Electrospun Nanofibrous Scaffolds of Polycaprolactone/Gelatin Reinforced with Layered Double Hydroxide Nanoclay for Nerve Tissue Engineering Applications

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ABSTRACT: Nerve tissue engineering (NTE) is an effective approach for repairing damaged nerve tissue. In this regard, nanoparticle-incorporated electrospun scaffolds have aroused a great deal of interest in NTE applications. In this study, layered double hydroxide (LDH)-incorporated polycaprolactone (PCL)/gelatin (Gel) nanofibrous scaffolds were fabricated by an electrospinning technique. The physicochemical, mechanical, and biological properties of the scaffolds were examined. Also, the phase identification, morphology, and elemental composition were studied using X-ray diffraction, scanning electron microscopy, and energy-dispersive X-ray spectroscopy, respectively. The results revealed that the inclusion of LDH nanoparticles into the PCL/Gel scaffold has improved its mechanical strength and elongation at the break, while the degradation rate was enhanced in comparison with the pure PCL/Gel mat. The LDH-enriched electrospun PCL/Gel scaffolds exhibited a considerable impact on cell attachment and proliferation. The gene expression results showed that the neuron-specific (γ) enolase (NSE) gene expression was significantly decreased in the scaffolds containing 1 and 10 wt % LDH compared to the scaffold without LDH, whereas in the scaffold with 0.1 wt % LDH, a slight increase in expression was observed. It can be deduced that electrospun PCL/Gel scaffolds containing LDH with optimum concentration can be a promising candidate for nerve tissue engineering applications.

INTRODUCTION

Nerve tissue engineering is a rapidly growing field of research offering a unique and encouraging approach to nerve healing and regeneration. The main goal in neural tissue engineering is to produce a three-dimensional polymeric scaffold with nerve cells, creating functional tissue ideal for implantation. Extracellular matrix (ECM) as a natural scaffold has a pivotal role in cellular regulation, whereas tissue engineering scaffold plays a crucial role in the regeneration of tissue. Since nanofibrous scaffolds structurally mimic the native ECM, they can provide a favorable microenvironment for cells to migrate, attach, and grow.† Among many manufacturing technologies, electrospinning is a widely used method with a relatively uncomplicated and versatile approach for producing nanofibrous scaffolds. The large surface area provided by nanofibers increases the cell contact area, thereby accelerating interaction between the cells and matrix.6 Polylactic acid (PLA), a synthetic aliphatic polyester, is a promising biodegradable biomaterial utilized as a neural guide to improve nerve tissue regeneration. By tailoring the physical and chemical properties of PLA, its degradation, biocompatibility, mechanical strength, and surface bioactivity can be modified.7 On the other hand, blending synthetic and natural polymers enhances bioactivity and cell attachment; also, the degradation rate of the blended matrix can be modified based on its application.5 The hydrolyzed form of collagen, gelatin, is a natural biopolymer that is widely used in medical and pharmaceutical fields due to its protein-based structure, biodegradability, biocompatibility, and commercial availability. Hence, gelatin can be combined with PCL to improve cell adhesion and biodegradation rate. Plenty of research has demonstrated the application of electrospun scaffolds based on PCL/gelatin in the fields of wound dressing and soft tissue engineering such as nerve, cartilage, and skin.6 Furthermore, remarkable attention is focused on the combination of inorganic nanoparticles with polymeric scaffolds for biomedical applications to tailor the physical, biological, and mechanical properties of polymers to meet the high demands of certain applications in the fields of tissue engineering and drug delivery systems. Nanoparticles offer some particular advantages over pristine polymers such as greater surface area, higher aspect ratio, and high surface charges.7,8 Different nanoparticles such as calcium...
phosphates, bioglass, hydroxyapatite, graphene oxide, gold, magnesium oxide, silica, and metal oxides in combination with PCL/gelatin polymeric matrices have exhibited superior properties and characteristics (thermal, physical, chemical, biological, mechanical, and optical) compared to pristine polymers. In this regard, layered double hydroxides (LDHs) have attracted considerable attention due to their wide area of applications. LDH, also known as anionic nanoclays, has been investigated in a broad range of fields such as biomedicine, biosensors, bioimaging, and mostly tissue engineering and drug delivery systems. There have been numerous studies on bone tissue engineering; however, there are limited nerve tissue engineering studies. Recently, LDH nanoclay has been incorporated into polymers, such as polycaprolactone, polypropylene, polyvinyl alcohol, polylactic acid, etc. Nevertheless, there are still no reports about the addition of LDHs to polycaprolactone/gelatin nanofibrous scaffold for nerve tissue engineering applications.

