Chemical Engineering Journal 279 (2015) 47–55

Contents lists available at ScienceDirect

Chemical Engineering Journal

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

Hydroxyl functionalization of single-walled carbon nanotubes causes inhibition to the bacterial denitrification process

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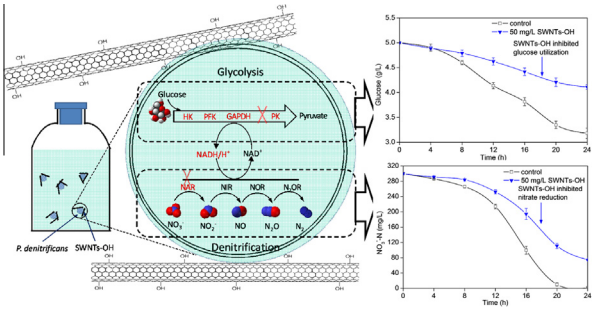
HIGHLIGHTS

- Effects of SWNTs-OH on denitrifying bacteria have not been reported.
- SWNTs-OH caused significant inhibition to the nitrate reduction.
- The utilization of carbon source was decreased by SWNTs-OH.
- SWNTs-OH affected the intracellular NADH/NAD^+ ratio.
- The activities of key enzymes were remarkably lowered by SWNTs-OH.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 1 January 2015
Received in revised form 25 April 2015
Accepted 2 May 2015
Available online 8 May 2015

Keywords:
Hydroxyl functionalized single-walled carbon nanotubes
Inhibition
Denitrification
Glucose metabolism
Enzyme activity

ABSTRACT

Functionalized groups are often attached to the surface of single-walled carbon nanotubes (SWNTs) to improve their outstanding characteristics for more extensive applications. However, the potential impacts of SWNTs modified by hydroxyl groups (SWNTs-OH) on denitrifying microorganisms are unclear. In this study, the effect of SWNTs-OH on denitrification was investigated by the use of *Paracoccus denitrificans* as a model denitrifying microbe. The presence of 50 mg/L of SWNTs-OH, compared with the pristine SWNTs, was found to remarkably decrease the denitrification efficiency from 99.3% to 75.0%. The investigation of the mechanisms showed that SWNTs-OH inhibited the key enzymes responsible for glycolysis due to the increased properties of the dispersibility, the combinative potential with enzyme proteins, the possibility to interact with membrane, and the generation of reactive oxygen species. The metabolism of *P. denitrificans* utilizing carbon source (glucose) was therefore severely disturbed, and subsequently the growth of bacteria and the generation of electron donor (NADH) for denitrification were declined. Further studies revealed that SWNTs-OH also decreased the activity of nitrate reductase. It seems that the release of SWNTs-OH into the environment will cause severe disturbance of nitrogen cycle in biosphere.

1. Introduction

Over the last decades, large numbers of engineered nanomaterials with specific physico-chemical characteristics have been manufactured, and the various applications of these nanomaterials are being developed [1–3]. Among a variety of nanomaterials, single-walled carbon nanotubes (SWNTs), a class of cylinder-shaped carbon nanomaterials with one layers of graphene, have prevailed for almost two decades since their first discovery in 1991 [4]. The special structure of SWNTs determines their exceptional electrical, chemical, mechanical, and thermal characters, which have led to their growing potential applications

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http://dx.doi.org/10.1016/j.cej.2015.05.005
1385-8947/

in many fields, such as composite materials science, electronics, water purification, and biotechnology [5,6]. Usually, carbon nanotubes show poor solubility and dispersity owing to the strong van der Waals interactions between tubes [7]. To improve their properties for desired applications, the attachment of surface functional groups to SWNTs is prevailing. Until now, plentiful inorganic or organic groups, even biomolecules (such as bovine serum albumin), are attached to the surface of SWNTs to realize special purposes [5]. Among the functionalized carbon nanotubes, SWNTs with hydroxyl functional groups (SWNTs-OH) are widely used for their improved biological, mechanical and other properties in extensive applications [8].

With the increasing manufacture and application of products associated with carbon nanotubes, more and more SWNTs will be released into the environment [9]. The released SWNTs might influence the activity of environmental microbes. In the literatures, plenty of studies had focused on the toxic effect of carbon nanotubes on the loss of viability of model microorganisms (such as *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*) [10–12]. Moreover, the effects of carbon nanotubes on microorganisms in the environment had attracted more attention. For example, the exposure of carbon nanotubes was found to cause the loss of functionality and influence on microbial community in sludge, aquatic sediments and soil [13–16]. Also, it was reported that the bacterial soil community was affected by SWNTs, which could disturb nutrient cycling in soil [17]. Shrestha et al. found that the metabolic activities of enzymes involved in cycling of nitrogen, carbon and phosphorus were influenced by multi-walled carbon nanotubes [18]. Due to the negative effects of carbon nanotubes on environmental processes, the influences of pristine and functionalized carbon nanotubes on nitrogen cycle should be investigated, because the toxicity of SWNTs are strongly affected by the functional groups [19].

