

Review Article

Effects of Graphene-Based Materials on the Behavior of Neural Stem Cells

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Neural tissue engineering is a research field aimed at rebuilding neurological defects resulting from severe trauma, vascular impairment, syringomyelia, spinal stenosis, malignant and benign tumors, or transverse myelitis. Of particular interest, neural stem cells (NSCs) and the effective differentiation and proliferation thereof are attractive research areas that have yielded widespread utility for implants or neural scaffold materials. Graphene and its derivatives have more effective and efficient physical, chemical, and biological abilities than other nanomaterials, and may act as new coating materials to promote neuronal proliferation and differentiation. Therefore, here, we review the recent progress of studies that examine the effect of graphene-based materials on NSCs. We specifically review how graphene and its derivatives influence NSC adhesion, differentiation, and proliferation. We also discuss the risks of graphene-based materials, including their anti-inflammatory effects, in the realm of neural tissue engineering as well as current challenges facing the field today.

1. Introduction

Spinal cord injury (SCI) is a debilitating and devastating physical dysfunction that is considered a major global issue for people of all ages [1]. To date, the repair of the central nervous system (CNS) after SCI remains a major medical challenge [2, 3], and thus the treatment and rehabilitation paradigms for SCI patients are a major priority for the international medical community. The application of neural precursor or stem cell transplantation to achieve a neuronal substitute effect is the primary strategy to solve the lacking regenerative capability of mature central nervous cells after SCI [4–7].

As multipotent cells in the CNS, neural stem cells (NSCs) can be self-renewing and play promising roles in the development of cell therapy for neural regeneration [8, 9]. However, transplanted cells cannot easily survive and play functional roles in damaged areas due to the often inhospitable microenvironment [10, 11]. Additionally, the delivery of NSCs also poses a problem because the effective transplantation requires a scaffold to enable cell adhesion and cell function

maintenance [12–14]. Along these same lines, suitable materials for guiding adhesion, proliferation, and differentiation of the NSCs recently have also become important for neural tissue regeneration.

Graphene has grown in popularity for stem cell-related applications due to its fascinating physico-chemical characteristics [15–18] that include excellent thermal, optical, electronic, magnetic, and mechanical properties [19–22]. Graphene can also be utilized as a biocompatible and arbitrary scaffold with single-atom thickness to facilitate stem cell adhesion, proliferation, and differentiation [23]. Although studies have been extensively performed on graphene in the fields of chemistry, physics, materials, biology, and interdisciplinary science, its applications in the field of neural regenerative medicine has not yet been widely considered. Nevertheless, rapid advances in the field of neuroscience have allowed graphene to be used as a neural tissue engineering material [24–26]; however, the potential neuroinflammatory effects of graphene are rarely reported and must be clearly identified before clinical applications.

Here, a brief review is provided, which describes the properties of graphene and its derivative materials as well as their toxicity and biocompatibility. We then discuss the recent progress regarding the effect of graphene and its derivative materials on NSCs in detail. Finally, according to the existing literature, we also discuss the anti-inflammatory effect of graphene on nerve tissue engineering.

1.1. Structure, Properties, and Preparation Methods of Graphene and Its Derivatives. Graphene has attracted increasing attention from the scientific community since its development [27]. As one of the thinnest 2D materials (only 0.35 nm thick), graphene is composed of a single layer of carbon atoms that are sp² hybridized [28]. To date, graphene has been crimped to form one-dimensional carbon nanotubes, warped to give zero-dimensional fullerenes, or stacked to prepare three-dimensional graphite [29].

The ultra-high strength of graphene can reach 130 GPa, which is 100 times higher than that of steel, and its outstanding thermal conductivity is nearly 5000 J m⁻¹ K⁻¹ s⁻¹ and three times higher than that of diamond. The rate of charge carrier mobility is as high as 1.5 × 10⁴ cm² V⁻¹ s⁻¹, which is 2 and 10 times higher than that of antimony indium and commercial silicon, respectively. Moreover, under certain conditions, the charge carrier mobility of graphene can be as high as 2.5 × 10⁵ cm² V⁻¹ s⁻¹. Graphene, thus, can act as an impermeable barrier that can support a pressure difference higher than 1 atm [27, 30]. Graphene can also absorb approximately 2.3 times of visible light and, thus, is slightly visible to the naked eye [31]. Furthermore, the high surface area of graphene, which is close to 2600 m² g⁻¹, makes it an attractive platform for anchoring large quantities of molecules [32]. Finally, as one of the most stretchable crystals, graphene can be deformed in the direction normal to its surface as well. All of these features make graphene well-suited for applications in mechanical, electrical, and optical industries [33, 34].

Functionalized graphene that contains many reactive groups is mainly used in biomedicine [35–37]. To aid in this process, defects can be created on the surface of graphene to reduce the hydrophobic interaction between graphene and cells/tissues. In this form, graphene becomes water-soluble and biocompatible and can then be widely used in various biomedical fields [38].

Graphene can be prepared using two primary methods, one physical and the other chemical [39–41]. Raw materials can easily be obtained for the physical method as simple graphite or expanded graphite can generally be used [42]. Graphene prepared via the physical method usually exhibits a large planar structure, high purity, and few defects. However, preparation at a large scale is challenging due to the low percentage yield and long production cycle. As for the chemical method, graphite or expanded graphite is directly added to an organic solvent or water with the aid of ultrasound, heating, or air flow [43–45]. Single or multilayer graphene solutions can be prepared this way. Other chemical methods include chemical vapor deposition (CVD) [46], crystal epitaxial growth [47], oxidation-reduction (including oxidation-modification-reduction) [48], and so on [49].

Graphene and its composite materials have caused the development of a broad set of “graphene-family nanomaterials” (GFNs), including graphene oxide (GO) [50, 51], reduced graphene oxide (rGO) [52–54], ultrathin graphite [55], and graphene nanosheets (GNS) [56]. Among them, GO sheets are separated using potassium permanganate and sulfuric acid to treat the graphite [57]. GO consists of graphene sheets with single-atom thickness and is a highly oxidized form of chemically modified graphene [58, 59], which is also full of polar reactive groups. Specifically, peripheral carboxylate groups can offer colloidal stability and pH-dependent negative surface charge [60]. Functionalized GO has a different wettability and large aromatic (π -configuration) interface and accordingly can interact with biological components through chemical bonding or physical absorption. For example, thiolated DNA-coated GO was used for the uniform and stable assembly of gold (Au)/carbon structures [61]. Graphene and GO have also been chemically modified by proteins or peptides, including avidin or diphenylalanine peptides, for similar effects and applications [62–64].

As an additional step, rGO can be prepared through the reduction of GO using thermal, chemical, or UV exposure techniques. For example, hydrazine was used to treat GO at 100°C to produce rGO [65] with a small number of oxygen on the surface. Because hydrazine was eliminated and pyrolyzed, the water solubility of rGO was reduced, which generated the hydrophobic feature [66]. The ascorbic acid reduction method is more biocompatible than the hydrazine reduction method because hydrazine is highly toxic and not suitable for biomedical applications. [67]. Cells that are cultured on a collagen scaffold coated with rGO and produced using ascorbic acid present high cell viability and nontoxicity [68]. One key feature of rGO is that it can exhibit high electron mobility (N400 S cm⁻¹) [69] that makes it a useful candidate for bio-nanointerface engineering with muscular and neural tissues.

