RETRACTED ARTICLE: Fluorescent labelling in living dental pulp stem cells by graphene oxide quantum dots

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ABSTRACT
Cellular labelling is possible to offer significant information after transplantation for the purpose of determining stem cell therapy’s efficacy. According to the research, it has been reported that graphene oxide quantum dots (GOQDs) are a kind of healthy biological labelling agent for stem cells which show little cytotoxicity. GOQDs’ interactions have been examined on the dental pulp stem cells (hDPSCs) of human beings for the purpose of investigating GOQD’s biocompatibility and uptake and explored GOQDs’ effects on hDPSCs’ metabolic activity and the proliferation. According to the outcomes, GOQDs have been accepted by hDPSCs in a time-dependent and concentration-dependent behaviour. Moreover, no important changes have been discovered within hDPSCs’ proliferation, viability as well as metabolic activity after treatment with GOQDs. Therefore, such resources have shown that GOQDs can be multifunctional agents for cell therapy, drug delivery as well as cell imaging and also as outstanding candidates for labelling stem cells.

Introduction
The stem cells of humans are representative to a revolutionary tool for drug discovery and disease modelling. The generation of the cell types which are relevant to tissues which exhibits the molecular and genetic background of patients provides the competence to improve useful and personal therapies [1]. Dental pulp stem cells (DPSCs) among other stem cells have been recognized to be a particular kind of adult stem cell source by Gronthos in 2000 [2]. Aside from their neural crest origin, DPSCs show embryonic markers which include Nanog, Sox2 as well as Oct4 and endow pluripotent features to them [3]. Such cells have the ability to be different from multiple cell types [4], which makes them potential candidates for regenerative medicine applications such as angiogenesis, nerve tissue regeneration as well as bone tissue engineering [5]. Aside from their outstanding stem cell properties, DPSCs protect their cell surface phenotype and stem cell features within the culture for long term [6]. Currently, DPSCs have turned to be significant resources within tissue engineering.

Currently, live cell imaging has turned to be a routine tool within biomedical and life science research. Moreover, it is vital to understand the organisal improvement through monitoring the interactions among and within the cells during their differentiation and growth. Nevertheless, a particular stem cell population’s enumeration and characterization seem to be hard since such cells are made up of a small percentage of cells in various kinds of milieu. As such, because of the shortage of accuracy offered by strategies with convenience, biolabelling techniques are at the attention’s centre for improving the characterization of stem cells with high rate of specificity and sensitivity. For this, many luminescent resources have been discussed [7].

Through the comparison with conventional fluorescence dyes for imaging, inorganic nanoparticles are core resources within nanotechnology and nanoscience. Graphene oxide quantum dots (GOQDs) have been a type of luminescent nanomaterials among them which is based on carbon with superior biocompatibility and outstanding optical-electronic features. GOQDs have attracted more and more attention because of their particular properties, such as easy preparation, robust chemical inertness, low toxicity as well as high luminescence. GOQDs’ thin sheet contains a large amount of functional groups which contain oxygen and grafted covalently at the edges or with the basal plane [1]. GOQDs have been adopted for biolabelling, bioimaging as well as other biomedical applications because of its photoluminescent (PL) properties. GOQDs have gained a lot of interest as functional resources with high chemical stability as well as particular optical properties [8]. In our previous study, fluorescent...
GOQDs have been adopted within our previous researches in the absence of any conjugation or coating with macromolecules and have adopted efficiently and directly as bioimaging probes for the bone marrow stem cells’ labelling. Additionally, GOQDs also show little cytotoxicity, where accordance with TEM (Japan) after sonicated in ice water bath (100 W, 30 min). In gaining by transmission electron microscope (TEM) (JEOL, Japan) after sonicated in ice water bath (100 W, 30 min). In accordance with TEM’s images, nearly 200 GOQDs’ lateral diameters had been measured by image J software. GOQDs’ size distribution as well as particle size within full cell culture medium had been evaluated by dynamic light scattering (Brookhaven Instruments Co, USA).

