Journal of Industrial and Engineering Chemistry 32 (2015) 282–291

Contents lists available at ScienceDirect

Journal of Industrial and Engineering Chemistry

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

Cytotoxicity of graphene oxide nanoparticles on plant growth promoting rhizobacteria

Sangiliyandi Gurunathan [a,b,*]

[a] Department of Animal Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea
[b] GS Institute of Bio and Nanotechnology, Coimbatore, Tamilnadu, India

ARTICLE INFO

Article history:
Received 15 April 2015
Received in revised form 12 August 2015
Accepted 18 August 2015
Available online 26 September 2015

Keywords:
Graphene oxide
Bacillus species
Rhizosphere
Ultraviolet–visible absorption spectroscopy
X-ray diffraction

ABSTRACT

This study was aimed at investigating the toxicological effects of GO on beneficial *Bacillus* soil microbes. Five bacterial isolates screened from the rhizosphere of a common pulse-growing agricultural field were identified as *Bacillus marisflavi*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megaterium*, and *Bacillus mycoides*. To study the effect of GO under *in vitro* conditions, GO was prepared and characterized by various analytical techniques. Our results suggest that GO decreases cell viability in a concentration- and time-dependent manner by regulating biochemical changes and demonstrate that GO nanoparticles can negatively impact beneficial bacterial communities in the soil.

Introduction

Recently, nanotechnology has produced numerous new nanomaterials with unique physical and chemical characteristics. Engineered nanoparticles (ENPs) have been used in cosmetics, antimicrobial agents, antimicrobial paints, electronic devices, medical devices, and textiles. ENPs are often designed to be extremely reactive, and they have characteristics, unlike some of their natural counterparts, that may be harmful to different life forms including microorganisms and animals [1]. Indeed, the increase of new ENP-based products promises a steady increase in ENP production, availability, and, ultimately, discharges into the environment [2]. ENPs can be classified by size, structure, toxicity, and chemical composition. ENPs affect the environment in a dramatic manner, which is due to dissolved-ion and high surface-to-volume ratio [3]. ENPs are finding increasing industrial application and ultimately enter into the natural ecosystems [4,5]. The potential toxicity of ENPs in these environments has been suggested by studies evaluating impacts on bacterial cultures [6], protozoa grazing on ENP-exposed bacteria [7], and hydroponically grown plants [8]; thus, ENPs may affect ecosystems through both population- and community-level effects.

Graphene and graphene-related nanomaterials are promising candidates for important biomedical applications and industrial applications owing to their unique versatility. Moreover, due to the expanding applications of nanotechnology, human and environmental exposures to graphene-based nanomaterials are likely to increase in the future. Because of the potential risk factors associated with the manufacture and use of graphene-related materials, the number of nanotoxicological studies of these compounds has increased rapidly in the past decade [9]. Several studies have reported on the application of carbon nanomaterials (CNMs), especially on their antibacterial properties [10]. Among the CNMs, single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) show noticeable antimicrobial activity to both Gram-positive and Gram-negative bacteria [11,12]. Furthermore, graphene oxide (GO) and reduced graphene oxide (rGO) nanosheets effectively inhibit the growth of *Escherichia coli* [13–15] and *Pseudomonas aeruginosa* [16]. Graphite-like nanoplatelets are used in many engineering applications for their excellent in-plane mechanical, structural, thermal, and electrical properties [17]. These excellent properties may be relevant at the nanoscale if graphite can be exfoliated into thin nanoplatelets, even to the level of single graphene sheets. GO is a graphene sheet with carboxylic groups at its edges and phenol hydroxyl and epoxide groups on its basal plane [18].

* Correspondence to: Department of Animal Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea. Tel.: +82 2 450 3687; fax: +82 2 544 4645.
E-mail address: gsangiliyandi@yahoo.com

http://dx.doi.org/10.1016/j.jiec.2015.08.027
1226-086X/

Soil is one of the ultimate recipients of nanomaterials in the ecosystem [19], and soil microorganisms that interact directly with the soil environment could be significantly affected when exposed to nanomaterials [20]. Since the change in activity of soil microorganisms that play an important role in nutrient cycling has been a sensitive indicator of the soil's response to environmental stressors such as heavy metals and antimicrobial agents [21,22], investigating the impact of GO on soil microorganisms will provide insights into how GO may alter key bacterial ecosystems. Soil microbial communities are responsible for many of the biogeochemical processes on Earth such as nutrient mineralization, nitrogen cycling, and organic carbon degradation [23,24]. Therefore, many ecosystem services, including clean groundwater supply, waste degradation, and agricultural production, are dependent on the well-being of the soil microbial community [1]. Changes in microbial activity and community composition can result from changes in nutrient and organic carbon availability, anthropogenic activity, and introduction of contaminants such as nanoparticles [1].

