Enhancement of Schwann Cells Function Using Graphene-Oxide-Modified Nanofiber Scaffolds for Peripheral Nerve Regeneration

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ABSTRACT: Peripheral nerve regeneration and functional recovery remain a significant clinical challenge. Biomaterials that can regulate biological behavior of Schwann cell (SC) and promote neural cell differentiation are beneficial for nerve regeneration and functional recovery. Graphene oxide (GO), as a bioactive nanomaterial, has attracted great attention in biomedical applications. In this study, the possibility of combining the excellent physicochemical properties of GO with nanofiber to develop a bioactive scaffold for nerve regeneration were explored. Briefly, GO was coated on the ApF/PLCL scaffolds. To demonstrate the potentials of this platform, in vitro and in vivo studies toward nerve regeneration were carried out. In vitro, GO-coated scaffolds could enhance SC biological behaviors including migration, proliferation, and myelination. The secretions from SCs cultured on GO-ApF/PLCL scaffolds could induce PC12 cells differentiation. Furthermore, GO-coated nanofibrous scaffolds proved to up-regulate focal adhesion kinase (FAK) expression of PC12 cell. In vivo, GO-ApF/PLCL nerve conduits could successfully repair a 10 mm sciatic nerve defect. These findings suggest that GO-based scaffolds efficiently modulate cell functions and promote nerve regeneration, indicating their potential for nerve regeneration applications.

KEYWORDS: ApF/PLCL nanofibrous scaffolds, GO, SC biological behaviors, FAK, peripheral nerve regeneration

1. INTRODUCTION

The regeneration and functional recovery of nerve is still a clinical problem in spite of surgical treatment.1 Up to now, autografts have remained the "gold standard", but they are restricted because of donor nerve unavailability.2−5 Therefore, to overcome this restriction, researchers have aided peripheral nerve regeneration by developing biomaterial-based nerve conduits.6,7 Although artificial nerve conduits have been proven to have the ability to bridge nerve gap, it is still an ongoing challenge to achieve the desired functional recovery. For the nerve regeneration of peripheral nerve, Schwann cells (SCs) are vital components and play a crucial role in nerve regeneration through the formation of Bungner bands and secretion of various growth factors to support axon growth.8,9 Therefore, modulating SC biological behaviors, including SC migration, proliferation, and myelination, is important to promote nerve regeneration. At present, despite the introduction of many techniques such as gene technique and external stimulation to modulate SC growth and enhance their response to injured peripheral nerve,20−14 it is still a challenge to develop a more accessible and simple method for up-regulating SC biological behavior to repair injured nerves.

Current studies have concentrated on a biomaterials approach for this purpose. Various materials have been fabricated and used in the research of nerve tissue regeneration.15−20 However, nerve regeneration and function recovery have been hampered for most of these materials because of their lack of bioactivity. Therefore, the improvement of biomaterials has great potential in promoting nerve regeneration and functional recovery.

GO has drawn considerable attention in biological applications because of its unique nanostructure and exceptional physicochemical properties such as abundant hydrophilic oxide functional groups, large surface area, excellent mechanical property, and topographical features.21−24 Because of these characteristics of GO, intensive research on GO for tissue engineering has been conducted. Earlier studies exhibited that using GO as a substrate could modulate the growth, interaction, and mobility of several cells, including embryonic stem cells, neural stem cells, induced pluripotent stem cells, and keratinocytes.25−28 GO was also found to regulate the differentiation and proliferation of neural stem cells.29−31 However, the application of GO in nerve tissue engineering has not been widely studied. The use of GO as a scaffold in peripheral nerve regeneration may provide a novel approach in nerve tissue engineering.

GO has great potential in promoting nerve regeneration and functional recovery. Graphene oxide (GO), as a bioactive nanomaterial, has attracted great attention in biomedical applications.
stem cell, and mesenchymal stem cell. Further studies showed that GO greatly promotes neural cell proliferation and differentiation. Several studies have suggested considerable potential of graphene oxide for nerve regeneration. Despite the recognized potential of GO in nerve regeneration, the poor implantable properties of pure GO determine the difficulty of fabricating tissue engineering scaffolds by pure GO via conventional fabrication technologies. Nanofibers with multiscale structure similar to extracellular matrix (ECM) have been extensively investigated as scaffolds for nerve regeneration because of their ability to create a suitable environment for neural cell growth. Up to now, present studies have focused on GO-incorporated into the nanofibers and GO-coated on the nanofibers. For example, electrospun PLGA/GO nanofibrous scaffolds enhance the growth of MSCs. The nanofibrous scaffolds coated with GO also promote neural cell growth. However, the GO modulates SC behaviors including SC migration, myelination, and neurotrophin secretion, which have not been reported. The mechanism of GO-promoted PC12 cell differentiation is also poorly understood. Furthermore, it is necessary to investigate the ability of GO-modified scaffolds to repair peripheral nerve defects in vivo. Therefore, it is necessary to design a bioactive scaffold for promoting nerve regeneration.

