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Research Article

Theanine-Modified Graphene Oxide Composite Films for Neural Stem Cells Proliferation and Differentiation

Zhiping Qi, Xue Chen, Wenlai Guo, Chuan Fu, and Su Pan

Department of Orthopedic Surgery, The Second Hospital of Jilin University, Ziqiang Street No. 218, Changchun TX: 130041, China

Correspondence should be addressed to Chuan Fu; fuchuan2015@163.com and Su Pan; spineps@sina.com

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The central nervous system (CNS) injury has been a worldwide clinical problem for regenerative medicine. Nerve tissue engineering is a new strategy for CNS injury. Among kinds of biomaterials, graphene oxide (GO)-based degradable composite materials are considered to be promising in the field of neurogenesis. In this study, GO and L-theanine (TH) were combined by chemical grafting to prepare a new PLGA/GO-TH composite material. X-ray diffraction (XRD), transmission electron microscope (TEM), Fourier-transform infrared spectra (FTIR), contact angle testers, and mechanical testers were performed to obtain characterization of composite materials. The protein adsorption efficiency of the PLGA/GO-TH films was then evaluated. Next, the effect of the composite films on neural stem cell (NSC) survival, proliferation, and differentiation was investigated. Our results indicated that L-theanine was successfully grafted onto GO. PLGA/GO-TH composite film can significantly improve NSC survival, proliferation, and neuronal differentiation. Our results demonstrated that the neurogenesis function of a novel PLGA/GO-TH composite film and its potential as a carrier for the further application in the CNS injury.

1. Introduction

The central nervous system (CNS) injury can always cause sensory and motor dysfunction, which has been difficult to overcome in the regenerative field [1]. Since nerve tissue engineering developed fast in the past years, neurobiological materials are chosen to treat CNS injury as a new strategy [2–5]. Graphene oxide (GO) is considered to be one of the most promising candidate biomaterials for neurogenesis. GO has excellent physical, chemical, and electrical properties; a large specific surface area; and other special physical and chemical properties [6, 7]. Studies have confirmed that GO can increase the neuronal differentiation rate of embryonic stem cells and neural stem cells, which is beneficial to nerve regeneration [8, 9]. Besides, there are many functional groups present on the surface of GO, facilitating the surface modification of GO with bioactive factor to further enhance the biological functions of GO.

Amino acids are the basic components of proteins. Amino acids contain several functional groups, which could enhance the adhesion of cells or proteins through hydrogen bonding or electrostatic attraction [10–12]. In consideration of these functional groups, amino acids could surface-modify some materials by chemical grafting [13]. As a type of amino acid extracted from green tea, L-theanine was identified to have a unique function in the nervous system. First, L-theanine had a clear protective effect on neuronal damage caused by cerebral ischaemia-reperfusion injury and β-amyloid intervention, which may be because of the inhibitory effect of L-theanine on the oxidation/nitrosation stress reaction and inflammatory factor production after central nerve injury [14–16]. In addition, L-theanine can promote the proliferation and neuronal differentiation of NSCs by upregulating the Slc38a1 gene and activating the mTOR signalling pathway [17].

To further enhance the bioactivity of GO to promote nerve regeneration, we stably combined L-theanine and GO by chemical grafting. Because of the potential cytotoxicity and nondegradability of high concentrations of GO, biodegradable polymers were used as another component to produce hybrid materials to compensate for this problem. Poly(lactic-co-glycolic acid) (PLGA) is an common degradable materials approved by FDA and has been widely used in nerve tissue engineering. In this study, the PLGA/GO-TH composite film was first

prepared, and its characterization was determined. Then, the lysozyme adsorption efficiency of the hybrid material was observed. Next, the effect of the PLGA/GO-TH film on NSC survival, proliferation, and differentiation was measured to evaluate the potential of the L-theanine-modified GO composite materials in nerve regeneration.

2. Materials and Methods

2.1. Materials. PLGA (LA : GA = 75 : 25, Mw = 85000 g mol − 1) was purchased from Changchun Sino Biomaterials Co., Ltd, China. GO was purchased from Chengdu Organic Chemicals Co., Ltd., China (thickness: 0.55–1.2 nm; diameter: 0.5–3 mm). L-theanine and lysozyme were purchased from Sigma (USA). NSC culture media components were purchased from PeproTech (USA). Primary antibody including Tuj-1 and GFAP were purchased from Abcam (USA).

2.2. Synthesis of the GO-TH Hybrid. The method for combining amino acids and GO by chemical grafting was followed as previously described. In brief, 0.1 g GO and 0.25 g EDC were first added to a flask, and the mixture was stirred for 1 h with vibration, followed by the addition of 0.3 g of L-theanine, and stirred at room temperature for 24 h. Then, the above mixture was placed into sediment, and the supernatant was replaced with clean distilled water, followed by ultrasonic concussion. This step was repeated 5 times to remove the impurity from the GO-TH hybrid solution, and the GO-TH hybrid was lyophilized and stored.

