In vitro toxicity evaluation of graphene oxide on A549 cells

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Abstract

Graphene and its derivatives have attracted great research interest for their potential applications in electronics, energy, materials and biomedical areas. However, little information of their toxicity and biocompatibility is available. Herein, we performed a comprehensive study on the toxicity of graphene oxide (GO) by examining the influences of GO on the morphology, viability, mortality and membrane integrity of A549 cells. The results suggest that GO does not enter A549 cell and has no obvious cytotoxicity. But GO can cause a dose-dependent oxidative stress in cell and induce a slight loss of cell viability at high concentration. These effects are dose and size related, and should be considered in the development of bio-applications of GO. Overall, GO is a pretty safe material at cellular level, which is confirmed by the favorable cell growth on GO film.

1. Introduction

Because of their unique physicochemical properties, graphene and its derivatives have attracted tremendous research interest (Allen et al., 2010; Geim, 2009; Rao et al., 2009). They hold great promise in electronics, energy, materials and biomedical areas (Allen et al., 2010; Geim, 2009; Neto et al., 2009; Rao et al., 2009). Graphene oxide (GO) is one of the most important graphene derivatives and has been extensively studied in recent years (Park and Ruoff, 2009). We reported that GO could be used to produce directly the graphene-based composites (Cao et al., 2010). Beyond that, GO has been also used in many areas, including hydrogen storage (Wang et al., 2009b), catalysis (Scheuermann et al., 2009), transparent film (Dikin et al., 2007) and electrode (Eda et al., 2008).

In particular, GO is a potential candidate for biological applications, such as drug delivery and bio-analysis (Liu et al., 2008; Lu et al., 2010; Sun et al., 2008; Yang et al., 2009b, 2010; Zhang et al., 2010a). For example, Liu et al. (2008) found that GO could deliver doxorubicin into cancer cells for the therapeutic purpose.

Many studies have shown that nanomaterials might have side-effects on health (Aillon et al., 2009; Oberdörster et al., 2005; Xia et al., 2009). For instance, we have reported the toxicity and retention of carbon nanotubes (CNTs) in vitro and in vivo (Deng et al., 2007; Jia et al., 2005; Wang et al., 2004, 2008, 2009a, 2009c; Yang et al., 2008a, 2008b, 2009a). The thorough understanding of the biological behavior of nanomaterials guarantees the sustainable nanotechnology (Aillon et al., 2009; Hussain et al., 2009; Nel et al., 2006; Oberdörster et al., 2005; Xia et al., 2009). However, for the newly developed graphene and its derivatives, such information is generally lacking to date.

Herein, we performed a systematic study on the toxicity of GO at cell level. The morphology, viability, mortality and membrane integrity of A549 cells, a human lung carcinoma epithelial cell line, were evaluated after GO exposure. The results suggest that, GO has no obvious toxicity to A549 cells, though GO induces the cellular oxidative stress even at low concentration and induce a slight decrease of the cell viability at high concentration. The transmission electron microscopy (TEM) investigation suggests that GO could hardly enter cells. The size of GO sheets has effect on the toxicity of GO at high concentration, that is larger sheets have better biocompatibility. The good biocompatibility of GO allows it to be used for various biomedical purposes in future. Preliminarily, we show the GO film is a good substrate for cell growth.

2. Materials and methods

2.1. Preparation and characterization of GO

Natural graphite powder (≤30 μm, with purity higher than 99.85 wt.%) was purchased from Sinopharm Chemical Reagent Co., Ltd., China. The preparation of GO followed the modified Hummer method (Hummers and Offerman, 1958; Kovtyukhova et al., 1999), which is described in Supplementary Data. The obtained

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Go suspension was further heated to 120 °C for 20 min to get GO mixture (m-GO). After cooling to room temperature, the suspension was centrifuged at 18,000 rpm for 50 min to obtain the s-GO (supernatant, GO with smaller size) and l-GO (residue, GO with larger size) samples.