Compared with other carbon-based materials, graphene and related materials have larger specific surface areas, which are suitable for the adhesion of cells and drug therapeutic carrier molecules. In addition, characteristics such as π - π stacking and electrostatic interactions contribute to efficient drug loading of partially dissolved drugs and make these materials suitable for drug delivery, gene therapy, and anticancer therapy. Another advantage of graphene and related materials is the ease of surface functional modification, including surface covalent and noncovalent modifications. Liu et al. [70] introduced covalent and noncovalent functionalization techniques in detail. The modification of noncovalent functional groups improved the dispersibility, biocompatibility, reactivity, and binding and sensing ability of these materials. For example, the formation of hydrogen bonds between GO surface-modified polar functional groups and water molecules to prepare GO colloidal suspensions is suitable for some potential biomedical applications [71], which is also the main advantage of GO over traditional carbon-based materials.

In tissue engineering, traditional regenerative transplant materials have limitations in repairing damage caused by

infections, tumors, and deformities. For example, materials such as hydrogels lack cell adhesion and have poor mechanical properties [72]. Calcium phosphate (CAP), calcium silicate (CS), and other materials lack tissue-inducing activity and delay the healing of functional modifiers. In addition, the biocompatibility, toxicity, and anticoagulation properties of implant materials are other important reasons that limit their application in tissue engineering [73]. Graphene and related materials have high mechanical strength, high specific surface area, and low density compared to other nanomaterials. Their uniquely high elasticity, flexibility, and adaptability to multisurface morphology make them suitable for structural enhancement of tissue engineering materials and improve tissue adhesion, differentiation, and cell function. The three-dimensional graphene scaffold material can better simulate the extracellular matrix environment and is more suitable for tissue regeneration. The advantages of this material include the following: (1) Three-dimensional graphene has a lower oxidative stress level and edge damage, and therefore exhibits higher biocompatibility; (2) These materials have higher specific surface areas, not only for enhancing the adhesion of cells, proteins, and nutrients but also for promoting the transport of cytokines, chemokines, and growth factors; (3) Graphene nanomaterials have more suitable mechanical properties and can imitate the extracellular matrix; (4) These materials also exhibit excellent electrical conductivity and can promote cell adhesion and migration, which is important for neural stem cell differentiation [74].

In the study of neural tissue, graphene can be used as a neural interface material, which can promote the growth of mouse hippocampal neuron protrusions, and neural stem cell differentiation to obtain a neural network. Although cell adhesion is improved in these cases, the mechanism is not clear [75]. Further research by Yang et al. found that only GO nanoparticles can significantly promote the differentiation of embryonic stem cells into dopaminergic neurons, while carbon nanotubes and graphene nanoparticles do not promote the differentiation of dopaminergic neurons [76].

1.2. Toxicity and Biocompatibility of Graphene and Its Derivatives. Current work on the toxicity and biocompatibility of graphene and its derivatives are usually contradictory and uncertain [77–79]. As such, there is not yet a unified conclusion regarding the potential negative side effects and hazards of graphene-based materials on both humans and environment [80]. In addition to brains, these tiny nanosized particles can easily enter the body via other routes and freely move as small molecules, which can quickly distribute into and infiltrate various organs, tissue, and cell systems [81–84]. Some studies have found that nanomaterials can also penetrate the blood-brain barrier (BBB) [85, 86] and indirectly reach brains. Kreuter found that the intravenous injection of polysorbate-80 coated doxorubicin nanoparticles penetrated the rat BBB after being engulfed by brain capillary endothelial cells [87]. Oberdorster et al. also confirmed that nanomaterials can enter the CNS through the olfactory nerve pathway or by being directly transported to the brain through sensory nerve endings [88]. Nanoparticles that enter into the nervous system can activate microglia to produce reactive

oxygen species (ROs), oxidative stress, inflammatory response, and neurotoxicity [89]. Nervous tissue, thus, can be significantly damaged and may or may not manifest as behavioral or neurological symptoms in the host.

In vitro studies show that cell viability was reduced when cells were exposed to graphene and its derivatives at a concentration of about $10 \mu\text{g mL}^{-1}$ [90–93]. ROS levels were increased by enhancing the activity of caspase-3 (apoptotic marker), and that cell metabolic activity was decreased when PC12 cells were exposed to a graphene solution ($0.1 \mu\text{g mL}^{-1}$) [94]. Nevertheless, the release of the lactate dehydrogenase enzyme during membrane damage was not increased when graphene concentrations were between 0.01 and $0.1 \mu\text{g mL}^{-1}$. Liao and coworkers further reported that dense graphene sheets induced high levels of ROS production in human skin fibroblasts [91]. The apoptosis and level of lactate dehydrogenase can be increased by graphene prepared using CVD, and ROSs in neural cells can be generated effectively [95]. Ren et al. reported that GO at a concentration of $50 \mu\text{g mL}^{-1}$ brought evident cytotoxicity in fibroblasts as well [96]. In general, ROS production seems to be a common trend related to graphene and GO experiments.

Despite the negative side effects, graphene-related materials also demonstrate promising beneficial effects to an array of cell types that are arguably more applicable to human biomedical therapies. Mesenchymal stem cells' (MSCs) adhesion, proliferation, and differentiation can be accelerated by graphene- and GO-coated substrates as reported by Lee et al. [97]. Morphological changes as well as cell enlargement and spread were found to occur for human adenocarcinoma HT-29 cells cultured on glass slides coated with GO [23] that has been proven to be a good candidate for cell attachment, growth, and proliferation [98].

In addition, graphene and GO can not only provide the culture for mouse induced pluripotent stem cells (iPSCs) but facilitate spontaneous differentiation as well. In comparison with iPSC's cultured on the graphene surface, those that are cultured on the GO surface presented higher rates of adhesion and proliferation [99]. Microbially rGO was also used to treat mouse embryonic fibroblast cells and showed very strong viability. In addition, in comparison with rGO by hydrazine, microbially rGO significantly facilitated the attachment and survival rate of cells grown on plates [100]. Similarly, after treatment with rGO by trimethylamine, mouse embryonic fibroblast cells presented in increased number and with significant attachment [101]. In mouse embryonic fibroblast cells, ALP activity can be enhanced by GO that was initially reduced by spinach leaf extract [100]. Similarly, compared with standard GO, significant biocompatibility was found for rGO that significantly increased the ALP activity in human breast cancer cells [102]. Conversely, GO effectively promoted the dopamine neuron-related gene expression and the dopamine neuron differentiation, in comparison with untreated cells [76].