2.3. Production of the PLGA/GO-TH Composite Film. GO is cytotoxic to some extent, so it is important to control its concentration when used with polymers. Therefore, the concentrations of GO were obtained by referring to lots of references about GO. Previous studies have demonstrated that 2% GO have the best biological activity, and there is no significant cytotoxicity [18]. Therefore, in this study, the ratio of PLGA to GO-TH is 98 : 2. The solution evaporation method was used to obtain PLGA/GO-TH composite film. First, 980 mg PLGA was dissolved in hexafluoroisopropanol and followed by the addition of 20 mg GO-TH. Then, the above solution was magnetically stirred and ultrasonically shaken to thoroughly mix. Part of the solution was spread on a siliconized slide for subsequent cell experiments, and another part was spread in a petri dish for characterization evaluation. The preparation process of the pure PLGA film and the PLGA/GO composite film was the same as that of the PLGA/GO-TH film.

2.4. Characterization. The FTIR spectra (Bruker Tensor 27) were used to evaluate the crosslinking between TH and GO. The morphologies of GO and GO-TH were observed by an H-7650B transmission electron microscope (Hitachi, Japan). XRD (D8 ADVANCE, Germany) was employed to identify the elemental composition. The water contact angle was used to measure the hydrophilia of the hybrid films. A universal mechanical testing machine (Instron 1121, UK) was chosen to determine the mechanical properties of the hybrid films.

2.5. Protein Adsorption Properties. The protein adsorption properties of PLGA, PLGA/GO and PLGA/GO-TH films were analysed by performing a bicinchoninic acid (BCA) protein assay using lysozyme as a model protein. Briefly, the films were first added into 10 mL of lysozyme solution (2 mg/mL), and liquid samples were taken to analyse the solution lysozyme concentration at certain time points. The amount of lysozyme loaded on the films was determined by the reduction in lysozyme concentration through BCA protein assay.

2.6. NSC Survival Assays. NSCs were suspension cultured as previously described [19]. Then, the NSCs were dissociated as single cells and cultured on glass, PLGA, PLGA/GO, and PLGA/GO-TH films. 100 μM H_2O_2 was added into each well and the culture medium was replaced after 24 h. The cell viability was determined by a CCK-8 assay. In brief, the medium in each well was replaced with 110 μl of CCK-8 solution for 2 h. Then, all the samples were transferred to a 96-well plate. The absorbance was determined by a microplate reader (Infinite M200, TECAN) at a wavelength of 450 nm.

2.7. Cell Proliferation Assay. NSCs were seeded on the surface of Matrigel-coated films at a density of 5×10^4 cells/cm2 in NSC growth medium. Cell proliferation was evaluated at 1, 4, and 7 days by an MTT assay. In brief, the stock solution was added to the medium, followed by the addition of acidified isopropanol after incubation at 37°C for 4 h. A full wavelength microplate reader (Infinite M200, TECAN) was used to observe the optical density (OD) at a wavelength of 540 nm in each well.

2.8. Cell Differentiation Assay. After NSCs were cultured in growth medium for two days, and then, the growth medium was removed and replaced to differentiation medium. Cell differentiation assay included immunofluorescence staining and real-time PCR, which was performed after cell cultured for 7 d. For immunofluorescence staining, the cells were fixed with 4% paraformaldehyde for 30 min, extracted with 0.1% Triton X-100 for 10 min, and blocked with 5% goat serum for 2 h. Then, primary antibodies including tuj-1 (1 : 300 dilution) and GFAP (1 : 500 dilution) were added followed by incubation. The staining result was obtained by a laser scanning microscope (LSM 780, ZEISS). For RT-PCR, after the total RNA purity was according with the standard for following procedure, reverse-transcription and amplification were performed., followed by normalized to housekeeping gene. The genes and the primer sequences are shown in Table 1.

2.9. Statistical Analysis. All quantitative data was analysed with OriginPro 8.0, which presented as the mean ± standard deviation. Statistical differences were analysed by one-way analysis of variance. A p value of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Characterization of the GO-TH Hybrid. The microstructure of the prepared material was observed by TEM, as shown in Figure 1(a). GO exhibited a natural stretch, showing a flaky and pleated pattern that is similar to a flaking pleated

Table 1: Genes and the primer information.

Gene	Primer sequence (5′-3′)
Tuj-1	F GATCGGAGCCAAGTTCTG; R GTCCATCGTCCCAGGTTC
GFAP	F GCACTCAATACGAGGCAGTG; R GGCGATAGTCGTTAGCTTCG
GAPDH	F TCGCCAGCCGAGCCA; R CCTTGACGGTGCCATGGAAT

