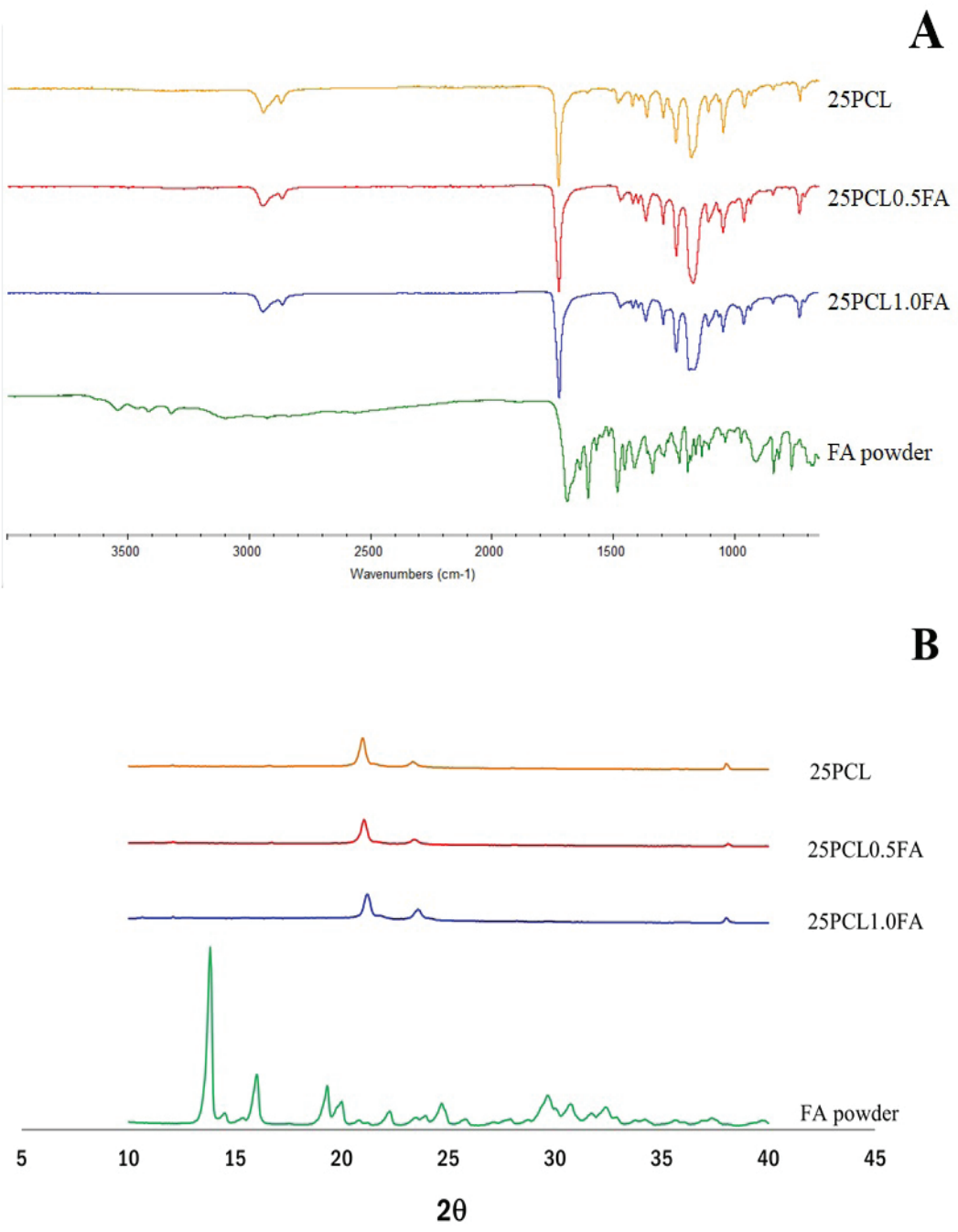
In this part of the study, rat Schwann cells were used to evaluate the cell proliferation on the 25PCL, 25PCL0.5FA, and 25PCL1.0FA nanofibrous scaffolds. The percentages of cell numbers at 1, 2, 4, and 7 days of culture, compared with the control, are presented in Figure 4. As can be seen, the 25PCL nanofibrous scaffold showed a significant reduction in cell numbers on days 1 and 2 post-seeding, compared to the control. In contrast, the 25PCL0.5FA nanofibrous scaffold exhibited a significant increase in cell numbers on day 2 post-seeding, while the 25PCL1.0FA nanofibrous scaffold exhibited a significant increase in cell numbers on both day 1 and day 2 post-seeding, compared to the control.

