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Graphite Oxide Nanoparticles with Diameter greater than 20 nm are Biocompatible with Mouse Embryonic Stem Cells and can be used in a Tissue Engineering System

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Introduction

The treatment of many degenerative diseases or tissue injuries mostly requires restoration or re-establishment of normal functions of the tissues. Tissue regeneration is achieved by replacing damaged tissue and/or stimulating the body's own repair mechanisms to heal previously irreplaceable tissues or organs. Regenerative medicine holds a future promise of providing functional and vital tissues or organs for the diseases that inherently have limited regenerative potential. For example, myocardial infarction, the current leading cause of death in the US, leads to significant cardiomyocyte loss, myocardial tissue damage, and

impairment of myocardial function.^[1] Current treatments of myocardial injury only attenuate the disease progression without facilitating myocardial repair. The natural regenerative potential of myocardial tissue remains suboptimal due to the limited mitotic capacity of cardiac cells and the decreased cellularity after injury. The only definitive treatment for advanced heart failure following a heart attack is heart organ transplant, but it is greatly limited by the availability of the organs.^[2] Therefore, multiple efforts in pre-clinical research have specifically employed stem cells to restore the function and regenerate the myocardial tissue.^[3-12] The damaged tissue can be replaced by a combination of living cells and biomaterials that aid in the cell survival, growth, mechanical support, and functions *in vivo*. In many cases, the viable stem cells can be transplanted into the biomaterials for targeted therapeutic applications. Embryonic stem cells (ESCs) derived from the inner cell mass of the blastocysts possess the highest pluripotency and the ability to regenerate functional tissues. The ESCs are capable of differentiating into cardiomyocytes to generate a large reservoir of ESC-derived cardiomyocytes (ESC-CMs) for the regeneration of cardiac tissue.^[13, 14] However, transplantation of the ESC-CMs *in vivo* is hindered by suboptimal cell survival post transplantation, compromising their restorative potential. In addition, transplantation of the ESC-CMs into a beating heart encounters significant challenges in cell engraftment post-transplantation.^[15] It is, therefore, of great clinical motivation to increase post-transplantation cell survival and engraftment.

Currently, there are multiple biomaterial strategies to be utilized in conjunction with ESC therapy to address the survival and engraftment problems, but most types of biomaterials exhibit different challenges. It is difficult for the biomaterials to be delivered along with the ESCs through an endovascular catheter directly into the injured myocardium. Therefore, our selection criteria for a biomaterial candidate include the following: 1) biocompatibility with ESCs without affecting their pluripotency, survival, or proliferation; 2) nano-scale to be deliverable with the cells; and 3) cross-link or bind to hydrophobic biomolecules such as pro-survival molecules or extracellular matrix molecules to facilitate engraftment. Graphite Oxide (GO), a two-dimensional, single-layered sheet with both sp^2 and sp^3 carbon, has in recent years shown promise for biomedical use.^[16-19] GO is naturally water-soluble but lacks solubility in buffered solutions due to a charge-screening effect.^[20] This is a barrier for biomedical applications, which take place in buffered solutions or serum; however, covalent attachment of branched polyethylene glycol (PEG) allows excellent solubility for GO in buffered solutions with minimum toxicity both *in vitro*^[17] and *in vivo*.^[21] Importantly, GO possesses intrinsic fluorescence in the visible and near-infrared (NIR) regions,^[17] which has helped to trigger the research into its biomedical potential. By attaching targeting peptides such as RGD to GO, selective cancer cell uptake was shown by NIR imaging.^[17] The NIR region was selected for imaging because of the low endogenous absorption, scattering, and autofluorescence.^[22] The high NIR absorption of GO has made it attractive as a photosensitizing agent for *in vivo* photothermal therapy.^[23] Of note, a wide range of biomolecules can be conjugated to GO through a PEG linker to track GO *in vivo* through PET imaging,^[24] attach targeting peptides for selective cell uptake,^[17] and link chemotherapeutics to GO for drug delivery *in vitro*.^[20] Manufactured GO has a range of sheet diameters from several hundred nanometers up to 10 microns. The PEGylation process of GO previously used long periods of bath sonication, resulting in small sheets ranging in

size from 5-50 nm.^[16] By tailoring the length of the bath sonication and the harshness of the subsequent centrifugation (used to remove multi-layered GO sheets), we are able to design GO with varying average sizes. Previous studies have shown that by using a graphene oxide (chemically reduced GO) surface, mesenchymal stem cell growth and differentiation were enhanced.^[25]