In this study, we aimed to fabricate novel LDH-incorporated electrospun PCL/gelatin nanofibrous scaffolds for application in nerve tissue engineering. In this regard, the effects of LDHs on the physical, biological, and mechanical properties of nanofibrous scaffolds were evaluated. Attachment, viability and proliferation, and differentiation of SH-SYSY were explored by electron microscopy (SEM), MTT assay, and gene expression through real-time PCR.

**Materials and Methods**

**Materials.** Poly (ε-caprolactone); (Mn = 70,000–90,000 gmol⁻¹) and gelatin were purchased from Sigma-Aldrich. Other chemicals and reagents were supplied from Merck (Germany) without further purification.

**Preparation and Characterization of LDHs.** In this study, a co-precipitation route was employed for the preparation of LDH. As previously reported, an aqueous solution containing MgCl₂·6H₂O and AlCl₃·6H₂O with a constant molar ratio of 3:1 was utilized. A fresh solution of Na (OH) (0.2 M) was directly added dropwise under continuous stirring and an N₂ atmosphere. The pH of the mixture was controlled and kept at 10, and the final mixture was stirred overnight. Eventually, the white precipitate was collected by centrifugation at 3900 rpm for 15 min and rinsed three times with deionized water. The obtained precipitate was dried in an oven at 50 °C. The phases of the resultant powder were identified by X-ray diffraction (XRD) analysis. An X-ray diffractometer system (D4 Bruker) with monochromatic radiation (Cu/Kα) was used to appraise the test. Forty kilovolt voltage and 30 mA current were used for scans. Dynamic light scattering (DLS) was used to characterize the size of as-synthesized LDHs. The test was performed by a Nano ZS (red badge) ZEN 3600 (MALVERN) test machine. The morphology of the synthesized LDH particles was characterized by transmission electron microscopy (TEM) using a Philips EM208 (Netherlands) at an acceleration voltage of 200 kV. For sample preparation, the freshly prepared LDH nanoparticles were dispersed in alcohol with ultrasonication for 30 min, and then a droplet was dropped on a copper grid coated with amorphous carbon film.

**Fabrication of Nanofibrous Scaffolds.** The polymer solution (10 wt %) was provided by dissolving PCL and gelatin with a weight ratio of 50:50 in 2,2,2-trifluoroethanol (TFE) under continuous stirring overnight at room temperature. LDHs with different concentrations (0, 0.1, 1, and 10 wt %) were dispersed completely in the polymer solution and kept under ultrasound conditions for 1 h. In the following step, 5 mL of each formulation was ejected over a stainless steel 21G blunt-end needle at a mass flow rate of 1 mL/h. A driving voltage of 12 kV was applied to the tip of the needle. The distance between the needle tip and the collector was arranged at 15 cm. For collecting nanofibers, a rotating collector was used. Eventually, electrospun samples were dried under a vacuum for 24 h to extract the remaining solvent.

**Characterization of Scaffolds.** The morphology of the fibers was assessed by SEM (Siemens, D5000-Germany). SEM micrographs were recorded at 20 kV. A thin layer of gold coating was applied on the surface of the samples by a vacuum sputter coater (EMITECH K450X, UK). The distribution of the LDH compound, aluminum, and magnesium elements within the fibers was obtained by an energy dispersive X-ray analysis (ZEISS, Germany). EDX was conducted at accelerating voltages of 1–20 kV and a magnification of 280×. ImageJ software was utilized to estimate the average fiber diameters. The tensile mechanical properties of the samples were determined by a SANTAM universal tensile machine (STM 20, Iran) at 25 °C. In the following step, samples (1 × 5 cm) were cut and located between the jaws of the grips. The pullout rate was fixed at 5 mm/min, and a load cell of 10 N was applied for all the measurements. To evaluate the degradation behavior of the scaffolds, an accelerated degradation test was used. Typically, samples were prepared and incubated in sodium hydroxide solution (0.5 M) at 37 °C. The solution of sodium hydroxide was gently withdrawn at a predefined time (48 h), and the scaffold was washed three times with deionized water. Finally, the scaffolds were frozen using liquid nitrogen and kept for further characterization.