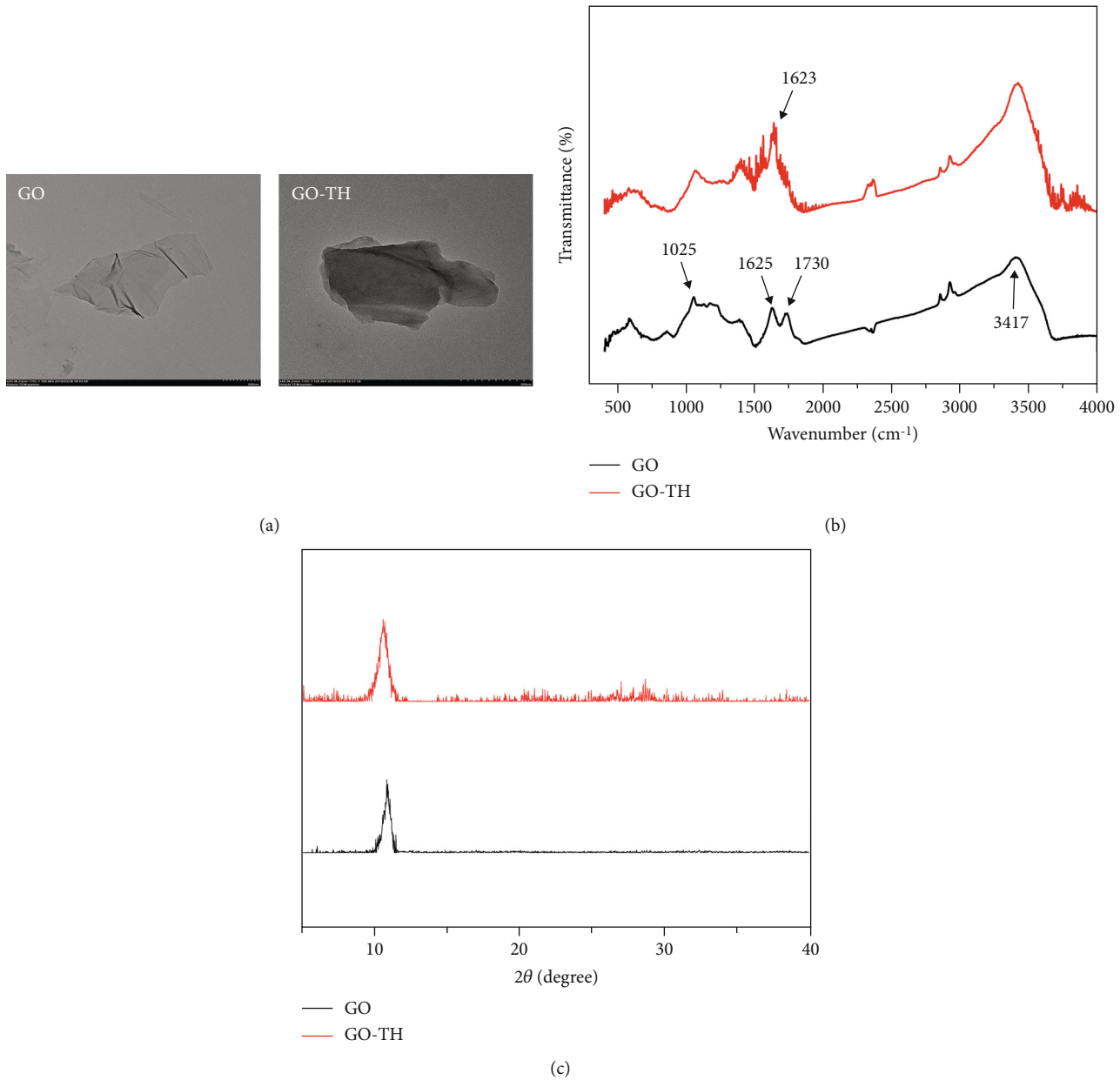

Figure 1: Characterization of the GO-TH hybrid. (a). TEM images. (b). FITR spectra of GO and GO-TH. (c). XRD of GO and the GO-TH.

sheet. After grafting L-theanine, the surface roughness of the GO-TH hybrid significantly increased, and the amino acid molecules were observed on the surface. This morphological change may be caused by the grafting of amino acids onto the surface of the GO. This rough surface may contribute to the enhancement of the material's adsorption capacity [20, 21]. FTIR was employed to confirm the interaction between GO and L-theanine. As shown in Figure 1(b), for the spectra of GO, the peaks at 1052 cm-1, 1730 cm-1, and 3417 cm-1 are attributed to the C—O band, C=O stretching band, and O—H stretching band of carboxylic acid and hydroxy groups, respectively. For the spectra of GO-TH, the C=O stretching of the amide and carboxylate groups in the range of 1620-1630 cm-1 and the O—H and N—H stretching peaks

at 3420 cm^{-1} confirmed the successful covalent functionalization of GO by the amino acid molecules. XRD was used to determine GO and the GO–TH hybrid. As shown in Figure 1(c), the single diffraction peak at 2θ was 11.6°, and the interlayer distance was 0.76 nm in the GO group, which indicated the oxygen-containing functional groups of GO. Compare with GO, GO–TH show two XRD peaks with a similar XRD. Due to the introduction of L-theanine, GO-TH showed a diffraction peak at 2θ =10.4°, corresponding to a d-spacing of 0.99 nm. The large interlayer distance may be attributed to the TH molecules that are inserted between the neighboring GO sheets in the GO–TH membranes.