Our long term research goal is to develop an optimal ESC therapy strategy to treat the injured myocardium. For the forgoing reasons, the two objectives of this study are: 1) to examine the effects of GO particles on mouse ESC viability, proliferation, and gene expressions *in vitro* and 2) to identify the optimal size and concentration of GO particles with mouse ESCs. We hypothesize that GO particles will be biocompatible with mouse ESCs and will not alter or hinder the ESC survival, growth and pluripotency. This study identified an optimal range of GO size and concentration to be used in conjunction with mouse ESCs.

Results

Spectroscopic Characterization of the As-Fabricated Graphite Oxide Nanoparticles

Atomic force microscopy (AFM) images of readily synthesized GO nanoparticles clearly showed three different size ranges (**Figure 1A-C**). Characteristic flat sheet-like structure with irregular circumferential shapes was clearly visible. To confirm the sizes of the GO nanoparticles, the size distribution was evaluated by dynamic light scattering. A Gaussian distribution of the GO apparent diameters was found, with polydispersity 0.208, 0.281, and 0.305 of small, medium, and large GO particles, respectively. The median diameters were found to be 11.3 nm, 37.5 nm and 289.0 nm, and the mean diameters were found to be 12.6 nm, 40.8 nm and 327.6 nm for small, medium and large GO, respectively (**Figure S4**). Therefore, the distinct size ranges of GO particle were confirmed.

GO-uptake of Mouse Embryonic Stem Cells

The uptake of GO in all three sizes were monitored with fluorescence microscopy as the GO particles showed auto-fluorescence signals. After 24 hours of culture, uptake of GO particles was found in all groups, with large GO group showing the strongest intensity. Presence of GO within cells continued to be observed throughout the 7 day culturing time. When normalized with the average fluorescence intensity per particle, no significant difference was found among all three sizes. Interestingly, a concentration-dependent difference in the amount of GO particles within the cells was found (**Figure 2A, Figure SI 1-3**).

Effects of GO on Mouse ESC Viability

The majority of the cells remained viable as shown in the Live/Dead staining images (living cells stained green while dead cells stained red). However, mESCs treated with small GO showed observably higher cell death compared to medium and large GO groups, as well as the control group (**Figure 2B-D**). The difference in cell proliferation was found to be significant between the control group and the small GO group at high concentrations at both day 1 and day 7 ($p < 0.01$) while the difference was only statistically significant between the control group and the low concentration small GO at day 7 ($p < 0.01$).

The positive expression of our reporter gene is also indicative of the cell viability and the related cellular and molecular biology at the gene level. As shown in the BLI results, small GO at high concentrations significantly decreased luciferase expression over time compared to the control group starting at day 3 ($p = 0.014$) and continuing after day 7 ($p = 0.0271$), based on Tukey-Kramer post hoc analysis. The small GO at low concentration caused observable decrease in luciferase expression over time compared to the control group, agreeing with the cell proliferation results. BLI signals increased over time for all the other groups (**Figure 3A-B**).

Furthermore, MRI reporter gene expression and its efficacy in molecular and cellular MRI detection of cell viability were also evaluated. Positive T2* signal in the control group indicated positive expressions of our reporter gene. Cells treated with small GO in both concentrations demonstrated diminished reporter gene expressions while large GO had no detrimental effect and medium GO had intermediate effect on the gene expression. The MRI signal on day 7 is shown (**Figure 3C**).

Effects of GO on Mouse ESC Growth and Pluripotency Gene Expression

Cells treated with GO in all sizes and both concentrations proliferated over time. However, mESCs receiving small GO showed the least proliferation compared to the control, medium, and large GO groups (Figures 2B and 3A). For each size, higher concentration of GO treatment decreased proliferation. No statistical difference was found between medium and large GO groups, based on Tukey-Kramer post hoc analysis.