The three GO samples were characterized by TEM (JEEM-200CX, JEDL, Japan), atomic force microscopy (AFM, SPM-9600, Shimadzu, Japan), Fourier transform infrared spectroscopy (FTIR, Avatar 370, Thermo Nicolet, USA), Raman spectroscopy (Renishaw Invia Plus laser Raman spectrometer, Renishaw, UK) and X-ray photoelectron spectroscopy (XPS, AXIS Ultra instrument, Kratos, UK). The particle size distribution and zeta potential in water were measured by Nanozetasizer (Zetasizer 3000 HS, Malvern, UK).

GO was dispersed in ultra-pure water to prepare the stock solution (1.0 mg/mL). The stock solution was sonicated for 1 h (40 kHz, 50W) and diluted to different concentrations with F-12K culture medium just prior to the cell exposure.

2.2. Cell culture

A549 cell line is one popular cell line in nanotoxicology studies with a cell cycle time of 22 h (Pulskamp et al., 2007; Herzog et al., 2007). A549 cells were kindly provided by Dr. Y. Zhong at Shanghai University, China. A549 cells were cultured in F-12K culture medium supplemented with 10% (v/v) fetal bovine serum (Lanzhou National Hyclone Bio-Engineering Co. Ltd., China) at 37 °C in a humidified atmosphere of 5% CO2/95% air.

2.3. Cell morphology and ultrastructure

A549 cells were plated in the 96-well plates (5 × 103 cells per well) and incubated for 24 h. m-GO, s-GO and l-GO were introduced separately to cells with a predetermined concentration in culture medium. Cells cultured in the medium without adding GO were taken as the control. The cell morphology was recorded under an optical microscope at 24 h postexposure.

To investigate the cellular ultrastructure of GO-treated A549 cells, thin-sections of cells were investigated under TEM. A549 cells were plated in the 6-well plates (1 × 105 cells per well) and incubated for 24 h. GO samples were introduced to the cells with a final concentration of 200 μg/mL. Cells without GO exposure were taken as the control. After 24 h exposure, the cells were washed with ice-cold PBS for three times. After the centrifugation (4000 rpm × 10 min), cells were collected, prefixed with 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in a graded alcohol series, embedded in epoxy resin, and cut with an ultramicrotome. Thin-sections poststained with uranyl acetate and lead citrate were inspected with TEM.

2.4. Cell viability

The cell viability was evaluated by CCK-8 assay (Dojindo Molecular Technolo-

gies, Inc.). A549 cells were plated in the 96-well plates (5 × 103 cells per well) and incubated for 24 h. m-GO, s-GO and l-GO were introduced separately to cells with different test concentrations (10, 25, 50, 100 and 200 μg/mL) in culture medium. Cells cultured in the medium without adding GO were taken as the control. After 24, 48 and 72 h incubation, the cells were washed with D-Hanks buffer solution. Two hundred microliters of CCK-8 solution was added to each well and incubated for an additional 3 h at 37 °C. The optical density (OD) of each well at 450 nm was recorded on a Microplate Reader (Thermo, Varioskan Flash). The cell viability (% of control) is expressed as the percentage of (ODsample - ODblank)/(ODcontrol - ODblank), where ODsample is the optical density of the cells exposed to GO sample, ODcontrol is the optical density of control sample and ODblank is the optical density of the wells without A549 cells.

In a separate experiment, to test the effect of the adsorption of culture medium by GO on the toxicity, the GO samples (10, 25, 50, 100 and 200 μg/mL) were incubated in culture medium (cell-free) at 37 °C under 5% CO2/95% air for 24 h. Then, the mixtures were centrifuged at 4000 rpm for 5 min to remove precipitate (GO). The GO free supernatant were collected and introduced to A549 cells (5 × 103 cells per well). After 24 h incubation, the cell viability was assayed by CCK-8 assay.

