Material Characterization and Bioanalysis of Hybrid Scaffolds of Carbon Nanomaterial and Polymer Nanofibers

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ABSTRACT: The interconnected porous structures that mimic the extracellular matrix support cell growth in tissue engineering. Nanofibers generated by electrospinning can act as a vehicle for therapeutic cell delivery to a neural lesion. The incorporation of carbon nanomaterials with excellent electrical conductivity in nanofibers is an attractive aspect for design of a nanodevice for neural tissue regeneration. In this study, nanoscaffolds were created by electrospinning poly(ε-caprolactone) (PCL) and three different types of carbon nanomaterials, which are carbon nanotubes, graphene, and fullerene. The component of carbon nanomaterials in nanofibers was confirmed by Fourier transform infrared spectroscopy. The fiber diameter was determined by scanning electron microscopy, and it was found that the diameter varied depending on the type of nanomaterial in the fibers. The incorporation of carbon nanotubes and graphene in the PCL fibers increased the contact angle significantly, while the incorporation of fullerene reduced the contact angle significantly. Incorporation of CNT, fullerene, and graphene in the PCL fibers increased dielectric constant. Astrocytes isolated from neonatal rats were cultured on PCL-nanomaterial nanofibers. The cell viability assay showed that the PCL-nanomaterial nanofibers were not toxic to the cultured astrocytes. The immunolabeling showed the growth and morphology of astrocytes on nanofiber scaffolds. SEM was performed to determine the cell attachment and interaction with the nanoscaffolds. This study indicates that PCL nanofibers containing nanomaterials are biocompatible and could be used for cell and drug delivery into the nervous system.

1. INTRODUCTION

Traumatic brain injury and spinal cord injury are two of the leading causes of lifelong physical and mental disability.1–3 Because of the central nervous system’s (CNS) complexity, very little spontaneous regeneration, repair, or healing occurs. Considerable work has been undertaken to activate the endogenous repair system in order to restore a wounded nervous system. However, limited success has been achieved. The exogenous repair technology involving cell and bioscaffold transplantation provides an alternate promising therapeutic approach.4 The hierarchical fibrous structures realigning at the nanometer scale imitate the extracellular matrix. The microporous, nonwoven poly(ε-caprolactone) (PCL) nanoscaffolds generated by electrostatic fiber spinning are biocompatible and biodegradable.5 The interconnected porous structures of the PCL nanofibers can support cell adhesion and are suitable for the purpose of tissue engineering.6

Previous studies have shown that that electrospun nanofibers support neurite outgrowth and glial cell migration.7–9 Astrocytes are star-shaped glial cells that are contained in the brain environment by building up the microarchitecture and maintaining the blood—brain barrier. Astrocytes maintain brain homeostasis, control neural cell metabolism and synaptic activity, and assist in glial signaling.10–13 In case of injury or disease, the death or survival of astrocytes affects the ultimate clinical outcome and rehabilitation through neuron genesis and

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compared with PCL, PCL-CNT, and PCL-fullerene. It was reported that PCL-graphene nanomaterials in nano fibers, PCL-graphene fibers, PCL-CNT fibers, and PCL-fullerene fibers; (B) quantification of contact angles of different nanofibers: PCL-graphene compared with PCL, PCL-CNT, and PCL-fullerene fibers, $p < 0.01$; PCL-CNT, compared with PCL and PCL-fullerene fibers, $p < 0.01$; PCL, compared with PCL-fullerene fibers, $p < 0.01$.

Figure 1. Contact angle measurements of nanofibers: (A) droplet profiles and contact angle evaluation on different films of dense PCL nanofibers, PCL-graphene fibers, PCL-CNT fibers, and PCL-fullerene fibers; (B) quantification of contact angles of different nanofibers: PCL-graphene nanofibers, PCL-CNT nanofibers, and PCL-fullerene nanofibers, $p < 0.01$; PCL-CNT nanofibers, compared with PCL and PCL-fullerene nanofibers, $p < 0.01$; PCL nanofibers, compared with PCL-fullerene nanofibers, $p < 0.01$.

reorganization. Successful cell culturing on fabricated artificial polymeric scaffolds is important for the purpose of tissue engineering.