3.2. Properties of Composite Film. The hydrophilicity and mechanical properties of composite materials play an essential role in cell interactions with the substrate [22–24]. In this study, the contact angle and tensile strength of the composite film were evaluated, as shown in Figures 2 and 3. We found the contact angles of the PLGA/GO and PLGA/GO-TH samples were significantly lower than those of the PLGA sample, indicating that GO and GO-TH addition to PLGA can enhance its hydrophilicity ($p < 0.05$, Figure 2). There are a large number of hydrophilic groups on GO surface, such as OH, CAOAC, and COOH, which can greatly improve the hydrophilicity of hydrophobic materials. The decrease in contact angle can be explained by the hydrogen bond interaction between GO and the oxygen-containing groups in the water [25]. Furthermore, GO can improve the roughness of the material surface to some extent, which can also improve the hydrophilicity of the material [26]. More importantly, L-theanine is a hydrophilic amino acid, and its presence on the material surface may also improve the material's hydrophilicity to some extent [19]. The improved hydrophilic properties of biomaterials can promote cell adhesion and growth. Next, the mechanical strength of different composite films was measured. Our results showed that the tensile strengths of the PLGA film were significantly lower than that of PLGA/GO and PLGA/GO-TH ($p < 0.05$, Figure 3). Studies showed better mechanical properties could enhance the stability of neural scaffolds [23, 24]. Because of the interfacial interaction between the oxygen-containing functional portion of GO and the hydroxyl or amino groups of the substrate, the mechanical properties of composite films are better than pure PLGA films [27].

3.3. Lysozyme Adsorption Capacity of the PLGA/GO-TH Film. In this study, lysozyme was used as a model protein to study the adsorption capacity of composite materials for proteins. The results showed that after 120 min, the PLGA/GO film had significantly more protein adsorption than the pure PLGA film ($p < 0.05$, Figure 4), possibly because of GO binding to proteins through hydrogen bonding and π–π interactions [28, 29]. We also found that the protein adsorption capacity of the PLGA/GO-TH film was higher than that of the PLGA/GO film. Combined with the results of the TEM observation, we suspected that the modification of GO with L-theanine roughened the surface of GO; this modification could be beneficial to protein absorption. Moreover, the isoelectric point is believed to play an important role in protein absorption. Studies have demonstrated that electrostatic attraction makes a larger contribution to the kinetic adsorption of proteins than hydrogen bonding and π–π interactions [30]. In our study, PBS solution (pH = 7.4) was selected to dissolve lysozyme. The isoelectric point of L-theanine is 5.6, which increases the negative charge on the modified GO surface, while the isoelectric point of lysozyme is 10.5 and thus is positively charged in PBS. The attraction between the positive and negative charges eventually leads to a better protein adsorption effect on the PLGA/GO-TH film. The isoelectric points of commonly used nerve-related growth factors, such as IGF, BDNF, and bFGF, are between 9 and 10, which also suggests that PLGA/GO-TH composite materials have the potential to act as a carrier to adsorb nerve-related growth factors to further strengthen its nerve repair effects.

3.4. Cell Survival Rate. Central nervous system damage will cause reactive oxygen species (ROS) overload, and the subsequent oxidative stress reaction will further cause increased cell death in the injured area, which is the main reason for the failure of nerve repair after injury [31, 32]. Therefore, the neuroprotective effects of implanted materials against oxidative stress are one of the indicators for the evaluation of their potential for neural application. To verify whether the composites can provide neuroprotective effects on surface-grown NSCs, we observed the survival rate of NSCs on the surface of each group after H_2O_2 treatment. The results showed that there was no significant difference in the survival rate of NSCs in the glass, PLGA, and PLGA/GO groups. Although there was no significant difference between the PLGA/GO-TH, glass, and PLGA/GO groups, the survival rate of the PLGA/GO-TH group was significantly higher than that of the PLGA group ($p < 0.05$, Figure 5).

It was found that L-theanine can upregulate the expression of the antiapoptotic protein Bcl-2 and decrease the level of caspase-3 by through inhibition of the ERK and JNK pathways, thereby reversing the large amount of ROS produced by mitochondrial dysfunction [33, 34]. Central nervous system damage will cause reactive oxygen species (ROS) overload, and the subsequent oxidative stress reaction will further cause apoptosis and cell death. The mechanism of the antioxidant effect of L-theanine was mainly related to reversing the ROS overload [35, 36]. Furthermore, studies have confirmed that L-theanine also plays a positive role in the fight against oxidative stress in various cells, such as hepatocytes, neurons, and cardiomyocytes [34, 37]. In the present study, H_2O_2 was used to establish the oxidative damage cell microenvironment, and the survival of NSCs on different materials was observed. The results showed that there was no significant difference between the PLGA group and the PLGA/GO group, indicating that the addition of GO alone did not improve the survival rate of NSCs. The survival rate of NSCs in the PLGA/GO-TH group was significantly higher than that in the PLGA group, indicating that the addition of L-theanine on the composite film effectively increased the survival rate of NSCs, which further confirmed that L-theanine not only can resist neurons and cardiomyocytes but also had an antioxidant neuroprotective function in NSCs; this outcome further provides a basis

