Ecotoxicology and Environmental Safety 161 (2018) 507–514

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Bacterial toxicity of exfoliated black phosphorus nanosheets

Zhiqiang Xiong[a,1], Xuejiao Zhang[b,1], Siyu Zhang[b], Lei Lei[b,d], Wei Ma[b,d], Dengyu Li[b,d], Weidong Wang[a,*], Qing Zhao[b,*], Baoshan Xing[c]

[a] Heilongjiang Provincial Key Laboratory of Environmental Microbiology and Recycling of Argo-Waste in Cold Region, College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Daqing 163319, China

[b] Key Laboratory of Pollution Ecology and Environmental Engineering, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, China

[c] Stockbridge School of Agriculture, University of Massachusetts, Amherst, MA 01003, USA

[d] University of Chinese Academy of Sciences Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Keywords:
Black Phosphorus
Bacterial toxicity
ROS
Membrane damage

ABSTRACT

Newly emerged two-dimensional material, black phosphorus (BP) shows promising applications in many fields owing to its superior properties. Despite the biological effects of BP were studied, its environmental impacts have not yet received enough attention. In this study, the bacterial toxicity of exfoliated BP nanosheets was for the first time evaluated against two model bacteria strains, Gram-negative *Escherichia coli* (*E. coli*) and Gram-positive *Bacillus subtilis* (*B. subtilis*). By monitoring the bacterial growth curve and colony counting, the bacterial toxicity of BP nanosheets was examined. Higher toxicity was induced for Gram-negative *E. coli* compared to Gram-positive *B. subtilis* after 6 h treatment, which was reversed at 12 h due to membrane self-healing of *E. coli*. The bacterial toxicity followed a time- and concentration-dependent fashion, with a maximum bactericidal efficiency of 91.65% and 99.69% for *E. coli* and *B. subtilis*, respectively, after 12 h exposure. Reactive oxygen species (ROS)-dependent oxidative stress and membrane damage were the main bactericidal mechanisms as proved by fluorescence microscopy, flow cytometry, scanning electron microscopy (SEM), and Lactate dehydrogenase (LDH) assay. This study indicates the potential environmental risk of BP nanosheets and the data from this work will guide their safety applications in the future.

1. Introduction

Since the successful isolation of graphene from monocrystalline graphitic films, two-dimensional (2D) nanomaterials have received considerable attention owing to their outstanding properties (Bonaccorso et al., 2010). Among them, graphene is the most studied and has been proved to be a good conductor (Bonaccorso et al., 2010; Hyun et al., 2013; Tassin et al., 2012). However, the bandgap of graphene in its natural state is zero, which impedes its application as a semiconductor (Meric et al., 2008; Zhang et al., 2009; Schwierz, 2010; Zhou et al., 2007). In contrast, a newly emerged 2D material, black phosphorus (BP), has a tunable bandgap (0.3–2 eV) from bulk to monolayer form, which is superior to other 2D materials (Castellanosgomez et al., 2014; Cai et al., 2014; Zhang et al., 2014). Due to the remarkable optical and electronic properties of 2D BP, more attention has been paid to the exfoliation techniques (Hao et al., 2016; Zhang et al., 2015; Guo et al., 2016; Zhao et al., 2015), the methodologies to enhance its stability (Ryder et al., 2016; Sun et al., 2015; Zhao et al., 2016), and its applications in electronics (Hanlon et al., 2015; Buscema et al., 2014; Xia et al., 2014), photonics (Guo et al., 2016; Rodrigues et al., 2016; Xia et al., 2014), and therapeutics (Sun et al., 2016; Wang et al., 2015). Given the promising applications of BP in the biomedical field, its toxicity towards mammalian cells and mice has been extensively studied (Mu et al., 2017; Zhang et al., 2017). In our previous study, the cytotoxicity of BP nanosheets was found to be related with the concentration, size, and cell type (Zhang et al., 2017). Moreover, BP quantum dots could induce lipid peroxidation, reduction of catalase activity, DNA breaks, and bone marrow nucleated cells damage in the in vivo studies (Mu et al., 2017). Despite the potential health risk of BP has been assessed, its ecotoxicological effects are scarcely demonstrated, which should be of great concerns before the massive production and application.