In this study, the electrospun composite Antheraea pernyi silk fibroin (ApF)/[poly(l-lactic acid-co-caprolactone]) (PLCL) was used. It has potential for nerve regeneration. GO was coated on the surface of ApF/PLCL scaffolds, and the morphology, hydrophilicity, and mechanical properties were characterized. SC biological behaviors on the scaffolds were studied. The capability of scaffolds to promote the differentiation of PC12 cells was evaluated. The effects of these scaffolds on the FAK expression of PC12 cell were also detected. Furthermore, in vivo studies were performed to study whether GO-modified scaffolds have a beneficial effect on peripheral nerve regeneration.

2. MATERIALS AND METHODS

2.1. Materials. P(LLA-CL) (LA/CL = 50/50) was obtained from Jinan Daigang Bioengineering Co. Ltd. (China). Chinese tussah silk worm A. pernyi silk cocoons were purchased from Liaoning province (China). The regenerated ApF was prepared as in previous study. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was acquired from Shanghai Co., Ltd. (China). Graphene oxide (Thickness: 0.8–1.2 nm; single layer ratio: ~99%; diameter, 50–200 nm) was obtained from...
Nanjing XFNano Tech Co., Ltd. (Nanjing, China). The cells for in vitro analysis were provided by Institute of Biochemistry and Cell Biology (Shanghai, China). Nerve growth factor (NGF) was obtained from Univ-bio Co. Ltd. (China).

2.2. Preparation of GO-Coated AfP/PLCL Scaffolds. The schematic of GO-coated AfP/PLCL scaffolds is illustrated in Figure 1A. There are three steps in the fabrication process: electrospinning, cross-linking, and coating. Briefly, the preparation and cross-linking of the electrospun AfP/PLCL scaffolds had been described in our previous study.37 The scaffolds were soaked in various concentrations (0.5, 1.0, 1.5, and 2 mg/mL) of GO solution for 20 min, and washed three times. Finally, the prepared scaffolds were dried in a vacuum oven. Five alternating cycles were performed. The scaffolds coated in the different concentration of GO solution were named as GO-Afp/PLCL-1, GO-Afp/PLCL-2, GO-Afp/PLCL-3, and GO-Afp/PLCL-4, respectively.

2.3. Characterizations. The morphology of the prepared scaffolds was viewed using SEM (HITACHI TM-100, Japan) and CANON-SSD digital camera (Japan). The nanofiber diameters were analyzed by ImageJ Software (National Institutes of Health, USA). The porosity of scaffolds was evaluated via liquid displacement. The scaffolds were characterized by Fourier transform infrared (FTIR, Nicolet Nexus 670 FTIR spectrometer), Raman (Via-Reflex Renishaw plc, England), and X-ray photoelectron spectroscopy spectra (XPS, Kratos Analytical, UK). The GO quantification was determined through measuring the weight of AfP/PLCL and GO-coated AfP/PLCL scaffolds. The water contact angle of these nanofibrous scaffolds was evaluated via a contact angle analyzer (OCA40, Data physics, Germany). The mechanical properties of scaffolds were tested using a universal testing machine (HSK—S, Hounsfield, UK). The tensile strength, elongation, and Young’s modulus were calculated according to the literature.38

2.4. Schwann Cell Growth on Scaffolds. The rat Schwann cells (SCs) were maintained in DMEM (Gibco, USA) with 10% FBS (Gibco, USA) and 1% penicillin streptomycin (PS). The medium was changed every 2 days. The cells were seeded at a density of 1.0 × 10^4 cells per well and allowed to adhere for 24 h. After 24 h of culture, the medium was changed with 400 uL fresh medium containing 50 ng/mL NGF. After being cultured for 5 days, PC12 cells were fixed. The samples were stained with Anti-Beta III Tubulin antibody (1:100, Abcam, USA). Cells morphology was observed and the percentage of differentiated cells and the neurite lengths on the differentiated cells were measured according to a previously described method41 to characterize the PC12 differentiation level. Furthermore, to assess the effects of GO coated onto the AfP/PLCL scaffolds on the FAK expression of PC12 cell, the protein expressions of FAK were analyzed by Western blotting according to the protocol described previously.52