Materials and methods

Characteristics of GOQDs

GOQDs (product number: XF074) had been bought from XFNANO, INC (Nanjing, China). GOQDs’ images had been gained by transmission electron microscope (TEM) (JEOL, Japan) after sonicated in ice water bath (100 W, 30 min). In accordance with TEM’s images, nearly 200 GOQDs’ lateral diameters had been measured by image J software. GOQDs’ size distribution as well as particle size within full cell culture medium had been evaluated by dynamic light scattering (Brookhaven Instruments Co, USA).

Cell isolation and characterization

The third molars of healthy humans (the patients who are from 18- to 22-years old) extracted for orthodontic causes had been adopted within the research. The experimental protocols had been authorized by the Department of Cariology and Endodontics, Nanjing Stomatological Hospital, Nanjing, China. Informed consent had been gained from all the patients before the extraction of tooth. Dental pulp tissues had been separated gently from the teeth which were extracted freshly after thoroughly rising within phosphate-buffered saline (PBS), digested and minced with 4 mg/mL dispase (Gibco, Grand Island, NY, USA) and 3 mg/mL collagenase type I (Invitrogen Life Technology, Carlsbad, CA, USA) at 37°C for 1 hour. Single-cell suspensions had been gained from the mixtures which were digested through a 70 μm cell strainer and had been seeded into six-well plates (Costar, Cambridge, MA, USA) within the Dulbecco’s modified Eagle’s medium (DMEM; Gibco, USA) added by 20% of antibiotics (Invitrogen), and normal tissues [10–12]. The near-infrared-emitting luciferins and bright chemiluminescent metal’s expanded colour palette has significantly developed the sensitivity of the cells’ in vivo tracking and enabled real-time cellular chemiluminescence imaging with high spatial resolution [12]. Nevertheless, the excitation light’s requirements for fluorescence detection, upconversion nanoparticles as well as semiconductor quantum have been globally adopted for live cell imaging because of their photostability and colour tenability and so on. Nevertheless, such inorganic resources usually include heavy metals and have metabolism issues potentially. It may sometimes cause serious issues such as autofluorescence from the specimen, photo-dependent biological phenomena’s perturbation and phototoxicity [13]. For the purpose of overcoming such problems, it has been suggested that GOQDs have superior features of targeted delivery, emission within the NIR scope, nice photostability, big Stokes shift as well as high fluorescence intensity, which enables them to be a great fluorescent agent for living cell imaging [14].

The stem cell scholars’ demand for a long time has been observing the activities which are relevant to pathological and physiological procedures as well as the bio-molecular dynamics in real time. There has been no systematic work on using GOQDs to the best of our knowledge as NIRF imaging agent to improve the performance of stem cell labelling for in vitro and in vivo.

Cell culture and co-incubation with GOQDs

DPSCs had been cultured in 5% CO2 at 37°C within the DMEM (Gibco, USA) including 100 mg/mL streptomycin (Gibco, USA), 100 U/mL penicillin as well as 10% FBS (Gibco, USA). Medium had been replaced every day. GOQDs had been diluted and sonicated to various kinds of concentrations (0, 50, 100, 150, 200, 300, 400 and 500 μg/mL) before being administered to cells. The time of exposure had been 24 h.

Cell viability assay

The viability of cells had been analysed by adopting a Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). Cells had been plated within a 96-well plate (Thermo Scientific, USA) at a density of 1.5 × 10^4 per well and incubated overnight. After exposing to GOQDs for 24 h at various kinds of concentrations, 10 μL CCK-8 solution had been replenished to all the wells and later on the cells had been incubated in 5% CO2 at 37°C for 2 h. The absorbance had been decided at 450 nm.