Aside from *in vitro* work, *in vivo* experiments have studied the biocompatibility of graphene and its derivatives and suggest that they present negligible harm in their interaction with cells, tissues, or bodies [33, 103]. Wang et al. used animal models to demonstrate that the injection of GO can

achieve a dose-dependent accumulation in the lungs and livers over long periods of time, suggesting *in vivo* toxicity [104]. These *in vivo* results have been explored to a larger extent to confirm toxicity in living organisms and characterized across an array of animals.

The functionalization of graphene dominates its toxicity or biocompatibility. Oxidation, for example, can be reduced by the functionalization of graphene with PEG. As determined from histological and hematological analysis, PEGylated graphene (20 mg kg⁻¹) did not present toxic effects in mice after 90 days of treatment [105]. One particularly exciting innovation in this area is the bottom-up approach to synthesize graphene quantum dots (GQDs) functionalized by amine. Such GQDs are superior to traditional quantum dots in terms of stable photoluminescence, chemical inertness, high water solubility, surface grafting, and result in low toxicity (over 90% in 48 h) and high cellular viability over time [106]. Recently, the role of graphene in bone tissue engineering was reviewed by Dubey et al. in detail [107]. Stem cell attachment and growth can be enhanced by graphene-based materials for osteogenic differentiation [107, 108]. In general, graphene-coated materials are nontoxic and can promote the attachment and proliferation of osteoblasts, fibroblasts, and MSCs [23, 109–113]. Graphene can also promote neurite sprouting and outgrowth more efficiently than tissue culture plates made of polystyrene [75]. Where most graphene-based material tests use 2D structures, 3D graphene foam is an important advancement in neural engineering because biocompatible scaffolds are necessary to facilitate NSC proliferation within a tissue volume [25]. Calcein-AM and EthD-I staining assays have been used to evaluate such 3D graphene films (3D-GFs) for cytotoxicity in comparison with 2D graphene film as controls (Figure 1). Nearly 90% of the cells in the 3D-GFs were viable for 5 days. However, there was almost no difference between the 3D-GFs and 2D graphene films for cell viability (lower inset in Figure 1). In addition, abnormal cell apoptosis on 3D-GFs was not found from a TUNEL assay. 3D-GFs thus are biocompatible, which is in agreement with previous studies, and offer the opportunity to treat large volumes of damaged tissue [75, 114, 115].

The quantity, geometry, and exposure time of graphene-based materials are major parameters affecting their toxicity. Among them, surface properties, such as functional groups or chemical structures, are specifically considered to be related to the toxicity of graphene-based materials. The size of compounds can also be controlled in chemical manners to achieve a similar goal. Aside from these physical properties, oxidative debris that may be generated by graphene-based materials can also induce cytotoxicity. The purity of graphene and its derivatives after functionalization, thus, must be considered before biomedical applications. Additionally, standard toxicity validation methods need to be established for clinical use.

1.3. Effect of Graphene of Nerve Cells. Graphene shows great potential in biomedical fields because of the previously mentioned unique properties. Graphene and its derivatives are usually combined with biocompatible polymers to prepare scaffolds for peripheral nerve regeneration. The role of gra-

phene is to improve the chemical and mechanical properties as well as the conductivity and hydrophilicity of the scaffolds.

Nano graphene oxide (NGO) was mixed into chitosan hydrogels to change their pore structures and improve mechanical strength. By doing so, the growth of nerve cells can be improved by up to 20% [116]. Additionally, an oligo polyethylene glycol fumarate (OPF) hydrogel was mixed with GO with cross-linkable bonds [117]. The chemical cross-linking was achieved with the functionalized graphene and OPF hydrogel before the *in situ* reduction. This technique improved the electrical conductivity and positive charge compared to the embedded graphene-influenced nerve cells. Furthermore, a small composition of acrylamide, sodium methacrylate, 2-methacryloxyethyl trimethyl ammonium chloride, and 2-sulfoethyl methacrylate was introduced into the network to enhance nerve cell activities [118]. The addition of carbon nanotube polyethylene glycol acrylate further facilitated the proliferation and spread of PC12 cells on the composite hydrogel [119]. Similarly, an electric field was used to align the orientation of silk fibroin and graphene in hydrogels to tune the adhesion, proliferation, differentiation, and extension of different nerve-related cells [120].

In addition to hydrogels, fibrous scaffolds with improved toughness and electrical conductivity via the addition of graphene were improved for nerve tissue engineering [121]. The attachment and spreading of PC12 cells were improved on a hybrid graphene/sodium alginate/polyvinyl alcohol scaffold because of its superior electrical and mechanical properties [121]. Graphene was also coated onto aligned and aminolyzed poly-L-lactide nanofibrous scaffolds to increase their roughness, resulting in the significantly improved proliferation of Schwann cells [122].

1.4. Effects of Graphene on the Adhesion of Neural Stem Cells.

Adhesion determines how cells proliferate, synthesize proteins, and form mineral deposits. NSCs can proliferate and differentiate into various cell types if they are cultured onto specific substrates under suitable conditions. Therefore, there is a need to achieve satisfactory adhesion of NSCs to different substrates for the proper spreading, proliferation, and maintenance of cellular function.

Graphene has been introduced into polymeric materials such as poly(L-lysine) (PLL) to significantly reduce the electrical resistance and the outgrowth of neurons and to stimulate adhesion [123]. As previously mentioned, NSCs can adhere very well to 3D-GFs [25]; in the culture medium of the 3D-GF, nearly no free-floating cells were found after cell seeding for 10 h. After culturing for 5 additional days (and even as long as 2 weeks), an extensive network of NSCs with strong filopodia/GF interactions was formed on these 3D-GFs, exhibiting very strong cell adhesion (Figures 2(a) and 2(b)). Furthermore, the cross-sectional fluorescence imaging of the 3D-GF scaffold clearly showed that cells were found both in the scaffold and on the surface, which allowed the 3D growth patterns of the cells to be determined. This strong cell adhesion to 3D-GFs has been confirmed for NSCs and human MSCs and is attributed to the unique surface properties of graphene materials [110, 114, 115]. It has also been suggested that the mechanical interlocking of the 3D-GFs

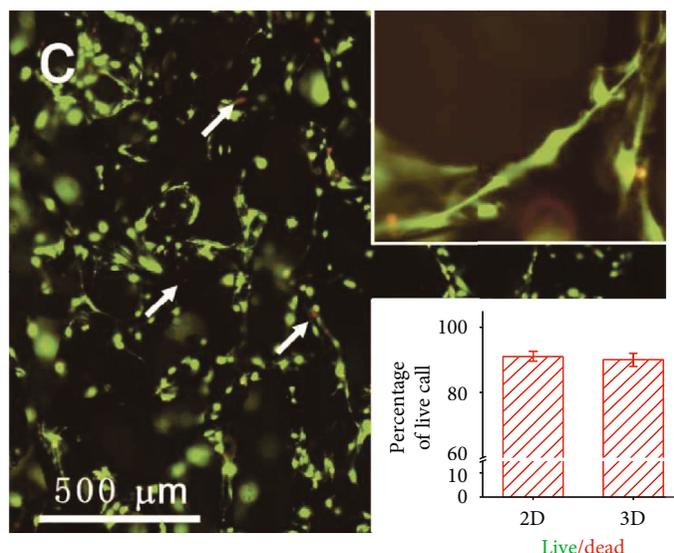


FIGURE 1: Cell viability of NSCs on 3D-GFs after 5 days of culture. Alive and dead cells are depicted in green and red, respectively. White arrows show the examples of dead cells. The lower right inset indicates the percentages of live cells on 2D and 3D graphene films. The figure was reproduced from Ref. [25] with permission.