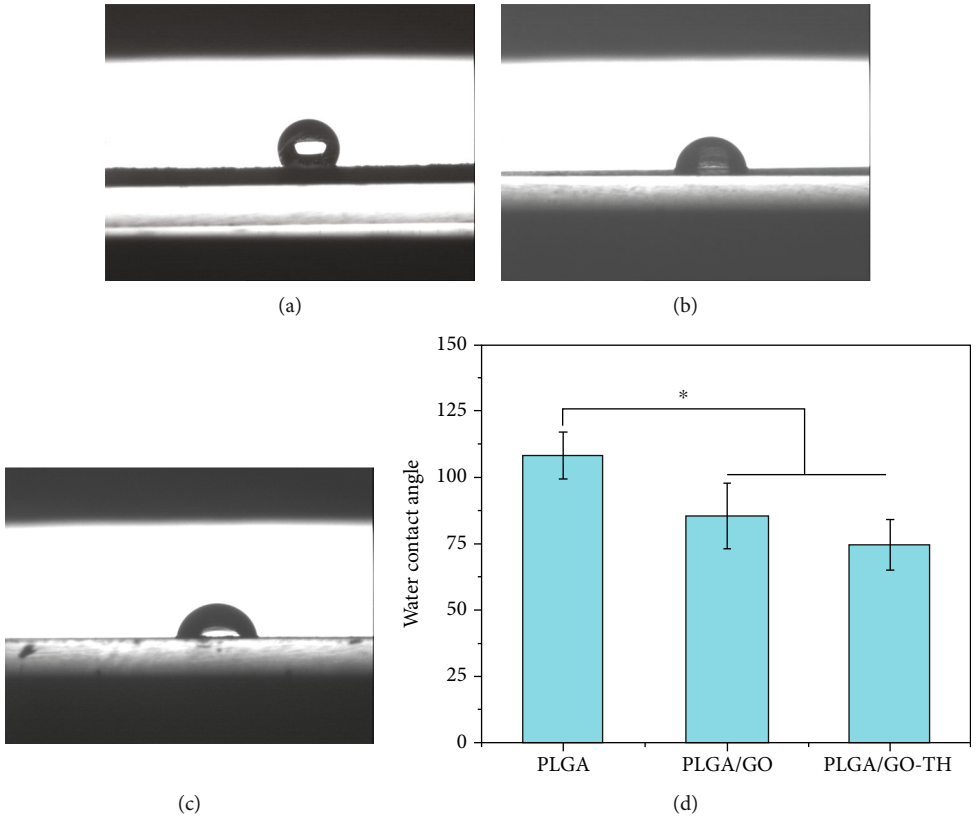

Figure 2: Water contact angle of pure PLGA film (a), PLGA/GO film (b), and PLGA/GO-TH film (c). (d) Average contact angle of PLGA film, PLGA/GO film, and PLGA/GO-TH film ($*p < 0.05$, $n = 3$).

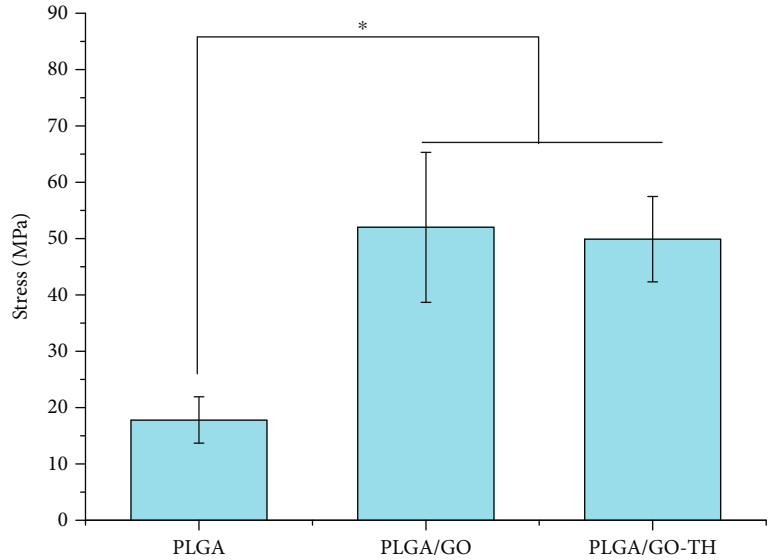

Figure 3: Mechanical properties of PLGA film, PLGA/GO film, and PLGA/GO-TH film. ($*p < 0.05$, $n = 3$).

for the in vivo application of PLGA/GO-TH composite film in nerve injury. In addition, our results suggested that there was no significant difference between the survival rates of the PLGA/GO-TH and PLGA/GO groups. A previous study indicated that the antioxidative effects of L-theanine on NSCs may be dose-dependent [37]. Therefore, we speculated that the amount of L-theanine grafting on the surface of the modified GO may be limited, which may not be sufficient for the NSCs to effectively survive under H_2O_2 treatment.