2.5. Cell mortality

The cell mortality was evaluated by Trypan blue assay (Beyotime Institute of Biotechnology, China). A549 cells were plated in the 6-well plates (1 × 105 cells per well) and incubated for 24 h. Then, GO was introduced to cells with different concentrations (10, 25, 50, 100 and 200 μg/mL) in culture medium. Cells cultured in the free medium were taken as the control. Twenty-four hours later, the supernatant was collected and the cells were detached with 300 μL Trypan–EDTA solution. The mixture of supernatant and detached cells was centrifuged at 1200 rpm for 5 min. Then, the residue was added with 800 μL Trypan blue solution and dispersed. After 5 min staining, cells were counted using cytomter. The dead cells were stained with blue color. Cell mortality (%) is expressed as percentage of the dead cell number/the total cell number.
3. Results

3.1. Characterization of GO

Except for the size, the three GO samples, m-GO, l-GO and s-GO, are very similar. Fig. 1 shows the representative AFM images of the three GO samples. Most of GO sheets exist as single or few layers. The thickness of the GO layer is around 0.9 nm according to AFM measurement (Fig. S1). Both large and small sheets are presented in m-GO (430 ± 300 nm). The size of l-GO sheets (780 ± 410 nm) is larger than that of s-GO sheets (160 ± 90 nm). In aqueous suspension, the average hydrodynamic diameter \( D_h \) is 588 nm for m-GO, 556 nm for l-GO, 148 nm for s-GO, according to Nanosizer measurements (Table S1). Three GO samples have very similar FTIR spectra (Fig. S2). The broad absorption at 3400 cm\(^{-1}\) suggests the existence of –COOH and –OH groups. The absorption at 1720 cm\(^{-1}\) corresponds to C=O bonds. The oxygen contents based on XPS analysis are 33.1% for l-GO, 37.0% for s-GO and 35.8% for m-GO (Fig. S3). The \( I_D/I_G \) values in Raman spectra, which indicate the defect content, are very close among the three samples (Fig. 1d and Table S1).

3.2. Cell morphology

The morphology is one important indicator of the status of cells. The cell morphological changes after GO exposure were recorded to demonstrate the effect of GO on A549 cells directly (Fig. 2). There is no obvious difference between the GO-treated cells and the control cells. Most cells adhere to the substrate tightly and are in normal spindle-shape.

We also checked the influence of GO on the cell attachment following Wang et al.’s method with some modifications (Wang et al., 2010). Compared with control cells, GO-treated cells do not show any difference in their adhesion to the culture dish (Fig. S7).

3.3. Cell viability

The cell viability is assayed to estimate the toxicity of GO samples quantitatively by CCK-8 assay (Fig. 3), in which the formation of formazan dye depends on the mitochondria activity. As a whole, the viability loss is dose-related. At higher GO concentrations, the viability loss is observed. Size is another factor on viability. The influence of l-GO and m-GO on the viability of A549 cells is tiny. Even at the highest concentration of 200 \( \mu g/mL \), more than 80% of the cell viability remains. However, s-GO induces more viability loss than l-GO and m-GO. At 200 \( \mu g/mL \) of GO, the cell viability is 67% at 24 h postexposure. Culture period has little influence on the viability. Similar results were obtained from 24, 48 and 72 h exposure (Figs. 3, S4 and S5). Therefore, the followed experiments were performed by 24 h exposure.

Nutrient depletion induced by nanomaterial adsorption is a well recognized reason for the nanotoxicity. Therefore, we tested the influence of culture medium adsorption on the toxicity of GO. F-12K medium was pre-treated with GO samples separately for 24 h and the supernatants were collected for A549 cell culture. If the cells did not survive in the GO-pretreated culture medium, we could conclude that the adsorption of nutrients on GO contributes to the toxicity. But the cells grew just as well as the control cells. The cell viability does not decrease along with GO concentrations (Fig. 4).
Fig. 2. Optical microscopy images of GO-treated A549 cells. (a) l-GO; (b) s-GO; (c) m-GO; (d) the control.

Fig. 3. The viability of A549 cells after exposed to GO for 24h.

This reveals that the adsorption of nutrients on GO sheets from medium does not affect the status of cells under our experiment condition.

3.4. Cell mortality

While viability shows the activity of cell mitochondria, the mortality indicates the death of cell. Here, the cell mortality is monitored by Trypan blue exclusion assay, in which the dead cells are stained into blue while the live ones remain unchanged. The mortality is expressed by the ratio of dead cells in all cells. While there is the viability loss induced by GO exposure, compared to the control, no mortality increase of A549 cells is observed after GO treatment (Fig. 5). The mortality remains around 1.5% upon the exposure, nearly the same as that of the control (1.4%).