In Figure 5, the SEM images depict the morphology of rat Schwann cells cultured on a glass slide (A), 25PCL (B) and 25PCL1.0FA (C) nanofibrous scaffold for a duration of 4 days. The cells on the glass slide displayed a stellate-pattern morphology and spread in a non-directional manner. Conversely, cells cultivated on both the 25PCL and 25PCL1.0FA nanofibrous scaffolds exhibited alignment parallel to the oriented fibers. Notably, there was no discernible difference in cell morphology between the 25PCL and 25PCL1.0FA nanofibrous scaffolds.



SEM=scanning electron microscopy, PCL=polycaprolactone

Figure 1 SEM images at a magnification of 7500x, diameter distributions and degree of alignments of 25PCL (A, D, G), 25PCL0.5FA (B, E, H) and 25PCL1.0FA (C, F, I) nanofibrous scaffolds



FTIR=fourier transform infrared spectroscopy, PXRD=powder X-ray diffraction, PCL=polycaprolactone, FA=folic acid

Figure 2 FTIR spectra (A) and PXRD pattern (B) of the 25PCL, 25PCL0.5FA, 25PCL1.0FA nanofibrous scaffolds and FA powder

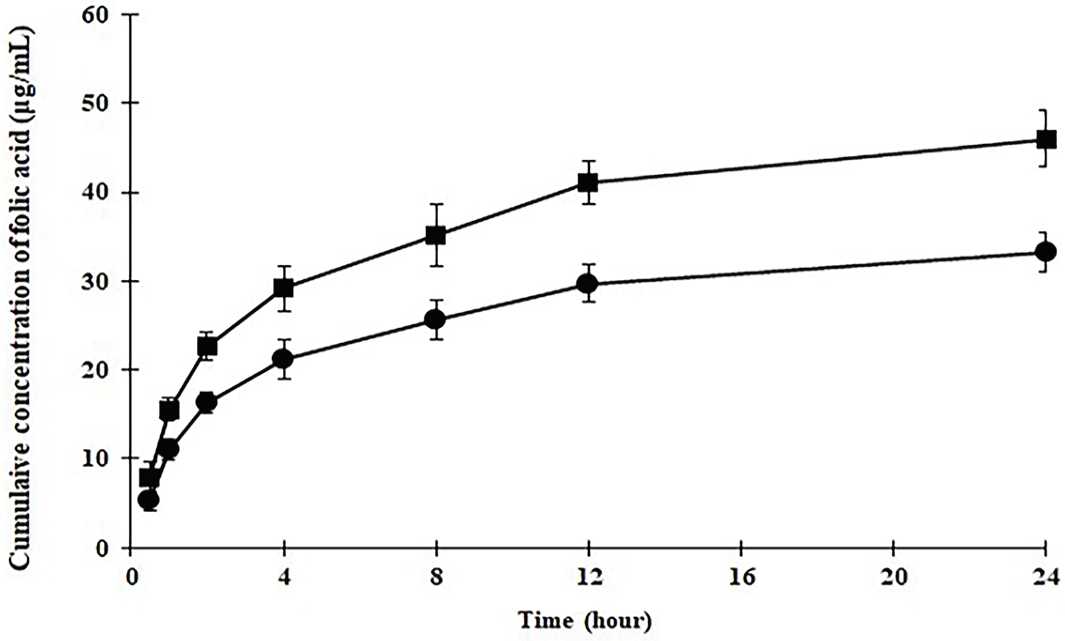
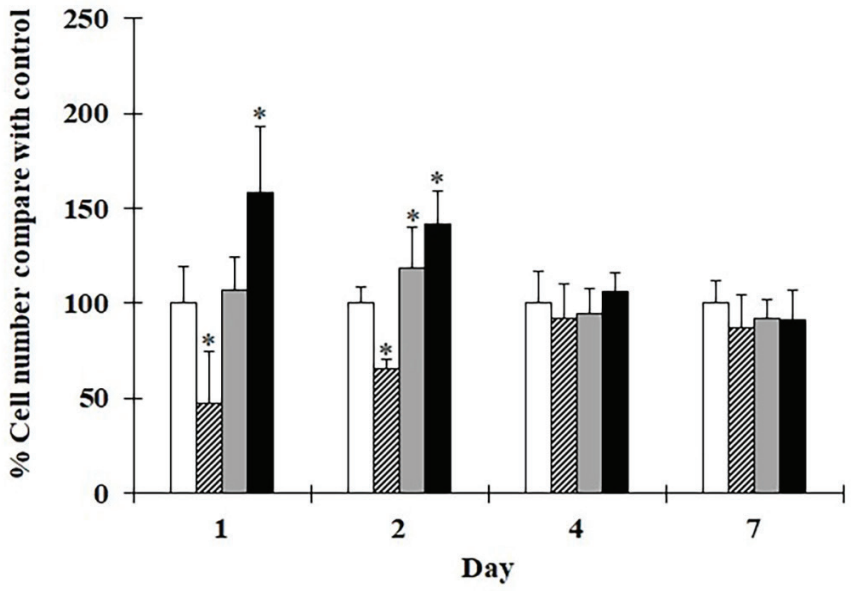
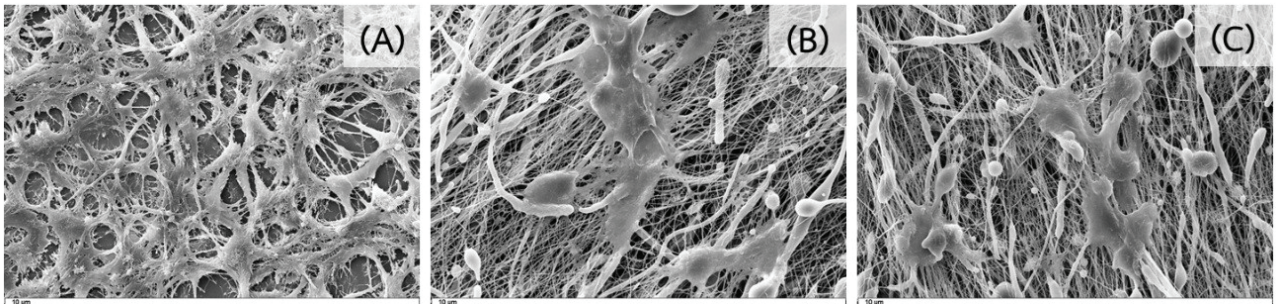


Figure 3 Release characteristics of (●) 25PCL0.5FA and (■) 25PCL01.0FA nanofibrous scaffolds



*statistical significance p-value<0.05