Real Time PCR results showed that all mESCs continued to express all pluripotency genes over 7 days of culturing time with the presence of GO. No statistically significant difference was found in between groups and compared to control (Figure 3B).

Discussion

In this study, we have evaluated the biocompatibility of GO nanoparticles on the mouse ESCs. This is the first report on the biocompatibility of GO nanoparticles on pluripotent stem cells. The growth, viability and gene expression of pluripotency markers of the ESCs are ascertained. The study data demonstrate that the mouse ESCs incorporated GO nanoparticles in all 3 sizes. There is a direct correlation on the concentration and the GO nanoparticle uptake, indicating that the uptake is dose-dependent. Possible mechanism of the GO nanoparticles taken up by the cells appears to occur through endocytosis. By demonstrating the biocompatibility of GO with stem cells, the first step towards their use as a substrate for *in vivo* implantation of a GO-stem cell tissue construct is achieved. GO nanosheets make excellent candidates as the stem cell substrates are easily loaded with hydrophobic drugs^[18, 20] and they possess ultra-high surface areas for loading, in excess of 1900 cm²/gram.^[27]

In addition, mouse ESCs exposed to all groups of GO proliferate over time. Specifically, small GO nanoparticles with diameter less than 20 nm suppress cell growth, show less viability signal, and increase cell death when compared to GO nanoparticles that have medium or large sizes. Higher concentrations of GO in all sizes also have detrimental effects

on the growth and proliferation of mouse ESCs. Furthermore, the expression of pluripotency markers for mouse ESCs is maintained with the GO nanoparticle. Thus, GO particles with a diameter larger than 20 nm in 0.005 mg/ml concentration are found to be biocompatible and can be safely used with mouse ESCs. The GO nanoparticles with diameter less than 20 nm might be in similar size or of physical properties that can participate in mechanisms involving the inhibition of cell survival.

Specifically, the live/dead cell data indicate that there is an inverse relationship between GO average sheet size and toxicity to stem cells. A recent study has also seen a similar relationship between GO sheet size and toxicity to A549 lung basal epithelial cells.^[28]

The study found that smaller GO had a higher rate of radical oxygen species (ROS) generation in cells, inducing oxidative stress known to cause toxicity to the cells. While the study did not examine GO below 10 nm in diameter, we predict that the trend of smaller GO diameter correlating with increased ROS generation would continue. This is due to the decreasing C:O ratio of GO at smaller sizes^[29] and the increased oxidation of GO at the edge sites, resulting in more oxygen functional groups and directly increased ROS.

This work demonstrates the potential of using biocompatible GO nanoparticles to facilitate the engraftment and survival of ESCs post-transplantation. The chemical properties of the GO particles allow for the development of a construct involving the cross-linkage of pro-survival biomolecules for the guided growth and survival of ESCs. Future studies will focus on evaluating the applicability of GO nanoparticles *in vivo* with ESCs.

Experimental Section

Cells and Cell Culture

Mouse ESC line, E14, was derived from inbred mouse strain 129/Ola and maintained on a confluent feeder layer of irradiated mouse embryonic fibroblasts CF-1 line (MEF, GlobalStem, Inc.) in high glucose Dulbecco's Modified Essential Medium (DMEM, Gibco, Carlsbad, CA) with 15% fetal bovine serum (FBS, Hyclone, Logan, Utah), 1% non-essential amino acids, 1% penicillin/streptomycin, 0.05 mM β -mercaptoethanol (β -ME, Millipore, Billerica, MA) and 1000 U/ml leukemia inhibitory factor (LIF, Millipore). To evaluate the effects of GO on mouse ESCs, cells were transduced with a reporter gene construct designed to over-express luciferase and HA/myc surface tags in lentiviral vector for multi-modality BLI and MRI detection of cell viability and related molecular processes. In the studies, cells were cultured on gelatinized surface in media previously conditioned by mouse embryonic fibroblasts (MEF). Cells derived from 20-60 passages were used for the study. The cultures were grown at 37°C and 5% CO₂ in a humidified normoxic environment.

