Graphene oxide-based substrate: physical and surface characterization, cytocompatibility and differentiation potential of dental pulp stem cells

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ABSTRACT

Objective. The aim of this study was to evaluate the cytotoxicity and differentiation potential of a graphene oxide (GO)-based substrate using dental pulp stem cell (DPSC).

Methods. GO was obtained via chemical exfoliation of graphite using the modified Hummer’s method and dispersed in water-methanol solution. 250 μL of 1.5 mg/mL solution were added to a cover slip and allowed to dry (25°C, 24 h). GO-based substrate was characterized by Raman spectroscopy, AFM and contact angle. DPSC were seeded on GO and glass (control). Cell attachment and proliferation were evaluated by polymeric F-actin staining, SEM and MTS assay for five days. mRNA expression of MSX-1, PAX-9, RUNX2, COL I, DMP-1 and DSPP were evaluated by qPCR (7 and 14 days). Statistical analyses were performed by either Mann–Whitney, one or two-way Anova followed by and Tukey’s post hoc analysis (α = 0.05).

Results. Peaks at 1587 cm\textsuperscript{-1} and 1340 cm\textsuperscript{-1} (G and D band) and ID/IG of 0.83 were observed for GO with Raman. AFM showed that GO was randomly deposited and created a rougher surface comparing to the control. Cells successfully adhered on both substrates. There was no difference in cell proliferation after 5 days. Cells on GO presented higher expression for all genes tested except MSX-1 and RUNX2 for 7 days.

Significance. GO-based substrate allowed DPSC attachment, proliferation and increased the expression of several genes that are upregulated in mineral-producing cells. These findings open opportunities to the use of GO alone or in combination with dental materials to improve their bioactivity and beyond.

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1. Introduction

Graphene oxide (GO) is a highly oxidized form of graphene prepared by oxidation of graphite. It can remain exfoliated in water or organic solvents as single or multilayer atomic sheets [1]. This amphiphilic compound has reactive oxygen functionalities, such as epoxy and hydroxyl (−OH) groups on the basal planes and carboxylic acid (−CO(OH)) groups at the edges [1,2].

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These allow for easy functionalization thus increasing the interest to use GO as a platform for substrate modifications, gene and drug delivery. In addition, GO can be used to enhance physical, mechanical and biological properties of biomaterials [3–5].

The most commonly used method of manufacturing GO from graphite is the Hummer’s method. This technique makes use of a combination of potassium permanganate (KMnO₄) and sulfuric acid (H₂SO₄) to insert oxygen atoms between graphene sheets forcing them apart. This results in a suspension that can be filtered to isolate flakes [3] which can be added to materials or deposited on substrates via different methods (e.g. electrical, dip or spin coating) [2,6,7].

As GO presents a high density of oxygen moieties that allow a wide range of reactions [2], it can be combined with other biomaterials to further improve their bioactivity. The addition of GO to hydroxyapatite creates a composite with improved corrosion resistance and higher adhesion strength to titanium sheets. Furthermore, the GO/hydroxyapatite composite presents higher cell compatibility as compared to the titanium and hydroxyapatite substrates used as controls [8]. Gelatin-based material presented increases of 84% in tensile strength and 65% in Young’s modulus when reinforced with 1 wt% GO sheets. The gelatin/GO composite also displayed improved bioactivity and increased calcium phosphate nanocrystals formation [9]. Nanocomposites consisting of calcium phosphate nanoparticles and GO microflakes have demonstrated a synergistic effect in accelerating stem cell differentiation into osteoblasts [10]. GO-modified β-tricalcium phosphate significantly enhanced the proliferation, alkaline phosphatase activity and osteogenic gene expression. In addition, the GO-modified bioceramic increased the rate of bone formation in vivo [11].

As shown previously, GO can enhance physical and mechanical properties of biomaterials and stimulate the differentiation of stem cells towards mineral-secreting cells. Hence, dental materials such as mineral trioxide aggregate, calcium hydroxide and implant materials may benefit from the GO-mediated improvements observed. Nonetheless, its ability to sustain dental stem cells viability and differentiation remains largely unknown. Our objective was to evaluate the cytotoxicity and differentiation potential of a GO-based substrate on dental pulp stem cells (DPSCs). The hypothesis is that the GO-based substrate will allow stem cell proliferation and increase the expression of the genes assessed.

