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The effects of graphene nanostructures on mesenchymal stem cells

Yahfi Talukdar [1], Jason T. Rashkow [1], Gaurav Lalwani, Shruti Kanakia, Balaji Sitharaman*

Department of Biomedical Engineering, Stony Brook University, Stony Brook, NY 11794-5281, USA

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ABSTRACT

We report the effects of two-dimensional graphene nanostructures; graphene nano-onions (GNOs), graphene oxide nanoribbons (GONRs), and graphene oxide nanoplatelets (GONPs) on viability, and differentiation of human mesenchymal stem cells (MSCs). Cytotoxicity of GNOs, GONRs, and GONPs dispersed in distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)] (DSPE-PEG), on adipose derived mesenchymal stem cells (adMSCs), and bone marrow-derived mesenchymal stem cells (bmMSCs) was assessed by AlamarBlue and Calcein AM viability assays at concentrations ranging from 5 to 300 μg/ml for 24 or 72 h. Cytotoxicity of the 2D graphene nanostructures was found to be dose dependent, not time dependent, with concentrations less than 50 μg/ml showing no significant differences compared to untreated controls. Differentiation potential of adMSCs to adipocytes and osteoblasts, – characterized by Oil Red O staining and elution, alkaline phosphatase activity, calcium matrix deposition and Alizarin Red S staining – did not change significantly when treated with the three graphene nanoparticles at a low (10 μg/ml) and high (50 μg/ml) concentration for 24 h. Transmission electron microscopy (TEM) and confocal Raman spectroscopy indicated cellular uptake of only GNOs and GONPs. The results lay the foundation for the use of these nanoparticles at potentially safe doses as *ex vivo* labels for MSC-based imaging and therapy.

1. Introduction

Carbon nanoparticles such as zero dimensional (0D) fullerenes, one dimensional (1D) carbon nanotubes, and recently two dimensional (2D) graphene [1] have been investigated for applications in therapeutics [2–5], bioimaging [6–8], and regenerative medicine [9]. Mesenchymal stem cells (MSCs) are an important class of adult or somatic stem cells, found in various tissues including bone marrow and adipose tissue. MSCs are multipotent cells that differentiate readily into osteocytes, chondrocytes, or adipocytes; express phenotypic characteristics of endothelial, neural, smooth muscle, skeletal myoblasts, and cardiac myocyte cells; and support hematopoietic stem cells or embryonic stem cells in culture [10–12]. MSC therapies are currently being widely investigated to repair, regenerate, and restore damaged tissues [13,14], with some of these therapies in clinical trials [15,16].

Nanoparticles have been employed to deliver growth factors/genes into MSCs to manipulate their differentiation [17,18]. They have also been used as contrast agents/nanoprobes for stem cell tracking [19,20] to locate the site of stem cell activity, and determine the efficacy of the therapy. Recent reports have indicated that graphene nanoparticles show excellent efficacy as delivery agents of genes and biomolecules as well as multimodal imaging agents [21–23], and thus could be suitable as multifunctional agents for MSC imaging and therapy.

The development of graphene nanoparticles for MSC applications necessitates thorough examination of their effects and interactions with these cells to identify potential therapeutic doses. To date, very few studies have investigated the cytotoxicity of graphene nanoparticle formulations with specific focus on progenitor cells, or MSCs [24,25]. Zhang et al. examined the toxicity of graphene quantum dots (GQDs), single reduced graphene sheets with diameters in the range of 5–10 nm, on three progenitor cell types: neurospheres cells, pancreas progenitor cells, and cardiac progenitor cells [24]. Akhavan et al. employed umbilical cord-derived MSCs and investigated the size-dependent cytotoxicity of graphene oxide nanoplatelets and reduced graphene oxide nanoplatelets (prepared using the modified Hummer's method) [25].

* Corresponding author. Department of Biomedical Engineering, Bioengineering Building, Rm 115, Stony Brook University, Stony Brook, NY 11794-5281, USA. Tel.: +1 631 632 1810; fax: +1 631 632 8577.
E-mail addresses: balajisitharaman@yahoo.com, balaji.sitharaman@stonybrook.edu (B. Sitharaman).
[1] These authors contributed equally to the work.