Due to their morphological and chemical versatility, carbon-based nanomaterials have been investigated for their potential application in neural tissue engineering. When neurons derived from developing embryonic rat brain were cultured on multiwalled carbon nanotubes (MWCNTs) coated with a bioactive molecule, the cells produced multiple neurites with extensive branching. Graphene is composed of two-dimensional sp²-hybridized carbon sheets, which have two exposed surfaces and thus provide more surface area than CNTs and fullerene. The electrical conductive property of carbon nanomaterials in nanofibers also provides opportunity of in vivo application of electrical stimulation for neural tissue regeneration. Electrospun carbon nanotube and poly(L-lactic acid) composite nanofibrous scaffolds can support growth of olfactory ensheathing glial cells. It was reported that electrospun graphene-silk fibroin composite scaffolds supported the survival and growth of the cultured Schwann cells. Although these studies have reported the interaction of neural cell and carbon-nanomaterial-loaded nanofibers, studies are lacking to characterize the nanofibers containing three different nanomaterials and study the astrocyte growth on those scaffolds.

In this study, nanofibers were fabricated by electrospinning the hybrid materials of PCL and nanomaterial including carbon nanotube, graphene, and fullerene. The physical and chemical properties of the PCL-nanomaterial hybrid nanofibers were characterized, and the astrocyte growth on these nanofibers was investigated. The cell viability and morphology of astrocytes on nanofibers were studied. This study indicates the feasibility of applying those nanoscaffolds in neural tissue engineering.

2. RESULTS

2.1. Contact Angle of Electrospun Fibers. The goniometric tests showed that the PCL scaffold exhibited a contact angle of $111.1 \pm 0.1^\circ$ (Figure 1). When 0.2% graphene or CNTs were incorporated into the PCL nanofibers, the contact angles of the PCL-graphene (144.9 ± 0.6°) and PCL-CNT (132.0 ± 1°) nanofibers increased significantly ($p < 0.01$), compared with that of PCL fibers. However, the incorporation of fullerene into the PCL nanofibers significantly reduced the contact angle (100.7 ± 0.1°, $p < 0.01$).

2.2. Nanofiber Diameter Alteration with Incorporation of Nanomaterials. Fiber diameters of the nanoscaffolds with 0.2% nanomaterials, shown in Figure 2A–D, vary from 80 to 200 nm. The fiber diameters of PCL, PCL-graphene, PCL-CNT, and PCL-fullerene are 123.2 ± 78.5, 116.7 ± 46.9, 165.1 ± 69.9, and 186.6 ± 50.1 nm, respectively (Figure 2E). Diameters of the PCL-CNT fiber and PCL-fullerene fiber are significantly higher than those of the PCL fiber and PCL-graphene fiber ($p < 0.05$). However, the diameters of PCL and PCL-graphene nanofibers are not significantly different.

The energy dispersive X-ray spectroscopy (EDS) results in Figure 3 show the elemental composition of the four different nanofiber scaffolds. Carbon and oxygen are the main elements found in various nanofibers. No compositional difference between the fibers and the bead-like globules was found. Gold was also detected because gold was used to coat the samples to improve the SEM image quality.

2.3. Chemical Characterization of Nanofibers. FTIR analysis provides a good indication of the chemical composition of materials, and this analysis was necessary to confirm the presence of nanomaterials in the scaffolds. Prior to assessing the scaffolds, FTIR analysis of pure base PCL was carried out. Figure 4 shows FTIR results of the nanoscaffolds. Figure 4A shows prominent peaks at 2944, 2864, 2150, 2022, 1710, 1418, 1365, 1293, 1240, 1174, 1107, 1046, 961, and 732
1. The peak at 2944 indicates the C–H stretch. The long peak at 1710 indicates the carbonyl stretch. The peak at 1471 reveals C–H scissoring. The peak at 1240 shows the C–O stretch. The peaks at 961 and 732 show the C–C stretch.