2. Materials and methods

2.1. Graphene oxide and sample preparation

GO was prepared according to the modified Hummer’s method [12]. In detail, 5 g of graphite (Lanka Graphite Limited, Canada) and 2.5 g of NaNO₃ were mixed (10 min, ice bath) with 12 mL H₃PO₄ and 108 mL H₂SO₄. 15 g of KMnO₄ were subsequently added to the mix and allowed to react for 2 h in an ice bath. The suspension was stirred at 40 °C and 98 °C for 60 min each while deionized water was added to a total volume of 400 mL. After 5 min, 15 mL of H₂O₂ were added. The suspension was centrifuged, washed with deionized water and 5% HCl solution and dried in a vacuum oven at 180 °C for 24 h. GO was dispersed in a water-methanol (1:5) solution at a concentration of 1.5 mg/mL. Finally, 250 μL of the solution were added to a cover slip (22 mm × 22 mm) and allowed to dry at 25 °C for 24 h.

2.2. Sample characterization

Raman characterization was performed for both GO and glass (control) at room temperature, with an excitation laser source of 532 nm (WITEC CRM 200 Raman spectrometer, Germany). The laser power was kept below 0.1 mW to prevent overheating of the sample. The ID/IG intensity ratio is correlated with crystallite size (La) as described in the Eq. 1 where A is a constant for a fixed laser excitation energy.

\[
\frac{ID}{IG} = \frac{A}{La}
\]  

Surface topography was characterized using tapping mode atomic force microscope (AFM, Bruker AXS, Germany) equipped with a ScanAsyst (n = 3). A silicon nitride cantilever was used with a resonance frequency of 40–90 kHz and a spring constant 0.4 N/m.

The surface wettability was investigated via contact angle measurements (VCA Optima, USA) at atmospheric conditions and room temperature (n = 5, 3 readings per sample). A 15 μL droplet of deionized water was automatically dispensed on the substrates. The image of the liquid droplet was obtained in real time acquisition mode using a CCD camera and the angle between the tangent line and the base line was determined.

2.3. DPSC culture

The use of human cells in this study was approved by NUS Institutional Review Board (Approval Number: NUS 2094). Human dental pulp stem cells from single donor (DPF003, Allcells, USA) were sorted by fluorescence-activated cell sorting analysis (FACS, BD Fortessa, BD Biosciences, Germany) for CD 34, CD73, CD90, CD105 (Millipore, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, USA), supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

2.4. DPSC attachment, proliferation and differentiation

DPSCs (5 × 10⁵ cells, passage 3) were seeded onto the surface of GO and glass substrates and left undisturbed for 24 h. Morphology and proliferation were evaluated for 5 days through scanning electron microscopy (SEM) and fluorescence microscopy. Briefly, for SEM cells were fixed (4% paraformaldehyde and for fluorescence with 2.5% glutaraldehyde, room temperature for 20 min) and washed with deionized water. For SEM, samples were sputter-coated with 200 Å of gold and observed under SEM (Olympus FEI 650 Scanning Electron Microscope, USA) at an operating voltage of 2 kV. For fluorescence, cells were permeabilized with 0.1% Triton X-100 in 1x phosphate buffered solution (PBS, Invitrogen) for 5 min at room temperature and washed with PBS. Blocking solution was applied for 30 min at room temperature followed by
staining with TRITC-conjugated phalloidin (1:1000, Merck Millipore, USA) for 60 min. Samples were imaged using a confocal microscope (FV1000, Olympus Optical, Japan).

Cell proliferation was assessed for 5 days using MTS assay. Cells were washed with PBS and 20 μL of the reagent (CellTiter 96 AQueous One Solution Assay, Promega, USA) mixed with 100 μL of DMEM were added to the wells. Samples were incubated at 37 °C for 2 h in a humidified 5% CO₂ atmosphere. The amount of soluble formazan produced by cellular reduction of MTS was measured by microplate reader (InfiniteM200, Tecan, Germany) at a wavelength of 490 nm.