Figure 4 Cell numbers (%) compared with a control of rat Schwann cells on (▨) 25PCL (▩) 25PCL0.5FA, (■) 25PCL01.0FA nanofibrous scaffolds, and (□) controls. Each value represents the mean±standard deviation of triplicate samples



SEM=scanning electron microscopy, PCL=polycaprolactone, FA=folic acid

Figure 5 SEM images at a magnification of 1,000x of rat Schwann cells on (A) glass slide, (B) 25PCL nanofiber scaffold, and (C) 25PCL1.0FA nanofiber scaffold after 4 days of cell culture

Discussion

The preparation of fibers via electrospinning involves various parameters that affect the morphological and physicochemical properties of the resulting fibers, such as diameter, polymer concentration, and electrical field intensity. Therefore, it is necessary to control these parameters appropriately to optimize the scaffold characteristics[25]. This study focuses on the development of a PCL nanofibrous scaffold containing FA. The results indicate that electrospinning PCL loaded with 0.5–1.0% w/w FA did not significantly affect the central diameter of the resulting nanofibers, which ranged from 210.07±55.71 to 227.36±63.76 nm. These findings align with previous work by Parin et al., who prepared nanofibers from polyvinyl alcohol loaded with FA via electrospinning and found that increasing the FA concentration led to a higher viscosity of the solution and larger fiber diameter[26].