Although many studies have focused on the antibacterial activity of GO, to our knowledge, none has reported the effect on soil bacteria, which are plant growth-promoting rhizobacteria (PGPR) that induce resistance against a broad spectrum of pathogens, colonize roots, and promote plant growth through direct action or *via* biological control of plant diseases [25]. Among several bacterial species, *Bacillus* species competitively colonize the roots of plant and can act as bio-fertilizers. Kumar et al. [26] reported that diversified populations of aerobic endospore-forming bacteria (AEFB), specifically *Bacillus* species, occur in agricultural fields and contribute to crop productivity directly or indirectly. *Bacilli* can survive under adverse environmental conditions for extended periods due to many physiological traits such as multilayered cell walls, stress-resistant endospore formation, and secretion of peptide antibiotics, peptide signal molecules, and extracellular enzymes. Multiple species of *Bacillus* and *Paenibacillus* promote plant growth through phytohormone production, solubilization and mobilization of phosphate, siderophore production, antibiosis (*i.e.*, production of antibiotics), inhibition of plant ethylene synthesis, and induction of plant systemic resistance to pathogens [27–29].

Corredor et al. [30] reported that carbon-coated magnetic nanoparticles could penetrate living plant tissues and migrate to diverse regions of *Cucurbita pepo*. On the other hand, silver nanoparticle treatment altered bacterial populations, eliminating *Bacillus thuringiensis* SBURR1 and making *Bacillus amyloliquefaciens* SBURR5 the dominant species [31]. Recent studies have reported that most carbon-based nanomaterials are cytotoxic to bacteria [32–34]. SWNT bacterial toxicity has environmental implications due to the impact of SWNT micropollutants on aquatic ecosystems [35]. Du et al. [36] reported that the extensive use of pristine graphene oxide (PGO) increases its environmental release and it regulates the bacterial community and exhibits property changes in soil. This report highlighted the critical interactions of PGO and soil which deserve comprehensive consideration in assessing the risks of nanomaterials. Although nanoparticles have been used as antibacterial agents, extensive use and increasing demand for nanoparticles will lead to their accumulation in the environment, especially in landfills and their water effluents [37]. However, non-target effects on the populations of microbes that play beneficial roles in the environment could have negative consequences on element cycling, pollutant degradation, and plant growth [37,38]. Therefore, an appropriate risk assessment of the effect of GO requires documentation, which is not currently available. This primary step is essential to understanding the impact of applying GO to the environment, particularly for rhizobacteria. Therefore, this study focuses on the effect of GO on various species of *Bacillus*. We first synthesized and characterized GO nanoparticles, then systematically analyzed the antibacterial activities of GO against various *Bacillus* species.

Materials and methods

Materials

Natural graphite (Gt) powder, NaOH, $KMnO_4$, $NaNO_3$ anhydrous ethanol, 98% H_2SO_4, 36% HCl, 30% hydrogen peroxide (H_2O_2) aqueous solution, DTNB [5,50-dithio-bis-(-2-nitrobenzoic acid)], and glutathione (GSH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Luria-Bertani (LB) agar was purchased from USB Corporation (Santa Clara, CA, USA). Mueller Hinton Broth (MHB) or Mueller Hinton Agar (MHA), silver nitrate, and crystal violet were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were purchased from Sigma–Aldrich unless otherwise stated.

Isolation and identification of bacteria

Bacillus strains were isolated and identified as described previously [39]. Briefly, soil samples were collected from an agricultural field at Coimbatore, Tamil Nadu, India, in sterile Falcon tubes and transferred to the lab under aseptic conditions on ice. The rhizosphere sample (1 g) was suspended in 100 mL 50 mM phosphate buffer (pH 7.0), serially diluted in 50 mM phosphate buffer (pH 7.0), and plated on LB agar (USB Corporation, USA) containing 10 g tryptone, 5 g yeast extract, 10 g/L NaCl, and 15 g/L agar. The inoculated plates were incubated at 37 °C for 24 h. Rough and abundant colonies with waxy growth and irregular spreading edges were obtained [39]. After the incubation period, the bacterial colonies were observed and further sub-cultured in the same medium to obtain pure colonies. Based on preliminary investigation, five isolates (GS 8–12) were selected and maintained on LB agar slants at 4C for further use. Characterizations of bacteria were carried out as previously described [40,41]. Morphological and physiological characterization of the strictly aerobic isolate was performed according to the methods described in Bergey's Manual of Determinative Bacteriology. To identify *Bacillus* species, 16s rRNA gene sequence analysis was performed according to the previously reported method [42].

Synthesis of GO

Synthesis of GO was followed as described previously [43]. In a typical synthesis process, 2 g Gt powder was added to 350 mL cooled (0 °C) H_2SO_4, and then 8 g $KMnO_4$ and 1 g $NaNO_3$ were added gradually while stirring. The mixture was transferred to a water bath set at 40 °C and stirred for 60 min. Deionized water (250 mL) was slowly added, and the temperature was increased to 98 °C. The mixture was maintained at 98 °C for a further 30 min, and the reaction was terminated by adding 500 mL deionized water and 40 mL 30% H_2O_2 solution. The color of the mixture changed to brilliant yellow, indicating the oxidation of Gt to Gt oxide (GtO). The mixture was then filtered and washed with dilute HCl to remove metal ions. Finally, the product was washed repeatedly with distilled water until pH 7.0 was achieved, and the synthesized GtO was further subjected to ultrasonication for 1 h to obtain GO. To remove un-exfoliated GO particles, the mixture was centrifuged for 10 min at 5000 rpm to obtain a GO suspension, which was gradually sprayed on a glass surface maintained at 90 °C then separated layer-by-layer from the surface to achieve powdered GO nanosheets. This simple and efficient procedure formed multilayer GO powder.