**Cell Culture Study.** Human neuroblastoma SH-SYSY cells were supplied from the Bonyakhte Cell Bank of Iran. Cells were incubated at 37 °C in a humidified environment with 5% CO₂ and cultured with DMEM/F-12 medium supplemented with 10% FBS and 1% pen/strep [100 U/Gibco]. The 80–100% confluent cells were harvested with 0.5% trypsin and counted with a hemocytometer and used for further tests. Before cell seeding, the scaffolds were sterilized using UV irradiation for 1 h, washed several times with PBS, and incubated with DMEM/F-12 for 24 h. Sterilized scaffolds were located in 96-well plates. 10,000 cells per well in 200 μL of complete cell culture medium were seeded on the scaffolds and incubated for 24 h. At each predefined time interval, the scaffolds were cautiously washed three times with PBS and then fixed for 3 days in a glutaraldehyde solution (2.5%). The scaffolds were dehydrated with a series of ethanol dilutions. The morphological characteristics of the fixed cells were observed through SEM. The most widely known cell viability assay, MTT (3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich)), was accepted to measure the in vitro cytotoxicity of the scaffolds. In this regard, SH-SYSY cells were cultured with a seeding density of 20 × 10³ in the vicinity of the scaffolds. In the following step, at each time point, MTT solution (5 mg/mL) was injected into the wells and incubated for an extra 4 h at 37 °C and 5% CO₂. The crystallized formazan substance was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich), and the optical densities were measured at 570 nm using STAT FAX 2100, USA Microplate Reader.

**Real-Time PCR.** The mature neuronal marker neuron-specific enolase (NSE) gene was selected to assess the differentiation of SH-SYSY cells seeded on the nanofibrous scaffolds. To induce and initiate differentiation, the seeded cells
on samples were cultured in a neuroinductive medium supplemented with retinoic acid (10 μM). The total duration of 5−7 days was considered for neurocyte differentiation. In the next step, RNA was extracted from SH-SYSY cells that were seeded on all scaffolds on the 7th day with an RNX-PLUS Kit (Sinacolon, Iran) according to the manufacturer’s instructions. Subsequently, cDNA was synthesized using a cDNA Synthesis Kit (Yekta Tajhiz Azuma, Iran) for use in quantitative RT-PCR. qRT-PCR was performed using SYBER Green in Corbett Research 6000:0809010, and the results were analyzed by Rotor-Gene 6000 Series Software 1.7. The sequence of primers is listed in Table 1. The qRT-PCR cycle including denaturation, annealing, and elongation phases occurred at 95, 60, and 72 °C for 10, 15, and 15 s, respectively, and was repeated 35 times. The GAPDH gene was preferred as a housekeeping gene, and data processing to calculate relative expression was performed using the Pfaffl method.

### Results

#### LDH Characterization

To confirm the successful synthesis of LDHs, phase detection was conducted using the XRD test. The X-ray diffraction spectrum of LDH is presented in Figure 1a. The significant peaks of LDH ((003), (006), and (110)) are consistent with the JCPDS 00-035-0965 standard pattern. The XRD pattern inferred that sample is pure and stable. Figure 1b shows the DLS measurement of LDH nanoparticles with a Z-average of 370 (d nm). The TEM micrograph of LDHs is shown in Figure 1 (top right); the LDHs are very properly separated and well-shaped in a hexagonal form.

#### Electrospun Fiber Characterizations

Figure 2 displays the SEM micrographs of the PCL/Gel/LDH scaffolds. The mean fiber diameter was estimated by measuring at least 100 fibers using ImageJ Software. The results indicate that the addition of LDH to the PCL/Gel matrix decreased the mean fiber diameter. The maximum and minimum average fiber diameters are 285 ± 12 and 160 ± 8 nm, which belong to the pure PCL/Gel and PCL/Gel/LDH (0.1%) scaffolds, respectively. Also, the mean fiber diameters of 230 ± 9 nm and 250 ± 7 nm correspond to PCL/Gel/LDH (1%) and PCL/Gel/LDH (10%), respectively. The micrographs illustrated uniform nanofibers with smooth surfaces and no beads. Only in the PCL/Gel/LDH (10%) sample was the formation of the beaded structure observed, indicating the agglomeration of LDH nanoparticles within the PCL/Gel matrix. The EDX spectrum of the PCL/Gel/LDH (10%) scaffold is presented in Figure 3.