3.5. Membrane integrity

When the membrane is damaged, the intracellular LDH molecules would be released into the culture medium. Therefore, LDH level out of cells reflects the cell membrane integrity. Interestingly, GO exposure does not induce, but restrains LDH leakage (Fig. 6). The LDH levels of GO-treated cells are even slightly lower than that of the control cells (7.5%). For example, at GO concentration of 200 μg/mL, the LDH leakage level is around 6% for the GO-treated cells. In contrast, the size of GO samples has ignorable influence on the LDH leakage.
3.6. Cell apoptosis

GO does not induce any apoptosis or necrosis of A549 cells (Fig. 7). The apoptosis level is not relevant to the dose or the size of the GO samples. At the concentration of 200 µg/mL, the apoptosis rates (1.1–2.4%) are still comparative with that of the control cells (1.5%). In contrast, the positive control cells, which treated with 200 mM H2O2 for 30 min, show much serious apoptosis (32.4%) and necrosis (54.3%).

3.7. Ultrastructure investigation

The ultra-section of A549 cells was observed under TEM for the uptake of GO and the changes of ultrastructure. All GO treated cells show similar structures to the control cells (Fig. 8). GO exposure does not have any obvious impact on the ultrastructure of A549 cells. We did not find any GO sheets inside cells, either.

3.8. ROS level

The ROS generation is one commonly proposed toxicological mechanism of nanoparticles. The GO exposure induces oxidative stress in A549 cells even at low concentrations (Fig. 9). GO with higher concentrations induces more ROS. Among the three GO samples, s-GO causes the most serious oxidative stress. For example, at 200 µg/mL, the ROS level for s-GO treated cells is 3.9 times of control, while it is 2.6 for l-GO treated cells and 2.1 for m-GO treated cells. However, for the positive control, the ROS level is 12.0 times of control, much higher than that of GO-treated cells under the same condition. There is no meaningful difference between the cells exposed to l-GO and m-GO.

In the F-12K medium (cell free), GO induces the GO dose-dependent ROS generation. Higher GO dose brings on the higher level of ROS (Fig. 10). However, it is l-GO, not s-GO or m-GO,
3.9. Cell growth on GO substrate

The cells grow very well on the GO film. The density and morphology of the cells cultured on GO film are comparative to those of the cells cultured in normal culture dish (Fig. 11). The thickness of the GO film is around several tens of micrometers. Due to the dark brown color of GO film, the contrast of Fig. 11a is not as good as that of the control.

4. Discussion

Nanomaterials have unique physicochemical properties and are applied in various areas. However, their biological properties in organisms will finally determine their destiny in future. Compared to available results of carbon based nanomaterials, such as fullerene, CNT, carbon nanofibre and carbon nanoparticle (Jia et al.,
A systematic study was performed to evaluate the toxicity/biocompatibility of GO to A549 cell, a widely used model cell line for the toxicity study. The results collectively indicate that GO is highly biocompatible, which is consistent with the GO drug delivery studies (Liu et al., 2008; Lu et al., 2010; Sun et al., 2008; Yang et al., 2009b; Zhang et al., 2010a). In addition to the literature reporting the good biocompatibility of GO, there is literature reporting that GO has higher toxicity to cells and animals at high concentrations (Agarwal et al., 2010; Hu et al., 2010; Wang et al., 2010). For example, Wang et al. found that GO is toxic to human fibroblast cells at the concentration of 50 μg/mL and higher. The inconsistency might come from the GO synthesis/film preparation, and the testing models. The good biocompatibility of GO sheets is also reflected by the cell growth on GO film. Unlike Agarwal’s report (Agarwal et al., 2010), we found that A549 cells grew very well on the GO film without obvious toxicity. Our study suggests that GO could be used as the cell growth substrate.

