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Potential of Collagen/PLA-Based Nanofibrous Scaffold to Support PC12 Cells and Neural Repair

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Abstract

Objective: This study attempts to modify Polylactic Acid (PLA) with the natural polymer Collagen (Coll), to develop materials such as an electrospun scaffold that have better mechanical stability and biocompatibility. Retinoic acid (RA), a bioactive material that promotes nerve growth, is to be added to the nanofiber scaffolds as part of this project.

Methods: One of the most important methods we employed in this work to create nanofibrous scaffolds was electrospinning.

Results: The synthesized nanofiber scaffold exhibited a diameter of 255 ± 40 nm and a tensile strength of 175 ± 10.4 N, providing sufficient support for native peripheral nerve repair. The inclusion of Coll enhanced the scaffold's hydrophilic behavior (contact angle: 56 ± 4°), ensuring stability in aqueous solutions. In addition, cell adhesion and proliferation are demonstrated to be improved by PLA composite nanofibers based on Collagen, while PC12 cell adhesion and proliferation are further improved by RA.

Conclusion: Based on their biodegradability, robust mechanical properties, and porous structure, these scaffolds are excellent choices for nerve tissue engineering, according to our findings. The significant increase in PC12 cells' adhesion and proliferation upon the addition of RA demonstrates the cells' potential for nerve repair.

Keywords: Collagen, polylactic acid, all-trans-retinoic acid, PC12 cells, tissue repair

Introduction

Sensory and motor impairments resulting from peripheral nervous system dysfunction are experienced by countless individuals worldwide. Current therapeutic goals primarily focus on symptom stabilization and disease spread reduction, leaving the pursuit of the ideal nerve restoration procedure in ongoing research.[1] One of the most promising approaches to address neurological abnormalities is tissue engineering, offering a unique avenue for repairing and replacing damaged tissues. The choice of suitable biomaterials is paramount in tissue engineering, facilitating the healing process in the presence of progenitor cells.[2-5] Various studies in brain tissue engineering have explored both organic and synthetic polymers.[6,7]

Recently, significant attention has been directed towards techniques for producing nanofiber-based scaffolds within the field of tissue engineering. Polymer nanofibers possess distinctive properties that render them invaluable tools in tissue engineering applications.[8] Their high surface-to-volume ratio and microporous structure make them particularly well-suited for cell adhesion, migration, and proliferation. Numerous methods exist for polymer production,[9] with electrospinning standing out as the most popular technique for creating nanofibers, especially in tissue engineering applications.[6–9]

Polylactic acid (PLA), a synthetic polymer, has gained prominence in the production of tissue engineering scaffolds. PLA is known for its biocompatibility and biodegradability. Previous research has demonstrated that conduits constructed from PLA fibers support cell and axon migration in peripheral nerve lesions.[10-14]

Collagen (Coll), a natural polymer containing extracellular matrix (ECM) proteins, can serve as an effective substitute for ECM.[15-18] Despite collagen's hydrophilicity and propensity for proper cell adhesion, it lacks the mechanical strength and degradation speed necessary for nerve growth. However, by blending natural biopolymers with biodegradable synthetic polymers, one can adjust the scaffold's degradation rate and physical and mechanical properties.[7,18-22]

Previous research findings emphasize the crucial role of scaffold topography in influencing cell morphology and guidance. In this context, aligned nanofibrous scaffolds have demonstrated significant enhancements in the function and repair of nerve cells under laboratory conditions when compared to randomly structured ones.[20] Electrospun fibers have also proven capable of closely mimicking the physical structure and mechanical properties of peripheral nerves.[18]

A research study is currently underway to investigate the modification of PLA (Polylactic Acid) with various extracellular matrix proteins, including collagen, in pursuit of improved materials for creating effective electrospun scaffolds. This research aims to harness the advantageous properties of collagen, a biopolymer, by blending it with PLA, resulting in the development of novel materials characterized by enhanced biocompatibility and increased mechanical stability.[19–22] Yang et al., have also emphasized the significance of combining PLA and Coll for electrospinning, as it enhances their physiochemical characteristics, making them more suitable for biomedical applications.[23]

A previous study has reported noteworthy advancements in using electrospun PLA fibers modified with Coll for repairing injured peripheral nerves, surpassing the capability of pure electrospun PLA scaffolds. Synthetic polymers like PLA, despite their mechanical strength, often exhibit poor neuron adhesion due to their surface chemistry. However, the combination of natural polymers with synthetic ones can significantly enhance surface chemistry and related properties. Coll, as the most abundant protein in the human body, plays a

vital role in extracellular matrix (ECM) production, structural integrity, and tissue support.[23,24]

