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Interactions of multiwalled carbon nanotubes with algal cells: Quantification of association, visualization of uptake, and measurement of alterations in the composition of cells

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ABSTRACT

Carbon nanotubes (CNTs) are considered promising materials in nanotechnology. We quantified CNT accumulation by the alga *Desmodesmus subspicatus*. Cells were exposed to radiolabeled CNTs (^{14}C-CNTs; 1 mg/L) to determine uptake and association, as well as elimination and dissociation in clear media. Attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) was used to detect effects of CNTs on algae. CNT-cell interactions were visualized by electron microscopy and related to alterations in their cell composition. A concentration factor of 5000 L/kg dry weight was calculated. Most of the material agglomerated around the cells, but single tubes were detected in the cytoplasm. Computational analyses of the ATR-FTIR data showed that CNT treated algae differed from controls at all sampling times. CNT exposure changed the biochemical composition of cells. The fact that CNTs are bioavailable for algae and that they influence the cell composition is important with regard to environmental risk assessment of this nanomaterial.

1. Introduction

Carbon nanotubes (CNTs) are considered promising materials in nanotechnology. They are used in different manufacturing sectors, for example electronic and medical applications, composite material development, aerospace technology, and energy storage (Klaine et al., 2008). Investment in nanotechnology research and development as well as nanoparticle production volumes are increasing rapidly worldwide (Nowack and Bucheli, 2007; Helland et al., 2008).

Based on the assumption that this will lead to increasing CNT release in the environment, a lot of research has been dedicated to investigating the possible (eco)toxicity of this nanomaterial (Petersen et al., 2011). In contrast to the large number of reported effect studies, little data are available on the bioaccumulation of CNTs by organisms (Jackson et al., 2013). This is consistent with the limited number of analytical methods available to differentiate carbon-based nanoparticles from organic matrices (Gottschalk et al., 2013a). For this reason special analytical techniques have to be applied to detect these materials and to measure their concentration in biological samples (Perez et al., 2009; Petersen and Henry, 2012; von der Kammer et al., 2012). Using mainly microscopic techniques, it was observed that CNTs clogged the filter apparatus and covered the carapace of daphnids (Roberts et al., 2007), precipitated on the gills of rainbow trout (Smith et al., 2007), or filled the gastrointestinal tract of several aquatic invertebrates (Roberts et al., 2007; Petersen et al., 2009; Templeton et al., 2006) after exposure of the organisms *via* aqueous dispersion of CNTs. It remains to be clarified whether nanotubes are bioavailable, *i.e.*, that they are accumulated in exposed organisms. Incorporation of CNT material through absorption across external tissues and the gut epithelium has not been reported so far (Petersen et al., 2011).

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Whereas uptake of CNTs in epithelial tissues of multicellular organisms could not be shown, internalization of nanotubes in mammalian cells was repeatedly observed (Al-Jamal et al., 2011; Lacerda et al., 2007; Thurnherr et al., 2011). In clinical studies, CNTs are investigated for their biocompatibility when used as drug delivery agents on the one hand, and for their risk to provoke (pulmonary) toxicity on the other. Although CNTs may act as free radical scavengers (Petersen et al., 2013; Ivana et al., 2006), there is also evidence from *in vitro* and *in vivo* experiments that CNTs induce the production of reactive oxygen species (ROS), which may elicit oxidative stress at the cellular level (Thurnherr et al., 2011; Manna et al., 2005; Pulskamp et al., 2007). Subsequent indirect consequences as lipid peroxidation causing disruption of cell membranes and DNA damage were previously observed following exposure of CNTs to different cells (Lindberg et al., 2009; Tyurina et al., 2011; Yamashita et al., 2010; Guo et al., 2011). CNTs were shown to be present in cells of different unicellular organisms such as protozoan and bacteria held in laboratory cultures (Thurnherr et al., 2011; Zhu et al., 2006a; Cheng et al., 2009a). *Escherichia coli* and *Stylonychia mytilus*, were reported to incorporate CNTs from their surrounding medium (Zhu et al., 2006a; Kang et al., 2008), and several studies showed unicellular algae to interact with CNTs (Long et al., 2012; Wei et al., 2010; Schwab et al., 2011). Inhibition of algal growth following to CNT exposure was repeatedly related to light absorption by the nanomaterial causing shading of the cells and to agglomeration and physical interactions of algae with CNTs rather than to a specific mode of toxic action (Long et al., 2012). Most effects were only observed at high CNT concentrations (>10 mg/L), which are not assumed to be environmentally relevant. In modelling surveys, surface water concentrations in the ng/L range were predicted (Gottschalk et al., 2009). Due to agglomeration and the high affinity of the hydrophobic CNTs to sediments, the material tends to settle (Schwab et al., 2011; Gottschalk et al., 2013b; Mueller and Nowack, 2008).