Graphite Oxide Synthesis

Graphite Oxide (GO) was synthesized using a modified Hummer's Method.^[26] In order to control the size of the GO, varying levels of sonication and centrifugation were used. Initially, 10 ml of GO solution at 0.2 mg/ml GO was brought to pH 11 through the addition of sodium hydroxide (J.T. Baker Inc.). Small and Medium GO samples were bath sonicated for 3 hours, while the large GO sample was stirred overnight. 1 ml of 70% HCl (12.1 N,

Fisher Scientific) was added to each sample, which caused aggregation. All three GO samples were washed with water by centrifugation 3x, then run 2x through centrifuge filtration using a 100 kDa MWCO centrifuge filter (Millipore). All GO solutions were then re-suspended in 10 ml of water at a concentration of 0.2 mg/ml. GO concentration was determined using a mass extinction coefficient of 61.5 L/g*cm at 230 nm.^[16, 17] Small GO was bath sonicated with 10 mg of 10 kilodalton, amine terminated 6 armed Poly(ethylene) glycol (6PEG-NH₂) (Laysan Bio) for 5 minutes. 2 mg of N-(3-Dimethylaminopropyl-N'-ethylcarbodiimide) hydrochloride (EDC, Sigma-Aldrich) was added, followed by 1 hour of bath sonication. 6 additional mg of EDC were added followed by overnight stirring. Then, 2 ml of 10x Phosphate Buffer Saline (10x PBS, 0.8% NaCl, 0.02% KCl, and 0.02 M PO₄) were added and the solution was centrifuged at 22,000g for 6 hours. Medium GO was synthesized using the identical procedure steps as the small GO, except bath sonication with 6PEG-NH₂ and EDC lasted for 20 minutes and centrifuged at 22,000g for 30 minutes. Large GO was bath sonicated with 10 mg 6PEG-NH₂ for 1 minute initially; 2 mg of EDC were added followed by 1 hour of stirring. Then, 6 additional mg of EDC were added followed by overnight stirring. 2 ml of 10x PBS were added and the solution was centrifuged at 7200g for 5 minutes.

All GO solutions were washed 6x after centrifugation with a 100 kDa centrifuge filter at 4000g to remove unreacted 6PEG-NH₂ and EDC while bringing the GO to the desired concentration.

GO Spectroscopic Characterization

Graphite Oxide absorption spectrum in water was determined by UV-Vis-NIR (Cary 6000i). Atomic force microscopy (AFM) was used to show the size of GO as well as confirm that only single layered sheets remained in solution after synthesis. To prepare the GO for AFM, 50 µL of a 0.05 mg/ml GO solution was placed on 1 cm silicon substrate for 20 minutes. 1 µL of 70% HCl was added to help adsorb the GO to the substrate. Dynamic Light Scattering (DLS) was used to determine the hydrodynamic radius of the GO solutions using a Brookhaven Instruments 90Plus Particle Size analyzer and a 1 cm quartz cuvette. 0.05 mg/ml GO solutions were used for all DLS measurements.

Experimental Design: To Examine the Effects of Graphite Oxide on Mouse ESCs

The mouse ESCs were cultured onto gelatinized surface without the presence of MEF. Culture media pre-conditioned with MEF was used in the study to maintain mESC pluripotency. Cells were seeded at 25,000 cells/cm² at day 0. GO particles of three size ranges (small: d=3-20 nm; medium: d=20-75 nm; large: d=125-700 nm) were dissolved in media at 0.005 mg/ml (low) or 0.01 mg/ml (high) concentration. Control group received only the media without GO particles and experimental groups received media containing GO of different size ranges and concentrations, resulting in a total of six experimental groups: Small (size)/Low (concentration), Small/High, Medium/Low, Medium/High, Large/Low, Large/High. Cells were exposed to GO for a total of 7 days and media was changed daily. At specific time points, GO particles taken up by the cells (photoluminescence microscopic imaging, n=6), cell number (Picogreen DNA quantification assay, n=6), cell

viability (Live/dead stain, n=3), reporter gene expression (BLI intensity as luciferase expression, n=3) and pluripotency gene expression (rt-PCR, n=6) were measured.