CNT is the closest material of graphene (Geim and Novoselov, 2007). The toxicity of CNTs is heavily influenced by their functionalization degree (Sayes et al., 2006). For example, carboxylation
of CNTs makes CNTs abundant in oxygen atoms, and decreases their toxicity. GO contains many oxygen atoms in the forms of carboxyl groups, epoxide groups and hydroxyl groups (Dreyer et al., 2010). The functionalization degree of GO is generally higher than that of carboxylated CNTs according to the oxygen content. Therefore, the good biocompatibility of GO is generally expected to this regard. Comparing to the very recent toxicity results of graphene (Zhang et al., 2010b), we find that GO has much lower toxicity, which is indicated by results of the viability assay and LDH leakage assay. This supports the phenomenon obtained from CNTs studies, i.e. functionalization decreases the toxicity of CNTs. Another aspect might contribute to the high biocompatibility is the two-dimensional structure of GO. The distinct difference between GO and CNTs is GO's two-dimensional structure and CNTs' one-dimension (Geim and Novoselov, 2007). Although the effect of shape on the toxicity is still unknown in detail to date, many previous results show that the shape affects the biological fate of nanomaterials (Oh et al., 2010; Simon-Deckers et al., 2009).

In addition, GO is not observed inside the A549 cells, GO more possibly interact with the cells on the cellular surface or via other pathway indirectly. The interaction on the cellular surface may be reflected by the membrane integrity evaluation. Surprisingly, the LDH leakage levels of cells treated with high concentration GO are lower than that of the control cells. It could hardly be regarded that GO exposure improves the membrane integrity, but most likely the leakage tunnels are partially blocked by GO covering. This hints that GO might partially block the substance exchange of A549 cells. The reduced LDH leakage might be a distinct character of sheet-like GO, since it is not reported in the toxicity study of fullerene, CNTs and other carbon nanoparticles.

As for the indirect interaction, one possibility is that GO absorbs the nutrients in culture medium and then the depletion of nutrients induces the oxidative stress and toxicity to A549 cells. Such toxicity mechanism has been reported in the study of CNTs (Guo et al., 2008; Liu et al., 2009). Guo et al. reported that the depletion of nutrients by the absorption onto CNTs led to severe toxicity to HepG2 cells (Guo et al., 2008). The theoretical calculations have predicted the absorption of amino acid and other biological molecules onto graphene (Qin et al., 2010; Rajesh et al., 2009). We mixed GO and culture medium for 24h, then centrifuged the mixture to precipitate GO. The supernatant was used to culture cells. No toxicity to A549 cells was found (Fig. 4), compared with the cells incubated with the normal culture medium. Therefore, the absorption of nutrients from the culture medium does not influence A549 cells under the experimental condition in this study.

Another possibility is that GO influences the cell adhesion ability of A549 cells. However, GO shows ignorable influence on the cell adhesion ability of A549 cells (Fig. 57). The unaffected adhesion ability is also indicated in the GO film evaluation. Our results clearly suggest that cells adhere to GO membrane steadily.

Although GO hardly enters A549 cells and the mortality/apoptosis of GO-treated A549 cells is the same as that of the control cells, GO induces statistically significant ROS generation, even at low concentration. Oxidative stress is a well recognized toxicological mechanism of various nanoparticles (Lewinski et al., 2008; Li et al., 2008a; Pulskamp et al., 2007; Yang et al., 2008b).
At low dose, GO induces ROS generation, but no obvious toxicity is observed. Similarly, oxidative stress was observed while no toxicity of CNTs to cells presented in Pulskamp et al.’s study (Pulskamp et al., 2007). The oxidative stress may contribute to the slight viability decrease of GO at high concentration. The oxidative stress induced by GO is moderately low when comparing to fullerene and CNTs (Lewinski et al., 2008). Further, ROS generate when incubating GO with the culture medium alone (cell free) and ROS level is GO concentration dependent. Hence, the intracellular ROS is most likely induced by the external ROS. To make such a mechanism clear, more efforts are required.

5. Conclusions

In summary, the toxicity of GO to A549 cells was evaluated by various cytotoxicity methods. GO hardly enters cells and shows slight decrease of the cell viability at high GO dose. The effect of GO on A549 cells is dose and size related. Our results are essential for the biomedical applications and safety assessment of GO and would stimulate more toxicology evaluations of graphene and its derivatives.

Conflict of interest

There are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2010.11.016.

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