Biomaterials 34 (2013) 2017–2023

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Myoblast differentiation on graphene oxide

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ARTICLE INFO

Article history:
Received 1 October 2012
Accepted 26 November 2012
Available online 19 December 2012

Keywords:
2D carbon materials
Graphene oxide
Myogenic differentiation
Myotube formation

ABSTRACT

Graphene-based nanomaterials have received much attention in biomedical applications for drug/gene delivery, cancer therapy, imaging, and tissue engineering. Despite the capacity of 2D carbon materials as a nontoxic and implantable platform, their effect on myogenic differentiation has been rarely studied. We investigated the myotube formation on graphene-based nanomaterials, particularly graphene oxide (GO) and reduced graphene oxide (rGO). GO sheets were immobilized on amine-modified glass to prepare GO-modified glass, which was further reduced by hydrazine treatment for the synthesis of rGO-modified substrate. We studied the behavior, including adhesion, proliferation, and differentiation, of mouse myoblast C2C12 on unmodified, GO-, and rGO-modified glass substrates. According to our analyses of myogenic protein expression, multinucleate myotube formation, and expression of differentiation-specific genes (*MyoD*, *myogenin*, *Troponin T*, and *MHC*), myogenic differentiation was remarkably enhanced on GO, which resulted from serum protein adsorption and nanotopographical cues. Our results demonstrate the ability of GO to stimulate myogenic differentiation, showing a potential for skeletal tissue engineering applications.

1. Introduction

Graphene, a 2D monolayer of sp^2-bonded carbon atoms, has been extensively studied in diverse fields for its excellent physical, chemical, and mechanical properties. 2D carbon nanomaterials include graphene oxide (GO), a highly oxidative form of graphene obtained by chemical exfoliation of graphite, reduced graphene oxide (rGO) prepared by chemical or thermal reduction of GO, and graphene sheets produced by chemical vapor deposition. Graphene-based materials are now utilized for various biomedical applications, such as drug/gene delivery [1,2], photothermal cancer therapy [3–5], imaging [6], and biosensing [7–10] because of their unique chemical, physical, and optical properties with facile functionalization. Recently, the capacity of graphene-based materials as a nontoxic and implantable platform has been studied for tissue engineering [11]. Graphene derivatives influence cellular behaviors, such as adhesion, proliferation, and differentiation. For example, fibroblasts, osteoblasts, and human mesenchymal stem cells (hMSCs) well attached to graphene-based substrates and developed good focal adhesion [12,13]. Pristine graphene induced the osteogenesis of hMSCs [14,15] and the differentiation of human neural stem cells into neurons [16]. GO, the oxidized derivative of graphene, stimulated the differentiation of hMSCs to adipocytes [14] and the differentiation of induced pluripotent stem cells (iPSCs) toward endodermal lineage [17]. According to the literature [18], the cytotoxicity and genotoxicity of graphene derivatives were influenced by the physicochemical properties, such as size and reduction state, according to the analysis of reactive oxygen species (ROS) generation, DNA fragmentation, and chromosomal aberrations. Graphene-based materials showed dose-dependent cytotoxicity to mammalian cells and the effect was not significant at a low concentration [18,19]. When paper-like structures of graphene derivatives were fabricated by a simple filtration method, they showed a good biocompatibility to mammalian cells [20–22]. In addition, antibacterial effect was observed after the exposure of microorganisms to graphene-based materials [23,24]; this effect can be useful to prevent infection in biomedical applications.

This paper first reports on myogenic differentiation on graphene-based materials. The regeneration of skeletal muscle tissues aims for the development of artificial scaffolds in skeletal tissue engineering to treat a variety of muscle diseases and traumatic injury [25]. Different materials, such as extracellular matrix components and biodegradable synthetic polymers, have been utilized for skeletal tissue engineering, but their use was limited because of low stability and inflammatory responses [26]. Because graphene-based materials exhibit high stability in aqueous solution

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0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2012.11.052

[27,28] and low inflammatory reactions [29], we investigated the behavior of myoblasts on graphene-based materials. Considering that key physicochemical properties of graphene that influence cell behaviors are not clear yet, we used both GO and rGO to investigate the effect of reduction states of graphene derivatives on myogenic differentiation. The properties of GO and rGO were characterized using atomic force microscopy and X-ray photoelectron spectroscopy. We further investigated the behavior of myoblasts on GO and rGO, including adhesion, growth, and differentiation through viability assay, immunofluorescence staining, and gene expression analysis.