GO-uptake of Stem Cells

Uptake of GO nanoparticles by mouse ESCs was examined by near infrared (NIR) Photoluminescence Microscopic Imaging. Briefly, cells treated with GO nanoparticles were trypsinized and free GO was washed away by PBS. Cells were then plated onto an 8-well chamber slide (Lab-Tek™ Chambered Coverglass, 1.0 Borosilicate). Microscopic fluorescence imaging was done using a 658-nm laser diode excitation with an 80 μ m diameter spot focused by 100x objective lens (Olympus). The resulting NIR photoluminescence was collected using a liquid-nitrogen-cooled, 320 $\times$ 256 pixel, two-dimensional InGaAs camera (Princeton Instruments) with a sensitivity ranging from 800 to 1,700 nm. The excitation light was filtered out using a 900 nm long-pass filter (Thorlabs) so that the intensity of each pixel represented light in the 900 – 1,700 nm range. The NIR photoluminescence images were taken at a fixed exposure time of 3 s. For bright field white light images, a fiber optic illuminator (Fiber-Lite) was used for illuminating the sample in the trans-illumination mode and the images were taken using the same filters at a fixed exposure time of 3 ms. Matlab 7 was used to process the images for any necessary flat-field correction and overlaid with each NIR fluorescence image with its corresponding bright field white light image.

Cell Growth

Total DNA per sample (n=6) was measured using the PicoGreen® dsDNA assay (Molecular Probes) following the manufacturer's suggested protocol. Briefly, at each time point, cells were lysed with 0.1% Triton-X100 and collected using a cell scraper. The amount of DNA/sample was correlated to fluorescence intensity measured with a microplate reader (Tecan, Maennedorf, Switzerland), at an excitation and emission wavelength of 485 nm and 535 nm, respectively. Cell number was converted by a factor of 8 pg DNA/Cell.

Cell Viability

Cell viability was evaluated by Live/Dead® Viability/Cytotoxicity staining (n=3, Invitrogen) and reporter gene expression by BLI and MRI. For Live/Dead stain, membrane-permeant calcein AM is cleaved by esterases in live cells to yield cytoplasmic green fluorescence, and membrane-impermeant ethidium homodimer-1 labels nucleic acids of membrane-compromised cells with red fluorescence. Briefly, stock solutions of calcein AM and EthD-1 were thawed in dark, and diluted in PBS to produce a working solution with 2 μ M of calcein AM and 4 μ M of EthD-1. The working solution was then added directly to the cell monolayer until complete coverage. Cells were allowed to incubate in Live/Dead working solution in dark at room temperature for 30 minutes. Qualitative Live/Dead images were examined under fluorescence microscopes.

For optical BLI to evaluate luciferase expression, D-luciferin was added to the culture media of reporter gene transduced ESCs (ESC-RGs) at a concentration of 15 mg/L. Non-transduced ESCs were used as negative control. Cells were imaged using IVIS-Spectrum (Caliper, Mountain view, CA) for 30 min with 1-min acquisition intervals. Bioluminescence

was quantified in units of average photons per second per centimeter squared per steradian ($\text{P}\cdot\text{s}^{-1}\cdot\text{cm}^{-2}\cdot\text{sr}^{-1}$) using Living Image 2.5 software (Caliper, Mountain view, CA).

To assess *in vitro* MR viability signal of mouse ESCs, the cells were labeled with 20 μL of either SPIO-conjugated antibody against myc or HA (SPIO-myc-MAb or SPIO-HAMAb, Miltenyi Biotech, Auburn, CA). The SPIO-myc- and SPIO-HA-MAb consist of a superparamagnetic iron oxide core with polysaccharide coating, linked covalently to monoclonal antibodies against myc and HA antigens. The mean diameter of the SPIO is approximately 50 nm. After labeling the cells with SPIO-HA- and SPIO-myc-MABs, all cells were washed twice with PBS (1 mL) and centrifuged at 600 RPM for 5 min. The cells were then suspended in 200 μL of PBS and placed in a phantom made of 0.7% agarose. The phantom was placed in the iso-center of MRI coil and scanned using Signa 3.0 T Excite HD scanner (GE Healthcare System, Milwaukee, WI). A GRE sequence using the following parameters optimized T2*-weighted imaging to maximize the signal from SPIO (TR 100 ms, TE 20 to 60 ms, FA 45°, matrix 128×128 , NEX 1, FOV 12, slice thickness 1 mm). The images were analyzed using ImageJ 1.41 software (NIH, Bethesda, MD).

