Synthesis of amphiphilic reduced graphene oxide with an enhanced charge injection capacity for electrical stimulation of neural cells†

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Advanced neural research demands new electrode materials with high performance. Herein, we have developed a facile approach to synthesize amphiphilic reduced graphene oxide (rGO) and demonstrated its performance in electrically stimulating neural cells with high charge injection capacity. Synthesis of the amphiphilic rGO features covalent functionalization and simultaneous thermal reduction in a one-step manner. The covalent functionalization of methoxy poly(ethylene glycol) (mPEG) chains on the rGO surface not only provides a high dispersibility in various solvents, enabling convenient post-treatment processes, but also allows for an enhancement in double-layer charging capacitance. Calcium imaging of PC12 neural cells on the amphiphilic mPEG–rGO films has revealed a predominant increase in the percentage of cells with higher action potentials, derived from double-layer capacitance enhancement in charge injection. These results suggest that the new amphiphilic mPEG–rGO material is capable of providing a much safer and efficacious solution for neural prostheses applications.

Introduction

Electrical stimulation of neural cells is widely employed in emerging technologies for prostheses and medical treatments of spinal cord injury, stroke, sensory deficits, and neurological disorders.1 The development of electrode materials is a critical factor because they interface the neural cells with the physical facilities. An ideal candidate for an electrode material needs to be safe and biocompatible in the first place, and efficient in mediating the charge transfer from electron flow in the electrode to ion flow in the cells.2 The charge injection can be conducted by a capacitive effect involving the charging and discharging of the double layer at the electrode–electrolyte interface, or a Faradaic reaction in which surface-conﬁned species are oxidized and reduced.3 Although the Faradaic reaction may provide a higher current density, the method is accompanied by the generation of complicated chemical species which are unfriendly to the biological cells in many cases. Therefore, capacitive charge injection is in principle a more desirable approach than the Faradaic reaction.4

Recent developments in carbon nanomaterials offer new opportunities to design stimulation electrodes with high capacitive charge injection capabilities.5 Graphene, single or few-layered two-dimensional (2D) sp2-bonded carbon sheets, has been widely explored for adoption in electronic and optoelectronic devices,6 heat storage,7 photodynamic therapy8 and sensors.9 Compared with conventional inert electrode materials (e.g. platinum), the unique properties of graphene make it an excellent candidate to address the requirements for advanced neural research. (1) Biocompatibility and bioactivity. It was reported that graphene can promote the sprouting and outgrowth of neurites of hippocampal cells10,11 and induce stem cells to preferentially differentiate into specific lineages.12–14 Moreover, owing to its strong noncovalent interactions with biomolecules, graphene can act as a substrate to preconcentrate the biomolecules for regulation of cell behavior and fate.15–17 (2) Mechanical compliance with the neural tissues. For example, the in-plane Young’s modulus of monolayer graphene is around 1 TPa, about five times stronger than steel.18 Yet graphene can be bent to large angles without breaking or variations in the electric resistance.19 This combination of strength and flexibility is highly desirable for robust and stretchable electrodes in neural prostheses. (3) Electrical conductivity. Graphene has been proven to possess high conductivity for charge transport and enables efficient electron transfer from the electrode
surface to the electrolyte.28 However, few studies have involved the electrical stimulation of neural cells on graphene platforms,23 leaving scarcely any data about the effect of surface functionalization on the charge injection capability as well as the consequence of neural cell behaviors.

In this work, we developed a facile one-step method to synthesize amphiphilic reduced graphene oxide (rGO) for application of neural prosthesis materials. The amphiphilic functionalization of the rGO surface with methoxy poly(ethylene glycol) (mPEG) not only provided a high dispersibility in various solvents, enabling convenient post-treatment processes, but also offered an enhanced double-layer charging capacitance. Moreover, a proof-of-concept study was conducted on the amphiphilic mPEG–rGO film as a conductive platform for electrical stimulation of pheochromocytoma (PC12) neural cells (a model cell system for a variety of neural functions). Calcium imaging of the PC12 neural cells on amphiphilic mPEG–rGO films revealed that the percentage of the cells with higher action potentials increased significantly, contributed to by the effect of the double-layer capacitance enhancement for charge injection. These results suggested important applications of amphiphilic mPEG–rGO for neural prostheses featuring high effectiveness and biosafety.