Cell cycle analysis

DPSCs which had been pretreated with GOQDs had been immobilized within 70% ethanol at –20°C for 24 h, PBS had been used to wash cells and 50 μg/mL propidium iodide (PI) which contains RNase (100 μg mL−1) had been used to stain the cells which was then incubated for 30 min at 37°C and then a FACS Calibur (BD Biosciences, Mountain View, CA) had been used to measure the cells. Cell clumps and debris had been eliminated. Flowjo 7.6.1 software had been adopted to quantify the populations of the G1, G2 and S.
**CLSM and TEM images**

Trypsin had been adopted to detach DPSCs which were re-cultured onto collagen-coated 14 mm cover glass with the density of $5 \times 10^4$ cells per well in a plate with 24 wells. To eliminate the interference of the FBS during endocytosis, PBS had been used to first rinse cells for two times after the incubation for 12 h and then 200 µg/mL GOQDs was replenished within RPMI 1640 media. PBS had been used to wash the cells for three times before the fluorescence imaging and then the cells had been immobilized for 20 min with 4% paraformaldehyde (pH: 7.4) and the cover glass had been immobilized onto microscope slide. Fluorescence microscope (Nikon ECLIPSE, TS100) as well as confocal laser scanning microscope (CLSM; A1R, Nikon, Japan) had been used to take the cell images. PBS was used to wash DPSCs treated with GOQDs for three times and then DPSCs had been immobilized in phosphate buffer with 2.5% glutaraldehyde at 4 °C for 1 h. Later on, the cells had been subjected to 1% osmium tetroxide for 1 h, treated with 2% uranyl acetate solution for 1 h. Later on, ethanol-water solutions had been used to wash the cells for dehydration for some times. At last, cells had been placed into resin for the purpose of getting resin block. The resin block had been sliced to ultrathin sections which had been transferred onto cooper grid. Uranyl acetate was used to stain the sections for TEM measurement (FEI, USA, Tecnai G2 Spirit BioTWIN).

**AlamarBlue™ assay**

For the purpose of measuring the cells’ metabolic activity as mentioned before, an AlamarBlue™ assay had been used and 3000DPSCs per cm² had been seeded per well for 24 h in a plate with 96 wells and then exposed to 0, 50, 100, 150, 200, 300, 400 and 500 µg/mL GOQDs for another 24 h. The medium had been changeable every 48 h. PBS had been adopted to wash the cells for two times 6 days later. The cells were also replenished with dye, which resulted from the changes in the solution’s colour from blue to pink. A microplate reader was used to measure the absorbance.

**Photoluminescent**

The cells had been seeded at $2 \times 10^4$ cells per mL onto the plates with 384 wells overnight. GOQDs’ various kinds of concentrations (0, 50, 100, 150, 200, 300, 400 and 500 µg/mL) had been replenished, and the plates had been incubated for 24 h at 37 °C. The supernatant had been removed after incubation and 0.1 M PBS solution had been used to wash the cells for three times for the purpose of washing off graphene quantum dots (GQDs) which were attached to DPSCs’ surface and removing residual GOQDs. Later on, lysis buffer (10% dodecyl phenyl sodium sulfonate and 0.1% NH4Cl solution) had been replenished to all the wells, and the plates had been incubated at 37 °C overnight. A microplate reader had been used to detect GOQDs’ fluorescence intensity.

**Statistical analysis**

One-way ANOVA had been used to perform statistics within GraphPad Instant software (GraphPad Software). It was reported that information was means ± standard deviation and statistical importance had been stipulated at 5% and 1% $p$ values (**$p < .01$; *$p < .05$) had been regarded highly and statistically important.

**Results**

**Characterization of GOQDs**

A nice colloidal stability had been exhibited by the GOQDs within the physiological media like PBS. A transparent dispersion without colour was formed which emitted blue light under the light irradiation of UV (Figure 1(A)). PL spectrum (Figure 1(B)) presented the excitation wavelength of 340 nm. The transmission and high-resolution TEM images (Figure 1(C)) presented that GQDs included a nice crystal graphene structure and were dispersed well. The lateral size was 5.0 ± 0.8 nm on average (Figure 1(D)).