Drug molecules like Retinoic acid (RA) and their metabolites play a pivotal role in nerve growth and repair. *In vitro* studies have demonstrated RA's ability to induce post-mitotic neuronal phenotypes in various stem cells and nerve cells. Additionally, RA, when combined with nanofibrous topography, has shown to enhance neuronal differentiation towards nerve cells.[25-30]

This study aims to design a suitable substrate in the form of nanofibers, using collagen and PLA polymers via the electrospinning process, to promote the growth and proliferation of nerve cells. Furthermore, Retinoic acid has been incorporated into the nanofiber scaffolds as a bioactive substance to enhance the prospect of nerve healing. The effects of Coll/PLA and Coll/PLA/RA on neural cells were thoroughly examined in this research endeavor.

Materials and Methods

Fabrication of Nanofibrous Scaffold

Collagen (Coll) type-I was extracted from rat tails, while poly (lactic acid) (PLA) and all trans-Retinoic Acid (RA) were sourced from Sigma Aldrich (USA). Solutions of 10% w/v were prepared seperately for the Coll/PLA nanofiber scaffolds. PLA was dissolved in a mixture of chloroform and DMF (1:9 ratio), while collagen was dissolved in 90% acetic acid. These mixtures were mixed for an hour at room temperature. Subsequently, both solutions were combined and stirred for 30 to 40 minutes at room temperature to form the final Coll/PLA mixture. The combined Coll/PLA solution was then loaded into a syringe and electrospun. The electrospinning parameters were set as follows: voltage of 18 kV, a needle tip to collector distance of 10 cm, and a flow rate of 0.6 ml/h.

For the Coll/PLA/RA nanofiber scaffolds, separate 10 percent w/v PLA solutions were prepared in chloroform and DMF. This PLA solution was mixed at room temperature for an hour. The PLA solution was then combined with a 0.3 percent formulation of RA obtained from a stock solution of 3 mg/ml in MeOH. This mixture was agitated at room temperature for two hours. Afterwards, a separate 10% w/v collagen solution was dissolved in 90% acetic acid and stirred for an hour. Both solutions were then combined and stirred for an additional 30 to 40 minutes at room temperature to create the final Coll/ PLA/RA blend. The Coll/ PLA/RA solution was drawn into a syringe and electrospun using the specified conditions. Approximately 5–6 mL of solution was used for each electrospun scaffold. All scaffolds were subsequently dried in preparation for further characterization.

Cross-Linking of Scaffolds

To cross-link the fabricated scaffolds, nanofiber scaffolds were placed in an enclosed chamber with 25% glutaraldehyde (GA) vapors for 2 hours at room temperature.[31,32]

Characterizations of Scaffolds

Morphological characteristics

The morphology of nanofibrous scaffolds was examined using a scanning electron microscope (SEM, XL 30; scaffold Amsterdam, Netherlands). To enhance imaging resolution, a thin layer of gold was applied to the samples. Using Image J (Image J; US National Institutes of Health, Bethesda, MD, USA), the average diameter and porosity of the fibers were calculated. Measurements were taken from twenty-five fibers in each SEM image, including the middle, two ends, and at three separate sites.[14,15,33]

Contact angle measurement

The electrospun nanofiber scaffolds' hydrophilicity was assessed using an optical water contact angle measurement device (OCA-15-plus, Data physics). For measuring the water contact angle, electrospun scaffolds on coverslips were placed directly on a testing plate. Following the application of 10 µl of deionized water to the scaffolds, observations were made in triplicate and recorded.[14,15]

Mechanical testing

Uniaxial mechanical properties of the scaffolds were evaluated through Universal testing machine (UTM) (Zwick Roell Z050 Germany). The device grips were moved at a speed of 5 mm/min throughout the samples. Tensile tests were conducted on three scaffold samples from each group at room temperature. Data including ultimate tensile strength, elongation at break, and elastic modulus were assessed from stress-strain curves in triplicate.[34,35]

Degradation analysis

Samples with an initial weight (W_0) were incubated in phosphate buffer saline (PBS) (pH = 7.4) at 37°C with an agitation rate of 50 rpm for 4-weeks to evaluate changes in the scaffold content in aqueous solutions. The PBS solution was not renewed during the experiment. At each interval, PBS was removed from samples, and they were washed with distilled water, dried and weighed (W_t). The remaining mass percentage was calculated using the following equation for samples (n = 3).[36,37]

$$\text{Remaining mass \%} = W_t/W_0 \times 100$$

In-vitro Cell Study

The cytotoxicity and biocompatibility of the composite scaffold samples were examined using the Alamar Blue® assay. PC12 Cells were cultured in a 25 cm^2 flask of RPMI supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C, 95% humidity, and 5% CO_2. Cells (passages 3–4) were seeded at a density of 4×10^5 cells per well onto the scaffold surfaces in 48 well plates containing 300 µl of cell suspension in complete media for days 1, 3, 5, and 7. The medium was removed from the plates at each time point, and each well was treated with the assay and incubated for 4–6 hours at 37°C. Absorbance was measured at a wavelength of 630 nm and a reference wavelength of 570 nm. Four samples were analyzed for each time point, and mean data were calculated.[34]