2.5. qRT-PCR and Western Blotting Analysis. To investigate the functional recovery of the regenerated sciatic nerve, Masson staining of Triceps surae muscle (TSM), triceps weight, and electrophysiological analysis were performed. A positive area percentage of collagen from Masson staining was calculated by Image-Pro Plus software. The TSM were carefully dissected out and collected both in experimental and normal side. Finally, the TSM weight percentage was calculated:

\[ \text{TSM weight ratio(\%)} = \frac{\text{TSM(experimental leg)}}{\text{TSM(normal leg)}} \]

For electrophysiological analysis, the regenerated sciatic nerve was stimulated and the NCV was recorded according to a previous study.32

2.6. NGF Secretion Analysis. We evaluated the secretion of NGF in PC12 cells cultured on AfP/PLCL group and GO-AfP/PLCL group. The conditioned media were harvested at 2, 5, and 7 days of culture. The concentrations of NGF in the conditioned media were measured by ELISA kit (Boster, China).

2.7. PC12 Cell Differentiation and FAK Expression. PC12 cells were cultured in DMEM 1640 medium which contains 15% HS, 2.5% FBS and 1% PS in a humidified atmosphere at 37 °C. For the differentiation study of PC12 cells, the cells were seeded on the nanofibrous scaffolds at a density of 1.0 × 10^5 cells per well and allowed to adhere for 24 h. After 24 h of culture, the medium was changed with 400 uL fresh medium containing 50 ng/mL NGF. After being cultured for 5 days, PC12 cells were fixed. The samples were stained with Anti-Beta III Tubulin antibody (1:100, Abcam, USA). Cells morphology was observed and the percentage of differentiated cells and the neurite lengths on the differentiated cells were measured according to a previously described method41 to characterize the PC12 differentiation level. Furthermore, to assess the effects of GO coated onto the AfP/PLCL scaffolds on the FAK expression of PC12 cell, the protein expressions of FAK were analyzed by Western blotting according to the protocol described previously.52

2.8. Preparation of GO-AfP/PLCL Nerve Conduit and Animal Implantation. The preparation of GO-AfP/PLCL conduit is illustrated in Figure 6A. Briefly, (1) a 2 mm diameter poly(tetrafluoroethylene) (PTFE) stick was used as a collector to fabricate a hollow AfP/PLCL conduit; (2) cross-linked AfP/PLCL conduit was fabricated with 75% ethanol vapor; (3) GO was coated onto the AfP/PLCL conduit as described above. SEM was used to observe the cross-section morphology of GO-AfP/PLCL conduit. For animal implantation, all surgery procedures were performed in accordance to the Institutional Animal Care guidelines. Male Sprague–Dawley (SD) rats were used for the implantation of nerve conduits. All SD rats were randomly divided into three groups: autograft, AfP/PLCL, and GO-AfP/PLCL nerve conduits groups. The sciatic nerve defect of 10 mm length was produced and AfP/PLCL and GO-AfP/PLCL nerve conduits were implanted to bridge the nerve gap, respectively. In addition, the 10 mm nerve was reserved and rebrided in the autograft group. After 12 weeks, all the rats were sacrificed.

2.9. Functional Assessment of the Regenerated Nerve. To investigate the functional recovery of the regenerated sciatic nerve, Masson staining of Triceps surae muscle (TSM), triceps weight, and electrophysiological analysis were performed. A positive area percentage of collagen from Masson staining was calculated by Image-Pro Plus software. The TSM were carefully dissected out and collected both in experimental and normal side. Finally, the TSM weight percentage was calculated:

\[ \text{TSM weight ratio(\%)} = \frac{\text{TSM(experimental leg)}}{\text{TSM(normal leg)}} \]

For electrophysiological analysis, the regenerated sciatic nerve was stimulated and the NCV was recorded according to a previous study.32

2.10. Morphological Evaluation of Regenerated Nerves. After 12 weeks, the regenerated nerves were fixed and treated with hematoxylin eosin (HE), toluidine blue (TB), and Luxol fast blue (LFB) staining, respectively, and the photographs were observed using light microscopy. The number of positive cells and myelin positive areas from TB and LFB staining were calculated. For axon and myelin morphology studies, the samples were stained with lead citrate and uranylacetate. Finally, a transmission electron microscope (TEM) was used to observe the regenerated nerve, and the thickness and diameter of myelin were analyzed using ImageJ software.