Experimental

Preparation of rGO

Yellow-brown GO was prepared from natural graphite powder (300 mesh, Alfa Aesar) by a modified Hummers method.22,23 Hexamethylenediamine diisocyanate (HMDI)-activated methoxy poly(ethylene glycol) 5000 (A-mPEG 5k, see Fig. S1 in the ESI†) was synthesized according to previously reported protocols.24 The mPEG–rGO was accomplished by a so-called one-step synthesis method. Briefly, anhydrous graphene oxide foams (10 mg) were added into a 25 mL round-bottom flask equipped with a magnetic stir bar, followed by addition of 10 mL anhydrous N,N-dimethylformamide (DMF). The flask was then sonicated for 30 min under nitrogen. The A-mPEG 5k (0.5 g) was next loaded and the mixture was heated to 160 °C with magnetic stirring under nitrogen for 1–5 h. The product was centrifuged and washed with DMF and ethanol six times. The control was obtained by substitution of A-mPEG 5k with pure mPEG 5k, with the other conditions unchanged. The rGO_{N,N},H_{x} was synthesized by using hydrazine as a reducing agent (more details are available in Section 2 of the ESI†). Their powders were obtained by drying the ethanol solution under vacuum. Their films were prepared by filtration of the aqueous solution through a 0.25 μm-pore cellulose membrane, followed by a transfer to a solid conductive substrate for cell culture.

Characterization

The morphology and average thickness of the films were characterized by atomic force microscopy (AFM) (Digital Instrument Dimension 3100, Veeco). The structural properties of the rGOs were measured by X-ray diffraction (XRD) (D8 Advance, Bruker) with a Cu Kα source (λ = 1.5406 Å). Fourier transform infrared spectroscopy (FT-IR) spectra were measured by a Nicolet 6700 Fourier transform infrared spectrometer (Thermo) and samples were dispersed in pressed KBr disks. X-ray photoelectron spectroscopy (XPS) (Axis Ultra DLD, Kratos, UK) was performed by utilizing an Al Kα X-ray source operated at 40 eV. The reduction quality was examined by a Raman spectrometer (lamRAM HR800, HORIBA, France). Thermogravimetric analysis (TGA) (TG/DTA 6200, Seiko) was performed under a nitrogen flow in a Pt crucible on sample sizes from 2 to 3 mg. The electrochemical experiment was performed with a CHI620c Electrochemical Analyzer (CHI, Austin TX). A glass carbon (GC) electrode (Φ = 3 mm) served as the working electrode, a platinum wire as the auxiliary electrode, and an Ag/AgCl electrode as the reference electrode.

Culture of PC12 cells

PC12 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 μg mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37 °C in a humidified atmosphere with 5% CO₂.

Cell viability assay

Cell viability of mPEG–rGO was evaluated with tissue culture polystyrene (TCPs) as a control using a LIVE/DEAD® viability/cytotoxicity kit for mammalian cells (Invitrogen, USA) according to the manufacturer’s instructions. The percentage of live cells was calculated by counting the number of calcine-AM positive cells over the total cell number. Additionally, the details of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay are described in the ESI†. The results are given as the mean ± the standard error of the mean.

Electrical stimulation & calcium imaging

To seed PC12 cells on the rGO films, a cloning ring (O.D.10 mm & height 10 mm) was mounted on the substrate. An electrical lead was loaded directly onto the dry area of the graphene substrate by using silver paste and copper wire. The input stimulation was applied with the aid of a function generator (S3K, Grass Technologies, USA) that gave flexibility in the applied electrical stimulus signal. The cells were further stained with Fluo-4 AM dye before stimulation (2.5 μM, Dojindo Laboratories, Japan). A series of 1–100 ms monophasic anodic pulses were applied with durations of 10 s. The stimulation potential was limited to lower than 0.6 V to avoid any undesired effects of Faradaic electrolyte reactions. Time-lapse calcium levels in live PC12 cells were measured using fluorescence microscopy.