Cell Adhesion Study

Cell growth and adhesion were assessed on Coll/PLA and Coll/PLA/RA scaffolds. The scaffolds were placed at the base of a 48-well cell culture cluster that had undergone UV irradiation sterilization. The macrophages were diluted to 4×10^5 cells per well in 2 mL of RPMI, with 10% fetal bovine serum added as a supplemental, and the mixture was incubated at 37°C. Cells

were grown in a well without a scaffold as a control. After incubation for 24–72 hours, the scaffolds were removed and rinsed in cold PBS. To fix the attached cells, the scaffolds were submerged in a 4% (v/v) formaldehyde in PBS solution. After a 20-minute immersion, the scaffolds were washed three times with alcohol ranging from 60% to 100%. The electrospun scaffolds were then carefully placed on glass slides and allowed to air dry. Finally, gold sputtered scaffold specimens were used for SEM analyses.[29,33-38]

Results

Morphology and Porosity of Nanofibers

SEM was employed to examine the morphology of electrospun nanofiber. Figure 1A and 1B depict the Coll/PLA nanofibers, while Figure 1C and 1D showcase the Coll/PLA/RA nanofibers, both exhibiting an average diameter of 237 ± 57 nm and 255 ± 40 nm, respectively.

Hydrophilicity Test

Contact angle measurements were conducted for both Coll/PLA alone and the mixed Coll/PLA/RA material. The contact angle of scaffolds provides insights into their hydrophilicity. As shown in Table 1, both scaffolds exhibited a hydrophilic behavior when in contact with an aqueous solution.

Tensile Testing

Table 2 provides details on the mechanical properties of the nanofibrous scaffols. Stress-strain results for two groups of the electrospun scaffolds are included, highlighting Young's modulus, tensile strength, and strain percentage at break during elongation.

Degradation Analysis

To assess scaffold stability in an aqueous solution, a degradation test was applied. Figure 2 illustrates the progression of degradation, which initiated during the first week after treatment. This degradation analysis offers insight into the stability and degradation rate of the fabricated construct within the microenvironment of the native ECM.

Cell Study

The cytotoxicity of scaffolds was evaluated using Alamar Blue® assay, as illustrated in Figure 3. The interaction of nerve cells (PC12) with composite scaffolds was evaluated for up to seven days. The results indicate that all tested samples exhibited no cytotoxicity over the course of a week, demonstrating the biocompatibility of Coll-based PLA scaffolds with nerve cells.

Cell Adhesion Study

Figure 4 displays SEM analyses of PC12 cell attachment to Coll/PLA scaffolds at days 1 and 7. The findings demonstrate that planted cells are widely dispersed and exhibit strong adhesion to Coll/PLA scaffolds.

Statistical Analysis

One-way ANOVA was used by the GraphPad Prism 9 program to statistically assess the results. The information was

Table 1. **Contact angle measurements of Coll/PLA & Coll/PLA/RA scaffolds**

Sample	Average contact angle
Coll-PLA	51 ± 06°
Coll-PLA-RA	56 ± 04°

Table 2. **Tensile properties of Collagen/PLA, and Collagen/PLA/RA nanofibrous scaffolds**

Sample	Young's modulus (MPa)	Tensile strength (MPa)	Strain at break (%)
Coll/PLA	9 ± 0.16	11.5 ± 0.5	230 ± 11.2
Coll/PLA/RA	6.7 ± 0.23	8.5 ± 0.8	175 ± 10.4