3.5. Proliferation of NSCs on the PLGA/GO-TH Film. We found the cell proliferation in different composite film

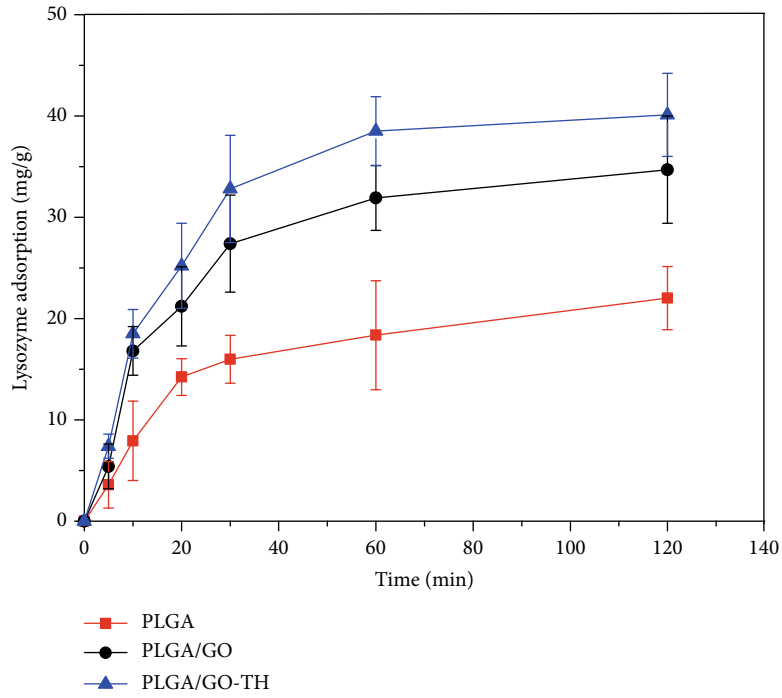

Figure 4: Lysozyme adsorption capacity of the PLGA film, PLGA/GO film, and PLGA/GO-TH film.

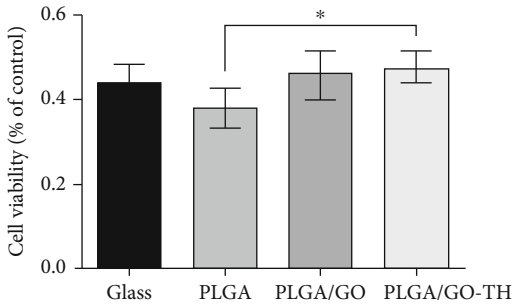

Figure 5: The NSC survival rate of the different groups ($*p < 0.05$, $n = 3$).

groups and a glass group as a control on days 1, 4, and 7. As shown in Figure 6, the results showed no significant difference in the OD values of the 4 groups on the first day. The OD values of the PLGA/GO-TH and PLGA/GO group were significantly higher than those of the other two groups on the 4th day. The OD values of the PLGA/GO-TH group were the highest among the four groups at 7 days ($p < 0.05$, Figure 6). There was no significant difference between the OD values of the glass and PLGA groups on the 7th day, both of which were significantly lower than that in the PLGA/GO and PLGA/GO-TH groups.

Our results show that the proliferation of NSCs on the surface of the PLGA film was lower than that on the composite films containing GO, possibly because PLGA itself is hydrophobic and not conducive to the adhesion of NSCs [38]. After the addition of GO, the hydrophilicity of the GO-based material was obviously improved, subsequently promoting the adhesion and growth of cells. Moreover, GO can adsorb proteins or cells through its specific chemical groups and hydrogen bonds; although, the proliferative effect of GO was not obvious over a short time based on our results. However, on the 4th and 7th day, the composite material mixed with GO still promoted NSC more proliferation than the pure PLGA material. We also found that the number of NSCs grown on the PLGA/GO-TH composite film was significantly higher than that on the PLGA/GO composite film on the 4th and 7th days. Amino acids are the basic substances for constituting proteins and are also well-known for ensuring the normal physiological activities of organisms, and playing a specific role in cell adhesion, proliferation, and differentiation [39–41]. Current studies have found that some amino acids can promote the proliferation of NSCs, such as serine and glutamate [42, 43]. L-Theanine is considered safe for humans as no toxic effects have been reported so far, though the regulation for its ingestion varies among countries. Because l-theanine is the precursor of glutamate, it can be used as an additional source of glutamate in the body, which can be integrated into the cytoplasm and stimulate the glycolysis process of cells and tissues [44]. This is one reason why l-theanine can improve cell proliferation. The results of this study further confirmed the proliferative effects of the PLGA/GO-TH composite film on NSCs and indicated that the introduction of L-theanine could have a positive effect on the proliferation of NSCs; these results are consistent with those from the previous study [17].