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**Table 1. The Sequence of Primers Used in this Study**

| gene name | primer sequence | melting temperature (°C) |
|-----------|-----------------|-------------------------|
| NSE forward | AGGTGCAGAGGTCTACCATAC | 58 |
| NSE reverse | AGCTCCAAGGGCTTACACGTAC | 58 |
| GAPDH reverse | ACCAAATCGCTTGGATCCCA | 58.3 |
| GAPDH forward | TCTGCTCCTCCTGTTGGCA | 57.8 |

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**Figure 1.** (a) XRD pattern of LDH. (b) DLS plot of the synthesized LDH and TEM micrograph of the LDH nanoparticle (top right).
Two peaks are related to magnesium (yellow dots) and aluminum (green dots) elements in the LDH, revealing the successful distribution of LDH in the PCL/Gel matrix. Also, the two high peaks correspond to carbon and oxygen elements within PCL. A significant peak that appeared in the spectrum is attributed to the gold coating.

**Mechanical Characterization.** The stress–strain curves for PCL/Gel/LDH nanofibrous scaffolds are shown in Figure 4. The highest tensile strength was achieved in the sample containing 1 wt % LDH, whereas the highest strain was gained in the sample containing 0.1 wt % LDH. The addition of LDH to the PCL/Gel matrix resulted in an increase in strain at break and tensile strength. Also, the sample containing 10 wt % LDH showed a significant decline in tensile strength typically due to the stress concentration and agglomeration of nanoparticles.

**In Vitro Degradation.** PCL has been found to have a slow degradation rate. In this regard, the impact of LDH on the degrading profile of PCL/Gel scaffolds was studied under accelerated conditions using a 0.5 M NaOH solution. It was found that the addition of LDH accelerates the degradation of the PCL/Gel, which could be ascribed to PCL/GEL/LDHs' reduced hydrophobicity as compared to the pristine PCL/Gel scaffold. Since PCL mostly degrades by hydrolytic deterioration, adding LDH may enhance the degradation rate by increasing water absorption. The percent of weight loss for each scaffold (PCL/GEL/LDH (0%), PCL/GEL/LDH (0.1%), PCL/GEL/LDH (1%) and PCL/GEL/LDH (10%)) is shown in Figure 5a. Also, Figure 5b shows the morphology of samples after degradation, in which the breakage of fibers can be observed. Although the highest degradation rate was observed in the sample containing 10 wt % LDH, the presence of LDH
supported the scaffold’s structural integrity during the degradation process.

**Cell Attachment and Viability Study.** The morphology of SH-SYSY cells seeded on PCL/Gel and LDH-incorporated PCL/Gel nanofibers after 1 and 3 days of cell culture was investigated through SEM analysis (Figure 6). SH-SYSY cells with spindle-shaped morphology are normally attached to the surface of scaffolds after 1 day. The results demonstrate that after 3 days of cell seeding, neural cells extension could be detected on the surface of nanofibers. Electrospun nanofibers provide a microenvironment similar to the natural extracellular matrix and conduct cells toward migration and proliferation. It can be observed that cell migration and proliferation were enhanced in PCL/Gel/LDH nanofibers, which could be attributed to the outstanding biological features of LDH. The presence of LDH in nanofibers leads to more hydrophilicity, resulting in higher protein adsorption of the scaffold. MTT assay was employed to study the cell proliferation of SH-SYSY cells on the PCL/Gel and PCL/Gel/LDH nanofibrous scaffolds. As illustrated in Figure 7a, the proliferation of cells on the LDH-incorporated scaffolds is significantly higher than on PCL/Gel scaffolds, indicating that the LDH-containing scaffolds might have accelerated the proliferation of cells.

**Gene Expression.** To investigate the effects of LDH on the differentiation of SH-SYSY cells, real-time RT-PCR was employed. The specific enolase (NSE) gene was chosen to study the differentiation of SH-SYSY cells seeded on nanofibrous scaffolds with and without LDH. The neuroinductive
medium containing retinoic acid was used to induce the differentiation process. The scaffolds were cultured in the induction medium, and after 7 days, the expression of the NSE neural marker was examined. The results indicated that in the

Figure 6. SEM micrographs of SH-SYSY neuroblastoma cells cultured on electrospun scaffolds for 1 and 3 days. (a, b) PCL/Gel, (c, d) PCL/Gel 0.1% LDH, (e, f) PCL/Gel 1% LDH, and (g, h) PCL/Gel 10% LDH scaffolds.
scaffolds containing 1 and 10 wt% LDH, the gene expression was significantly reduced compared to the scaffold without LDH, whereas in the scaffold with 0.1 wt% LDH, a slight increase in expression was observed (Figure 7b).

