Ecotoxicology and Environmental Safety 140 (2017) 103–108

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Interference of single walled carbon nanotubes (SWCNT) in the measurement of lipid peroxidation in aquatic organisms through TBARS assay

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ARTICLE INFO

Keywords:
Carbon nanotubes
Fluorescence
Interference
Nanotoxicology
TBARS

ABSTRACT

Nanomaterials (NM) exhibit unique properties due their size and relative area, but the mechanisms and effects in the living organisms are yet to be unfold in their totality. Potential toxicity mechanisms concerning NM as carbon nanotubes include oxidative stress generation. Several fluorimetric and colorimetric methods have been systematically used to measure NM toxicity, and controversial results have been reported. One of the problems can be related to the interference effects induced by NM, leading to artifacts that can lead to misleading conclusions. In present study, it was performed *in vitro* assays with two aquatic species: the zebrafish *Danio rerio* and the polychaete *Laeonereis acuta* to evaluate the potential interference capacity of single-wall carbon nanotubes (SWCNT) in a fluorometric method (TBARS assay) to measure lipid peroxidation. Obtained results indicated that gills and brain of zebrafish presented a lowered fluorescence only at extremely high concentrations (50 and 500 mg/L). Determinations in anterior, middle, and posterior body regions of *L. acuta* showed a quite different pattern: high fluorescence at low SWCNT concentrations (0.5 mg/L) and lowering at the highest (500 mg/L). To eliminate matrix effect of biological samples, tests employing the standard for TBARS assay, 1,3,3-tetramethoxipropane, were run and the results showed again higher fluorescence values at low concentrations (0.5–5 mg SWCNT/L), a technique artifact that could lead to misleading conclusions since higher fluorescence values implicate higher TBARS concentration, implying oxidative stress. Using the colorimetric FOX assay with cumene hydroperoxide as standard presented remarkable better results since no artifacts were observed in the same SWCNT concentration range that employed with the TBARS technique.

1. Introduction

Nanomaterials (NM) are substances of natural origin or manufactured that possess at least 50% of their particles in one dimension under 100 nm, dimension where significant changes in the chemical and physical properties arise (Colvin, 2003; Oberdörster et al., 2005). Due to size compatibility and physic-chemical properties, NM can interact with biomolecules from cells and organelles (Verma and Stellacci, 2010). Besides, NM show the capacity to cross cellular barriers and interact with several biomolecules that may lead to altered biochemical and physiological mechanisms, creating pathological conditions (Eckert et al., 2013).

Among these NM, carbon nanomaterials (CNM) have increased their production and utilization in different products, so, the release into environment is expected as showed through mathematical models (Sun et al., 2014). The CNM family is composed by carbon allotropes with different chemical structure, and include fullerenes (C_{60}), nanotubes, graphenes (and their derivatives) and nanodiamonds (X Liu et al., 2011; Y Liu et al., 2011; Zhu et al., 2015). Because of the difference between chemical structures, these NM can exert different role in biological systems. In fact, several nanotoxicological studies has been done with considerable progress for the comprehension of toxicity mechanisms of

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http://dx.doi.org/10.1016/j.ecoenv.2017.02.034
Received 20 May 2016; Received in revised form 22 February 2017; Accepted 23 February 2017

CNM (Templeton et al., 2006; Smith et al., 2007; Fraser et al., 2011). Ecotoxicological studies with CNM have shown oxidative damage or triggering of antioxidant responses in fish (Shvedova et al., 2012; da Rocha et al., 2013), augment mortality rate in crustaceans (Templeton et al., 2006; Roberts et al., 2007), accumulation in the digestive tract of annelids and crustaceans (Petersen et al., 2008, 2009), and reproductive impairment in fish and annelids (Cheng et al., 2007; Scott-Fordsmand et al., 2008).

Oxidative stress has been considered as a key mechanism of nanomaterials toxicity (Nel et al., 2006; Shvedova et al., 2012), although some contradictory results are found in the literature, including in assays using CNM as carbon nanotubes (Wu et al., 2015). Some discrepancies of results may be related to physic-chemical properties of CNM, once that these NM may interfere with some methodologies used to evaluate their toxicity. A study of Zhu et al. (2008) reported lower levels of lipid peroxidation in brain and gills of fish *Carassius auratus* after exposure to fullerene C_{60} (nominal concentrations: 0.4–1.0 mg/L), results that suggests an antioxidant effect of this CNM. However, in the same study, lower levels of reduced glutathione (GSH) were registered in the same organs of C_{60}-exposed fish, a result that can be interpreted as a pro-oxidant effect of this CNM.

The measurement of reactive oxygen species (ROS) concentration is usually performed with fluorometric methods that employ dichlorodihydrofluorescein diacetate (H_2DCF-DA) as fluorophore. Some studies showed that H_2DCF-DA can interact with CNM, leading to erroneous interpretation of the obtained results (Martin et al., 2011; Aranda et al., 2013; Kong et al., 2013). Particularly for C_{60}, Lyon et al. (2008) found that exposure of 200 μM H_2DCF-DA to 10 mg/L of this CNM increased the fluorescence in 346% to respect the control group even in absence of cell esterases that cleave the acetate groups, allowing H_2DCF to react with several kinds of ROS and producing fluorescence.