Gene expression was obtained based on the ΔΔCq method for calculating relative gene expression from quantification cycle (Cq) values obtained by quantitative real-time PCR (qPCR) analysis. The DPSCs from each substrate were harvested after 7 and 14 days and total RNA was isolated (Purelink RNA Mini Kit, Invitrogen). Following this, cDNA was synthesized (iScript RT Supermix, Bio-Rad, USA) and the expression of the target and housekeeping genes (GAPDH and β-actin) were quantitatively assessed. Relative expression was calculated against GAPDH. The oligonucleotide primer sequences are shown in Table 1.

All tests were performed in triplicates.

2.5. Statistical analysis

Data for contact angle and qPCR were analyzed with Mann-Whitney and cell proliferation with two-way ANOVA and Tukey’s test. The global significance level was set at 0.05 (SigmaPlot 11, SPSS, USA).

3. Results

DPSCs characterization is shown in Fig. 1. At least 97% of the cells were positive for the mesenchymal stem cells-related markers assessed.

The spectrum obtained by Raman and ID/IG ratio (0.83) exhibit the typical response of GO (Fig. 2A). The G and D bands are the most prominent in GO samples and absent in

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo sapiens msh homeobox 1 (MSX-1)</td>
<td>Forward</td>
<td>5′-ACACAAAGACGAACGTAAAGCC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CACATGGCCTGTGATAGTC-3′</td>
</tr>
<tr>
<td>Paired box 9 (PAX-9)</td>
<td>Forward</td>
<td>5′-GGAGGACTTCTGCTGAAACGG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CGCCTGATCTCAGCCGTTC-3′</td>
</tr>
<tr>
<td>Runt-related transcription factor 2 (RUNX2)</td>
<td>Forward</td>
<td>5′-CATCTCCAGCCTCAGAGAATAA-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GTCTGGGACCAACCTCAGAGG-3′</td>
</tr>
<tr>
<td>Collagen type I (COL I)</td>
<td>Forward</td>
<td>5′-CTCCAGCTTTGACATGAG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-AGTTCCCAACTGAGAAGTC-3′</td>
</tr>
<tr>
<td>Dentin matrix acidic phosphoprotein 1 (DMP-1)</td>
<td>Forward</td>
<td>5′-TGGAGTGCAGTGCAGCAGG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CCATTTCCCCTGCACACCACAGC-3′</td>
</tr>
<tr>
<td>Homo sapiens dentin sialophosphoprotein (DSPP)</td>
<td>Forward</td>
<td>5′-ATGAGGAATGATGACACGGC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-AGTCCCTTCACACTGTGCACCA-3′</td>
</tr>
<tr>
<td>GAPDH</td>
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<td>5′-CAGGCTGTGCTATCCCTGTA-3′</td>
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<td>5′-CGGCTGATGTCACACGGTC-3′</td>
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<tr>
<td>β-Actin</td>
<td>Forward</td>
<td>5′-CTCCTTCAGATGTGACAGCC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CAGGCTGTGCTATCCCTGTA-3′</td>
</tr>
</tbody>
</table>

Fig. 1 – Dental pulp stem cells characterization via FACS.
the control. The G band (~1587 cm⁻¹) is an indicator of the graphitization and arises from the stretching of the C–C bond in graphitic materials whereas the D band is associated with the disorder of the system and structural defects. The contact angles were statistically similar for both substrates (Fig. 2B).

The three-dimensional AFM topography of the substrates can be observed in Fig. 3. The surface of glass (A) was smooth while the presence of GO created a rougher surface (B and C). The GO flakes were randomly deposited and no pattern was observed.

Cell attachment and proliferation were assessed by fluorescence microscopy (Fig. 4), SEM and MTS assay (Fig. 5). DPSCs were capable to attach and proliferate on both substrates. Fig. 5 shows that cells are successfully anchored to the rough surface provided by GO after 3 (Fig. 5A and B) and 5 days (Fig. 5C and D). Although the control (glass) showed higher absorbance for 3 days, there was no significant difference in cell proliferation for both substrates after 5 days in culture (Fig. 5E).