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Graphene nanoparticles, depending on the synthesis method, can exhibit different morphologies, chemical properties, and physical properties. Graphene nano-onions (GNOs) are spherically shaped concentric layers of graphene. Graphene nanoribbons (GONRs), synthesized using multiwalled carbon nanotubes, are ribbon-shaped graphene stacks. Graphene nanoplatelets (GONPs), synthesized using graphite as the starting material, are disc-shaped multi-layered graphene. Reports indicate that graphene nanoparticles, depending on their morphology and synthesis method, show diverse responses on cells and tissues [26–29]. Thus, it is necessary to systematically investigate the effects that graphene nanoparticles with different morphologies, synthesized by various methods, have on MSC viability and differentiation. In this study, the dose- and time-dependent effects were investigated of three graphene nanoparticles – GNOs, GONRs, and GONPs – which were water-dispersed with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)] (DSPE-PEG), on the viability and differentiation of human MSCs.

2. Materials and methods

2.1. Nanoparticle synthesis and characterization

2.1.1. Synthesis of nanomaterials

GNOs were purchased from Graphene Laboratories Inc. (NY, USA). GONRs were synthesized from multi-walled carbon nanotubes (MWCNTs) possessing outer diameters between 20 and 30 nm (Cat. No. 636487, Sigma Aldrich, New York, USA) using a modified longitudinal unzipping method [30]. GONPs were synthesized from graphite flakes using a modified Hummer's method [21]. Nanoparticles were dispersed in a 1.2 mg/ml solution of DSPE-PEG and bath sonicated for 1 h to ensure homogenous stable dispersions before treating the cells.

2.1.2. Raman spectroscopy

Raman spectroscopy was performed using a WITec alpha300R Micro-Imaging Raman Spectrometer equipped with a 532 nm Nd-YAG excitation laser. Spectra were recorded between 50 and 3750 cm^{-1} at room temperature.

2.1.3. Transmission electron microscopy

Nanomaterials were dispersed in ethanol:water (1:1) by probe sonication (Cole Parmer Ultrasonicator LPX 750) using a 1 s "on", 2 s "off" cycle. The samples were subjected to ultracentrifugation (5000 rpm for 5 min), and the supernatant was dropped onto lacey carbon grids (300 mesh size, copper support, Ted Pella, USA). HRTEM imaging was performed using a JOEL 2100F high-resolution analytical transmission electron microscope at the Center for Functional Nanomaterials, Brookhaven National Laboratory, New York. Samples were imaged at an accelerating voltage of 200 kV.

2.1.4. Thermogravimetric analysis

Thermogravimetric analysis (TGA) was performed on GNOs, GONRs, and GONPs using a Perkin–Elmer Diamond 500 (Waltham, MA, USA) instrument at the Center for Functional Nanomaterials, Brookhaven National Laboratory, New York. The samples were heated from 50 °C to 800 °C with the heating rate of 10 °C/min under the argon flow of 20 ml/min.

2.1.5. Zeta potential and hydrodynamic diameter

Zeta potential and hydrodynamic diameter of GNOs, GONRs, and GONPs dispersed in DSPE-PEG were measured at 25 °C using Malvern Zetasizer Nano ZS instrument at the Center for Functional Nanomaterials, Brookhaven National Laboratory, New York. The electrophoretic mobility values were calculated based on three separate measurements of 20 runs each. The zeta potential values were calculated from the electrophoretic mobility by the software using the Helmholtz–Smoluchowski equation. The hydrodynamic diameter of nanoparticles was calculated by measuring the velocity of particles under Brownian motion using the Stokes–Einstein equation.

2.2. Cell culture

Adult human MSCs were isolated from lipoaspirate (Lifeline Cat No. FC-0034) and normal bone marrow (Lonza Cat NO. PT-2501). StemLife™ MSC medium (Lifeline, Cat No. LL-0034) was used for cell cultures, with a media change every 2–3 days. The cells were incubated at 37 °C and 5% CO_2 throughout the experiment. Passages 4 through 8 were used for the studies.

2.3. Viability

Adipose-derived human MSCs (adMSCs) and bone marrow-derived MSCs (bmMSCs) were used for the viability studies. Cells were plated in 96-well plates with 5000 cells/well. Twenty-four hours after plating, the cells were treated with a 10% volume of DSPE-PEG (control), or of GNOs, GONRs, and GONPs at concentrations of 0 μg/ml, 5 μg/ml, 10 μg/ml, 50 μg/ml, 100 μg/ml and 300 μg/ml. Viability was assessed 24 or 72 h after treatment with alamarBlue and Calcein AM assays.