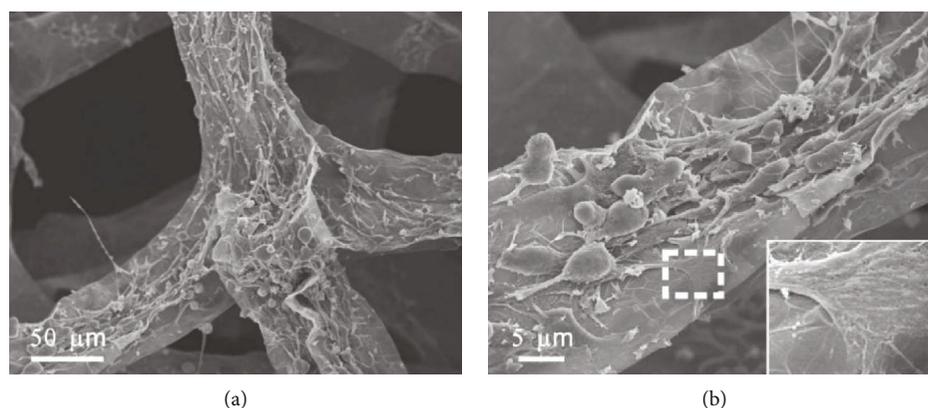


FIGURE 2: SEM images of NSCs cultured on three-dimensional graphene films (3D-GF) under a proliferation medium (low- (a) and high- (b) magnifications). The interaction between cell filopodia and the 3D-GF surface is shown in the inset. The figure was reproduced from Ref. [25] with permission.

with cells was improved by the ripples and wrinkles on their surface, subsequently facilitating the adhesion [124]. Furthermore, the porous structure of the 3D-GFs provides additional irregular surfaces and an increased surface area overall for NSC adhesion.

Graphene films have also been reported to be biocompatible neural interface materials [75, 114, 115]. Very recently, graphene coated with peptides offered a cytocompatible substrate for the adhesion and spreading of retinal ganglion cells [125]. The biocompatibility of graphene substrates for primary cultures of mouse hippocampal neurons was studied as well [75], as shown in Figure 3. These and other studies have shown that graphene can facilitate neurite sprouting and outgrowth in the early developmental stage. Similar to the previous paragraph, substrates with coated or patterned graphene effectively promote the adhesion and neurite outgrowth of PC-12 cells. Compared with a glass coverslip without graphene-coated layers, more cells were found adhered to

one with FBS-covered graphene after incubation for 3 days [126]. Similarly, more hNSCs were observed on graphene than on a pure glass substrate after cell seeding for 10 h (Figure 4), suggesting rapid hNSC attachment [114].

1.5. Effects of Graphene on Proliferation and Differentiation of Neural Stem Cells. NSCs are a widely involved stem cell type in the field of neural tissue regeneration and can differentiate into neurons, oligodendrocytes, and astrocytes. Currently, therapies for neural regeneration have become increasingly attractive; however, inducing human NSCs to differentiate into neurons is still a significant issue [25, 127]. Graphene is a strong candidate for tissue engineering and can be used as a substrate for the differentiation of stem cells or components of implantable devices. To date, limited investigations have been reported regarding the effects of graphene on the proliferation and differentiation of nervous systems. Nevertheless, there are various reports on how other nanomaterials

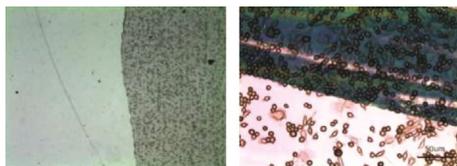


FIGURE 3: Adhesion of neural cells on biomimetic substrates with graphene. The figure was reproduced from Ref. [75] with permission.

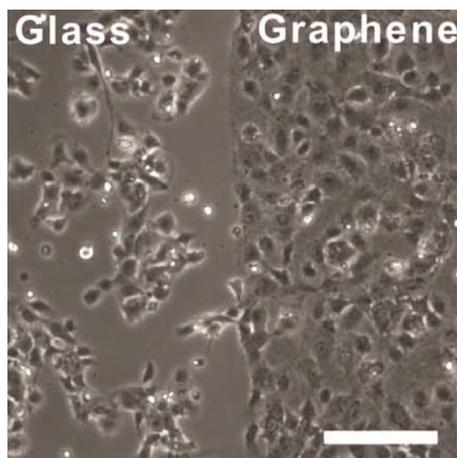


FIGURE 4: A bright-field image of hNSCs at the boundary area between glass (left) and graphene (right) after cell seeding for 10 h. The graphene regions appear slightly dark. The scale bar represents $200\ \mu\text{m}$. The figure was reproduced from Ref. [114] with permission.

such as carbon nanotubes (CNTs) affect the interaction of nervous structures [128].

In one of the few reported cases, a recent study showed that a graphene substrate was used to enhance the differentiation of hNSCs into neurons [114], as shown in Figure 5. Since graphene had a positive interaction with differentiated neurons for electrical stimulation, its unique surface property can facilitate the differentiation of hNSCs into neurons over glia. The effect of GO size on the cellular fate of mouse NSCs was studied [129], and the results showed that the ability of mNSCs to self-renew was improved by GO with a hydrodynamic size of 663 nm. The expression levels of Tuj1 and GFAP were also enhanced when using a GO size of 4651 nm.

Seeding cells on graphene-based materials, such as fluorinated graphene, also appears to be an effective enhancement of neuronal differentiation [115]. As shown in Figure 6, a similar treated graphene material, reduced graphene oxide/titanium oxide (rGO/TiO₂), in the form of heterojunction film was used as a biocompatible stimulator to effectively carry out flash photo stimulation of hNSCs into neurons rather than glia [130]. Stronger proliferation of hNSCs was also found on the GO/TiO₂ than rGO/TiO₂ even though the surface morphologies of the GO and rGO sheets on the TiO₂ layer were similar. Therefore, the chemical composition of graphene sheets, thus, is the dominant factor for facilitating proliferation.

Moreover, arrays of hybrid structures of graphene-nanoparticles have been fabricated by Solanki et al. [131] to create highly aligned axons for adult hNSCs differentiation and growth. In this case, GO was used with positively charged silica nanoparticles to create arrays of graphene-nanoparticle hybrids as substrates for human NSCs. The presence of the underlying silica nanoparticle monolayer increased the average length of axons of hNSCs that differentiated on the SiNP-GO material. The alignment of axons, thus, appears to be exclusively caused by the presence of GO within the ECM instead of the cellular density of the hNSCs.