**DISCUSSION**

The successful performance of neural tissue engineering primarily depends on the control of cell behavior and patterning and tissue regeneration by creating a synthetic scaffold that precisely resembles the native extracellular matrix and acts as a framework for 3D cell culture. An ideal nerve tissue engineering scaffold should possess biocompatibility, tunable biodegradability, flexibility, adequate porosity for angiogenesis, and tailored mechanical properties to support functional tissue regeneration. The synthetic fibrous scaffold accurately mimics the native microenvironment of cells in terms of migration and growth. Also, it can exert influence on cellular function by coordinating the signaling factors that interact with cells. The electrospinning technique is an extensively used method for the mass production of fibers in different dimensions and alignments. It also allows the inclusion of nanoparticles into fibers to manipulate cellular function for enhancing nerve regeneration. In the current study, we have developed electrospun nanofibers based on PCL/Gel/LDH made by electrospinning. PCL/Gel blend is a favorable biomaterial for nerve scaffolds and protects nerve regeneration over several months. One of the advantages of the electrospinning technique over other fabrication routes is producing highly interconnected porous structures that facilitate accurate vascularization and cell colonization. PCL as a widely used synthetic polymer lacks functional groups, which results in weak cell–polymer interaction and low hydrophilicity. Oppositely, gelatin as a natural and bioactive polymer shows weak mechanical properties. The idea behind blending these polymers is to provide a synergistic effect and develop superior features. Meanwhile, the incorporation of bioactive nanoparticles such as LDHs into a soft polymeric matrix leads to reinforcing the mechanical properties and elevating hydrophilicity while the biological property of polymeric matrix is altered. Numerous researches have been conducted to evaluate the effects of ceramic nanoparticle addition on the physicochemical and biological properties of electrospun nanobioglass blended polymers for nerve tissue engineering. It is reported that collagen/PCL nanofibers containing nanobioglass with tailored degradation rates could provide favorable support for nerve regeneration. In another study, graphene-doped electrospun PCL/Gel scaffolds have been developed. It was shown that the addition of graphene improves the hydrophilicity and degradation rate of the scaffold. Also, the antibacterial effect along with biocompatibility has made the graphene-incorporated scaffold a promising candidate to be used in nerve tissue engineering. In recent decades, LDH nanoclay has been used in drug delivery systems in vitro and in vivo. In addition to the application in the controlled release of therapeutic agents, few studies have demonstrated the cellular interaction of LDH nanoclay and activating related signaling pathways. LDH has also been incorporated into polymers as a reinforcing agent to enhance physical and mechanical properties. Also, LDH influences the cellular function and differentiation of stem cells by acting in many signaling pathways. Studies have demonstrated the effects of LDH addition on the physical, mechanical, and biological properties of polymer matrices. The results showed better cell interaction with the surface of scaffolds and stimulating stem cell differentiation cascade. Previous studies revealed that the PCL/gelatin blend is a promising candidate in nerve tissue engineering and acts as a suitable scaffold for supporting neurocyte outgrowth. To the best of our knowledge, there are no reports on PCL/Gel/LDH nanofibrous scaffolds or nerve tissue engineering.

In the present study, we hypothesized that the incorporation of LDH into PCL/Gel nanofibrous scaffolds provides an appropriate construct for nerve tissue regeneration. In this regard, our findings demonstrated that LDH was successfully synthesized by the co-precipitation method as confirmed by XRD analysis. The morphology of PCL/Gel/LDH nanofibrous scaffolds was observed using SEM. As the micrographs revealed, uniform bead-free nanofibers with smooth surfaces were formed. The results showed that the mean fiber diameter was decreased by the addition of LDHs to the PCL/Gel matrix compared to the pristine PCL/Gel fibers. The minimum fiber diameter was achieved in the PCL/Gel/LDH (0.1%) sample. By increasing the concentration of LDH to 10 wt%, the mean fiber diameter was increased to 250 nm in which bead formation was observed. One possible reason for the decrease in fiber diameter is related to the electrical conductivity of the electrospinning solution. As the electrical conductivity of the polymer solution increases, the diameter of the nanofibers might be remarkably decreased. Typically, the minimum nanofiber diameter can be achieved by the solution with maximum electrical conductivity. The solutions with exceeding electrical conductivity result in the formation of beads and broad diameter distribution. The addition of ion salts to the electrospinning solution has been found to enhance the uniformity of the nanofibers and produce....