The objective of the present study was to link CNT-cell interactions to CNT specific effects in algae. To our knowledge, we quantitatively measured the CNT accumulation of unicellular algae for the first time. Thereto, the green alga *Desmodesmus subspicatus* was exposed to radiolabeled CNTs (^{14}C-CNTs) to determine algal uptake and association of CNTs with cells, as well as elimination and dissociation of the material from the algae over time. CNT-cell interactions were visualized by means of transmission electron microscopy (TEM). The results were related to alterations in the cell composition detected by attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy. The latter method was previously shown to allow analysis of subcellular spectral alterations in the chemical and biochemical composition of microorganisms due to stress by toxic chemicals, even at subcytotoxic concentrations (Llabjani et al., 2009; Bi et al., 2007; Riding et al., 2012a). The combined results of the present paper give new insights on the bioavailability of CNTs.

2. Material and methods

2.1. Synthesis of unlabeled and ^{14}C-radiolabeled carbon nanotubes

The synthesis was described elsewhere (Maes et al., 2014a). In brief, multiwalled CNTs (MWCNTs) were synthesized by means of catalytic chemical vapour deposition (CCVD) of benzene in H_2 at 700 °C using cobalt as catalyst (Bucholz et al., 2006) and N_2 as carrier gas. The process was carried out in cooperation with Bayer Technology Services GmbH (BTS, Leverkusen, Germany) using a small-scale fluidized sand batch reactor. For the production of ^{14}C-labelled CNTs, labelled ^{14}C-benzene (1850 MBq/mmol) was purchased from Hartmann Analytic GmbH (Braunschweig, Germany). Before synthesis, ^{14}C-benzene was diluted (1:10 v:v) with unlabeled benzene. The obtained ^{14}C-CNT agglomerates were labelled at the carbon framework and had a high specific radioactivity, *i.e.*, 1.3 MBq/mg CNTs. The CNTs were washed with a 12.5% hydrochloric acid solution in order to remove excess catalyst, giving the product a C-purity of more than 95%. Characterization of the material by means of electron microscopy showed that agglomerates had a median size of 500 μm and consisted of single tubes with 3–15 walls (4 nm inner and 5–20 nm outer diameter) and a length of less than 1 μm.

2.2. Preparation of CNT test suspensions for exposure of the green alga D. subspicatus

A total of 1.4 mg of MWCNT agglomerates were weighed on a microbalance (0.0001 mg readability; Mettler-Toledo UMX2, Mettler-Toledo GmbH, Germany) and transferred to a beaker containing 0.7 L of the suggested medium for testing the toxicity of chemicals to algae in OECD Guideline 201 (OECD201, 2006). The nanomaterial was dispersed in water by means of ultrasonication with a micro tip (Sonoplus HD 2070, Bandelin, Germany) set to a rhythm of 0.2 s pulse at 70 W followed by 0.8 s pause for 5 min. The procedure was repeated four times or until no more agglomerated CNT material was macroscopically visible. This method resulted in the presence of small agglomerates and single tubes of 0.2–1.0 μm mean tube length (both referred to as CNTs) in the medium, as was visualized by TEM [see Supporting Information (SI) Fig. S1]. In advance of experiments with ^{14}C-CNTs, the concentration and homogeneity of the test dispersion were verified by measuring the amount of radioactivity in replicate samples directly after sonication. Six samples of 4 mL were mixed with 16 mL of scintillation cocktail (Insta-Gel Plus™, Perkin Elmer, Germany) each, and subjected to liquid scintillation counting (LSC; LS 5000 TD, Beckmann Instruments GmbH, Germany). The limit of detection of this method amounts to 1 Bq, which corresponds to about 1 ng ^{14}C-CNTs. Therefore, a rather high test concentration was needed to perform uptake and elimination experiments.

The CNT dispersion (50 mL) was added to an algal suspension of the same volume in a gas wash flask, in order to obtain an exposure concentration of 1 mg (^{14}C-)CNTs/L. *D. subspicatus* cells were harvested from an in-house stock culture. The algal suspension was finally diluted to $2*10^6$ cells/mL to obtain an initial density of 10^6 cells/mL in the CNT-algal suspension. The bottles were placed in a climate chamber (20 ± 1 °C) at a light intensity of 70 μE/(m^2 s). The test medium was aerated using Pasteur pipettes connected to an air-blowing pump (Hailae ACO-961, 10 W, *Hailea* Group, Germany) to provide the algae with CO_2 and to prevent sedimentation. To reduce evaporation, the flasks were covered with paper tissue.

2.3. Quantification of CNTs accumulation by algae

Algae were exposed to 1 mg ^{14}C-CNTs/L for 24, 48, and 72 h. For each sampling time, four flasks were prepared, as described above. A method to separate algal cells from CNTs was developed in advance. Filtration was no option due to the presence of CNT agglomerates of similar size as the algal cells in suspension. Good results were however obtained using density gradient centrifugation. This method was previously successful in separating different algae from each other (De Jonge, 1979). A colloidal silica suspension (Ludox® TM-40: 40% in distilled water; Sigma–Aldrich, Germany) was used, of which two mixtures with tap water were prepared: one of 3:2 v:v to separate compact CNT agglomerates from the algae and one of 2:3 v:v to isolate the cells from less dense agglomerates. The density of Ludox, algae, and MWCNT agglomerates amounts to 1.3 kg/L (at 25 °C), less than 1.3 kg/L, and between 1 and 2 kg/L, respectively (De Jonge, 1979; Arnold et al., 2005). Preliminary