3.6. Differentiation of NSCs on the PLGA/GO-TH Film. To further explore the effect of PLGA/GO-TH composite film on the differentiation of NSCs, tuj-1 was selected as a specific expression gene of neurons, and GFAP was selected as a specific expression gene of astrocytes, which underwent quantitative and qualitative analysis using PCR and

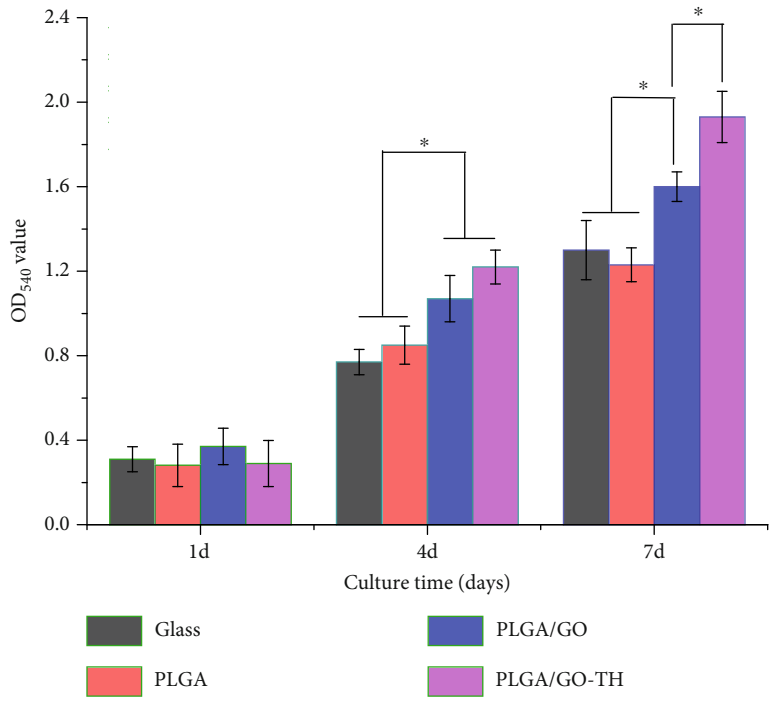

Figure 6: Cell proliferation of the glass, PLGA, PLGA/GO, and PLGA/GO-TH groups at 1, 4, and 7 days in vitro. ($*p < 0.05$, $n = 3$).

immunofluorescence, respectively. The PCR results showed that there was no significant difference in the expression level of tuj-1 between the glass control group and the PLGA group, both of which were significantly lower than that of the PLGA/GO group. The expression of tuj-1 in the PLGA/GO-TH group was higher than that in the rest groups ($p < 0.05$, Figure 7(a)). The GFAP expression levels in the glass control group and the PLGA group were significantly higher than those in the PLGA/GO and PLGA/GO-TH groups ($p < 0.05$, Figure 7(b)), and there was no significant difference in GFAP expression between the PLGA/GO group and the PLGA/GO-TH group. As shown is Figure 8, the immunofluorescence analysis showed that the neurons and astrocytes derived from cultured NSCs spread on the surface of different materials, and the change in the trend of the number of positive cells in each group was basically corresponding to the PCR results.

Our results indicated GO composites have a positive effect on the neuronal differentiation of NSCs, which is consistent with previous studies [8, 9]. Studies have shown that GO-based materials have electrical properties related to ion channel opening and signal transmission between neurons, so they can spontaneously initiate neuronal activity [45, 46]. Considering that the physiological activities of neurons are closely related to bioelectricity, GO-based composite biomaterials have unique advantages in the field of neural tissue engineering. Some studies have suggested that the unique structure of GO nanocomposites increases the contact and communication between cells and the interaction between cells and matrix materials, which may also have a positive effect on the growth and neuronal differentiation of NSCs [47]. After the modification of GO by L-theanine, we found that the new PLGA/GO-TH composite film further promotes the differentiation of NSCs into neurons. A previous study demonstrated that L-theanine can effectively induce NSCs to differentiate into neurons and relatively inhibit their differentiation into astrocytes, which may be due to the upregulation of Slc38a1 gene expression by L-theanine to activate the mTOR signalling pathway [17]. This study further demonstrated that the GO-based composite film after the grafting of L-theanine facilitated NSC neuronal differentiation. For central nervous system injury, NSCs should be induced to differentiate into neurons as much as possible while avoiding their differentiation into astrocytes [48–50]. How to find a solution to induce the NSC neuronal differentiation rate in the injured site has always been a problem in the process of nerve repair, and the PLGA/GO-TH composite materials produced in this study will create a new way to solve this problem. However, the long-term effects of PLGA/GO-TH composite materials in the organism should be taken into account when they were applied in nerve repair. The degradation product of PLGA is acidic, which may cause an inflammatory response in the surrounding tissues. Furthermore, the optimum range of water contact angles for cell culture substrates is between 5° and 40°, and the proliferation rate of cells can be improved if they grow on the materials with such a water contact angle. The hydrophilicity of PLGA/GO-TH composite materials is still relatively limited and therefore cannot maximize cell adhesion and proliferation. In the future work, we will further study and solve these problems.

