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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 ($\alpha = 0.05$).
Results. Peaks at 1587 cm^{-1} and 1340 cm^{-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.

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 (—C(O)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 ($KMnO_4$) and sulfuric acid (H_2SO_4) 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 $NaNO_3$ were mixed (10 min, ice bath) with 12 mL H_3PO_4 and 108 mL H_2SO_4. 15 g of $KMnO_4$ 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 H_2O_2 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 CRM200 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.

$$\frac{ID}{IG} = \frac{A}{La} \quad (1)$$

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 (5×10^3 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