Aquatic Toxicology 147 (2014) 76–83

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Co-exposure of the organic nanomaterial fullerene C_{60} with benzo[a]pyrene in *Danio rerio* (zebrafish) hepatocytes: Evidence of toxicological interactions

Josencler L. Ribas Ferreira[a,b,d,*], María Noelia Lonné[e], Thiago A. França[a], Naiana R. Maximilla[a], Thiago H. Lugokenski[f], Patrícia G. Costa[c], Gilberto Fillmann[c], Félix A. Antunes Soares[f], Fernando R. de la Torre[g], José María Monserrat[a,b,d,h]

[a] *Universidade Federal do Rio Grande-FURG, Instituto de Ciências Biológicas (ICB), Campus Carreiros, Av. Itália km 8 s/n (96200-970), Rio Grande, RS, Brazil*
[b] *Programa de Pós Graduação em Ciências Fisiológicas, Fisiologia Animal Comparada, Instituto de Ciências Biológicas (ICB), FURG, Brazil*
[c] *Laboratório de Microcontaminantes Orgânicos e Ecotoxicologia Aquática (CONECO), Instituto de Oceanografia (IO), FURG, Brazil*
[d] *Rede de Nanotoxicologia (MCTI/CNPq), Nanotoxicologia ocupacional e ambiental: subsídios científicos para estabelecer marcos regulatórios e avaliação de riscos, Rio Grande, RS, Brazil*
[e] *Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina*
[f] *Universidade Federal de Santa Maria (UFSM), Departamento de Química, Santa Maria, RS, Brazil*
[g] *Universidad Nacional de Luján, Departamento de Ciencias Básicas, Buenos Aires, Argentina*
[h] *Instituto Nacional de Ciência e Tecnologia de Nanomateriais de Carbono (CNPq), Brazil*

ARTICLE INFO

Article history:
Received 31 May 2013
Received in revised form 4 December 2013
Accepted 7 December 2013

Keywords:
Nanotoxicology
BaP
Synergistic effect
Delivery
GST

ABSTRACT

Compounds from the nanotechnology industry, such as carbon-based nanomaterials, are strong candidates to contaminate aquatic environments because their production and disposal have exponentially grown in a few years. Previous evidence shows that fullerene C_{60}, a carbon nanomaterial, can facilitate the intake of metals or PAHs both *in vivo* and *in vitro*, potentially amplifying the deleterious effects of these toxicants in organisms. The present work aimed to investigate the effects of fullerene C_{60} in a *Danio rerio* (zebrafish) hepatocyte cell lineage exposed to benzo[a]pyrene (BaP) in terms of cell viability, oxidative stress parameters and BaP intracellular accumulation. Additionally, a computational docking was performed to investigate the interaction of the fullerene C_{60} molecule with the detoxificatory and antioxidant enzyme πGST. Fullerene C_{60} provoked a significant ($p < 0.05$) loss in cellular viability when co-exposed with BaP at 0.01, 0.1 and 1.0 μg/L, and induced an increase ($p < 0.05$) in BaP accumulation in the cells after 3 and 4 h of exposure. The levels of reactive oxygen species (ROS) in the cells exposed to BaP were diminished ($p < 0.05$) by the fullerene addition, and the increase of the GST activity observed in the BaP-only treated cells was reduced to the basal levels by co-exposure to fullerene. However, despite the potential of the fullerene molecule to inhibit π GST activity, demonstrated by the computational docking, the nanomaterial did not significantly ($p > 0.05$) alter the enzyme activity when added to GST purified extracts from the zebrafish hepatocyte cells. These results show that fullerene C_{60} can increase the intake of BaP into the cells, decreasing cell viability and impairing the detoxificatory response by phase II enzymes, such as GST, and this latter effect should be occurring at the transcriptional level.