2.4. AlamarBlue

Viability of adMSCs and bmMSCs treated with various concentrations (0–300 μg/ml) of graphene nanoparticles was determined using an alamarBlue assay. Untreated cells were used as a positive control. Cells treated with ice-cold methanol were used as a negative control. Twenty-four or 72 h after treatment, the culture media were removed from the wells. After washing the wells with phosphate buffer solution (PBS), 100 μl of media were added. 10 μl of alamarBlue (Life Technologies, St. Louis, MO, USA) reagent was added to the wells. After incubating for 2 h, fluorescence was measured at an excitation wavelength of 530 nm and emission wavelength of 580 nm.

2.5. Calcein AM

Viability of cells treated with different concentrations (0–300 μg/ml) of graphene nanoparticles was evaluated with a Calcein AM assay. At each time point, culture media were removed, and each well was washed with 100 μl of PBS to remove nanoparticles. Next, the PBS was completely removed and 100 μl of 0.05% Calcein AM reagent was added to each well and incubated for 30 min at room temperature. The fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.6. Adipogenic differentiation

adMSCs were used for the differentiation studies. adMSCs were plated in 24-well plates at a density of 20,000 cells/well. The cells were treated with DSPE-PEG and either 10 μg/ml or 50 μg/ml of GNOs, GONRs, or GONPs. After incubation with the nanoparticles for 24 h, the wells were washed with PBS to remove nanoparticles and treated with adipogenic and osteogenic differentiation media (Lonza, Cat No. PT-3004 & PT-3002). For adipogenic differentiation, the wells were treated with three rounds of adipogenic induction media, three days each round, and then incubated with the adipogenic maintenance media for a total of 21 days, with a media change every three days. Adipogenic differentiation was characterized with Oil Red O staining and elution and quantification of this dye. For osteogenic differentiation, the cells were treated with the osteogenic media for 14 days. Alizarin red S staining, alkaline phosphatase activity, and calcium matrix deposition were analyzed as markers of osteogenic differentiation.

2.7. Oil Red O

A well-established protocol was used for staining triglycerides and esters within the cells. Oil Red O stain was used to determine differences in adipogenic differentiation between the groups [31]. After incubation, the culture media were removed from the wells, and the cells were washed with PBS. The wells were then treated with 1 ml of 4% paraformaldehyde solution for 10 min, followed by 60 min of incubation with 1 ml of fresh 4% paraformaldehyde solution. The paraformaldehyde was then removed, and the cells were washed with water, followed by a 60% isopropanol solution. After drying at room temperature, the wells were filled with a 0.5 ml Oil Red O working solution made of two parts Oil Red O stock solution (0.35% solution in isopropanol) with three parts isopropanol. The wells were then incubated for 10 min. Excess Oil Red O dye was removed by incubating with a 100% isopropanol solution for 10 min. The wells were then washed immediately with double-distilled water four times. They were then imaged using a BX-51 Olympus microscope (Hamburg, Germany). The concentration of Oil Red O dye eluted was quantified by optical absorbance (wavelength = 500 nm). Optical absorbance of elute from each well was measured in triplicates (Varioskan Flash, Thermo Electron, Finland) and compared to the 100% isopropanol controls.

2.8. Osteogenic differentiation

2.8.1. Alizarin Red S

The well-established Alizarin Red S staining method was used to characterize a mineralized matrix due to osteodifferentiation of adMSCs [32]. Alizarin Red S solution (40 mM) was prepared in water and adjusted to a pH of 4.1 using 0.5 N ammonium hydroxide. After incubation with differentiation media, media were removed from the wells and the cells were washed with PBS. The cells were fixed using 1 ml of a 4% paraformaldehyde solution for 15 min. After fixation, the wells were washed twice with deionized water, and 1 ml of Alizarin Red S was added to each well. Following 20 min of incubation with light shaking, the wells were washed four times with deionized water while shaking for 5 min. The wells were imaged using a BX-51 Olympus microscope (Hamburg, Germany).

2.8.2. Cellularity

The number of cells per well was determined using QuantiFluor Dye Systems (Promega, WI, USA). A standard curve of double-stranded DNA was used to determine DNA content in each well. Subsequently, a standard curve of a known number of adMSCs was used to determine the number of cells per well corresponding to the calculated DNA content. 100 μl of sample or standard were added to a 96-well plate. 100 μl of 1x TE buffer were added with 100 μl of a QuantiFluor dye working solution. The 96-well plate was incubated in the dark at room