Figure 4B shows the FTIR analysis of PCL-graphene scaffolds. A previous study reported that pristine graphene shows up in the 2900 peak region. With the increase in percentage of graphene, the peak at 2930 keeps increasing. Figure 4B shows that the length of the C–H stretch increases at peaks 2944 and 2864, whereas the C–C stretch increases at peak 732.

Figure 4D suggests an increase in the C–H stretch with the increase in fullerene concentration at peaks 2944 and 2864. The C–C stretch at peak 732.42 also seems to increase. Small peaks at 525 and 572, which were not present in the original PCL, also emerge. Figure 4CD shows an extra peak at 2350, which is actually background CO2. The pristine MWCNTs show up at around peak 1560. As can be seen in Figure 4C, peaks that were initially not present in PCL show up in subsequent PCL-CNT scaffolds, thus confirming the presence of CNTs.

2.4. Dielectric Characterization. The PCL electrospun fibers showed a dielectric constant of 2.13 ± 0.17. Incorporation of CNT, fullerene, and graphene in the PCL fibers increased dielectric constant. The increase of the carbon nanomaterial amount in the nanofibers increased dielectric constant. The dielectric constants of the PCL-CNT (0.2%), PCL-fullerene (0.2%), and PCL-graphene (0.2%) are 3.11 ± 0.16, 3.89 ± 0.17, and 6.54 ± 0.14, respectively. The dielectric constant of PCL-graphene (0.2%) is significantly higher than...
those of PCL, PCL-CNT (0.2%), and PCL-fullerene (0.2%) (Figure 5). Having higher dielectric constants of electrospun nanocomposite fibers may be beneficial for cell–nanofiber communications because the dielectric constant values of these fibers are close to those of the axonal membrane and myelin sheath of neurons.

2.5. High Viability of Astrocytes on Nanoscaffolds. The viability of astrocytes on the nanoscaffolds was studied by monitoring cell metabolic activity using the alamarBlue assay (Pierce Biotechnology, Rockford, IL). Cell viability was presented as the ratio of the reduction of alamarBlue reagent in cells on nanofibers to that of cells cultured on a cell culture dish. The ratios for PCL, PCL-graphene, PCL-CNT, and PCL-fullerene nanofibers are 1.09 ± 0.1, 1.20 ± 0.1, 1.11 ± 0.1, and 0.94 ± 0.2, respectively (Figure 6). The difference between cell
viability on different types of nanofibers is not statistically significant. This study suggested that the PCL-nanomaterial nanofibers did not show significant toxicity to astrocytes.

2.6. Morphological Study of Astrocytes on Nanofibers. Both immunostaining and SEM imaging studies were performed to investigate the morphology of astrocytes grown on nanofiber scaffolds. Immunocytochemistry of GFAP labeling showed the multipolar morphologies of the astrocytes on nanofibers. These morphologies did not show a significant difference on nanofibers containing different nanomaterials (Figure 7). SEM studies were performed to confirm the morphology of astrocytes on nanofibers observed by means of a fluorescence microscope and demonstrated the interaction of cell processes with nanofibers (Figure 8).

3. DISCUSSION

Almost all biological structures including human tissues and organs are characterized by well-organized hierarchical fibrous structures at a nanometer scale. The basic architecture of neural tissue such as the brain or spinal cord very closely resembles that of an electrospun scaffold. It has been suggested that the dual-scale scaffold, wherein the microporous structure facilitated cell infiltration and multicellular organizations in the pores, and the nanofibrous structures promoted cell differentiation and cell–matrix adhesion.24 The electrospinning process is capable of fabricating a nearly ideal tissue-engineered scaffold that mimics the in vivo microtissue structure. By carefully controlling the process parameters, it is possible to control the fiber diameters within a certain range. The high surface area and extracellular-like physical environment provided by nanofibers compared to that of other non-fibrillar surfaces may have led to an increase in cellular attachment and the observed cell polarity.