1. Introduction

The fate of products and effluents from the nanotechnology industry has been a growing matter of concern because their production and disposal have exponentially risen in the last few years (Kahru and Dubourguier, 2010). The current data about the actual risks to humans and to the environment are not conclusive, and this is mainly due to the lack of information concerning their mechanisms of toxicity, actual concentrations and chemical behavior in the environment (Christian et al., 2008; Aschberger et al., 2011). However, the novel chemical and physical properties arising from the nanoscale greatly enhance the reactivity of the nanoparticles with biomolecules, making the nanomaterials potentially toxic and capable of harming the environment (Kahru and Dubourguier, 2010). On the other hand, it must also be considered that some works show low toxicity levels of carbon nanomaterials (such as

* Corresponding author at: Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande-FURG, Cx. P. 474, CEP 96200-970, Rio Grande, RS, Brazil. Tel.: +55 53 32935175.
E-mail address: josenclerf@gmail.com (J.L.R. Ferreira).

0166-445X/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquatox.2013.12.007

fullerenes) in fish, at least with respect to oxidative stress parameters (Fraser et al., 2011; Henry et al., 2011).

Despite the debate concerning the actual toxicity level of the nanomaterials, especially in the aquatic environment, there is a consensus that nanomaterials may potentially affect biological systems not only *per se*, but also through interaction with other compounds (Christian et al., 2008; Henry et al., 2011). Considering their high reactivity, a question arises about what can happen when nanomaterials are in the presence of other toxic molecules. One of the first attempts to investigate this issue was conducted by Limbach and Wick (2007), who measured the oxidative stress in human lung epithelial cells induced by nano-silica doped with a number of metals. This study found higher damage in the treatments with cobalt- and manganese-doped silica nanoparticles than in metals or silica alone. Because nano-silica facilitated the uptake of the metals by the cells, this mechanism was so-called the "Trojan horse" effect. This type of delivery mechanism displayed by nanomaterials has been investigated in a few additional nanotoxicological studies, mainly with metallic nanoparticles. For example, Fan et al. (2011) showed that nano-TiO_2 enhanced copper bioaccumulation and toxicity in the crustacean *Daphnia magna*, even at low nanomaterial concentrations. It was also found that nano-TiO_2 enhanced arsenate toxicity in *Ceriodaphnia dubia* (Wang et al., 2011) and, when doped with the lanthanide Ce(IV), it caused deformation in the cell morphology of a human hepatocyte cell line (Mao et al., 2010).

Studies investigating co-exposure with carbon-based nanocompounds, such as nanotubes and fullerenes, are less common. Fullerene C_{60} is a worldwide produced nanomaterial with a unique cage-like molecular structure made solely of carbon. Although highly hydrophobic, due to its electronic configuration it can form strong C_{60}—H_2O bonds when in colloidal water suspensions (Andrievsky et al., 2002; Khokhryakov et al., 2006), resulting in stable nano-aggregates that can promote deleterious effects in biological systems (Murdock et al., 2008; Ehrenberg et al., 2009).

C_{60} has been widely investigated in terms of the chemical and physical interactions with a range of molecules and devices looking for applications as nano-probes, nano-sensors and nano-electrodes (Nakashima et al., 1998; Cho et al., 2005; Goyal et al., 2005) and in medicine (Partha et al., 2008; Pinteala et al., 2009; Ganji et al., 2010; Tarabukina et al., 2010; Adini et al., 2011; Santos et al., 2011). Despite being poorly studied, the uptake rate and toxicity of other environmental contaminants seem to be somehow affected when co-exposed to fullerene. Baun et al. (2008) indicated that co-exposure with fullerene C_{60} enhanced the toxicity of phenanthrene to the microcrustacean *Daphnia magna* and to the algae *Pseudokirchneriella subcapitata.* This was due, at least in part, to the high adsorption of phenanthrene molecules onto C_{60} nano-aggregates, which facilitated phenanthrene uptake. Similarly, Costa et al. (2012) observed that arsenic (As^{III}) uptake was higher in zebrafish hepatocytes co-exposed to fullerene (1 mg/L).