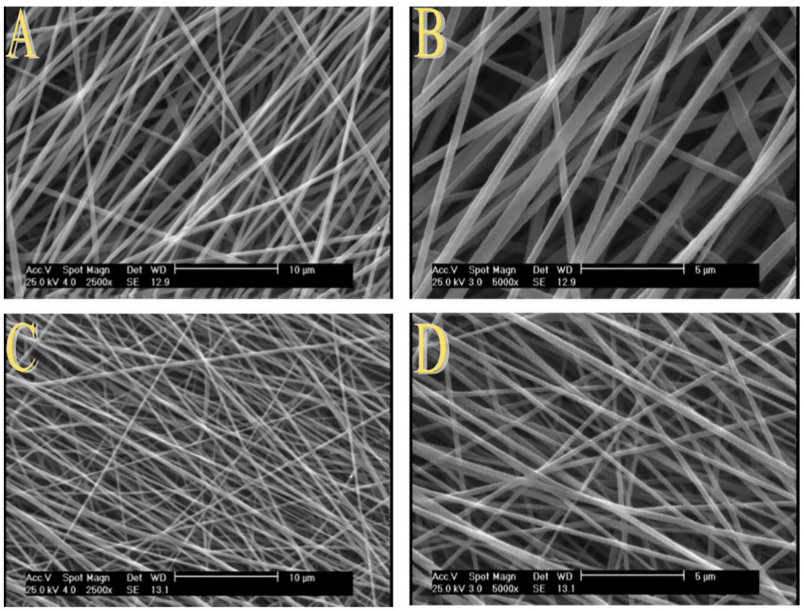

Fig. 1 **SEM images of aligned Coll/PLA/RA nanofibers, scale bar = 10 µm & 5 µm.**

presented as mean SD of the means (n = 3). The statistical threshold for significance was set at $P < 0.05$.

Discussion

Peripheral nerve injury remains a challenging clinical problem. Even though autograft can offer suitable treatment, their limitations, including a shortage of donors, graft size mismatches, and the risk of disease transmission, pose significant disadvantages.[39,40] Neural tissue engineering, with the ability to design scaffolds to guide regenerating axons, holds promise for supporting natural nerve growth along its axis. In recent years, various techniques have been employed to create scaffolds by leveraging different material properties, including electrical, mechanical, and magnetic attributes, to combine synthetic and natural polymers for tissue regeneration. Among these techniques, electrospinning stands out as a widely used method to fabricate nanofibrous scaffolds that cater to specific tissues needs, including fiber alignment and the incorporation of bioactive molecules.[17,25,26]

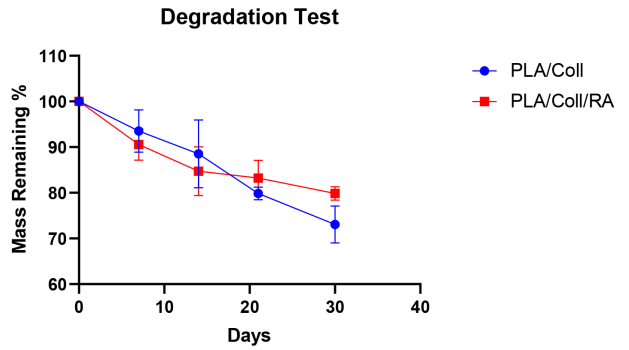

Fig. 2 **Degradation analysis of Coll/PLA & Coll/PLA/RA scaffolds.**

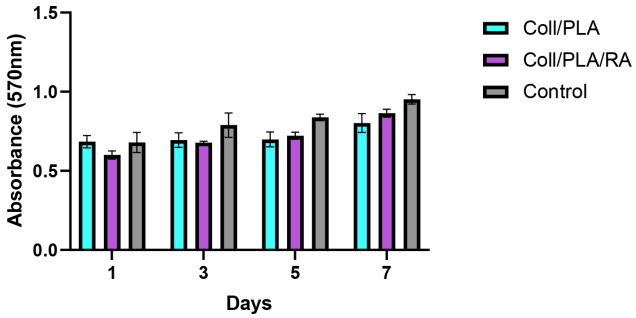

Fig. 3 **Alamar blue assay of PC12 cells on Coll/PLA & Coll/PLA/RA scaffolds.**

Polylactic acid (PLA) has garnered attention as an organic compound due to its low allergenic potential, minimal toxicity, and predictable degradation rate. Collagen, a natural polymer containing extracellular matrix (ECM) proteins, represents a promising substitute for ECM because of its hydrophilic nature and strong cell adhesion properties. However, collagen lacks mechanical strength and degradation rate conductive to nerve growth. By combining natural biopolymers with biodegradable synthetic polymers, tit becomes possible to adjust the scaffold's degradation rate and its physical and mechanical properties.