Nanomedicine shows great potential for treating various causes of damage in the central nervous system. Nanofiber scaffolds have the capacity to provide structural support for regenerating, guiding, and supporting cell growth from migration or transplantation. Nanomaterials can assist in cell regeneration and healing due to their unique chemical components and nanostructure. This study aimed to generate nanomaterial scaffolds that resemble the extracellular matrix by electrospinning and to characterize the biocompatibility of these nanoscaffolds. In this study, the artificial PCL scaffolds encapsulating three types of nanomaterials—carbon nanotubes, graphene, and fullerene—were successfully fabricated. In general, the diameters of the nanofibers range from 80 to 200 nm, which is close to the actual architecture within the CNS. We found that, under the same fabrication condition, the diameters of PCL-CNT and PCL-fullerene composite nanofibers are higher than those of PCL and PCL-graphene nanofibers.

In the contact angle study, the shape of the liquid drop depends on the surface tension of the liquid, gravity, and density difference between the liquid (water), substrate (electrospun scaffold), and surface free energies. It was shown that the contact angle of PCL fiber varies from 70 to 120°. The process of electrospinning has been shown to

![Figure 7. Immunocytochemical images of astrocytes labeled with anti-GFAP antibody and Hoechst dyes: cell culture dish, PCL fibers, PCL-graphene fibers, PCL-CNT fibers, and PCL-fullerene fibers. Scale bar, 50 μm. The high magnification images of cells pointed with arrows are shown in the bottom row. Scale bar: 25 μm.](image1)

![Figure 8. SEM images of astrocytes on nanoscaffolds: (A) PCL fibers, (B) PCL-graphene fibers, (C) PCL-CNT fibers, and (D) PCL-fullerene fibers.](image2)
increase the hydrophobicity of fibers. The water-repellent properties of PCL electrospun materials are due to their rough, rippled surfaces, which trap air between the ripples. This trapped air prevents liquid, such as water, from penetrating the ripples, forcing it to remain perched on top as intact droplets.\textsuperscript{23–28} It was suggested that the hydrophobic materials act as an excellent medium for drug delivery over an extended period of time. The hydrophobicity lowers the degradation kinetics and increases the time for hydrolysis, thereby increasing the time for cell attachment before degradation.\textsuperscript{28,30}

In this study, we observed that the contact angle increased with the increase in the incorporation of graphene and CNT in nanofibers. However, fullerene significantly decreased the contact angle when it was encapsulated in the nanofibers. FTIR analysis is widely used to study bond types and to identify unknown substances. The test can also aid in understanding the mechanism of chemical reactions by measuring unstable substances. The results of this study clearly indicate the peak location of the nanomaterials and an increase in the peak intensities with the increase in nanomaterial concentration.\textsuperscript{19,31,32}

The signal transmission between axons of different neurons in the nervous system is analogous to the wires that connect different points in an electric circuit. However, its basic mechanism is quite different. In an electrical circuit, the wire maintains both ends at a constant electrical potential difference and the current is caused by the movement of electrons. The intracellular domain and the extracellular space of axon are separated by the plasma membrane, and therefore, the signal conducts due to a transient potential difference that appears across this membrane. The electrical signal transmission along the axonal membrane depends on the cell membrane across this membrane, and therefore, the signal and the current is caused by the movement of electrons. The mechanism is quite different in the nervous system.\textsuperscript{33}

The dielectric constant of axon myelin depolarization and repolarization. The dielectric constant of the axonal membrane depends on the cell membrane across this membrane, and therefore, the signal and the current is caused by the movement of electrons. The mechanism is quite different in the nervous system.\textsuperscript{33}