Authors like Liu et al. (1999) stated that fluorescence generated by fluorophores can be absorbed by several molecules, that reduce the measured fluorescence, a condition defined as inner filter effect. This situation can lead to erroneous conclusions, if for example, ROS is measured in tissues of organisms exposed to molecules presenting inner filter effect, because the lowering of ROS levels would lead, for instance, to the conclusion of an antioxidant effect. Also, Zhao and Liu (2012) warned about the high absorption both at excitation and emission wavelengths in fluorescence spectroscopy studies with mixtures of NM and biomolecules.

Taking into account that some techniques for characterizing oxidative damage through lipid peroxidation and others for the measurement of antioxidants like GSH are fluorometric (Oakes and Van Der Kraak, 2003; White et al., 2003), present study aimed to evaluate the potential interference of single walled carbon nanotubes (SWCNT) in the measurement of thiobarbituric reactive substances (TBARS) by fluorometry. Some previously models employed in nanotoxicological studies were used in these assays: the polychaete *Laeonereis acuta* and the zebrafish Danio rerio (da Rocha et al., 2013; Fell Marques et al., 2013; Cordeiro et al., 2016). Also, a comparison was made between a fluorometric and a spectrophotometric protocol in terms of SWCNT interference.

2. Materials and methods

2.1. Single walled carbon nanotubes (SWCNT)

SWCNT with 10–30 nm diameter were purchased from SES Research (Houston – USA). The SWCNT were purified following Chen et al. (2004). After purification, SWCNT were dried at 50 °C and stored until use. The characterization of SWCNT was performed through Raman spectroscopy performed at room temperature in Via Renishaw Raman Spectrometer, in the range of 0–2500 cm^{-1} using a laser of 532 and 785 nm wavelength (Ibañez et al., 2014; Landois et al., 2014). This technique characterizes carbonaceous materials by identifying the types of links and provides information regarding the disorder of the crystal lattice of the material and identifying the various crystalline and amorphous forms present in the sample, and thereby exhibiting characteristic peaks in the spectra in the region between 1000 and 1800 cm^{-1}.

For utilization, the SWCNT was suspended in deionized water (8.34 mg/ml), vortexed three times for 10 s each and then sonicated (9.3 W, with an energy input of 16.7 kJ at 25 °C) for 15 min in an ultrasonic bath (ECO-SONICS, Brazil) before use in the *in vitro* assays.

2.2. Biological samples

For the tests, it was employed two animal species: the fish *Danio rerio* (brain and gills) and the polychaete *Laeonereis acuta* (anterior, middle, and posterior region). Brain and gills from zebrafish were selected because a previous study showed molecular responses in zebrafish brain after i.p. exposure to SWCNT (da Rocha et al., 2013) and Maes et al. (2014) reported multi-walled carbon nanotubes (MWCNT) accumulation in gills of this species. The use of vertebrate species (*D. rerio*) was approved by the Ethics Committee of Federal University of Rio Grande – FURG (CEUA-FURG n° Pq004/2013).

In the case of worms *L. acuta,* and due that a previous study that showed a gradient of antioxidant defenses along the worm body (Ferreira-Cravo et al., 2007), the anterior, middle, and posterior regions were analyzed separately. Permission for wild animal sampling (polychaete) was emitted by SISBIO (registration number 39368-1).

Details of how fish and worms were maintained in laboratory can be found in da Rocha et al. (2013) and Fell Marques et al. (2013), respectively. After dissection of gills and brain from zebrafish and anterior, middle, and posterior region of *L. acuta* (defined according Ferreira-Cravo et al., 2007), the tissues were frozen in liquid nitrogen and stored at −80 °C, for subsequent homogenization, centrifugation and analysis. Tissues were homogenized (1:5) in a solution containing KCl (1.15%) and butylated hydroxytoluene (35 μM). The five-different kind of homogenates (brain and gills from fish; anterior, middle, and posterior region from worm) were split into aliquots and each aliquot received one of the five SWCNT concentration assayed (0.5; 1.0; 5.0; 50; and 500 mg/L) plus a control group. All the six treatments were applied to the same animal sample, so the fluorescence differences should not be due to differences in the redox status of the sample, but due to interactions between the biological matrix and the SWCNTs. At least five independent experiments were performed using different organisms.

2.3. Measurement of lipid peroxidation through a fluorometric assay

The thiobarbituric acid reactive substances (TBARS) assay quantifies a by-product of lipid peroxidation, the malondialdehyde, that reacts with thiobarbituric acid (TBA) forming an abduct (MDA-TBA2). The fluorometric version of TBARS method was done following Oakes and Van Der Kraak (2003). Butylated hydroxytoluene (BHT) was used as an antioxidant for the samples and 1,3,3-tetramethoxipropane (TMP) as standard. Before TBARS measurements, 30 μl of each sample was incubated 5 min at room temperature with 30 μl of one of the different concentrations of SWCNT. After, tissue extracts (10 μl) were added to a reaction mixture containing 150 μl of 20% acetic acid, 150 μl of thiobarbituric acid (0.8%), 50 μl of Milli Q water and 20 μl of sodium dodecyl sulfate (SDS, 8.1%). After mixing, the samples were heated at 95 °C during 30 min. Then they let to cooled during 10 min and 100 μl of Milli Q water and 500 μl of n-butanol were added. After centrifugation (3000 × *g* during 10 min at 15 °C), the organic phase (150 μl) of each sample was placed in blank ELISA plate and read using a microplate reader, where the fluorescence was registered using an excitation wavelength of 520 nm and an emission wavelength of 580 nm.

To avoid tissue matrix effects in TBARS measurements, other *in vitro*