2. Materials and methods

2.1. Materials

Graphite powder, $KMnO_4$, 3-aminopropyltriethoxysilane (3-APTES), toluene, and dimethylformamide (DMF) were purchased from Sigma–Aldrich (St. Louis, USA) and used without further purification.

2.2. GO synthesis

GO was prepared from natural graphite according to the modified Hummer's method [30]. Briefly, graphite powder, $NaNO_3$, and H_2SO_4 were mixed together while stirring in an ice-bath. Then, $KMnO_4$ was added slowly while the system was maintained at 35 °C for 30 min. The mixture was further diluted with warm water and treated with H_2O_2 to remove the residual $KMnO_4$ until the bubbling disappeared. The resulting solution was centrifuged at 7200 rpm for 30 min three times, and 1 N NaOH was added to adjust the solution pH to near 7. GO powder was obtained by washing and drying the suspension in a vacuum oven.

2.3. Preparation of GO- and rGO-modified glass substrates

Glass substrates were cleaned with piranha solution. After washing and drying, the substrates were immersed in 10 mM 3-APTES in anhydrous toluene solution for 30 min at RT, and then washed with toluene, ethanol, and water. APTES-modified glass substrates were baked at 125 °C. GO sheets were immobilized by immersing APTES-modified glass into GO aqueous solution (1.5 mg/ml) for 1 h at RT, and then washed with water. GO-modified glass substrates were reduced by immersing into 20% hydrazine in DMF for 24 h at 80 °C, and then washed with DMF, ethanol, and water.

2.4. Characterization of GO- and rGO-modified glass substrates

The surface of GO- and rGO-modified glass substrates was observed using a Nanoscope IIID Multimode atomic force microscope (Digital Instruments Inc., USA) under ambient condition. The surface of unmodified, GO-, and rGO-modified glass was analyzed using X-ray Photoelectron Spectroscopy (XPS) (MultiLab 2000, Thermo Scientific, UK). XPS analysis was conducted with an Al K_α line as an X-ray source, and the spectra were taken at a pass energy of 0.1 eV. Raman spectra were obtained by accumulating 30 scans with a resolution of 2 cm^{-1} in the range of 1200–3000 cm^{-1} using the LabRAM HR UV/Vis/NIR (Horiba Jobin Yvon, France). The water-contact angle was determined using a contact angle analyzer (Surface Electro Optics Co., Korea).

2.5. Cell proliferation and morphology analysis

Cell culture media (DMEM) and sera (fetal bovine serum and horse serum) were obtained from Welgene (Korea), and antibiotic–antimyotic was purchased from GIBCO (Carlsbad, CA, USA). Mouse skeletal myoblasts C2C12 were maintained in growth media (GM; DMEM containing 10% FBS and 1% antibiotic–antimyotic). Cells were subcultured at least twice a week and were maintained at a humidified atmosphere with 5% CO_2. Unmodified, GO-, and rGO-modified glass substrates were placed in a 24-well plate, and C2C12 cells were seeded at a density of 5×10^3 cells/well. The cell morphology was analyzed using cytoskeleton staining after a 1-day culture. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, stained with TRICT-phalloidin (Sigma–Aldrich, USA), and mounted with Vectashield mounting solution containing DAPI (Vector Laboratories, USA). The actin filaments and nuclei were observed using a fluorescence microscope (Eclipse 80i, Nikon, Japan). The cell proliferation was analyzed by MTT assay after a 2- or 4-day culture. The assay was performed through the following steps: addition of MTT solution (5 mg/ml in PBS, pH 7.4), incubation for 3 h at 37 °C, dissolution of purple formazan using DMSO, and measurement of absorbance at 595 nm.