In this study, experiments were performed to test the toxicity of the nanofibers loaded with carbon nanoparticles. So, the cell viability assay (alamarBlue) was performed, and the study showed that the scaffolds were not toxic to the cells. It is well established that the glial fibrillary acidic protein is the principal 8–9 nm intermediate filament in mature astrocytes of the CNS. GFAP was established as a standard marker for astrocytes.\textsuperscript{35,36} This protein is important in modulating astrocyte motility and shape by providing structural stability to the astrocytic processes. It has been reported that GFAP expression by astrocytes exhibits both regional and local variability that is dynamically regulated by a large number of inter- and intracellular signaling molecules.\textsuperscript{10,37–39}

The immunostaining of astrocytes using anti-GFAP antibody showed astrocyte morphology on the scaffolds. We observed the multiple processes of the GFAP positive astrocytes grown on the nanoscaffolds. These images clearly show the growth of viable astrocytes on different PCL-nanomaterial composite nanofibers.\textsuperscript{10,41} SEM imaging confirmed astrocyte attachment and growth on the nanoscaffolds. The study also showed that the cell morphology was not significantly different on nanofibers containing different carbon nanomaterials. However, the cell density will affect cell contact and cell interaction. The cell surface receptors and molecules that regulate cell interaction will be investigated.

4. MATERIALS AND METHODS

4.1. Scaffold Fabrication by Electrospinning. PCL (molecular weight 70000, $T_m = 60 \degree C$, $T_g = -60 \degree C$, Scientific Polymer Products Inc., New York) nanofibers were generated by electrospinning a PCL solution. The solvent of the PCL solution consisted of a combination of acetonitrile and acetic acid in a 1:1 ratio (by volume). The solute consisted of a combination of PCL as the main ingredient and different types of nanomaterials, which were graphene (platelets, $\geq 98.48\%$, X-Y dimensions $\leq 5 \mu m$, $Z$ dimension $= 50–100 nm$, $\leq 2.2 g/cc$, Angstrom Materials Inc.), CNTs (catalytic multivalled, 5–15 $\mu m$, 140 nm diameter, 0.2 g/cc, MER Corporation), and fullerene (between 99.95%, spherical $= 0.7–1$ nm, clump of crystals $= 3$ nm, molecular weight 720.64 g/mol, 1.600 g/cc, SES Research). The percentage of nanomaterial in the nanofibers was 0.2% (by weight). The solvent-to-solute ratio was 85:15 (by weight), and the temperature of the solution was 50 °C in order to maintain a fixed viscosity. The nanomaterial-loaded PCL solution was prepared by magnetic stirring for 12–16 h at 50 °C. In the process of electrospinning, the nanofibers were collected on a stationary collector placed 25 cm from the syringe needle. The infusion speed of the solution in the syringe was 1 mL/h at 25 kV.

4.2. Surface Characterization by Goniometric Analysis. A static sessile drop method employing a goniometer (CAM 100, KSV Instruments Ltd., Helsinki, Finland) was used to characterize the nanoscaffold surface. This surface characterization was performed by capturing the image of a liquid drop and recording its shape as a function of time. The image was captured at a rate of 10 frames per second with 10 repetitions using a FireWire connectable CCD camera with 50 mm optics. Only the results having a standard deviation of less than 2° were included in the study. The contact angle, ranging from 1 to 180° with an accuracy of $\pm 0.1^\circ$, was measured, and the curve fitting was performed based on the Young–Laplace equation.
4.3. Characterization of Nanofiber Diameter by Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDS). A ZEISS SIGMA VP scanning electron microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) was used to take images of nanofibers. Fiber diameters in the SEM images were measured using NIH ImageJ software (National Institutes of Health, Bethesda, MD). To study the astrocytes grown on nanofibers, the samples were dehydrated with graded ethanol and dried with hexamethyldisilazane. Then the samples were coated with gold and studied using SEM.

Backscattered electron images in the SEM display compositional contrast that results from different atomic number elements and their distribution. EDS can identify the particular elements and their relative proportions in the carbon-nanomaterial-loaded nanofibers. Elemental analysis and chemical characterization were performed using a ZEISS SIGMA VP SEM. Data was collected from multiple locations including the fibers and the bead-like globules.