More recently, graphene-based nanomaterials were used for the design of hybrid nanofibrous scaffolds to guide the differentiation of NSCs into oligodendrocytes [132]. In combination with electrospun nanofibers, GO was coated onto nanofibers for the induced differentiation of NSCs into oligodendrocytes. The effect of the GO coating on NSC differentiation was studied systematically based on hybrid scaffolds having GO coatings of varying concentrations. Where the coating with a high concentration of GO promoted differentiation into mature oligodendrocytes, the change that depended on GO concentration was observed in the expression of key neural markers. Furthermore, oligodendrocyte differentiation may be promoted by the GO-coating on nanofiber scaffolds through specific microenvironmental interactions that activate intracellular signaling related to integrin. The previously described 3D-GFs also act as a robust scaffold for in vitro NSC culture [25]. These 3D-GFs are prepared using a vapor deposition method based on a nickel foam template and were found to strongly support NSC growth [133]. Additionally, in comparison with 2D graphene films, cells on 3D-GFs remained at a more active proliferation state with the upregulation of Ki67 expression. 3D-GFs can also facilitate the NSC differentiation toward astrocytes, especially neurons, and provide a more efficient conductive platform than the standard 2D graphene structure for electrical stimulation of differentiated NSCs [134]. Another report by Hong et al. showed that neural differentiation can be promoted by graphene-based substrates [126]. This group demonstrated that cells on a bare glass coverslip proliferated worse than those on a glass coverslip having FBS-covered graphene. These results indicate that a specific surface property related to graphene-patterned substrates provides biomimetic cues for promoting neural cell differentiation.

The induction of dopaminergic neural differentiation by graphene, GO, and carbon nanotubes for mouse embryonic stem cells (ESCs) has been investigated by Yang et al. [76]. After the induction of SDIA, GO effectively promoted dopaminergic neuron differentiation in a dose-dependent manner. The gene expression related to dopaminergic neurons can be enhanced with CNTs and graphene compared to control cells. GO's unique properties may possibly contribute to the mechanism of dopaminergic neuron differentiation. On a separate note, PA6 cells greatly interact with ESCs through covalent connections, hydrogen bonding, and electrostatic force, in addition to the adsorption of ascorbic acid. To date, limited reports have focused on the formation of neuronal

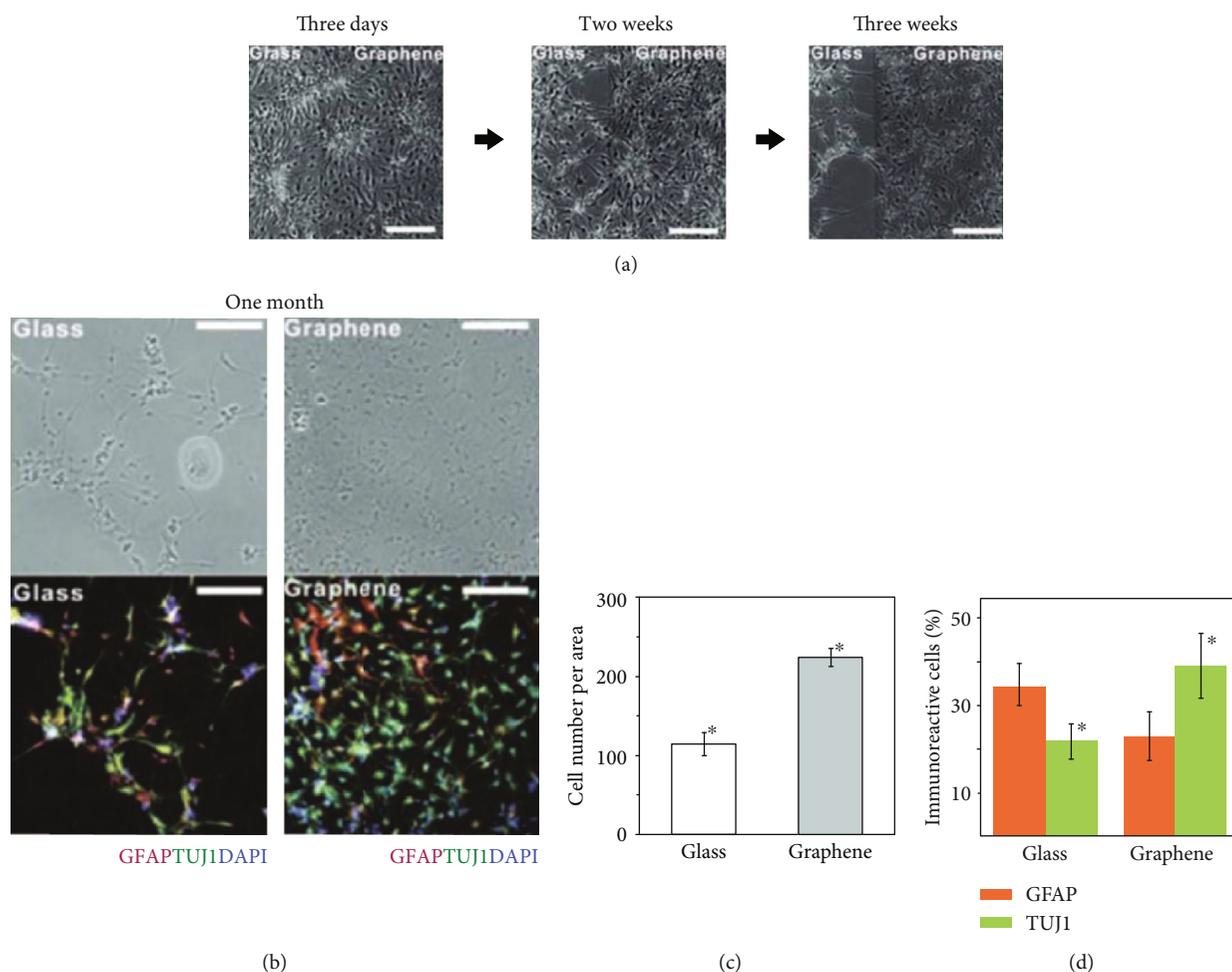


FIGURE 5: Graphene films enhanced neural-differentiation of hNSCs: (a) Bright-field images of hNSCs that were differentiated for three days (left), two weeks (middle), and three weeks (right). (b) Bright-field (top row) and fluorescence (bottom row) images of hNSCs that were differentiated on glass (left) and graphene (right) for one month. TUJ1 (green) was used for the immunostaining of neural cells. GFAP (red) was for astroglial cells, and DAPI (blue) was for nuclei. (c) After differentiation for one month, the numbers of cells per 0.64 mm^2 were counted for the graphene and glass regions. (d) Percentages of immunoreactive cells for TUJ1 (green) and GFAP (red) on glass and graphene. All scale bars represent $200 \mu\text{m}$. The figure was reproduced from Ref [114] with permission.