2.11. Immunohistochemistry and Immunofluorescence. For immunohistochemistry, the regenerated nerves were stained with several antibodies: S-100 antibody (Abcam), NF-200 (Abcam), and GFAP (Abcam) respectively. Finally, samples were observed by light microscopy, and positive areas were measured by the ImageJ software. For immunofluorescence, the samples were treated with S-100 and NF-200 antibody, respectively. DAPI (1:200, Life Technologies, America) was applied to stain the cell nuclei. Finally, samples were viewed by a laser confocal microscope (CLSM).

2.12. Statistical Analysis. All of the experimental data were expressed as means ± standard deviation. The significance of the experimental data was analyzed by one-way ANOVA statistical
analysis and a value of (*) \( p < 0.05\) and (**) \( p < 0.01\) were considered to be significant and highly significant, respectively.

3. RESULTS AND DISCUSSION

3.1. Fabrication and Characterization of Scaffolds.

The schematic of GO-coated scaffolds is illustrated in Figure 1A. The morphology of different scaffolds was viewed using SEM and digital photos (Figure 1B). Under the 10K magnification, all the scaffolds displayed a nanoscale structure and there was no significant difference between ApF/PLCL and GO-coated ApF/PLCL nanofibrous scaffolds. It was clear that the ApF/PLCL nanofibrous scaffolds were smooth under higher magnifications than GO-coated ApF/PLCL nanofibrous scaffolds. Notably, the existence of GO on the nanofibers surface does not destroy the nanoscale structure of nanofibers. As shown in digital photos, after GO coating of the white ApF/PLCL nanofibrous scaffolds, the samples turned brown and the intensity of the color increased with the concentration of GO, suggesting that GO was successfully coated onto the scaffolds. To evaluate the effect of GO coating on the fiber diameters and porosity, we characterized the average diameters and porosity of ApF/PLCL and GO-coated ApF/PLCL nanofibrous scaffolds in Table S1. The increase in diameter and decrease in porosity is due to the deposition of the GO on the scaffold surfaces.

The ApF/PLCL and GO-coated ApF/PLCL nanofibrous scaffolds were characterized by several techniques. The corresponding FTIR spectrum was shown in Figure 1C. The characterized peaks around 2942 and 1756 cm\(^{-1}\) were \(-\text{CH}_2\) and C=O stretching of the PLCL molecular skeleton, which were clearly observed in ApF/PLCL, GO-ApF/PLCL-1 GO-ApF/PLCL-2, GO-ApF/PLCL-3, and GO-ApF/PLCL-4. In addition, the significant absorption bands at 1630 cm\(^{-1}\) (amide I) were observed in these samples, which confirmed the existence of ApF.\(^{43}\) The characteristic peak at 3400 cm\(^{-1}\) is assigned to the stretching band of O–H in GO. This characteristic peak also appeared in ApF/PLCL and GO-coated ApF/PLCL nanofibrous scaffolds, but it does not indicate the presence of GO nanosheets in GO-coated ApF/PLCL nanofibrous scaffolds. To confirm the existence of GO nanosheets on the surface of scaffolds, we measured the Raman spectra of pure GO, ApF/PLCL and GO-coated ApF/PLCL nanofibrous scaffolds. As shown in Figure 1D, the Raman spectrum of GO-coated ApF/PLCL nanofibrous scaffolds presented two characteristic D band and G band of GO at 1352 and 1590 cm\(^{-1}\),\(^{44}\) suggesting the presence of GO on the ApF/PLCL nanofibrous scaffolds.

The scaffolds were also characterized using the high-resolution XPS spectra of C 1s (Figure S1). All the samples exhibited peaks at 284.6, 286.0, 287.1, and 288.5 eV, which are ascribed to C–C/C==C, C–O–C/C==O, C==O, and HO–C==O bands, respectively.\(^{49}\) Figure S1F shows the peak area ratios of the oxygen active functional groups peaks to C–C/C==C peak. To give a quantitative analysis on the chemical state variations of different samples at the same conditions, these peak area ratios of ApF/PLCL nanofibrous scaffolds were obviously higher than those of the GO-coated scaffolds, which owing to the presence of amino acids in ApF/PLCL nanofibrous scaffolds. Notably, with the increasing of GO content on the scaffold surfaces, the peak area ratios were increased, which were due to existence of oxygen active groups in GO.

Further evidence is given by the atomic percent of C, O, and the atomic ratio of O/C (Table 1), which were calculated from XPS spectra. The atomic percentage of C, O, and the O/C ratio increased after GO coating. However, the N percent show the opposite tendency. With the increase in GO content on the nanofibrous scaffolds, the atomic percentage of N atom decreased. These results also indicated that GO successfully coated the scaffold. In addition, GO loading was increased as GO concentration increased (Table 1). When the loading was above 1.18%, the GO content did not increase and indicated that the GO coating amount on the scaffolds was saturated.