The investigation of the electrospun PCL nanofibrous scaffold's functional group containing FA was conducted using Fourier-transform infrared spectroscopy (FTIR). The FTIR spectra of PCL and FA powder exhibited identical peaks at 1,731 cm−1 for C=O stretching and 2,998 cm−1 for C–H stretching, indicating that the electrospinning process did not affect the chemical structure of PCL and folic acid. This finding with a study by Yang et al. in which the FTIR spectrum of PCL with FA displayed characteristic absorption peaks at 2,934 cm−1, 2,841 cm−1 (ν C–H), and 1,739 cm−1 (ν C=O), which can be attributed to the absorption peaks specific to PCL chains[27]. The PXRD technique was employed to determine the crystallinity of the scaffold. Analysis of the PXRD pattern of the nanofibrous scaffold revealed that even after undergoing the electrospinning process, the PCL remained semi-crystalline. These experimental findings are consistent with previous study on the development of PCL nanofibers blended with polyvinyl pyrrolidone, which demonstrated that the PCL fibers exhibited semi-crystalline characteristics[24].

The current study examined the impact of different amounts of FA on the mechanical properties of electrospun PCL nanofibrous scaffolds. The findings revealed a significant increase in both tensile stress and Young's modulus of the fibers with the addition of FA. Moreover, the fibers became more flexible and required greater force to break, which may be attributed to the increased diameter of the fibers, resulting in greater resistance against deformation. These results are consistent with the research findings of Akhgari et al., who found that adding FA to Eudragit S100 improved the mechanical properties of electrospun nanofibers[28]. Furthermore, the drop shape analysis used to test the water wettability demonstrated that

the augmentation of FA concentration within the fibers led to increased hydrophilicity and greater water affinity. This phenomenon may be attributed to the inherent hydrophilicity of FA, which can potentially enhance the water wettability of PCL (24). These discoveries signify that incorporating FA into electrospun PCL fibers could potentially enhance the fibers' mechanical properties and wettability.

The content (%) study demonstrated that the solubility of FA in the solvent used for electrospinning was high, as indicated by FA loading into PCL fibers ranging from 99.25% to 102.49%. Moreover, the FA did not undergo degradation during the electrospinning process. The release of FA from the 25PCL0.5FA and 25PCL1.0FA nanofibrous scaffolds followed a Higuchi model, with respective r^2 values of 0.9229 and 0.9258. Initially, FA on the fiber surface was rapidly released upon immersion in PBS, and subsequently a gradual and sustained release of FA was observed for up to 24 h. This suggests that the presence of FA in the PCL scaffold may generate pores on the fiber, leading to a slow release of FA from the scaffold upon immersion in PBS[29].

In this study, we investigated the effects of incorporating FA into electrospun PCL nanofibrous scaffolds on the growth of rat Schwann cells. Our results demonstrated that PCL nanofibrous scaffold containing FA can better support the growth and alignment of Schwann cells compared to PCL without FA and control fibers during the first two days of culture. The hydrophilic nature of folic acid likely contributed to these results, as it facilitated directional growth along the electrospun fibers[19]. Overall, our findings suggest that folic acid-containing PCL fibers have potential applications as tissue engineering scaffolds for neural cells.

Conclusion

This research study successfully utilized the electrospinning technique to fabricate uniaxially aligned electrospun PCL incorporated with FA for nerve tissue engineering purposes. The experimental results found that the diameter of the PCL fibrous scaffolds containing FA fell within the nanometer range. The FTIR spectra indicated a well-mixed integration of FA with PCL. The PXRD pattern suggested that the FA present on the PCL nanofibrous scaffolds existed in an amorphous state. The electrospinning process retained approximately 100% of the FA content. The inclusion of FA in the PCL nanofibrous scaffolds notably enhanced the hydrophilicity of the scaffolds. Furthermore, the FA-incorporated PCL nanofibrous scaffolds exhibited a significant increase in rat Schwann cell proliferation during the initial two days of culture compared to the regular PCL nanofiber scaffolds. These findings support the potential application of electrospun PCL fibrous scaffolds containing FA as a promising scaffold material for nerve tissue engineering.

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Conflict of interest

The authors declare no conflict of interest.

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