Gene Expression

Gene Expression was measured by reverse transcription followed by real-time polymerase chain reaction (rt-PCR). Primers were designed for pluripotency genes *Sac4*, *Sox2* and *Nanog*. Total RNA was isolated (n=4) using the Trizol® extraction method (Invitrogen, Carlsbad, CA). The isolated RNA was reverse transcribed into cDNA using the SuperScript™ First-Strand Synthesis System (Invitrogen) and amplified using iQ SYBR Green Supermix (Applied Biosystems) and StepOne Plus Real-Time PCR Detection System (Applied Biosystems). All genes were amplified for 40 cycles. Specific gene expression was first normalized to GAPDH and then compared to the control groups.

Statistical Analysis

Data are presented as the mean $\pm$ standard deviation. A two-way analysis of variance (ANOVA) was performed. The Tukey-Kramer post-hoc test was used for all pair-wise comparisons and statistical significance was set at $p < 0.05$. All statistical analyses were performed using the JMP statistical software package (SAS Institute, Cary, NC).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Heidenreich PA, Trogon JG, Khavjou OA, Butler J, Dracup K, Ezekowitz MD, Finkelstein EA, Hong Y, Johnston SC, Khera A, Lloyd-Jones DM, Nelson SA, Nichol G, Orenstein D, Wilson PWF, Woo YJ. Circulation. 2011; 123(8):933. [PubMed: 21262990]