2.6. Myotube formation analysis with immunocytochemistry

C2C12 were seeded at a density of 3×10^4 cells/well. After 1 day, the cells were incubated in differentiation media (DM; DMEM containing 2% horse serum and 1% antibiotic–antimyotic) for a further 5 days to induce myotube formation. DM was changed every other day. After differentiation, cells were immunostained for myosin heavy chain (*MHC*) and *myogenin*. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 5% BSA solution, incubated with primary antibody (for *MHC*: 1:20, Developmental Studies Hybridoma Bank; for *myogenin*: 1:100, Santa Cruz) at 4 °C overnight, incubated with secondary antibody (Dylight 488-conjugated goat anti-mouse IgG; 1:100; Abcam) at RT for 1 h, and mounted with Vectashield mounting solution. The multinucleate myotubes were observed using a fluorescence microscope, and analyzed with ImageJ software.

2.7. Quantitative real-time RT-PCR

Real-time RT-PCR was used to analyze the expression level of *MyoD*, *myogenin*, *Troponin T*, and *MHC*. cDNA was directly synthesized from cultured cells using a FastLane cDNA synthesis kit (Qiagen, Germany). Real-time RT-PCR using Quantitect SYBR Green (Qiagen, Germany) was performed with a BioRad CFX96 Real-Time Detection System (Bio-Rad, CA, USA). Thermocycling conditions were as follows: 95 °C for 15 min, 40 cycles of denaturation (15 s, 94 °C), annealing (30 s, 55 °C), and extension (30 s, 72 °C). The primer sequences are listed in Table S1 (in Supporting information). The relative expression level of each gene compared with that of *beta-actin* was calculated and normalized by the value for cells cultured on unmodified glass substrates.

2.8. Protein adsorption on GO- and rGO-modified glass

Unmodified, GO-, and rGO-modified glass substrates were immersed in DM at 37 °C for 2 days. After washing and drying, the surface of the substrates was analyzed using XPS. The molar percentage of nitrogen was calculated for each sample.

2.9. Statistical analysis

All of the quantitative results were expressed as mean ± standard error of the mean (SEM). Statistical analysis was carried out by means of one-way analysis of variance (ANOVA). A p-value less than 0.05 was considered statistically significant.

3. Results and discussion

Fig. S1 shows the morphology of GO sheets synthesized by chemical exfoliation of graphite powder. GO had a thickness of approximately 0.80 nm, indicating a single-layer state of GO sheet [31], and the lateral dimension of GO ranged from several hundred nanometers to several micrometers. When GO sheets were immobilized on APTES-functionalized glass substrate, they exhibited a thickness of 4.5 nm with numerous wrinkles, implying that they were stacked in multiple layers (Fig. 1A). We obtained rGO-modified glass by chemical reduction of GO substrates using hydrazine. Similar to GO-modified glass, rGO substrates also had multiple-layered rGO sheets, which were confirmed by the thickness of 3.93 nm. GO- and rGO-modified glasses exhibited surface roughness (R_a) of 0.66 nm and 0.63 nm, respectively, indicating that the reduction process did not change the surface morphology of GO. We characterized the chemical reduction of GO into rGO using XPS. As shown in Fig. 1B, GO-modified glass exhibited a peak at 284.6 eV for a C–C bond and peaks at 286.2, 288.0, and 289.5 eV for C–O, C=O, and O–C=O, respectively. After hydrazine treatment, rGO-modified substrates showed high C–C peak at 284.6 eV and the intensity of the peaks for oxygenous groups was substantially reduced. A new peak appeared at 286.0 eV for a C–N bond, which should be generated during the reduction process [32]. According to our analysis using Raman spectroscopy (Fig. S2A), GO possessed a G band at 1607 cm^{-1}, whereas the G band of rGO was located at 1592 cm^{-1}. The location of the G band for rGO was close to the value of pristine graphite, indicating successful reduction of GO by hydrazine treatment. A D band appeared at 1348 cm^{-1} for GO and 1349 cm^{-1} for rGO, respectively, implying a reduction in the size of the in-plane sp^2 domain. The D/G ratio changed from 0.96 to 1.35 after reduction, indicating a decrease in the average size of the sp^2