Results and discussion

Scheme 1 illustrates the design and preparation of the amphiphilic reduced graphene oxide (rGO) composite coated electrode. The rGO amphiphilic functionalization was achieved by a new method featuring a one-step synthesis procedure allowing for tunable material properties. The surface modification was performed by conjugating an isocyanate group of A-mPEG 5k with surface hydroxyl functional groups of GO via the formation.
of amides or carbamate esters. Simultaneously, the GO sheet was thermally reduced during this reaction. Afterwards, the mPEG chain was covalently immobilized on the carbon surface of rGO to form an amphiphilic outer layer, consequently facilitating the dispersion of mPEG–rGO in both organic low polar and water-miscible high polar solvents. The as-prepared amphiphilic mPEG–rGO was filtered to form a film on a cellulose membrane (pore size 0.25 μm, diameter 47 mm), and then transferred to a conductive substrate for cell culture and electrical stimulation (Section 2 in the ESI†).

The morphology and structure of mPEG–rGO were studied by tapping-mode AFM. Fig. 1 shows the AFM images and cross-sectional analyses of single-layer GO and mPEG–rGO adsorbed on mica. The thickness of a single layer GO sheet was measured to be about 1.3 nm, consistent with previous reports. The thickness of a single layer of the mPEG–rGO sheet was 1.7 nm. Thus, the interlayer distance between mPEG–rGO was enlarged by the intercalated mPEG molecules. Because the one-step synthesis procedure was performed in a homogeneous solution with steady stirring, it was likely that the mPEG molecules attached onto both sides of the rGO sheets, thus forming a sandwich-like structure. The covalent bonding of the mPEG molecules onto the GO sheets was further verified by Fourier transform infrared spectroscopy [FT-IR, Fig. S2 in the ESI†].

Upon treatment with A-mPEG 5k, the C=O stretching vibration at 1730 cm⁻¹ of GO became obscured by the appearance of a strong absorption peak at 1564 cm⁻¹, ascribed to the stretching vibration of a carbamate or amide group. Significantly, the broad and intense peak of the O–H (3200–3700 cm⁻¹) stretching vibration was altered to the N–H stretching vibration at 3345 cm⁻¹, suggesting that the hydrogen-containing functional groups of GO were substituted by mPEG molecules after their reaction with A-mPEG 5k. Additionally, the isocyanate group signal (2270 cm⁻¹) was invisible in the mPEG–rGO spectrum, indicating that the treatment of GO with A-mPEG 5k resulted in chemical reactions and not mere adsorption/intercalation of the unreacted A-mPEG 5k.

To further investigate the structure of mPEG–rGO, UV-Vis absorption spectroscopy, XPS, Raman spectroscopy and TGA were employed in our experiments. The reduction of GO to rGO is shown in the UV-Vis absorption spectra (ESI, Fig. S3†) and was further confirmed by the results obtained by XPS (ESI, Fig. S4†) and by the Raman spectra (ESI, Fig. S5†). All the data supported that GO was deoxygenated and reduced, and the mPEG chains were immobilized on the carbon plane. Based on the TGA data (ESI, Fig. S6†) and the yield of mPEG–rGO (~120%), it was estimated that mPEG–rGO was composed of 20–50 wt% of mPEG.

A powder XRD pattern was utilized to investigate the reduction quality of mPEG–rGO (Fig. 2a). A typical broad peak near 10.5° (d-spacing ~8.4 Å) was observed for the GO powder. Compared with the parent GO, the mPEG–rGO peak displayed a dramatic shift to higher 2θ angles with the increase in reaction temperature. The mPEG–rGO peak reduced at 160 °C was finally increased to 23.5° (d-spacing ~ 3.8 Å), which is very close to that of rGO reduced by hydrazine (rGOH2N2). The results suggested that rGO was well ordered with two-dimensional sheets, although part of the graphene plane was occupied by the coupled mPEG chains. Additionally, the reaction process was monitored using XRD. As shown in Fig. S7 (ESI†), the GO peak (10.5°) quickly right-shifted to 23.4°, suggesting that the electronic conjugation within the graphene sheets was recovered during the reduction. There was little further increase of the peak position after 1 hour, indicating completion of the reduction. This experiment also indicated that the electrical and polymer conjugation level of graphene was chemically controllable, thus providing possibilities to tune the optical and electrical properties of the graphene sheets. We found that graphene sheets with different reduction levels can all form stable dispersions after amphiphilic functionalization (see Section 2 in the ESI†).