4.4. Chemical Characterization by Fourier Transform Infrared Spectroscopy (FTIR). FTIR measurements were performed using a ThermoNicolet Avatar 360 Fourier transform infrared spectrometer, which was configured for transmittance. Spectra were collected from the mid-infrared region (4000–400 cm⁻¹) using 90 scans at a resolution of 16 cm⁻¹ with the detector positioned perpendicular to the fiber direction. Three measurements were taken per sample for a total of three samples per set.

4.5. Dielectric Characterization. The effect of nanoparticles in the dielectric constant of PCL was measured using an Automatic Capacitance Bridge. It has a resolution of 0.8 aF.

4.6. Astrocyte Culture. The procedure for isolating the astrocytes from neonatal rats was approved by the Institutional Animal Care and Use Committee (IACUC) and completed at Wichita State University in Wichita, Kansas.⁴²,⁴³ In brief, cerebral cortices were isolated from the brains of neonatal rats (postnatal day P1-2 rats) after they were sacrificed. The cortex tissues were triturated gently through a 5 mL syringe with a needle. The tissue suspension was passed through a 70 mm nylon cell strainer (BD Falcon, Durham, NC), and the flow-through was collected with a 50 mL conical tube. The isolated cells were cultured for about 7–14 days. After reaching confluency, the cultures were shaken to remove macrophages and progenitor cells. The adherent astrocytes were cultured subsequently for cell–nanofiber interaction. The cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, Grand Island, NY). The medium was changed every 2–3 days, and the cell culture was incubated with 5% CO₂ at 37 °C.

4.7. Growth of Astrocytes on Nanoscaffolds and Viability Assay. The electrospun scaffolds were cut as circle membranes that can exactly fit and cover the bottom of the culture well of 24 well plate wells. Silicone O-rings (Ace O-ring, silicone, Sigma Aldrich, St. Louis, MO) were then used to fix the membrane to the bottom of the wells. Using the standard procedure, astrocytes were seeded into a 24 well plate with electrospun fibers attached to the bottom of the cell culture dish. About 50000 cells were seeded in each well, and the cells were incubated for 4 days. The viability and proliferation of astrocytes that were seeded on the nanofibers were studied by monitoring their metabolic activity using the alamarBlue assay (Pierce Biotechnology, Rockford, IL). Then they were incubated with an astrocyte culture medium containing 10% (v/v) alamarBlue reagent for 3 h. Absorbance was measured at wavelengths of 570 and 600 nm in a microplate reader (Synergy Mx Monochromator-Based Multi-Mode Microplate Reader, Winoski, VT).

4.8. Immunocytochemistry and SEM Imaging of the Nanofibers Seeded with Astrocytes. The cultured astrocytes on nanofibers were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were exposed to a blocking solution (10% horse serum and 1% bovine serum albumin) for 20 min. Then the cells were incubated with monoclonal anti-GFAP antibody (Millipore, Billerica, MA) to determine the cell phenotype. The secondary antibody was Alexa Fluor 488 donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA). Nuclei were stained with diamidino-2-phenylindole. The labeled cells were viewed using a Carl Zeiss fluorescence microscope.

To prepare the SEM test, the chemical dehydration procedure was performed for the nanofiber scaffolds seeded with astrocytes. First, the samples were washed with PBS and placed in 2% glutaraldehyde PBS for 2 h. The samples were then dehydrated with graded ethanol (50, 70, 90%, and pure ethanol) followed by hexamethyldisilazane treatment. The air-dried samples were then coated with gold before they were analyzed by SEM.

4.9. Statistical Analysis. Statistical analysis was conducted with one-way ANOVA. A p-value of 0.05 was considered to be statistically significant. Data were expressed as mean ± standard deviation.

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**Notes**
The authors declare no competing financial interest.

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