Bacteria play an important role in the ecosystem. In addition, as single cell organisms, bacteria are suitable models to evaluate the risks of substances to the ecological environment. The assessment of bacterial toxicity of 2D materials can provide us foundation information

* Corresponding authors.
E-mail addresses: wwdcyy@126.com (W. Wang), zhaoqing@iae.ac.cn (Q. Zhao).

[1] Zhiqiang Xiong and Xuejiao Zhang contributed equally to this work.

https://doi.org/10.1016/j.ecoenv.2018.06.008
Received 16 March 2018; Received in revised form 24 May 2018; Accepted 3 June 2018
0147-6513/

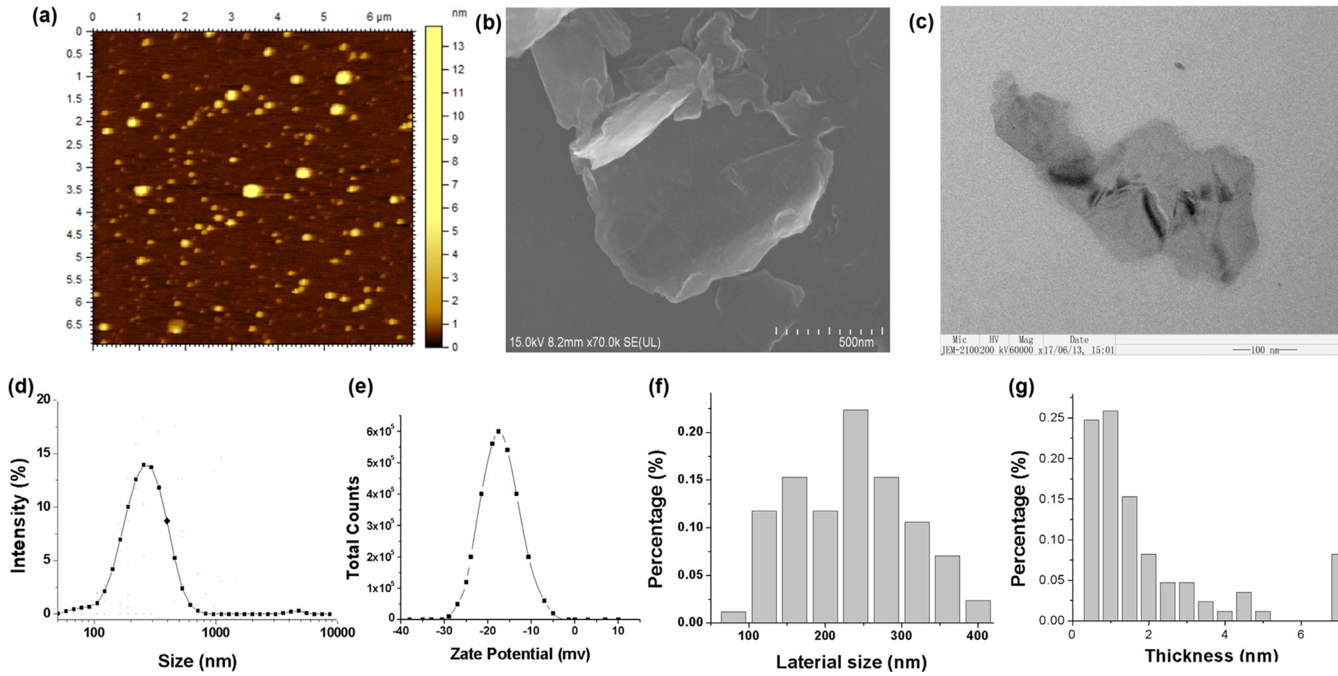

Fig. 1. Characterization of BP nanosheets. AFM (a), SEM (b) and TEM (c) image of BP nanosheets, hydrodynamic size distribution (d), and Zeta potential distribution (e); Statistical analysis of the lateral size (f) and thickness (g) of BP nanosheets by AFM measurements. The scale bars in (b) and (c) represent 500 and 100 nm, respectively.