2. Edwards LB, Keck BM. Clinical transplants. 2002;29. [PubMed: 12971435]
3. Kim PJ, Yang PC. Reviews on recent clinical trials. 2012; 7(3):204. [PubMed: 22540909]
4. Hung T-C, Suzuki Y, Urashima T, Caffarelli A, Hoyt G, Sheikh AY, Yeung AC, Weissman I, Robbins RC, Bulte JM. Circulation: Cardiovascular Imaging. 2008; 1(1):6. [PubMed: 19808509]
5. Arai T, Kofidis T, Bulte JWM, de Bruin J, Venook RD, Berry GJ, McConnell MV, Quertermous T, Robbins RC, Yang PC. Magnetic resonance in medicine. 2006; 55(1):203. [PubMed: 16315206]
6. Singla DK, Lyons GE, Kamp TJ. American Journal of Physiology-Heart and Circulatory Physiology. 2007; 293(2):H1308. [PubMed: 17416601]
7. Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, Reinecke H, Xu C, Hassanipour M, Police S. Nature Biotechnology. 2007; 25(9):1015.
8. Hendry Ii SL, van der Bogt KEA, Sheikh AY, Arai T, Dylla SJ, Drukker M, McConnell MV, Kutschka I, Hoyt G, Cao F. The Journal of Thoracic and Cardiovascular Surgery. 2008; 136(4): 1028. [PubMed: 18954646]
9. Gneccchi M, He H, Liang OD, Melo LG, Morello F, Mu H, Noiseux N, Zhang L, Pratt RE, Ingwall JS. Nature Medicine. 2005; 11(4):367.
10. Mirotsou M, Zhang Z, Deb A, Zhang L, Gneccchi M, Noiseux N, Mu H, Pachori A, Dzau V. Proceedings of the National Academy of Sciences. 2007; 104(5):1643.
11. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K. Cell. 2003; 114(6):763. [PubMed: 14505575]
12. Bearzi C, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A, Yasuzawa-Amano S, Trofimova I, Siggins RW, LeCapitaine N. Proceedings of the National Academy of Sciences. 2007; 104(35):14068.
13. Urbanek K, Rota M, Cascapera S, Bearzi C, Nascimbene A, De Angelis A, Hosoda T, Chimenti S, Baker M, Limana F. Circulation research. 2005; 97(7):663. [PubMed: 16141414]
14. Urbanek K, Torella D, Sheikh F, De Angelis A, Nurzynska D, Silvestri F, Beltrami CA, Bussani R, Beltrami AP, Quaini F. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102(24):8692. [PubMed: 15932947]
15. Smart N, Riley PR. Circulation research. 2008; 102(10):1155. [PubMed: 18497316]
16. Robinson JT, Tabakman SM, Liang Y, Wang H, Sanchez Casalongue H, Vinh D, Dai H. Journal of the American Chemical Society. 2011; 133(17):6825. [PubMed: 21476500]
17. Sun X, Liu Z, Welsher K, Robinson J, Goodwin A, Zaric S, Dai H. Nano Research. 2008; 1(3): 203. [PubMed: 20216934]
18. Yang X, Zhang X, Liu Z, Ma Y, Huang Y, Chen Y. The Journal of Physical Chemistry C. 2008; 112(45):17554.
19. Liu Z, Robinson JT, Tabakman SM, Dai H. Materials Today. 2011; 14(7-8):316.
20. Liu Z, Robinson JT, Sun X, Dai H. Journal of the American Chemical Society. 2008; 130(33): 10876. [PubMed: 18661992]
21. Yang K, Wan J, Zhang S, Zhang Y, Lee S-T, Liu Z. ACS nano. 2010; 5(1):516. [PubMed: 21162527]
22. Britton C. Annals of the New York Academy of Sciences. 1998; 838:29. [PubMed: 9511793]
23. Yang K, Zhang S, Zhang G, Sun X, Lee S-T, Liu Z. Nano Letters. 2010; 10(9):3318. [PubMed: 20684528]
24. Hong H, Yang K, Zhang Y, Engle JW, Feng L, Yang Y, Nayak TR, Goel S, Bean J, Theuer CP. ACS nano. 2012; 6(3):2361. [PubMed: 22339280]
25. Lee WC, Lim CHYX, Shi H, Tang LAL, Wang Y, Lim CT, Loh KP. ACS nano. 2011; 5(9):7334. [PubMed: 21793541]
26. Hummers WS, Offeman RE. Journal of the American Chemical Society. 1958; 80(6):1339.
27. Srinivas G, Burress J, Yildirim T. Energy & Environmental Science. 2012; 5(4):6453.
28. Chang Y, Yang S-T, Liu J-H, Dong E, Wang Y, Cao A, Liu Y, Wang H. Toxicology Letters. 2011; 200(3):201. [PubMed: 21130147]
29. Zhao J, Pei S, Ren W, Gao L, Cheng HM. ACS Nano. 2012; 4(9):5245. [PubMed: 20815368]

Figure 1.

Atomic force microscopy (AFM) images of individual, PEGylated GO sheets. A. Small GO sheets had an average size of ~10 nm, B. Medium GO sheets had an average size range of 20-75 nm, and C. Large GO sheets had an average size greater than 125 nm. AFM imaging confirmed that the majority of GO sheets were not aggregated, even after deposition on a silicon substrate.

Figure 2.

A. Fluorescence imaging of small GO uptake by mESC after 3 days, incubated at 0.01 mg/ml concentration, B. Cell proliferation over time, with * indicating statistical difference from the control group, $p < 0.05$ C. Live-dead stain after 7 days of culture with small GO sheets (live cells stain green while dead cells stain red), and D. 10x zoom of live-dead stain after 7 days of culture with large GO sheets.

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B

Control	Small/Low
Small/High	Medium/Low
Medium/High	Large/Low
Large/High	Acellular

C

D

Figure 3.

A. BLI intensity on days 1, 3, and 7, with * indicating statistical difference from the control group, $p < 0.05$ B. BLI of mESC viability on day 7, C. MRI of mESC viability on day 7, and D. Expressions of pluripotency genes Sac4, Sox2 and Nanog of mESCs after 7 days of culture.