networks developed by NSC differentiation and its activity on graphene films. However, artificial culturing substrates based on graphene have been developed for understanding how graphene interfaces influence the formation of NSC-differentiated neuronal network formation, network activity, and neural performance [135]. The combination of morphological observations, calcium imaging, and electrophysiological recordings shows that in addition to the support of functional neural circuit growth, graphene can improve electrical signaling and the neural performance of the network as a whole. Recently, similar results have shown that hNSCs on hydrazine-rGO differentiated into neurons more concretely than those on GO films due to the higher electron transfer capability of rGO [130]. In addition, better differentiation was reported on the ginseng-rGO films compared to traditional GO films due to higher hydrophilicity, higher biocompatibility, and the *II-II* attachment of ginsenoside molecules on the surface of rGO sheets [130]. Furthermore, cells on graphene films, which were stimulated by a pulsed laser, facili-

tated the self-organized differentiation of hNSCs into neurons [136]. The transcriptomic profiling of NSC differentiation regulated by 2D graphene was studied using next-generation RNA sequencing. Compared with conventional cell culture, the NSCs on graphene substrates showed greatly enriched and differentially expressed genes [137]. As shown in Figure 7, the healthy adhesion of cells was found on graphene films with extensive spreading. GC-MS based metabolic techniques was used to study the effect of graphene on proliferation and cell fate decision [138]. Amino acid incorporation and glucose metabolism were improved to facilitate NSC growth. Insulin-like growth factor 1 was immobilized on GO-PLGA electrospun nanofibers and NSC survival, proliferation, and differentiation were enhanced by GO [139]. As shown in Figure 8, in comparison with the control group, pretreatment with H_2O_2 greatly decreased cell viability. When GO was used instead, the cell survival rate was increased efficiently. In recent years, an electric field has been used to further promote NSC proliferation and neuronal

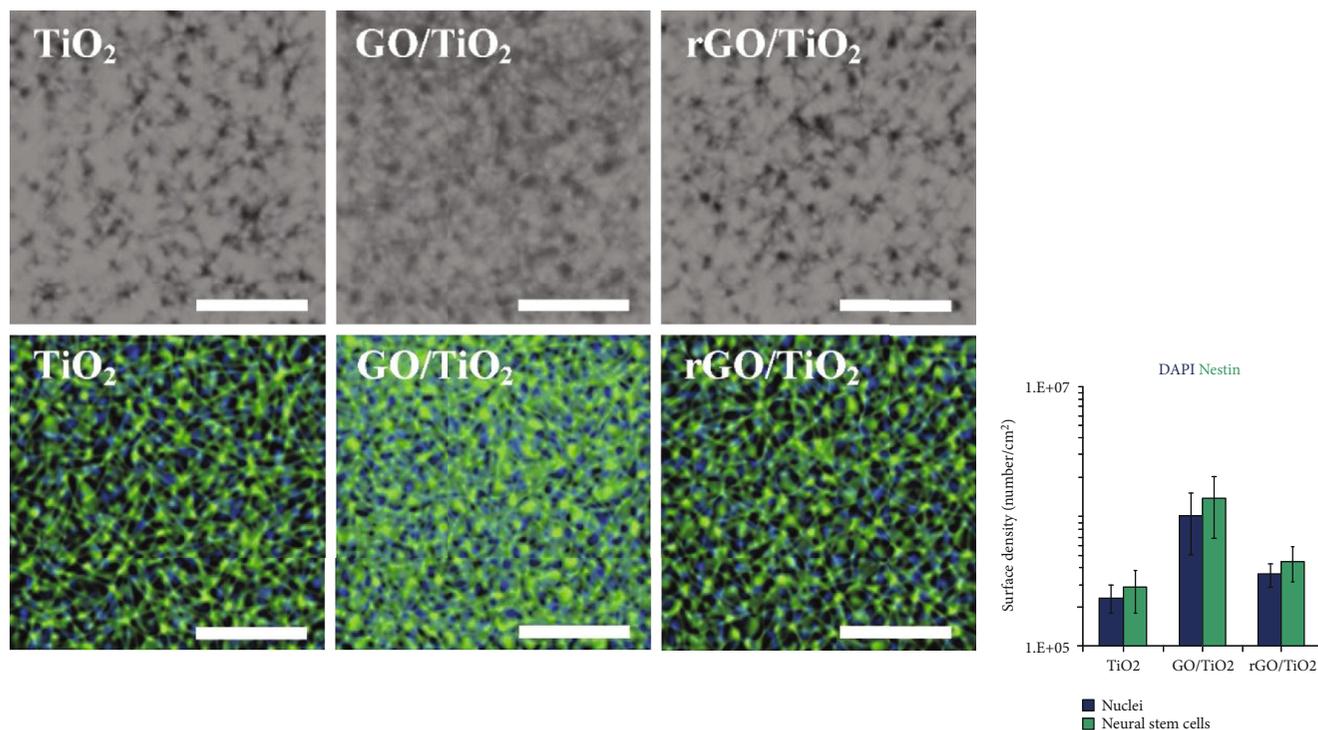


FIGURE 6: Bright-field (upper row) and fluorescence (lower row) images of proliferated hNSCs on TiO₂, GO/TiO₂, and rGO/TiO₂ annealed at 100°C for three days. Nestin (green) and DAPI (blue) are immunostaining markers for neural stem cells and for nuclei, respectively. Surface densities of the neural stem cells and nuclei on different samples were also presented quantitatively through cell counting ($n = 3$, $P < 0.05$). All scale bars represent 200 μm . The figure was reproduced from Ref. [122] with permission.

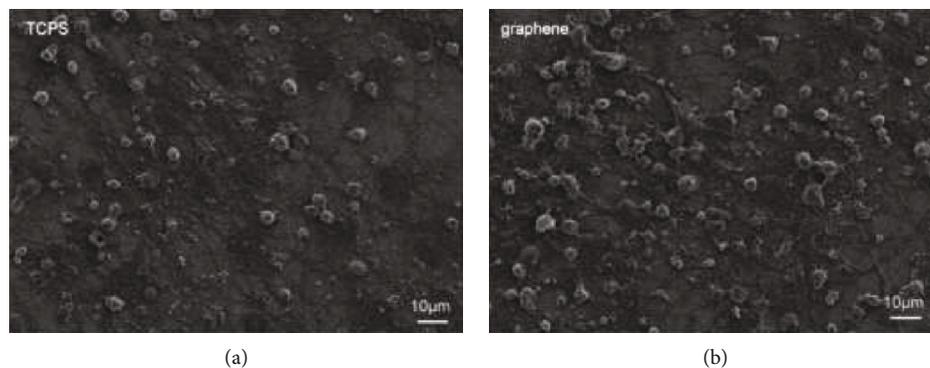


FIGURE 7: SEM images of NSCs grown on a TCP substrate (a) and a graphene substrate (b) for 21 days. This figure was reproduced from Ref. with permission [137].

differentiation of NSCs on PLGA/GO membranes [140]. Besides, the electrical stimuli from an inkjet-printed graphene electrode were applied to trigger the differentiation of MSCs into Schwann cell-like phenotypes [141].