In this study, we designed parallel electrospun PLA/COL fibers with a diameter around 300 nm. The prior reports have underscored the importance of topography in cell morphology and guidance. Aligned nanofibrous scaffolds, as seen in our study, have demonstrated significant improvement in the function and repair of nerve cells under laboratory conditions compared to random ones.[28] The cultivation of PC12 cells on the scaffold highlighted the ability of electrospun fibers to guide cell orientation and facilitate axonal extension.[23]

The nanofibrous scaffolds of Coll/PLA and Coll/PLA/RA, with a fine size distribution were successfully produced via electrospinning. Our morphology study revealed that rotational speed and polymer concentration could influence fibers orientation, with higher rotational rates leading to more uniform fibers. SEM analyses under controlled conditions demonstrated that 10% (w/v) Coll/PLA and Coll/PLA/RA solutions

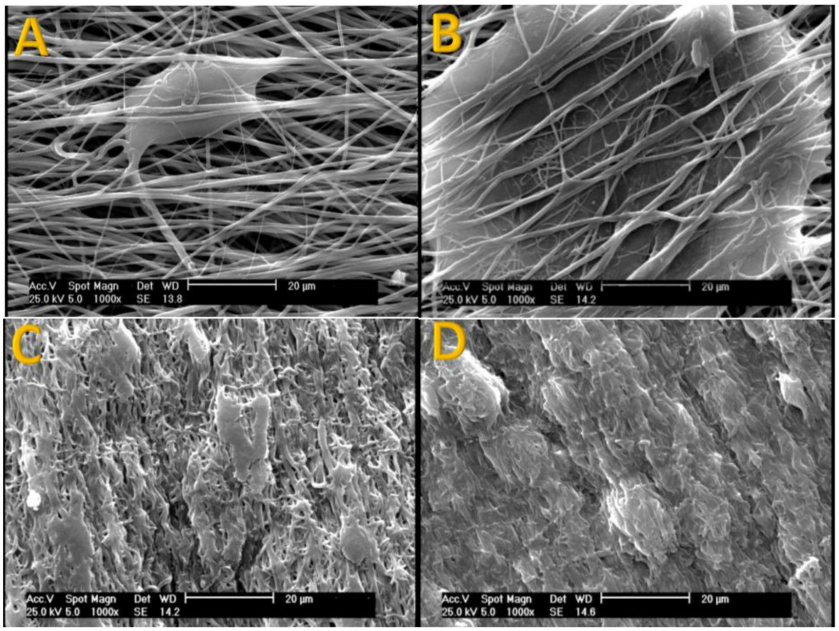

Fig. 4 **SEM images of cell adhesion. (A, B) Coll/PLA/RA with PC12 on Day 1, (C, D) Coll/PLA/RA with PC12 on day 7.**

could form aligned nanofibers. It is worth noting that while PLA is naturally hydrophobic, the addition of collagen to PLA nanofiber scaffolds increased their hydrophilicity.[41] The results revealed that the addition of Collagen to PLA nanofiber scaffolds increased the hydrophilicity of the scaffold. Our findings suggest that the enhanced tear resistance and improved tensile strength observed in PLA-based composite nanofibers make them a favorable choice for nerve regeneration applications. Moreover, degradation analyses confirmed that Coll/PLA/RA scaffolds degrade more slowly in an aqueous solution compared to Coll/PLA alone, rendering them a superior choice for regeneration applications.[39]

All-trans retinoic acid (RA) plays a crucial role in cellular differentiation and acts as a significant morphogen during somatic development through its interaction with nuclear receptors and transcription complexes. Previous studies have demonstrated RA' potential to reverse both functional and structural alterations in neuropathy, ultimately leading to nerve repair and regeneration. This effect is attributed to elevated levels of neural growth factor within nerve terminals.[40]

Our findings revealed that Coll-based PLA composite nanofibers can promote cell proliferation and attachment along the direction of aligned nanofibers.[42] Cell study results demonstrated no discernible difference in sensitivity between the nanofibers and the control, indicating that both Coll/PLA and Coll/PLA/RA are non-toxic to the nerve cells. Furthermore, the cell adhesion study showcased how PC12 cells adhered to and spread along the alignment of Coll/PLA/RA nanofibers, as shown in Figure 4. These results indicated that these nanofibers significantly enhance PC12 cell adhesion and proliferation. Overall, both scaffolds exhibit biocompatibility towards PC12 cells and represent viable options for peripheral nerve repair.

Conclusion

In this study, we have explored nanofibrous scaffolds as a promising solution for neural tissue engineering. These scaffolds, crafted from a blend of type I collagen (Coll), polylactic acid (PLA), and all-trans retinoic acid (RA), emulate the natural extracellular matrix's topographical features. Our findings reveal these scaffolds as excellent candidates for neural tissue engineering, featuring a porous structure, robust mechanical properties, and biodegradability. Importantly, the inclusion of RA significantly enhances PC12 cell adhesion and proliferation, underscoring their potential for nerve repair.

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Conflict of Interest

None.

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