about their impact once released into the ecosystem. In the previous studies, the bacterial toxicity of graphene (Akhavan and Ghaderi, 2010; Liu et al., 2011), $Ti_3C_2T_x$ (MXene) (Rasool et al., 2016), and molybdenum disulfide (MoS_2) (Pandit et al., 2016; Yang et al., 2014), has already been evaluated. In general, a few mechanisms are proposed to explain the bactericidal activity of 2D materials. First, physical damage of bacterial membrane upon contacting with the sharp edges of 2D materials, so-called nano-knives effect, can trigger the leakage of intracellular substances and eventually induce cell death (Pham et al., 2015). Moreover, oxidative stress resulted from either the production of reactive oxygen species (ROS) (Yang et al., 2014) or the charge transfer activity between bacterial membrane and 2D materials (Li et al., 2014) can interfere with bacterial metabolism, disturb the cellular functions, and ultimately inactive the bacterial cells. In addition, the unique 2D lateral structure can provide flexible barrier to separate bacteria from their surrounding nutrients and ambience by wrapping or trapping bacterial membranes (Carpio et al., 2012). Also, other mechanisms, such as extraction of lipid bilayers, interference of protein-protein interaction, and "self-killing" effect contribute to the 2D material-mediated antibacterial activity (Luan et al., 2015; Tu et al., 2013; Salas et al., 2010). Considering the unique features and great potentials of BP nanosheets, it is necessary to assess their environmental risks by studying the bacterial toxicity and the underneath mechanisms.

Herein, the bacterial toxicity of exfoliated BP nanosheets against two model bacterial strains, Gram-negative *Escherichia coli* (*E. coli*) and Gram-positive *Bacillus subtilis* (*B. subtilis*), was evaluated for the first time. BP nanosheets were prepared by direct exfoliation of BP crystals in oxygen-free Milli-pore water via tip ultrasonication. The bacterial toxicity was studied by growth curve analysis, colony counting, and live/dead cell staining. To understand the in-depth bactericidal mechanisms, the intracellular ROS production was detected by the fluorescence microscopy and flow cytometry. Moreover, scanning electron microscopy (SEM) and Lactate dehydrogenase (LDH) assay were applied to monitor the morphological changes and membrane damages of bacterial cells induced by BP nanosheets. This is a pioneer research for investigating the environmental impacts of BP nanosheets, which will guide BP's safety application in the future.

2. Materials and methods

2.1. Materials

Bulk BP crystals were obtained from XFNANO Materials Tech Co. Ltd. (Nanjing, China) and stored in nitrogen atmosphere under dark condition before use *E. coli* (*E. coli DH5α*) and *B. subtilis* (*B. subtilis* subsp. *subtilis strain* NCD-2) were provided by Heilongjiang Provincial Key Laboratory of Environmental Microbiology and Recycling of Argo-Waste in Cold Region (Daqing, China). Acridine orange/ethidium bromide (AO/EB) staining kit and 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) probe were purchased from Beijing Puyihua Tech Co. Ltd. (Beijing, China). Lactate dehydrogenase (LDH) Cytotoxicity Detection Kit was purchased from Yuchun Bio-tech Inc. (Shanghai, China).

2.2. Exfoliation and characterizations of BP nanosheets

BP nanosheets were produced according to the published method (Zhang et al., 2017). In brief, 10 mg of BP crystals was grinded into small pieces in the glove box. Then 10 mL oxygen-free Milli-pore water was added and the mixture was sonicated with a ultrasonic cell disruption (JY 88–11 N). 8 h later, the mixture was centrifuged at 800 rpm for 20 min to remove the un-exfoliated BP crystals. The concentration of BP nanosheets in the supernatant was determined to be 1036 ± 54 μg/mL by the inductively coupled plasma optical emission spectrometry (ICP-OES). The morphology of BP nanosheets were examined by atomic force microscope (AFM, Agilent 5100), scanning electron microscope (SEM, Hitachi, S-4700 OR FEI, Quanta 250), and transmission electron microscope (TEM, Tecnai F20, FEI, USA). The hydrodynamic diameter and zeta potential measurements were performed using Zetasizer Nano ZS (Malvern Instruments) by a He-Ne laser source with a wavelength of 633 nm at 25 °C.

2.3. Bacterial culture

The bacterial toxicity of exfoliated BP nanosheets was tested against Gram-negative *Escherichia coli* (*E. coli DH5α*) and Gram-