1.6. Antineuroinflammatory Effects of Graphene. Traumatic neural events include ischemia, hypoxia, and a number of bacterial and viral infections and all generally cause characteristic neuroinflammatory reactions. Astrocytes, microglia, and peripheral macrophages play great roles in mediating this response [142] and the development of new materials that cause minimal or no neuroinflammation is a primary objective for the field of nerve tissue engineering. Graphene

has been heavily used as a neural interface material; however, several issues still need to be clarified, i.e., whether graphene can provoke neuroinflammation and how neuroinflammation induction is affected by the topographical features of graphene. The pro- and/or anti-inflammatory responses of microglia in 2D or 3D-graphene culturing systems have been studied by Song et al. [142]. Although similar proinflammatory responses were found in microglia without LPS activation, significantly milder neuroinflammation was caused by 3D graphene films than 2D graphene films after LPS activation. Such inflammatory behaviors may therefore depend on the topographical features of graphene. Furthermore, the morphological transformation of microglia under

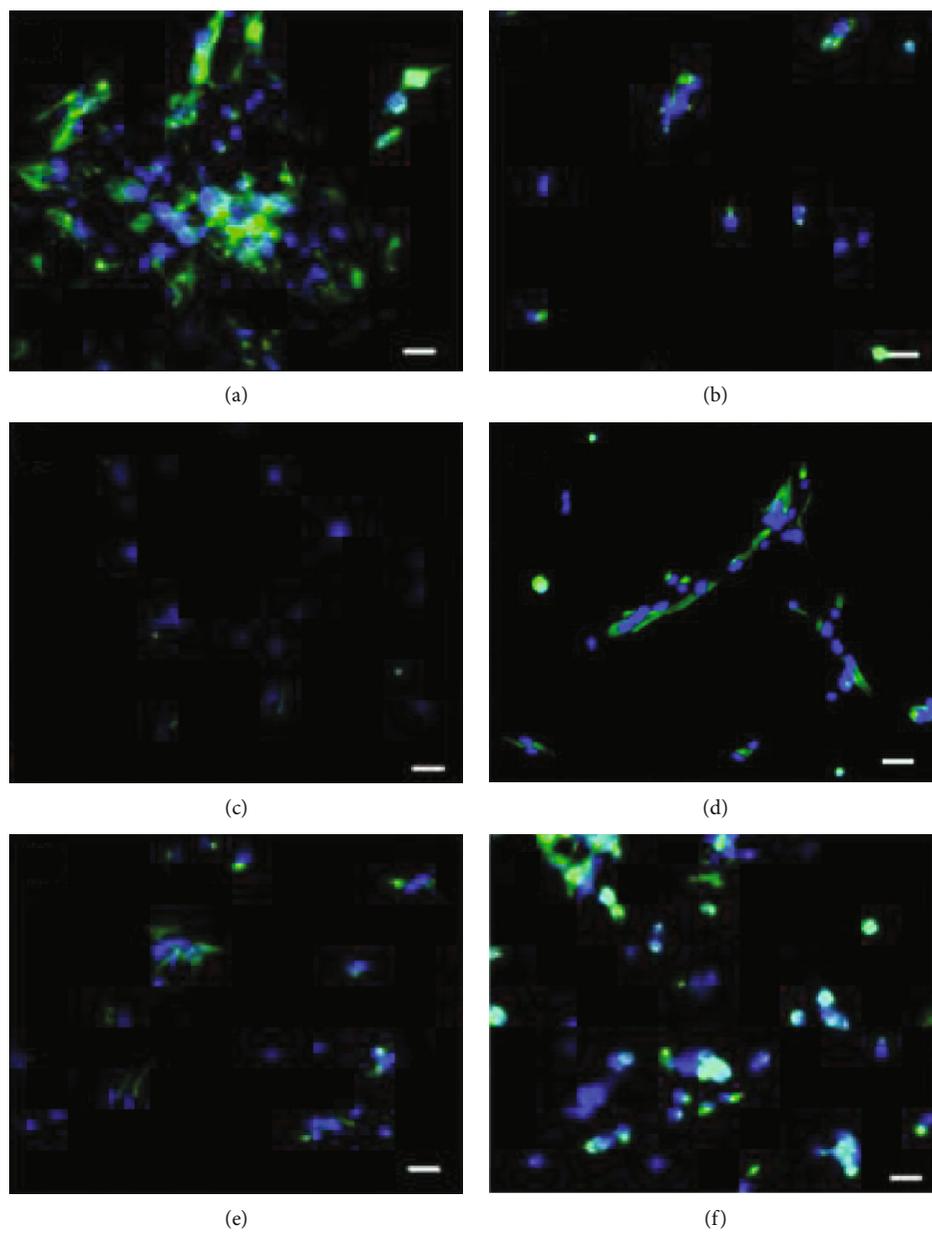


FIGURE 8: Continued.

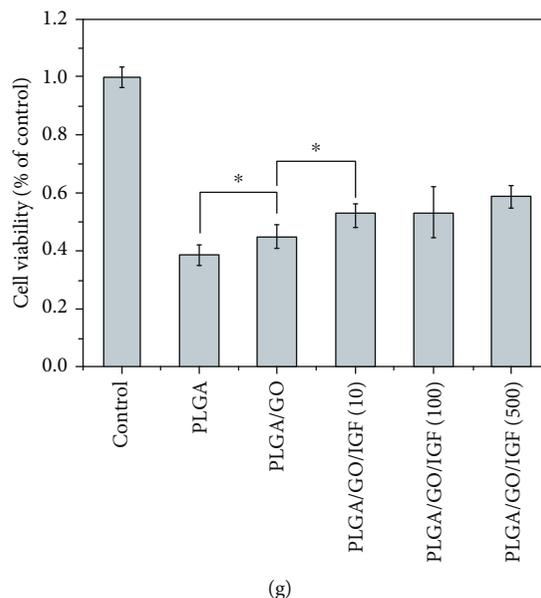


FIGURE 8: NSC morphologies and viabilities after pretreatment with H_2O_2 . Immunostaining images of NSCs on control (a), PLGA (b), PLGA/GO (c), and PLGA/GO/IGF-1 (10, 100, and 500 ng ml^{-1}) samples (d, f) after 24 h. Nestin for NSCs is shown in green, while DAPI for cell nuclei is shown in blue. All scale bar lengths are $100\ \mu\text{m}$. (g) The cell survival ratio was measured using a CCK-8 assay after pretreatment with H_2O_2 *in vitro*. This figure was reproduced from Ref. with permission [139].

overactivation may be limited by topographical structures of 3D-graphene, resulting in anti-inflammatory effects. Remarkably, in comparison with the conditioned mediums of tissue culture polystyrenes and 2D graphene, which caused much more cell death, conditioned mediums of 3D graphene facilitated NSCs and PC12 growth, suggesting that 3D graphene may facilitate neurogenesis.