For the applications of GO-coated ApF/PLCL scaffolds as nerve tissue engineering, the hydrophilicity of the materials is a pivotal property to cell attachment, proliferation and differentiation.\(^{46,47}\) The GO-coated ApF/PLCL nanofibrous scaffolds showed better hydrophilicity compared with ApF/PLCL nanofibrous scaffolds (Table S1) which was in accordance with the results from the other studies.\(^{25,48}\) This was due to the existence of oxygen active groups (COOH, OH, and C–O–C groups). Besides the changes in surface hydrophilicity, the mechanical strength of the GO-coated ApF/PLCL nanofibrous scaffolds was investigated (Figure S2). The mechanical properties of the GO-coated ApF/PLCL nanofibrous scaffolds significantly increased in comparison with ApF/PLCL nanofibrous scaffolds (Figure S2B–D), which suggested that coating with GO nanosheets enhanced the mechanical properties of the ApF/PLCL scaffolds.

3.2. SC Growth.

Cell migration and proliferation are vital for tissue regeneration.\(^{49}\) SCs have the ability to secrete various growth factors to promote peripheral nerve regeneration.\(^{50,51}\) Therefore, to evaluate whether the GO coated ApF/PLCL scaffolds satisfied the fundamental requirement in nerve tissue engineering, SCs were used to culture on the GO-ApF/PLCL scaffolds in vitro study and the SCs proliferation and migration were investigated. After 1, 3, and 5 days of culture, SCs proliferation on tissue culture plate (TCP), ApF/PLCL and GO-ApF/PLCL scaffolds was investigated by MTT assay. The SCs cultured on the scaffolds exhibited good proliferation (Figure 2A). However, compared to TCP and ApF/PLCL groups, the cells cultured on GO-coated ApF/PLCL nanofibrous scaffolds exhibited better proliferation on days 1, 3 and 5, especially in the GO-ApF/PLCL-4 scaffolds, indicating that the GO nanosheets coating ApF/PLCL nanofibrous scaffolds significantly promoted cell growth and proliferation.

The scaffold material is a critical factor that determines the fate of cell proliferation. The optimum content of GO onto the ApF/PLCL scaffolds exhibit the greatest extent of SC proliferation. It is important to further evaluate cell viability, migration and myelination in vitro and nerve regeneration in vivo. Therefore, GO-ApF/PLCL-4 scaffolds were chosen for further evaluation and termed as GO-ApF/PLCL. After 5 days, the morphology of the SCs on the ApF/PLCL nanofibrous scaffolds was smooth under 100X magnification (Figure 2B). The GO-ApF/PLCL-4 scaffolds exhibited good proliferation and cell migration (Figure 2C).

Table 1. Atomic Percent of C, O, N, and O/C Ratio of the Various Scaffolds, and GO Loading

<table>
<thead>
<tr>
<th>samples</th>
<th>C (%)</th>
<th>O (%)</th>
<th>N (%)</th>
<th>O/C</th>
<th>GO loading (%)</th>
</tr>
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<tr>
<td>ApF/PLCL</td>
<td>66.49</td>
<td>25.34</td>
<td>8.17</td>
<td>0.381</td>
<td>ND</td>
</tr>
<tr>
<td>GO-ApF/PLCL-1</td>
<td>68.76</td>
<td>27.44</td>
<td>3.79</td>
<td>0.399</td>
<td>0.48</td>
</tr>
<tr>
<td>GO-ApF/PLCL-2</td>
<td>68.27</td>
<td>28.18</td>
<td>2.21</td>
<td>0.412</td>
<td>0.78</td>
</tr>
<tr>
<td>GO-ApF/PLCL-3</td>
<td>69.33</td>
<td>28.46</td>
<td>2.67</td>
<td>0.41</td>
<td>0.97</td>
</tr>
<tr>
<td>GO-ApF/PLCL-4</td>
<td>67.68</td>
<td>30.49</td>
<td>1.84</td>
<td>0.45</td>
<td>1.18</td>
</tr>
</tbody>
</table>
scasffolds with or without GO nanosheets was evaluated by immunofluorescence and SEM images (Figure 2B). The SCs spread well on these samples. The cell cytoskeleton was further elongated and more cells proliferated on the GO-ApF/PLCL scaffolds than those on ApF/PLCL scaffolds. Similar results were further verified by the SEM images. As shown in Figure 2C, D, SCs migration was significantly enhanced on the GO-ApF/PLCL group in comparison with the ApF/PLCL group after 24 and 48 h. These results revealed the existence of GO to promote SC proliferation and migration.

