Ecotoxicology and Environmental Safety 132 (2016) 372–378

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Graphene oxide induces plasma membrane damage, reactive oxygen species accumulation and fatty acid profiles change in *Pichia pastoris*

Meng Zhang [a], Qilin Yu [a], Chen Liang [a], Zhe Liu [a], Biao Zhang [b], Mingchun Li [a,*]

[a] Ministry of Education, Key Laboratory of Molecular Microbiology and Technology, Department of Microbiology, College of Life Science, Nankai University, Tianjin 300071, PR China

[b] Tianjin Traditional Chinese Medicine University, Tianjin 300193, PR China

ARTICLE INFO

Article history:
Received 26 March 2016
Received in revised form
19 June 2016
Accepted 20 June 2016
Available online 1 July 2016

Keywords:
Graphene oxide
Unsaturated fatty acid
Reactive oxygen species
Membrane damage
Pichia pastoris

ABSTRACT

During the past couple of years, graphene nanomaterials were extremely popular among the scientists due to the promising properties in many aspects. Before the materials being well applied, we should first focus on their biosafety and toxicity. In this study, we investigated the toxicity of synthesized graphene oxide (GO) against the model industrial organism *Pichia pastoris*. We found that the synthesized GO showed dose-dependent toxicity to *P. pastoris*, through cell membrane damage and intracellular reactive oxygen species (ROS) accumulation. In response to these cell stresses, cells had normal unsaturated fatty acid (UFA) levels but increased contents of polyunsaturated fatty acid (PUFA) with up-regulation of UFA synthesis-related genes on the transcriptional level, which made it overcome the stress under GO attack. Two UFA defective strains (*spt23*Δ and *fad12*Δ) were used to demonstrate the results above. Hence, this study suggested a close connection between PUFAs and cell survival against GO.

1. Introduction

Graphene is the name given to a flat monolayer of carbon atoms tightly packed into a two-dimensional (2D) honeycomb lattice, and is a basic building block for graphitic materials of all other dimensionalities (Geim and Novoselov, 2007). Graphene research has been more and more popular because graphene has many characteristics which reach theoretically predicted limits, such as room-temperature electron mobility (Mayorov et al., 2011), high thermal conductivity (Balandin, 2011), optical absorption (Nair et al., 2008), complete impermeability to any gases (Bunch et al., 2008) and readily chemical functionalization (Elias et al., 2009; Nair et al., 2010). The numerous properties make it potentially promising for bioapplications (Novoselov et al., 2012). Before the scientists use it to fulfill its promise in the biomedical area, we must grasp its toxicity to organisms, firstly.

Many studies have demonstrated the toxicity of carbon nanomaterials, such as their acute toxic effects in primary cultures (Belyanskaya et al., 2009), oxidative stress, inflammation (Murray et al., 2009) and plasma membrane damage (Hirano et al., 2008). As with other nanomaterials, graphene nanomaterials lead to direct or indirect generation of intracellular ROS which can interfere with biochemical processes and induce cytotoxicity and genotoxicity (Zhang et al., 2014). Cellular ROS are generated endogenously from the mitochondria or formed with the exogenous sources. Generally, cells own an antioxidant defense system to eliminate free radicals. When excess ROS cannot be cleaned, oxidative stress occurs and results in ROS-mediated damage of many biomolecules, such as nucleic acids, proteins and lipids (Catala, 2012). Previous study showed that followed ROS accumulation by graphene exposure, apoptosis is activated through the MAPK and TGF-β signaling pathways (Li et al., 2012).

As one of the most important molecules in the cell, fatty acids, especially unsaturated fatty acids (UFAs), not only function as energy sources, but also are required for membrane synthesis, and further influence many cell processes, including cell growth and proliferation (Carracedo et al., 2013). The model industrial organism, *Pichia pastoris*, has four fatty acid desaturase (*FAD*) genes, including *FAD9A* and *FAD9B* (encoding Δ-9 desaturase), *FAD12* (encoding Δ-12 desaturase) and *FAD15* (encoding Δ-15 desaturase) and can synthesize three UFAs including oleic acid, linoleic acid and α-linolenic acid (Yu et al., 2012b). UFA synthesis is controlled by a series of desaturation processes with the desaturases above and the *FAD* gene transcription is regulated by a transcriptional factor, Spt23 (Yu et al., 2012a). Compared to the model organism *Saccharomyces cerevisiae* which only synthesizes one kind

Abbreviations: DCFH-DA, 2′,7′-dichlorofluorescein diacetate; FAD, fatty acid desaturase; FAMEs, fatty acid methyl esters; GO, graphene oxide; PI, propidium iodide; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; UFA, unsaturated fatty acid

* Correspondence to: Department of Microbiology, College of Life Science, Nankai University, Tianjin 300071, PR China.
E-mail address: nklimingchun@163.com (M. Li).

http://dx.doi.org/10.1016/j.ecoenv.2016.06.031
0147-6513/

of UFA (oleic acid), *P. pastoris* possesses a relatively complete system of UFA biosynthesis which can provide the evidence of the relationship between UFAs and the membrane damage induced by GO more fully. According to the reasons above, *P. pastoris* is an appropriate eukaryotic model organism for toxicity investigation of graphene nanomaterials associated with fatty acid synthesis and membrane damage.