**Cellular uptake of the GOQDs**

TEM was adopted to examine the GOQDs’ cellular internalization. The cells had been incubated for 12 h with GOQDs for the purpose of getting high extent of internalization. Figure 2(A–C) presented the TEM images of GOQDs’ cellular uptake. According to what black arrows suggested, GOQDs had been found within the cytoplasm. According to the enlarged image (Figure 2(D)), several small black dots had also been aggregated inside endoplasmic reticulum (ER) and within cytoplasm. It is not easy to define the diffusion of GOQDs into the nucleus with merely TEM images.

To verify visually the GOQDs’ location gained from the images of TEM, the GOQDs’ sub-cellular localization in detail had been further explored by fluorescence microscopy since the GOQDs’ PL property permits people to directly monitor the cellular uptake. GOQDs were used to incubate the DPSCs’ images for 24 h and then incubated for 72 h with the fresh media without GOQDs had been taken adopting CLSM. Since GOQDs’ maximum fluorescence emission had been centred at $\approx 460$ nm (excited at 340 nm), every image had been taken for a short exposure time for the purpose of excluding the cellular autofluorescence’s interference [15]. According to Figure 4, through combining the bright field images with the fluorescence images, it is obvious that several GOQDs were within the cytoplasm and several were in the inner nucleus (Figure 3(A–C)). When the fresh culture medium was used to incubate the cells for another 3 d without GOQDs, the entered GOQDs were accumulated within nucleus according to Figure 3(D–F).

**Quantification of GOQD uptake**

According to some researches before, GOQDs can penetrate into DPSCs in an easy and direct way by adopting confocal microscopy. A microplate was used for detecting GOQDs’ fluorescence intensity which was dissolved within PBS for the
purpose of determining GOQD uptake's efficiency and confirming the microscopic observation. The GOQDs had been emissive strongly with an emission wavelength of 460 nm and an excitation wavelength of 340 nm. According to Figure 4(A), the PL intensity created by the GOQDs which highly related with the concentrations of GOQD (0–500 μg/mL) ($R^2 = 0.9992$), which suggested that it had been a reliable and precise strategy for quantifying the GOQDs.

When the DPSCs had been incubated for 24 h with various GOQDs concentration (0–500 μg/mL), the microplate reader had been used to measure the resulting fluorescence. According to Figure 4(B), an increase which was dependent on dose existed within the intensity of fluorescence at 0–200 μg/mL although the intensity of the fluorescence was a little higher at the highest concentration tested (500 μg/mL) through the comparison with control group. It appeared that the growth of DPSCs was arrested by GOQDs at the highest concentration (i.e. 500 μg/mL) down to 46.8% after 7d exposure. The outcomes indicate that the DPSCs proliferation's certain inhibition had been caused by 500 μg/mL GOQDs.

### Biocompatibility of GOQDs

#### Proliferation of DPSCs upon exposure to GOQDs

DPSCs' proliferation which was incubated with GOQDs increased in a manner which was dependent on time (Figure 5(A)). Similar growth trends were shown by various kinds of concentrations. The cells' proliferation had been kept after 1d despite of the GOQDs’ presence. After 1, 3, 5, and 7d, 50, 100, 150 and 200 μg/mL GOQDs did not lead to DPSCs proliferation’s negative influence through the comparison with control group. It appeared that the growth of DPSCs was arrested by GOQDs at the highest concentration (i.e. 500 μg/mL) down to 46.8% after 7d exposure. The outcomes indicate that the DPSCs proliferation’s certain inhibition had been caused by 500 μg/mL GOQDs.