During the control of inflammatory cell infiltration, tissue engineering scaffolds should provide a supportive environment to facilitate endogenous nerve migration for the purpose of promoting neuronal regeneration under inhibitory conditions. The formation of a graphene polyelectrolyte multilayer was reported by Zhou et al. on electrospun PCL microfiber scaffolds. These scaffolds were used as an electroactive substrate for brain repair and were implanted in the striatum of adult rats to evaluate the inflammatory responses of microglia and astrocytes. From week 1 to week 3, a significant decrease of microglial growth and density was found in gP6 implants, suggesting that graphene reduced the microglial activation/macrophage infiltration. Graphene can also be constrained at a later stage of inflammation near the tissue/scaffold interface. 3D graphene on microfiber surfaces may reduce proinflammatory cytokine and secrete reactive oxide species [143]. The cycle of microglia activation that is self-propelling can also be reduced, which decreases the period of chronic inflammation. Surface modifications to PCL scaffolds using Graphene-LbL demonstrated suppression in the number of infiltrated macrophages as well as microglia and astrocyte activation after implantation. Compared to P6 implants, the number of microglia/macrophages was reduced greatly in gP6 groups by week 3 of culture. Glial scarring also could not be found in surrounding tissues and surface functionalization negligibly affected the onset time

of astrocyte activation. Nevertheless, gP6 scaffolds reduced the number of activated astrocytes between week 3 and week 7 in both tissue and implants [144].

2. Discussion

Many studies have shown that graphene-based scaffolds can promote adhesion, proliferation, differentiation, and the anti-inflammation of neural stem cells. However, the detailed mechanism of these functions, related to signaling pathways in molecular biology, still needs to be determined.

The behavior of NSCs is influenced by many factors inside and outside of cells, especially the specific microenvironment of NSCs and the metabolic status of cells. Some studies have shown that the metabolic pathway is the moderator of the destiny of an NSC regarding proliferation and differentiation. Nevertheless, the detailed mechanism supporting the moderator process is still not fully understood. Although previous studies have been reported, the underlying mechanisms of how graphene nanofiber scaffolds affect NSC metabolism are still poorly known, and more detailed studies are needed. It is a crucial task to explore the interactions between metabolism, related metabolites, and enzymes, and to propel more research on clinical applications.

By increasing the utilization of graphene in different biomedical applications, the biocompatibility of graphene and its derivatives *in vitro* and *in vivo* as well as its possible toxicity should be of high priority. The crucial factors of graphene nanofibers that can lead to toxicity include

- (1) Size, concentration, and shape. Small volumes and high doses may lead to significant toxicity, which results in DNA fragmentation and/or chromosomal

aberrations in living cells. The potentially sharp edges of graphene flakes can also cause physical damage to cell wall membranes and even the nucleus through direct contact

- (2) The graphene flakes may adversely affect blood circulation or the immune system by aggregation (for example, producing harmful free radicals) [145]
- (3) The aggregation of graphene nanofiber materials produces reactive oxygen free radicals (ROS) inside and outside of the cell, which can block the absorption of nutrients and damage human cells and tissues [26]. Therefore, some attractive approaches have been taken to solve these problems, such as using synthesis methods to control the content of graphene and its derivatives, which could effectively alleviate cytotoxicity. In another method, the surface of graphene and its derivatives can also be modified by PEG [146], fetal calf serum [147], or dextran [148] to solve the biocompatibility problem. At the same time, we realized that most studies only focus on *in vitro* studies. *In vitro* 2D or 3D stem cell culture environments are different from the complex 3D physiological conditions *in vivo*. As a result, the regeneration of cells and tissues based on graphene nanomaterials and the detailed *in vivo* evaluation are essential requirements to evaluate the potential for using graphene nanomaterials as implantable biomaterials in a clinical setting. However, there has been no systematic study on the safety of graphene nanomaterials, which is very important for future clinical applications in biomedicine

In addition, most studies have only focused on the external cells' behavior after exposure to graphene. Therefore, there should be more emphasis on the internal effects of intracellular processes [149, 150]. For example, it is necessary to perform more research on the mechanisms and signaling pathways involved in the development of stem cell differentiation and network function for explaining and controlling the interactions between graphene and cells. In a recent study, Paolo et al. used single-layer and multilayer graphene films to design the biosensor interface which allowed for adjustable neuronal communication and enhancement of the membranous neurons [151].

To be specific, when external stimuli (including thermal stimulation, optical stimulation, and electrical stimulation) are applied to graphene nanomaterials, more research is needed to explore the pathologic mechanisms caused by the interactions of materials and stimuli [113, 152]. Besides, implanted conductive GNFs should be stimulated in an animal model to fully understand neural tissue reconstruction and to evaluate its efficacy.

Another challenging problem involves the biodegradability of graphene-based materials. This problem will become more serious when efficient cell interactions and/or ion exchange between cells are hampered by graphene [153] and should be remedied. Some reports have shown that car-

boxylated derivatives may degrade under certain conditions, such as the photocatalytic reduction and degradation caused by using TiO₂ nanoparticles, and the photodegradation assisted by near-infrared light, which provides a noninvasive approach to circumvent this limitation [113, 154].

Nevertheless, biodegradation may cause diffusion and accumulation in the blood system. As a result, it is very important to analyze the cytotoxicity and genotoxicity of graphene *in vivo*.

3. Conclusion

Here, we present an update regarding the application of graphene and its derivatives to neural stem cells and tissue engineering. Over the previous few decades, the exploration of graphene has progressed greatly for tissue engineering applications. In recent studies, both graphene and its derivatives have been used as biocompatible substrates for promoting the adhesion, proliferation, and differentiation of neural stem cells. Additionally, graphene and its derivatives have been shown to exhibit certain anti-inflammatory effects in nerve tissue engineering as well.

Preliminary preclinical studies utilizing are encouraging, but various issues that include cytotoxic or genotoxic effects must be solved before graphene, and its derivatives can be used as large-area substrate coating materials for clinical applications of neural tissue engineering in the future. Furthermore, it will be necessary to clarify the signaling pathways underlying the effects of graphene on neural stem cell behaviors. Studies in this field are still rare, and many efforts are needed to reveal the mechanism that induces stem cell differentiation towards their various designated lineages. As a conductive material, graphene plays an important role in neural engineering. The use of electrical stimulation based on graphene-related materials for clinical applications has not been frequently successful, although various attempts using *in vitro* methods have been made. Furthermore, the effects and mechanisms of different electrical stimulation conditions on neurons should be better understood and before confidence is placed in the use of graphene-based materials for these applications. For deeper understanding and increased efficacy in nerve regeneration, *in vivo* electrical stimulation must be carried out using implanted conductive graphene-based materials. Although many unresolved issues and challenges exist, the landscape of neural tissue engineering and regenerative medicine based on graphene-based materials offers significant opportunities and promise for the eventual integration into biomedical applications and the clinic.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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