3.3. SC Myelination. SC myelination play a key role in nerve regeneration. Therefore, to investigate the influence of GO-ApF/PLCL scaffolds on regulating SCs’ myelination, SCs were cultivated on ApF/PLCL and GO-ApF/PLCL scaffolds for 5 days. The RT-qPCR and Western blotting analysis were carried out. NCAM is an indicator of SCs’ promyelination. It is expressed only in immature SCs and decreases in SCs’ myelination (Figure 3A). As shown in Figure 3B, NCAM gene expression decreased significantly, and the level of gene expression of NGF, PMP22, and Krox20 dramatically increased on the GO-ApF/PLCL scaffolds in comparison with that on ApF/PLCL scaffolds (p < 0.01). Western blotting analysis results also showed the lower NCAM and higher NGF protein expressions on GO-ApF/PLCL scaffolds. **p < 0.01.

Figure 2. SC proliferation and migration. (A) SC proliferation on TCP, ApF/PLCL, and GO-coated ApF/PLCL nanofibrous scaffolds. (B) Immunofluorescence and SEM images of SCs on ApF/PLCL and GO-ApF/PLCL scaffolds at 5 days. Scale bar = 100 μm, (C) Migration of SC on different nanofibrous scaffolds (scale bar = 500 μm). (D) Quantitative analysis of the migratory ability of SCs on the ApF/PLCL and GO-ApF/PLCL scaffolds (**p < 0.01).

Figure 3. SCs myelin gene expressions on ApF/PLCL and GO-ApF/PLCL nanofibrous scaffolds at 5 days. (A) Schematic of the gene expression changes from SCs during myelination. (B) Gene expression of SCs on ApF/PLCL and GO-ApF/PLCL nanofibrous scaffolds. (C) NCAM and NGF protein expression of SC measured by Western blotting and (D) the calculated corresponding protein levels (**p < 0.01).
scaffolds (Figure 3C, D). The above results suggest that the existence of GO has a positive effect on SC myelination.

Secretion of NGF from myelinating SCs can promote nerve regeneration via stimulating neurons outgrowth.40 To further evaluate the influence of GO-ApF/PLCL nanofibrous scaffolds on NGF secretion from SCs, we performed immunofluorescence staining, flow cytometry, and ELISA assay (Figure 4). NGF immunofluorescence staining suggested that higher amounts of NGF protein in SCs was synthesized and mature bipolar morphology of SCs was exhibited on GO-ApF/PLCL scaffolds (Figure 4A), which consistent with other literatures about myelinating SCs.52 Similar results can be obtained with the fluorescence intensity of NGF (Figure 4B, C). To quantify the amount of NGF protein, we performed an ELISA assay (Figure 4D). The content of NGF secreted from SCs on GO-ApF/PLCL was much higher than that on ApF/PLCL after 2 days (p < 0.01). After continuing cultured for 5 days, it was observed that the secretion of NGF increased significantly, especially on the GO-ApF/PLCL scaffolds. All the results above suggested that GO-ApF/PLCL scaffolds significantly increased NGF secretion from SCs.

3.4. PC12 Cell Differentiation on Different Types of Scaffolds. PC12 cells exhibit many neuronal properties including neurotransmitter synthesis and morphological differentiation.53,54 Herein, the PC12 cells differentiation was investigated by adding the medium suspension from SCs-cultured on the following groups: the normal medium of PC12 cells without the SCs medium (NM group); the medium from SCs-cultured on ApF/PLCL and GO-ApF/PLCL scaffolds, and the normal medium of PC12 cells without the SCs medium but with the existence of 50 ng/mL NGF (NM+NGF group). After 5 days of culturing, PC12 cells exhibited better differentiation in the GO-ApF/PLCL group in comparison to the NM and ApF/PLCL groups and exhibited a similar level of differentiation with NM+NGF group (Figure 5A). The average length of neurites in PC12 cells from the GO-ApF/PLCL group and NM+NGF group was significantly longer than that on the ApF/PLCL group (p < 0.01) (Figure S3B). In addition, similar conclusions were drawn from the proportion of differentiated PC12 cells results (p < 0.01). The above results show that the NGF secretion from SCs on the GO-ApF/PLCL scaffolds could induce PC12 cells differentiation.