![Scheme 1](image)

*Fig. 1* Tapping-mode AFM images and cross-sectional analysis of (a) GO and (b) mPEG–rGO on mica.

![Image](image)
We demonstrated excellent solubility of mPEG−rGO as prepared in various solvents, which was very important for the post-treatment process and potential functionalization of electrode materials.\(^{27,29,30}\) Fig. 2b shows the typical dispersions of mPEG−rGO in water, DMF, ethanol, acetone, tetrahydrofuran (THF), and chloroform, at 1 mg mL\(^{-1}\) concentrations. The black dispersion of mPEG−rGO in these solvents (except ethanol) contained no visible precipitate and was stable for weeks. A control sample was synthesized under the same reaction conditions, except that a tiny amount of mPEG molecules might be adsorbed on the graphene surface by use of the non-reactive pure mPEG 5k instead of A-mPEG 5k. The XRD pattern of the control demonstrated that it was reduced as its 2\(\theta\) peak was almost identical with that of mPEG−rGO (ESI, Fig. S8\(^{†}\)). However, the vials with the control in all of the solvents mentioned above contained visible precipitates, indicating poor dispersion. The reason was that the covalent bonding of the mPEG chain can facilitate the solubility of the carbon plane in these solvents.

The electrochemical properties of mPEG−rGO were evaluated by cyclic voltammetry using a potentiostat with a three-electrode system. The rGO, prepared by the conventional method of using hydrazine as a reducing agent (rGO\(_{N,H}^{}\)), was included in the experiments, providing additional information on the electrical properties of GO in the reduced formulation for comparison.\(^{25}\) Fig. 3a shows different cyclic voltammograms (CVs) of GO, rGO\(_{N,H}^{}\), and mPEG−rGO modified GC electrodes with 1.0 mM K\(_3\)Fe(CN)\(_6\) in the presence of 0.1 M KCl. After being modified with GO, the anodic and cathodic peaks almost disappeared, consistent with previous reports.\(^{26}\) This demonstrated that the presence of a GO layer blocked the diffusion of Fe(CN)\(_{6}^{}\)\(^{3−}\) into the film. For the rGO modified GC electrode, our experiments displayed well-shaped and repeatable CV curves. Significantly, at the mPEG−rGO modified GC electrode, the current density in the overall potential range increased significantly compared with that observed at the rGO\(_{N,H}^{}\) modified GC electrode, which was a key parameter for potential applications in neural stimulation. The CVs of rGO in PBS (Fig. 3b) verified that the mPEG−rGO modified GC electrode can provide much stronger charge injection ability with the same geometrical area in the potential window ranging from −0.2 to +0.8 V.

It’s notable that the surface modification of graphene with biological molecules (e.g. PEG) often enhances the cell viability and functions for biomedical applications.\(^{31}\) The morphology of PC12 cells cultured on a mPEG−rGO film was examined by scanning electron microscopy. Fig. S9 (ESI\(^{†}\)) illustrates that the PC12 cells formed a well-developed neural network and exhibited excellent cell adhesion on the mPEG−rGO film. In addition, the cells exhibited polygonal or fusiform morphologies, suggesting good phenotypic spreading of the PC12 cells on the mPEG−rGO film.\(^{22}\) Cytotoxicity of mPEG−rGO was evaluated by calcein-AM and EthD-I staining assays using a LIVE/DEAD\(^{®}\) Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, USA), with TCPS as the control. As shown in Fig. S10 (ESI\(^{†}\)), nearly 90% of the PC12 cells cultured on mPEG−rGO for 2 days were viable, while the cell viability difference between the mPEG−rGO film and the TCPS was negligible (confirmed with a MTT assay, Fig. S11 in the ESI\(^{†}\)). These data demonstrated good biocompatibility of mPEG−rGO, consistent with previous studies.\(^{33,34}\)