Gene expressions are shown in Fig. 6. For 7 days, there was up-regulation of all genes tested on GO except MSX-1 and RUNX2 that were similar to the control. Nonetheless, all the genes for GO were up-regulated as compared to the control after 14 days.

### 4. Discussion

Graphene oxide can be obtained by chemical exfoliation of graphite [1,3]. The nature of GO was confirmed by Raman spectroscopy. Here, the fingerprints for different groups and number of layers reflect changes in the electron bands and allow unmistakable identification of GO (Fig. 2). The D peak is due to first order resonance and is associated with the disorder of the system (structural defects generated by the presence of epoxy and hydroxyl groups on the carbon basal plane) while the G peak is an indicator of the plane optical vibrations of sp² carbon [13]. The ID/IG ratio diminishes for values of La in the range of few nanometers because of the sp² carbon hexagonal structure [14,15]. Likewise, the 0.83 ID/IG ratio is consistent with the values reported in the literature for graphene oxide [16]. The three peaks centered around 3000 cm⁻¹ correspond to the response of the 2D (~2700 cm⁻¹ corresponding to the stacking) due to a D–G combination (~2947 cm⁻¹, S3 mode) and 2D' at 3110 cm⁻¹ [17].

Graphene has been recognized as a hydrophobic material [2]. The water contact angle on graphite is of 98.3° and increases to 127° on graphene monolayer or a stack of a very few graphene layers [18]. The contact angle observed for GO in our study was approximately 60° and similar to the control (Fig. 2). This is related to the presence of oxygen-containing functional groups on the basal plane which makes GO hydrophilic [1,19]. The hydrophilicity of GO favors high levels of protein adsorption (e.g. insulin, serum and others) that may favor stem cell differentiation [2,20].

DPSCs present important similarities to bone marrow stem cells (BMSCs). They are analogous on the expression profiles for more than 4000 genes and present similar expression for a several markers such as fibroblast growth factor 2, alkaline phosphatase, type I collagen, osteocalcin and others [21,22]. DPSCs can be obtained from extracted teeth, under local

![Graphene Oxide Characterization](image-url)
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anesthesia without aesthetic damage. They can undergo both osteoblastic and odontoblastic differentiation which make this cell type an interesting model for tissue engineering research [21,23–25]. DPSCs were capable to proliferate on the GO-based substrate manufactured as observed in Figs. 4 and 5. It is also to be observed in Fig. 5A–D that the irregular surface provided by GO did not compromise cell anchorage. In addition, DPSCs presented the typical fibroblast-like appearance and similar proliferation after five days on both GO and glass (Fig. 5E).

Finally we have evaluated the DPSCs gene expression on the GO-based substrate. Except for MSX-1 and RUNX2 for seven days, there was up-regulation of all genes tested from cells cultured on GO for both time points (Fig. 6). This is rather exciting as no chemical inducers for differentiation were used and all of those genes are related to differentiation processes towards phenotypes that can secrete mineral deposits. For example, RUNX2 is essential for osteoblastic differentiation and skeletal morphogenesis acting as a scaffold for nucleic acids and regulatory factors involved in skeletal development. It can upregulate the expression of OCN which is a determinant of bone formation and a marker expressed in late stages of osteoblastic differentiation [26,27]. Interestingly, the substrate was also capable of increasing the expression of both DMP-1 and DSPP which are intimately related to odontogenic differentiation of stem cells from dental pulp [28,29].

To our knowledge, this study provides the first evidence that GO can induce high expression of odontogenic genes, which may have impact in the use of GO in biomaterials to induce odontoblastic differentiation or as a substrate for pulp biology research. Future studies must focus on the potential of GO to induce or increase differentiation of DPSCs into specific lineages.

5. Conclusion

GO is a derivative of graphene that can be obtained via chemical exfoliation from graphite. It presents several oxygen species which allow functionalization and combination with biomaterials. The GO-based substrate allowed stem cell attachment, proliferation and increased the expression of several genes that are upregulated in mineral-producing cells. These findings open new opportunities for the use of GO alone or in combination to improve bioactivity of dental materials and beyond.

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