Graphene and its derivatives were verified to promote neural cell differentiation.55−57 To determine whether GO-ApF/PLCL scaffolds can promote PC12 cells differentiation, cells were cultured separately on ApF/PLCL and GO-ApF/PLCL scaffolds. As shown in Figure 5A, after culturing for 5 days, the cells showed a dispersive and multipolar shape on all samples. However, longer neurites and more dispersive morphology were found on the GO-ApF/PLCL nanofibrous scaffolds in comparison to ApF/PLCL nanofibrous scaffolds. The neurite length and the percentage of differentiated PC12 cells on GO-ApF/PLCL scaffolds were significantly greater than that on ApF/PLCL scaffolds (p < 0.01 and p < 0.05, respectively) (Figure 5B). The enhanced PC12 cell differentiation could be attributed to surface properties (surface coating of GO). Recent reports indicate that graphene can promote neurite growth.59,60 FAK is a key adhesion molecule and plays a key role in neurite growth in neurons.61−62 To evaluate the influence of GO coated ApF/PLCL nanofibrous scaffolds on FAK expression from PC12 cell, FAK expression was detected by Western blot analysis (Figure 5C). The results showed higher FAK expression from PC12 cell cultured on GO-ApF/PLCL scaffolds than on ApF/PLCL scaffolds (p < 0.01). The GO has positive effects to the expression of key adhesion protein-FAK. That is, the GO coated on the ApF/PLCL nanofibrous scaffolds may promote differentiation of PC12 cells through specific microenvironmental interactions that can activate integrin-related intracellular signaling.

3.5. Preparation and Implantation of GO-ApF/PLCL Nerve Conduit. Given the excellent performance of GO-ApF/PLCL scaffolds in vitro, the ability of this scaffolds to
promote peripheral nerve regeneration in vivo still needs to be explored. To mimic axial orientation structure of peripheral nerve tissues, we prepared the GO-ApF/PLCL conduits with a 2 mm diameter and 10 mm length by axial orientation electrospinning (Figure 6A). The SEM images of conduit exhibit a framework constituted by axial-oriented nanofibers (Figure 6B). Finally, the ApF/PLCL, GO-ApF/PLCL, and autograft were implanted.

3.6. Functional Assessment of Regenerated Nerve. Twelve weeks after implantation, Masson staining, triceps weight analysis, and electrophysiological assessment were performed to evaluate functional recovery of regenerated nerves. The Masson staining of triceps surae muscles (TSM) in all groups were exhibited (Figure 7A). After 12 weeks, more collagen (blue region) was created on ApF/PLCL group than GO-ApF/PLCL and autograft groups (p < 0.01) (Figure 7B), which indicates that the TSMs of ApF/PLCL group had more severe atrophy than GO-ApF/PLCL group. The TSM weight ratio also confirmed that the motor function recovery in the GO-ApF/PLCL group was similar to that in the autograft group, and significantly better than that in ApF/PLCL group at week 12 (p < 0.05) (Figure 7C). Electrophysiological studies were performed to further measure the functional repair of sciatic nerve. NCV is a key index for the nerve conduction function. The electrophysiological analysis revealed that the NCV of the GO-ApF/PLCL group was significantly higher than that of the ApF/PLCL group (p < 0.01) (Figure 7D). Furthermore, the recovery level of electrophysiological properties in the GO-ApF/PLCL group was close to that in the autograft group. These remarkable differences between GO-ApF/PLCL and ApF/PLCL groups indicated that the presence of GO promoted the functional recovery of peripheral nerve.

3.7. Morphological Evaluation of Regenerated Nerves. As well-known, the function of nerve conduits is mainly to provide space for SCs and axons to connect the distal and proximal nerve. Therefore, the HE, TB, and LFB staining were performed separately to evaluation the histology and morphology of cross sections of the middle part of the regenerated nerves. After 12 weeks, there were no inflammatory signs in all groups from HE staining results. TB and LFB staining were performed separately to evaluate the histology and morphology of cross sections of the middle part of the regenerated nerves. After 12 weeks, there were no inflammatory signs in all groups from HE staining results. TB and LFB
staining showed that large amounts of SCs and regenerated nerve myelin were observed in GO-ApF/PLCL and autograft groups compared with ApF/PLCL group (Figure 8A). In addition, quantitative analysis of SC density and the positive area percentage of myelin from TB and LFB staining images is calculated, respectively. Those two data in the GO-ApF/PLCL group were similar to those in the autograft groups and better than those in ApF/PLCL group (*p < 0.05 and **p < 0.01) (Figure 8B).

The generated myelinated axons provide the structural basis for improving nerve regeneration. Therefore, to investigate the degree of myelination, we characterized the samples via TEM. The diameter and thickness of regenerated axons were similar to those in the autograft groups and better than those in ApF/PLCL group (p < 0.01) (Figure 8B).