Among the polycyclic aromatic hydrocarbons (PAHs), benzo[a]pyrene (BaP) is one of the most important due to its ubiquitous presence in most environments. It is produced mainly during the incomplete combustion of organic matter and in cigarette smoke (Rose and Levi, 2004). It is also a carcinogen and mutagen toxicant and reactive oxygen species (ROS) generator (Sasco et al., 2004; Naspinski et al., 2008). Its detoxification process includes metabolization by phase I enzymes that can produce electrophilic epoxides that can readily bind to DNA (Walker et al., 2001). BaP contamination can be harmful through the generation of oxidative stress (Palanikumar et al., 2012), the inhibition of retinoid synthesis (Alsop et al., 2007) and the formation of DNA adducts (Kurelec et al., 1991). The exposure of cultured cells to BaP can also cause changes in gene expression (Castorena-Torres et al., 2008), oxidative impairment (Winzer et al., 2001) and an increase of the carcinogenic risk by interaction with 17β-estradiol (Chang et al., 2007), among many other deleterious effects.

In order to investigate the influence of fullerene C_{60} upon the toxicity of an important environmental contaminant, such as BaP, the present work aimed to assess the oxidative stress parameters, cell viability and bioaccumulation of BaP in ZF-L cells, an established culture of hepatocytes from the zebrafish *Danio rerio* (Cyprinidae). This cell lineage was chosen because *Danio rerio* is a highly suitable biological model widely used in toxicology, including in studies with nanomaterials (Fako and Furgeson, 2009; Costa et al., 2012). Additionally, an *in silico* study was performed by computational docking to verify the hypothesis of the interaction of the fullerene C_{60} molecule with the antioxidant and phase II detoxificatory enzyme glutathione-S-transferase (GST).

2. Materials and methods

2.1. Preparation of the chemicals

2.1.1. Preparation and characterization of C_{60} suspension

In order to produce a homogeneous suspension of C_{60} nanoparticles, 200 mg of fullerene C_{60} in powder form (99% purity, SES Research, USA) was added to 1 l of ultra-pure Milli-Q water and stirred for two months under artificial light. After this period, the suspension was centrifuged at 25,000 × *g* and 15 °C for 1 h to remove the bigger aggregates and was then sequentially filtered by 0.45 and 0.20 μm nylon membranes. This methodology was based on the work of Lyon et al. (2006) where no organic solvent was employed because these solvents can release residual degradation products that affect the toxicity of the nanomaterial (Henry et al., 2007). The concentration of the suspension was determined by measurement of the total organic carbon content in a total organic carbon analyzer (TOC-V CPH, Shimadzu Corp., Japan). The characterization of the C_{60} suspension was performed by transmission electron microscopy (TEM) in a JEOL JSM 1200 EX II transmission electron microscope operating at 100 kV. For the TEM, aliquots of the C_{60} suspension (10 μl) were disposed onto 300 mesh TEM grids (SPI) that were coated with Formvar. The analysis was performed after 24 h to allow sample evaporation, according to previous studies (Britto et al., 2012; Costa et al., 2012; Ferreira et al., 2012). As previously reported for C_{60} suspensions prepared using the water-stirring method without the addition of organic solvents (Lyon et al., 2006; Britto et al., 2012; Costa et al., 2012; Ferreira et al., 2012), the ubiquitous presence of fullerene nano-aggregates in the nanometer range were seen in the C_{60} suspension analyzed by TEM (Fig. 1).

2.1.2. Preparation of BaP solutions

BaP solutions ranging from 0.01 to 10.00 μg/mL were obtained by dissolving benzo[a]pyrene (Fluka, purity ≥ 96%) in dimethyl sulfoxide (DMSO) (Synth, Brazil). The final concentration of DMSO in contact with the cells was 1% since Filgueira et al. (2007) showed that this DMSO concentration was not deleterious for an erythroleukemic cell line. In addition, the DMSO control group showed no effects in the analyzed variables (see Section 3).

2.2. Maintenance of the hepatocytes

Zebrafish hepatocytes (ZF-L lineage) purchased from the American Type Culture Collection (ATTC) were maintained in culture flasks with 10 mL of RPMI 1640 (Gibco) medium supplemented with 10% fetal bovine serum and a 1% antibiotic/antimycotic cocktail (streptomycin, amphotericin and penicillin) at 28 °C. For the exposure assays, cells were initially removed from the flasks with 0.125% trypsin, washed with phosphate buffered saline (PBS) and transferred to 24-well culture plates (0.5 mL per well, 10^6 cells/mL)