### Effect of GOQDs on the DPSCs metabolic activity

The influence of GOQDs on the metabolic activity was next examined when it had been proved that the influence of GOQDs on the proliferation of DPSCs was not detectable. An
Figure 2. TEM images of DPSCs incubated with (A) GOQDs (200 μg mL⁻¹) for 12 h, and (B) in cytoplasm and (C) in endoplasmic reticulum. (D) High magnification of the GOQDs in the endoplasmic reticulum.

Figure 3. CLSM images of the DPSCs incubated with GQDs (200 μg/mL) for 24 h (A–C) and re-incubated with fresh culture media without GQDs for another 3d (D–F). Excitation wavelength was 340 nm. Scale bars equal to 20 μm.
AlamarBlue™ assay had been adopted for the purpose of measuring the cells’ metabolic activity. According to what has been presented within Figure 5(C), the cell metabolic rate seems to be quite similar at the time of exposing to the concentrations that were tested (0–500 μg/mL). Through the comparison with control, no important differences existed. In other words, GOQDs did not affect profoundly on the metabolic activity of the DPSCs in vitro.

**Cellular retention**

Being a kind of effective cell labelling agent, it is significant that a proportion of the cells in the population keep their label throughout the experiment’s time course. For the purpose of investigating GOQDs’ cellular retention, DPSCs had been incubated for 24h with 200 μg/mL GOQDs and the culture medium had been changed to a normal one in the absence of GOQDs and cultured for 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 days (Figure 6(A)). With the increase in incubation days, the fluorescence signals within the cells had been reduced slightly. On one hand, the gradual reduction might be because of the DPSCs’ quick proliferation. On the other hand, the gradual reduction could be because of the excretion of GOQDs from cells, which causes the GOQD signal’s dilution. For the purpose of further characterizing GQDs’ exocytosis, the fluorescence intensity had been quantified by the microplate readers over time [16].

**Discussion**

The GOQDs’ retention, internalization as well as the cellular uptake mechanisms had been discussed by adopting the observations of TEM and confocal microscopy. The GOQDs’ cellular responses on DPSCs’ competence to self-renew had been explored by the use of immunocytochemistry. The discoveries showed in the research offer data on GOQDs’ mechanism and quantification within cellular uptake and present that the GOQDs show great potential applications within...
stem cells’ biolabelling and prove nice biocompatibility under a common dose.

According to the reports of some researchers, modified micrometer-sized GO sheets come into the cells through the endocytosis [15]. Moreover, the internalization of the single-walled carbon nanotubes with small size (50–100 nm long) through caveolae-mediated endocytosis [17–19]. To visualize the GOQDs’ location gained from the TEM images, the GOQDs’ sub-cellular localization in detail had been further explored by fluorescence microscopy since the DOQDs’ PL property permits people to monitor their cellular uptake in a direct way. The outcome consists with GOQDs accumulation’s observation on the TEM images in ER (Figure 2(D)).

The discoveries not only offered significant data towards carbon nanoparticle probes’ improvement for intracellular imaging application but also provided insights into the fundamental uptake mechanisms [20]. GOQDs possess intense emission and big absorptivity within NIR area, strong photo-bleaching resistance, and biocompatibility and big Stokes shift [21], as well as particular cell targeting competence [22], which renders them appropriate for imaging with detection’s high sensitivity. Beyond fluorescent probe materials’ superior properties [23], when they have been adopted within living organisms, it is also vital to know the interaction between cells and the materials [24].

Conclusion
The GOQDs’ intrinsic PL and small lateral size which had been gained by the Photo-Fenton reaction [24] made it possible to research the performance of their cell labelling. In addition, it has also been proved that the metabolism, proliferation as well as cell viability did not affected by GOQDs labelling, which suggests that GOQDs are possible to be bio-compatible with DPSCs. Totally, such kind of discoveries show a primary exploration of the stem cells’ labelling with GOQDs and indicate that GOQDs are possible to be an eco-friendly and useful probe with low-toxicity for biomedical imaging.

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