Nitrogen is a vital element existing in the soil, atmosphere, and organism, and the nitrogen cycle is one of the fundamental biogeochemical processes in the biosphere. In nitrogen cycle, the conversions between various chemical forms of nitrogen are of significant relevance to climate change, soil fertility, and water quality. Among the complex steps in nitrogen cycle, denitrification is the unique way to transfer nitrogen from nitrate to gaseous nitrogen to maintain nitrogen balance, and this process can result in the loss of nitrogen in aquatic and soil systems as well as the concomitant production of nitrous oxide (N_2O), a kind of greenhouse gas. Thus, any negative influence on denitrification will break the nitrogen balance and interfere with global climate [20,21].

It has been reported that denitrification can be disturbed by several environmental pollutants, such as heavy metal and engineering nanoparticles [22–26]. For example, the oxide nanoparticles (such as ZnO, SiO_2 and Al_2O_3 nanoparticles) have been observed in the literatures to inhibit the denitrifying reductase in activated sludge, which further led to more nitrate accumulation [24–26]. Also, the denitrification process of *Pseudomonas stutzeri* was reported to be negatively affected by quantum dots [23]. It is well-known that the denitrification process contains a series of redox reactions, in which nitrate was reduced step by step via acquiring electron, so the denitrification depends on electron transfer mostly. In electron transfer chain, the transformations of nicotinamide adenine dinucleotide (NAD^+) and reduced nicotinamide adenine dinucleotide (NADH) play vital roles between the carbon source metabolism and denitrification process [27]. In addition, many key enzymes are involved in the metabolism of carbon source and denitrification reactions. Both enzymes and NADH are biomacromolecule, and SWNTs have been reported to interact with some macromolecule (such as protein and collagen) [28,29]. Therefore, it is necessary to investigate the influence of SWNTs on the metabolism and function of denitrifying bacteria, especially from the aspect of enzymatic activity.

In this study the impact of SWNTs-OH on denitrification was investigated by using *Paracoccus denitrificans* as a model denitrifying microorganism. Also, the underlying mechanisms for SWNTs-OH significantly inhibiting denitrification process and carbon source metabolism were explored from the aspects of cell membrane integrity, oxidative stress response, reducing power generation, cell proliferation, and activities of key enzymes involved in glycolysis and denitrification reactions.

2. Materials and methods

2.1. Denitrifying bacteria

P. denitrificans (ATCC 19367), which was purchased from ATCC, was chosen as the test bacteria because it is widely appearing in the aquatic and soil environments and the denitrification metabolism is well understood [27]. Prior to exposure experiments, the microorganism was grown aerobically in Difco nutrient broth at 30 °C in a shaker with constant agitation (200 rpm) overnight. After cultivating for 24 h, the cells were harvested in the early stationary growth phase according to our previous publication [30]. Briefly, the cells were harvested by centrifugation at 5000 rpm for 10 min, washed by 0.1 M PBS buffer (pH 7.4) for 3 times, and then resuspended in the same buffer.

2.2. Preparations of SWNTs and SWNTs-OH bulk solution

SWNTs and SWNTs-OH were purchased from Nanjing XF Nanotech port Co., Ltd, China, and SWNTs-OH were oxidized from the pristine SWNTs by the manufacturer. For exposure experiment, the SWNTs and SWNTs-OH were treated according to the literature [31]. Nanotubes were placed into a 12 M HCl solution for 8 h to remove residual metal catalyst, washed with copious amounts of Milli-Q water until neutral pH, and then dried in an oven (60 °C) overnight to get powder SWNTs and SWNTs-OH. Before the following exposure experiment, 0.025 g prepared powder carbon nanotubes were added to 50 mL of Milli-Q water, and the stock suspension (500 mg/L) was ultrasonicated for 1 h (25 °C, 250 W, 40 kHz).

2.3. Characterizations of SWNTs and SWNTs-OH

The morphology and size of SWNTs and SWNTs-OH were determined by transmission electron microscopy (TEM) technology and images were collected using a FEI Titan FEG-TEM. Briefly, the powder of carbon nanotubes was well dispersed in ethanol, and a drop of suspension was placed on Cu grids. Then, the grid was allowed to dry prior to imaging. The Brunauer–Emmett–Teller (BET) method was used to measure the specific surface areas (SSA) of both SWNTs and SWNTs-OH, and the data were 280 and 244 m^2/g, respectively. The diameter distribution of SWNTs and SWNTs-OH was measured by dynamic light scattering (DLS) in Mastersize 3000 (Malvern, UK) and the zeta potential were determined by Zetasizer Nano ZS 90 (Malvern, UK).

Raman spectra for SWNTs and SWNTs-OH were obtained using a Jobin Yvon XploRA-fdu Laser Raman Spectrophotometer with 532 nm incident wavelength. The Raman spectra of both SWNTs and SWNTs-OH displayed the D and G band at 1342 and 1583 cm^{-1}, respectively. For each sample, the spectra curve was plotted and the I_D/I_G was calculated after the normalization of G-band.