4. Conclusion

In summary, we first successfully synthesized a PLGA/GO-TH composite film and then determined its characterization. We found that the PLGA/GO-TH composite film had a

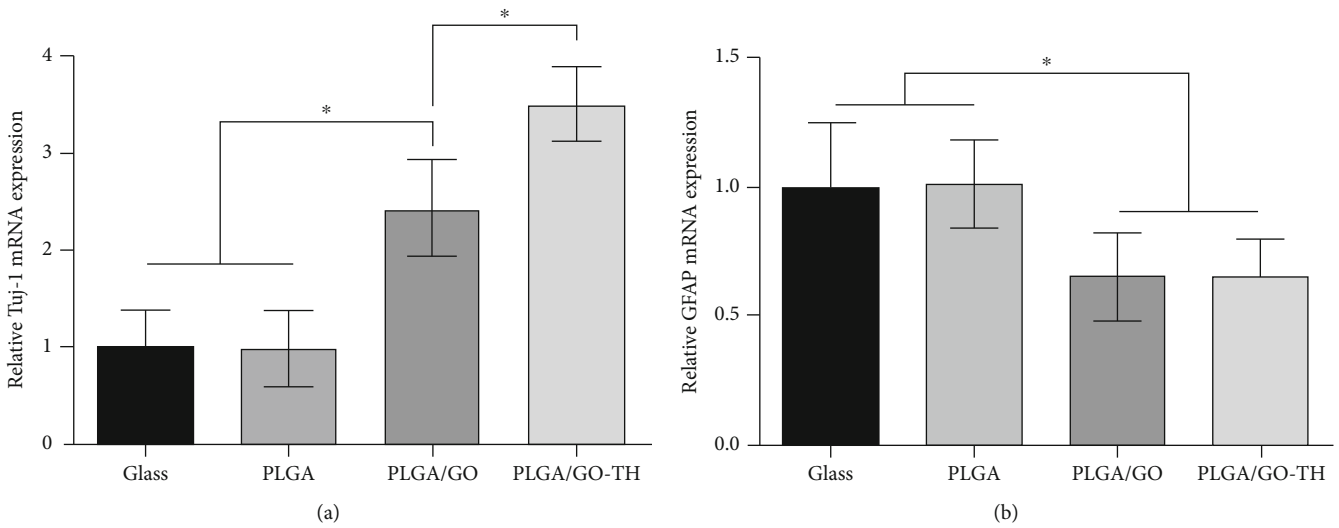

Figure 7: PCR analysis of NSCs cultured for 7 days. (a). Tuj-1. (b).GFAP.

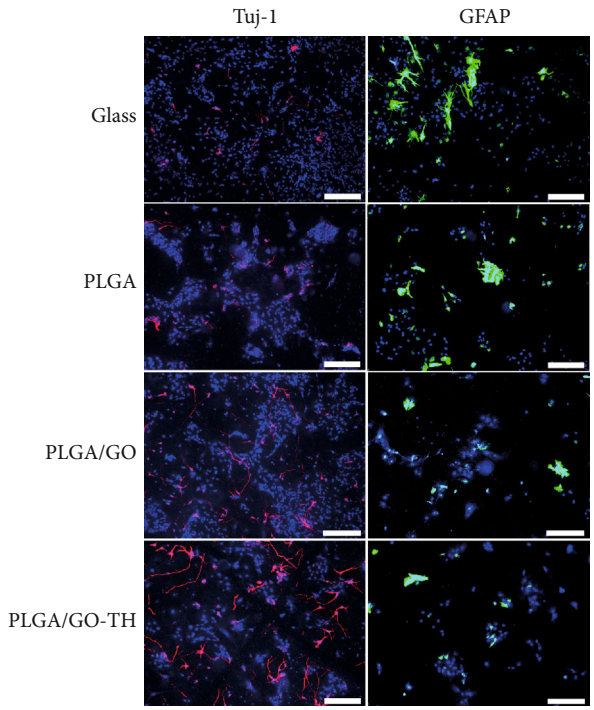

Figure 8: Immunofluorescence staining of NSCs cultured for 7 days under differentiation condition. The markers are shown in red for Tuj-1, green for GFAP, and blue for DAPI. All scale bar lengths are 200 μm.

satisfactory physical property and protein adsorption capability. Furthermore, our results indicated that the PLGA/GO-TH composite film can effectively enhance the survival of NSCs in the oxidative damage environment and have a clear promotion effect on the cell proliferation and neuron differentiation. However, the present study only focused on cell experiments. The application of the PLGA/GO-TH material in vivo should be further evaluated. In addition, the molecular mechanism of PLGA/GO-TH promotion of the proliferation and differentiation of NSCs into neurons should also be explored. Our findings revealed that the novel modified composite biomaterial PLGA/GO-TH has great potential as a candidate implant for nerve repair.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

There are no conflicts of interest to declare.

Acknowledgments

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