Figure 6. Preparation of GO-ApF/PLCL nerve conduit and animal implantation. (A) Schematic illustration of GO-ApF/PLCL nerve conduit preparation; (B) characterization of GO-ApF/PLCL nerve conduit; (C) optical images of nerve conduits at implantation, scale bar = 250 μm.

Figure 7. Functional recovery analysis of the regenerated sciatic nerve after implantation for 12 weeks. (A) Masson staining of TSMs, scale bar = 100 μm, and (B) the percentage of collagen acquired from Masson staining; (C) TSM weight ratio analysis at 12 weeks; (D) NCV of the regenerated nerves in various groups at 12 weeks (*p < 0.05 and **p < 0.01).
measured. Although the newly formed myelin sheath was produced in all the groups (Figure 8C), the diameter and thickness of myelin in the GO-ApF/PLCL group were significantly greater than the ApF/PLCL group \((p < 0.05)\) but still did not match the autograft group (Figure 8D).

3.8. Immunohistochemistry and Immunofluorescence. The peripheral nerve regeneration starts from the SCs adhesion, migration, and proliferation on the nerve conduits matrix.\(^{65}\) Therefore, it is vital for the nerve conduits to promote SCs adhesion, migration, and proliferation in vivo. To further investigate the recovery of regenerated nerve, three immunohistochemical indicators (S-100, NF-200 and GFAP) and the corresponding positive area of these indicators were studied. S-100 was used to investigate SC growth during the nerve regeneration.\(^{66}\) NF-200 is a key intermediate filament for neurons.\(^{67}\) GFAP plays a role in neural cell adhesion as well as myelination maintenance and cytoskeletal reorganization.\(^{68}\) As shown in Figure 9A, the positive expressions of all the measured indicators were generated in these groups and quantitative analysis results showed that the positive area of these indicators in the GO-ApF/PLCL and autograft groups were significantly more than the ApF/PLCL group \((p < 0.01)\). There was no significant difference between the GO-ApF/PLCL and the autograft group (Figure 9B). Similar results were obtained with the immunofluorescence staining of S-100 and NF-200 (Figure 9C). In summary, the above results show that the GO-ApF/PLCL nerve conduits promoted peripheral nerve regeneration compared with the ApF/PLCL group and the performance was close to that of the autograft.

This research demonstrated that GO-coated nanofibrous scaffolds created an ideal interface for SC growth, the PC12 differentiation, and related protein expression in vitro and promoted nerve regeneration and functional recovery in vivo. These observed effects are due to the combination of the
morphological structure of nanofibers and the physicochemical properties of GO. In previous studies, electrospun nanofibers have been demonstrated to provide ideal morphological cues for preparing nerve conduits and promote nerve regeneration because of their topological structure similarity to natural ECM. Surface topography and physiochemical of scaffolds play a key role in regulating cell behaviors and regulating related gene expression. GO coating with a unique nanostructure and physicochemical properties modifies the surface physicochemical properties of scaffolds without destroying the nanoscale structures of the nanofibers. Previous studies showed that GO modified scaffolds exhibit excellent biocompatibility and have demonstrated a great ability to promote neural stem cell growth. These excellent performances are attributed to its unique chemical structure, the rippled and wrinkled surface morphology, and oxygen-containing hydrophilic functional groups. Therefore, the combination of morphological features and physicochemical properties in a platform to regulate SC and PC12 cell biological behavior as well as promote nerve regeneration and functional recovery could be desirable. However, a precise mechanism of GO on the regulation of SC biological behaviors still needs to be explored.

5. CONCLUSION

In summary, a bioactive nerve scaffold was fabricated via coating GO on scaffolds. The effect of this scaffold in nerve regeneration was evaluated both in vitro and in vivo. GO-coated ApF/PLCL scaffolds significantly promoted SC migration, proliferation, and myelination; promoted the differentiation of PC12 cell; and proved to up-regulate FAK expression of PC12 cell in vitro. Through the evaluation of morphological and functional recovery of regenerated nerve, it was found that GO-modified ApF/PLCL could successfully repair a 10 mm sciatic nerve defect and exhibited a similar healing capacity to autograft. Therefore, GO-ApF/PLCL scaffolds, as bioactive substrates, are expected to perform better in peripheral nerve regeneration.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomater.8b01564.

Results of the average diameter, porosity, and contact angles; high-resolution XPS C 1s spectra; mechanical properties of scaffolds; and PC12 cell differentiation of GO-ApF/PLCL scaffolds are presented in the Supporting Information.
induced by SCs cultured media on the various scaffolds (PDF)

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**Notes**

The authors declare no competing financial interest.

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