Figure 7. (a) MTT results after 1, 3, and 5 days of culture. The control is SH-SYSY cultured on tissue cultured polystyrene (*p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001). (b) Real-time RT-PCR assay of NSE gene expression in SH-SYSY. The scaffolds were cultured in the induction medium for 7 days. Significant levels are *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001.

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bead-free nanofibers with the lowest diameters by increasing the charge-carrying capacity of the jet. Accordingly, by the addition of LDHs, the electrical conductivity of PCL/Gel solution increases, while the flow rate was maintained high enough. Hence, a slight amount of LDH would lead to thinner fiber, and a further increase in LDH concentration causes an increase in the mean fiber diameter with the broader distribution. Also, at a higher concentration of LDH, the formation of beads was observed because of the agglomeration of nanoparticles. EDX analysis revealed the uniform dispersion of LDH in PCL/Gel fibers. The uniformity of distributed particles in the matrix could be attributed to ultrasonic treatment before electrospinning. One of the most important parameters that should be considered in designing nerve tissue engineering scaffolds is mechanical stability, specifically during implantation. The major disadvantage of gelatin is its weak mechanical property. The heterogeneous blends of natural and synthetic polymers produce a nanofibrous scaffold with desirable properties. The synthetic polymeric parts of the biocomposite act as mechanical support, while the natural component plays a crucial role in cell attachment and proliferation. The mechanical mismatch between the scaffold and the nerve tissue may cause inflammation and irritation at the implant site. If the scaffold is too rigid, it might exert pressure on the newly formed tissue. Conversely, the too soft and delicate scaffolds are not suitable for implantation and the bearing of stresses during the surgical procedure. It is worth mentioning that the elasticity and stiffness of the scaffold are proposed to be important factors in cell responses and differentiation. The mechanical stimulation of stem cells exerted by a scaffold may result in differentiation to desired cell types. Our results demonstrated that fibers containing LDH nanoparticles possess higher tensile strength and elongation at break compared to the pristine PCL/Gel scaffold. Several reports have demonstrated the influence of nanoparticle addition on the mechanical performance of electrospun polymer blends. Mohamadi et al. showed that the inclusion of nanobioglass into PCL/collagen fibers significantly enhanced the tensile strength and Young’s modulus. Also, the elongation of PCL/collagen reduced remarkably mainly due to the addition of collagen compared with the pure PCL. However, our results showed that the incorporation of LDH nanoclay to the PCL/Gel matrix increased the elongation at break compared to the pure PCL/Gel. Also, the ultimate tensile strength was increased in the presence of LDH. A slight amount of LDH generates higher stretchability of nanofibrous scaffold that can be attributed to the alignment of fibers during the mechanical stretch. It is worth mentioning that the electrostatic forces and the possible interaction between PCL/Gel and LDH materials can be generated by the electrospinning process. Nonetheless, there is an optimum concentration for LDH as a reinforcing agent that causes optimum impact in terms of elongation at break and tensile strength values. These findings confirmed that PCL/Gel/LDH can be a suitable scaffold for nerve tissue engineering applications. The degradation of PCL is mainly caused by the hydrolysis of ester linkages. The hydrophobic effect of this polymer leads to poor water absorption and, consequently, a slow degradation rate. Pure PCL takes approximately 2–3 years in the human body to be degraded. The degradation mechanism is mainly based on surface degradation. It was shown that blending PCL with gelatin increases the degradation rate of the scaffold and preserves the integrity of nanofibers during the degradation process. Our findings showed that loading LDH into nanofibers provided relatively faster degradation of PCL/gel nanofibers. Owing to the positively charged layers of LDH and gelatin matrix, the hydrophilicity of nanofibers has improved. As a result, water adsorption into the fiber structure has been promoted and more hydrolysis in ester bonds has occurred. From the SEM micrographs, it could be deduced that the presence of LDH has supported the scaffold’s structural integrity. This could be assigned to a strong interaction between LDH and the PCL/Gel nanofibrous matrix. The effect of LDH on cellular attachments of PCL/Gel nanofibrous scaffolds was evaluated. The results revealed that the SH-SYSY cells attached and flattened on the surface of nanofibers and after 5 days, they migrated and proliferated on the surface of the matrix. In previous studies, cells cultured on scaffolds containing LDH nanoparticles showed more confluency and attachments. Also, PCL/Gel/LDH scaffolds provide better cellular interaction as induced by the LDH in PCL/Gel electrospun scaffolds. On the other hand, results of the MTT assay indicated that nanofibrous scaffolds did not show any cytotoxic effects on the viability of the cells after 5 days. Not only did scaffolds containing LDH show no cytotoxicity activity against SH-SYSY cells, significantly higher proliferation rate was also observed in the LDH-incorporated scaffolds. The synergistic effect of the inclusion of LDH into the PCL/Gel matrix leads to an increase in the proliferation and cell attachment of the scaffolds. Consistent with our findings, the addition of LDH facilitates cell interactions with the surface of fibers through chemical groups and positively charged layers. To evaluate the differentiation of SH-SYSY cells, seeded scaffolds were cultured in neuroinductive medium containing retinoic acid for 7 days. The specific enolase (NSE) gene expression was characterized by real-time PCR. It was found that gene expression was significantly reduced in the scaffolds containing 1 and 10 wt % LDH compared to the scaffold without LDH, whereas in the scaffold with 0.1 wt % LDH, a slight increase in expression was observed. The results indicate that there is an optimum concentration of LDH to increase the differentiation of neuroblastoma SH-SYSY cells. A higher concentration of LDH leads to increased viability and proliferation of cells instead of increasing differentiation. These effects might be observed due to the cancerous nature of SH-SYSY cells. Further studies need to be done to evaluate the effects of LDHs on the differentiation of stem cells toward neuronal lineage. Few researchers have demonstrated the effects of LDH nanoparticles on the differentiation of stem cells and the possible signaling pathways. Retinoic acid is known for its ability to induce neural differentiation. There are several reports on the inhibition of cell growth and enhancing the production of noradrenaline by retinoic acid in SH-SYSY cells. Molecular mechanisms involved in retinoic acid-induced neuroblastoma cellular differentiation have been extensively studied and are beyond the scope of this study. However, it was shown that embryonic stem cells (ES) can be maintained in an undifferentiated state in the presence of LDH nanoparticles by activating the PI3K/Akt signaling pathway. Also, LDH nanoparticles inhibit the spontaneous differentiation of ES. In this study, the same molecular mechanism can be proposed for the possible effects of LDHs on the differentiation of SH-SYSY cells. As the concentration of LDH increased, the NSE gene expression has been significantly decreased. This could be attributed to activating the PI3K/Akt signaling pathway. Moreover, positively charged LDH nanoparticles can interact with the negatively charged cell membrane and initiate the triggering of the PI3K pathway that is associated with the membrane receptor. Overall,
it can be concluded that there are possible interactions between LDH nanoparticles and retinoic acid that trigger multiple signaling pathways and determine cell fate. Further studies should be performed to confirm these findings.