In this study, we for the first time investigated the effect of synthesized graphene oxide (GO) on *P. pastoris*, and discussed the possible toxicity mechanisms of these nanomaterials to this model organism. Our results revealed that the graphene nanomaterials showed dose-dependent toxicity to *P. pastoris*. Moreover, we found the toxicity was mainly due to membrane damage and cellular ROS accumulation which may be overcome by increasing PUFA synthesis.

2. Materials and methods

2.1. Synthesis and characterization of graphene oxide (GO)

GO used in this study was synthesized by the modified Hummer's method (Hummers Jr and Offeman, 1958). Transmission electron microscopy was performed to observe the GO structure with a transmission electron microscope (TEM, Tecnai G2 F-20, FEI, USA). A Raman spectroscopy (Renishaw inVia, England) was used to examine the characteristic Raman shift of GO.

2.2. Preparation of GO solutions

10 mg of the GO were suspended in 1 mL SC medium (2% glucose, 0.67% yeast nitrogen base without amino acids, 0.2% complete amino acid mixture). The stock solution was sonicated for 30 min (AS3120, Autoscience, China) and subsequently diluted in SC medium to obtain the appropriate concentrations.

2.3. Strains and growth conditions

Pichia pastoris strain GS115 (*his*$^-$ *Mut*$^+$) used in this study was obtained from Invitrogen (San Diego, CA USA). The two mutant strains, *spt23*Δ and *fad12*Δ were constructed in our previous studies (Yu et al., 2012a, 2012b). Cells were grown in liquid SC medium at 30 °C.

2.4. Growth inhibition assays

Overnight cultured cells were suspended in fresh SC liquid medium to OD_{600}=0.1. Equal volume of cell suspension and prepared GO solutions with different concentrations (0, 250, 500, 1000, 2000, 4000 ppm) were mixed in glass tubes. The mixtures were cultured at 30 °C with stirring at 180 rpm for 12 h or 24 h. The cell number in each treatment group was counted using a hemocytometer. The percent of growth was defined as the cell number divided by that of the control without GO treatment × 100.

2.5. Cell death assays

In order to evaluate the cell death, yeast cells were treated with different concentrations of GO for 24 h, harvested and suspended in PBS buffer. The cell suspensions were stained with 10 μg/mL propidium iodide (PI, prepared in distilled water, Sigma, USA) for 5 min. After staining, the cells were observed and photographed with a fluorescence microscope (BX-51, Olympus, Japan). The numbers of PI-stained cells and total cells were counted and the percentage of PI-positive cells (dead cells) was calculated as below. The percentage of death cells=(the number of PI-positive cells/the number of total cells) × 100. At least 40 fields were determined.

2.6. Cellular ROS level detection

Cellular ROS levels were measured using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) –staining method. Cells with 24 h co-incubation with GO were harvested, washed and suspended in PBS buffer. The suspensions were then stained with 10 μg/mL DCFH-DA (dissolved in ethanol, Sigma, USA) at 30 °C for 30 min. Then the cells were washed twice and resuspended in PBS buffer. The fluorescence density (FLU) was examined by a fluorescence microplate reader (PerkinElmer, USA) and the cell numbers of each treatment were calculated with a hemocytometer.

2.7. Lipid analysis

Cells were treated with different concentrations of GO, harvested and washed in distilled water. Cellular total fatty acids were extracted and methyl esterified as previously described (Yu et al., 2012a). Briefly, dry yeast powder was incubated in 5 mL 5% (w/v) KOH/methanol for saponification at 70 °C for 5 h. After the pH was adjusted to 2.0 with HCl, total fatty acids were subjected to methyl esterification with 4 mL 14% (w/v) boron trifluoride in methanol at 70 °C for 1.5 h. Then, fatty acid methyl esters (FAMEs) were extracted with *n*-hexane after addition of saturated sodium chloride solution. The FAMEs were analyzed by gas chromatography using a GC-6890 (Agilent, USA) equipped with a flame ionization detector and a HP-INNOWAX capillary column (25.0 m × 0.53 mm × 0.20 μm). The temperatures of the injector and detector were 280 °C, respectively. The oven temperature was programmed starting at 160 °C, and then increased to 210 °C at a rate of 10 °C/min, held for 5 min, and then increased gradually to 240 °C at a rate of 2 °C/min, held for 15 min. Ultra-high purity N_2 was used as the carrier gas at a constant flow rate of 1.0 mL/min. The split ratio was 4:1, and the injection volume was 5 μL. Relevant FAMEs were identified by comparison of their peaks with those of standards (Cayman Chemicals, USA).

2.8. RNA extraction and Real-time PCR

Cells were treated with GO, harvested and washed. Total RNA was extracted and used for reverse transcriptional synthesis of cDNA as previously described (Yu et al., 2014). Real-time PCR was performed using SYBR Green qPCR Supermix (TransGen Biotech) according to the instructions. The transcriptional levels of the genes, *FAD9A*, *FAD9B*, *FAD12*, *FAD15* and *SPT23*, were normalized against the levels of *ACT1* gene in different samples. Primers used in the assay are listed in Table 1. All the results were performed in triplicate and repeated in three independent experiments.

2.9. Statistical analysis

Each experiment was performed with three replicates, and the value represents the mean ± standard deviation of three experiments. Significant differences between the treatments were determined using one-way ANOVA ($P < 0.05$). All statistical analyses were performed by Statistical Packages for the Social Sciences (SPSS, Version 20).

3. Results

3.1. Characterization of the synthesized GO

When examined by TEM, the synthesized GO showed thin films