Right before electrical stimulation, the PC12 neural cells on the rGOs were stained with Fluo-4 AM dye (2.5 \(\mu\)mol, Dojindo Laboratories, Japan). A series of 1–100 ms monophasic anodic pulses were administered with durations of 10 s using a function generator (S3K, Grass Technologies, USA) and the stimulation threshold was 0.6 V.\(^{24}\) Fig. 4a and b show that the fluorescence levels of the PC12 cells on the mPEG−rGO films increased during a stimulus, whereas the intensity change on the control film of rGO\(_{N,H}^{}\) was obscure. The relative change in fluorescence intensity \(\Delta F/F\) was plotted versus stimulation time in Fig. 4c. The typical cells exhibited fluorescence intensity increases of over 40–50% and 10–20% on the mPEG−rGO and rGO\(_{N,H}^{}\) films, respectively, via electrical stimuli. The cells with various \(\Delta F/F\) changes are illustrated in Fig. S12 (ESI\(^{†}\)), which
suggests a threshold ($\Delta F/F > 5\%$) for fluorescent differentiable responses to electrical stimuli. The statistical histogram (Fig. 4d) shows that the percentage of cells with large $\Delta F/F (>5\%)$ on mPEG–rGO was significantly higher than that on the rGON$_{H_4}$ and indium tin oxide films (Fig. S13 in the ESI†), indicating that the number of cells that evoked a strong action potential on mPEG–rGO was tremendously increased even under the identical stimulation voltage (see Fig. S14 in the ESI† for the significance analysis). This evidence supported that amphiphilic mPEG–rGO performed better than rGON$_{H_4}$ as an electrode material. We proposed that mPEG–rGO should enable electrical stimulation of cells at a higher current density owing

![Fig. 3](image1.png)

**Fig. 3** CVs recorded at the GO/GC, rGON$_{H_4}$/GC and mPEG–rGO/GC electrodes with 1 mM $K_3$Fe(CN)$_6$ in the 0.1 M KCl buffer (a) and with 1× PBS (b), scan rate: 50 mV s$^{-1}$.

![Fig. 4](image2.png)

**Fig. 4** Electrical stimulation of PC12 cells on mPEG–rGO. Fluorescence imaging of the cells pre-incubated with Fluo-4 AM dye on mPEG–rGO (a) and rGON$_{H_4}$ (b) films before (left) and after (right) electrical stimulation. Graph (c) plots the relative fluorescence intensity change, $\Delta F/F$, of the circled cells in panels (a) and (b) versus the stimulation time period. Histogram (d) depicts the percentage of cells in the different ranges of $\Delta F/F$. 

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to its elevated double-layer charging capacitance. In physiological solutions, the double layer at the electrode–electrolyte interface serves as the capacitor. According to the equation $Q_{\text{max}} = CV_{\text{max}} = \varepsilon_0\varepsilon_r(A/d)V_{\text{max}}$, a reasonable approach to increase $Q_{\text{max}}$ is to increase the relative permittivity ($\varepsilon_r$) which is contributed to by the mPEG chains. On the other hand, the amphiphilic functionalization may increase the wettable surface area ($A$) accessible by electrolyte ions, giving rise to a much higher double-layer charging capacitance. As a result, the mPEG–rGO electrode can provide an elevated current density, which plays an important role in regulating cell behavior during electrical stimulation. These results promise effective electrode materials for neural research using amphiphilic mPEG–rGO as a conductive scaffold.

Conclusions

In summary, we demonstrated an effective thermal reduction method to prepare amphiphilic mPEG–rGO sheets for electrical stimulation of neural cells. Taking advantage of the amphiphilicity of mPEG, the functionalized rGO can be well dispersed in a variety of solvents, including chloroform and water. Measurements of cyclic voltammetry supported that the amphiphilic mPEG–rGO modified electrode can provide much stronger charge injection ability than conventional rGO$_{N,H}$. Furthermore, calcium imaging of PC12 cells verified that mPEG–rGO was an efficient conductive platform to mediate electrical stimulation for neural cells featuring an enhancement of double-layer charging capacitance. Importantly, amphiphilic functionalization in this novel graphene composite renders good biocompatibility and high charge injection capability in the physiological environment, promising versatile applications in neural prostheses and neural tissue engineering.

Acknowledgements

This work was supported by the Major State Basic Research Development Program (2013CB932702, 2012CB932601), and by NSFC (21275106) and NSF of the Jiangsu Province (BK20130306); a project supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions, and the Open Project Program of State Key Laboratory of Molecular Engineering of Polymers (Fudan University, K2012-09). J.L. was supported by the “Youth 1000-plan” in the Recruitment Program of Global Experts.

Notes and references