**CONCLUSIONS**

In this study, we produced electrospun PCL/Gel/LDH nanofibrous scaffolds for application in nerve tissue engineering. Compared to PCL/Gel nanofibers, LDH-incorporated PCL/Gel scaffolds showed a higher degradation rate mainly due to the presence of positively charged layers in LDH structure and more hydrophilicity as a result of LDH inclusion. The mechanical results revealed that the inclusion of LDH into PCL/Gel nanofibers increases tensile strength and strain at break. Cell culture studies demonstrated that PCL/Gel/LDH mats support the cells with an adequate microenvironment promoting cell migration, attachment, and proliferation. Furthermore, loading LDH into nanofibers provided enhanced cell viability and proliferation compared to the pure PCL/Gel nanofibers. The results of RT-PCR demonstrated that the NSE expression was significantly reduced in the scaffolds containing 1 and 10 wt % LDH compared to the scaffold without LDH, whereas in the scaffold with 0.1 wt % LDH, a slight increase in expression was observed. The activation of signaling pathways is strongly dependent on the concentration of LDH nanoparticles within the fibers, and there is an optimum amount for LDH loading that could enhance differentiation or proliferation. Together, PCL/Gel/LDH nanofibrous scaffolds with improved physical and mechanical properties can promote the nerve cells’ growth and cell differentiation process.

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was financially supported by the National Institute of Genetic Engineering and Biotechnology grant no. 736.

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