Usefulness of fish cell lines for the initial characterization of toxicity and cellular fate of graphene-related materials (carbon nanofibers and graphene oxide)

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HIGHLIGHTS

- Current lack of knowledge about toxic mechanisms of graphene-related materials.
- Two fish cell lines were used to generate initial ecotoxicity data of GRMs.
- Carbon nanofibers (CNFs) showed higher toxicity than graphene oxide (GO).
- The presence of CNFs and GO inside vesicles was cell and size dependent.
- CNFs toxicity was inversely related to the graphitization degree.

ABSTRACT

Graphene-related materials (GRMs) are one of the most attractive materials from an application perspective, consequently their release into aquatic environments is highly likely. In the present work, the potential of fish hepatocytes (topminnow fish hepatoma cell line, PLHC-1) and macrophages (carp leucocyte cell line, CLC) to study the toxicity and intracellular fate of helical-ribbon carbon nanofibers (CNFs) and graphene oxide (GO) used in a variety of intermediate industrial products was evaluated, allowing a first ranking of GRMs according to their cytotoxicity. Cells were exposed to a concentration range of 0–200 μg ml⁻¹ of GRMs for 24 and 72 h and cell viability was assessed by measuring mitochondrial activity (AlamarBlue assay), plasma membrane integrity (5-carboxyfluorescein diacetate-acetoxymethyl ester assay) and lysosomal function (neutral red uptake assay). Results showed that both the cell type and the choice of endpoint determined the toxicity of GRMs. In both cell lines, CNFs appeared to have higher toxicity than GO and the highest degree of graphitization in fibers was associated with lower toxicity. Transmission electron microscopy revealed that CNFs were taken up into membrane-bound compartments of PLHC-1 cells in a size-independent manner, whereas in CLC, longer CNFs were encountered free in the cytoplasm and only the shorter CNFs were localized in membrane-surrounded vesicles. GO sheets were present within vesicles as well as free in the cytoplasm of both cell types. These findings contribute to the understanding of the toxicity and behaviour of these GRMs in living systems, therefore aiding in designing safer materials for the environment.

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1. Introduction

In the last years, graphene-related materials (GRMs) have become the subject of an extraordinary research interest. They include a wide range of carbon nanostructures, from two-dimensional sheets (e.g. graphene) and spherical forms (e.g. fullerenes) to tubular-shaped high aspect ratio materials (e.g. carbon nanotubes and nanofibers). Various technological and biomedical applications (Aschberger et al., 2010; Liu et al., 2018; Zhang et al., 2016) have been implemented on the basis of the exceptional properties (electrical conductivity, mechanical strength, chemical reactivity, etc.) of these materials.

Among the tubular-shaped nanomaterials (NMs), carbon
nanotubes (CNTs) have received much attention and a number of investigators have already demonstrated their ability to elicit toxicity (Boyles et al., 2015; Lam et al., 2006; Monteiro-Riviere et al., 2005; Poland et al., 2008; Smith et al., 2007). Structurally, CNTs are similar to carbon nanofibers (CNFs), since both are comprised of graphene layers. These stacked graphene layers are arranged concentrically in CNTs, while rolled along the fiber axis at a particular angle in the case of CNFs (Vera-Agullo et al., 2007; Weisenberger et al., 2009). Despite the increasing production and use of CNFs, only limited studies have assessed their adverse effects. Moreover, published studies have mainly focused on their pulmonary or dermal toxicities (Brown et al., 2007; Jensen et al., 2012; Lindberg et al., 2009; Magrez et al., 2006; Yokoyama et al., 2005). Nevertheless, the incorporation of these NMs into products inevitably leads to their release into the environment and aquatic bodies.

Ideally, the development of new NMs should be accompanied by toxicological testing and this information feeds back the development team so that necessary modifications can be implemented in the synthesis to create low toxicity products (Hurt et al., 2006). The so-called Safe-by-Design (SbD) approach requires not only an understanding of material intrinsic properties but also the behaviour of NMs in living systems (Fadeel et al., 2013). Testing the toxicity of these NMs is particularly challenging given their low solubility in aqueous media and the potential interferences associated with some assay reagents. Not surprisingly, existing literature on the outcomes of toxicological assessments varies greatly. Conflicting results may arise from differences in the physico-chemical properties of the NM tested (e.g. length, aspect ratio, surface modification and impurities), as well as the experimental design employed (e.g. dispersion method, dose levels, exposure duration, test models and the measured endpoints) (Johnston et al., 2010). Although CNTs and CNFs are structurally related, they may pose different toxicities as have been shown by several authors. According to Magrez et al. (2006), CNFs were more toxic in human lung tumor cell line (H596) than single-, or multi-walled CNTs. Contrary to these findings, Grabinski et al. (2007) found increased cytotoxicity in mouse keratinocytes (HEL-30) exposed to CNTs, but CNFs did not significantly affect the cell viability. The potential of CNFs to induce genotoxicity in Chinese hamster lung fibroblast cells (V79) was investigated by Kisin et al. (2011) and authors reported stronger effect on DNA damage by CNFs than caused by single-walled CNTs. Moreover, authors also revealed cellular uptake and generation of reactive oxygen species in the murine macrophage cells (RAW 264.7) following exposure to CNFs but not after administration of single-walled CNTs.

Unlike CNFs, graphene oxide (GO) is one of the most extensively investigated GRMs in in vitro toxicity studies (Bianco, 2013; Guo and Mei, 2014; Ou et al., 2016; Srikanth et al., 2018; Zhang et al., 2016). Despite this, opposite conclusions have been drawn from the toxicity results. Chang et al. (2011) evaluated the toxicity of GO on human lung epithelial cells (A549) and found neither obvious cytotoxicity, nor intracellular uptake by cells, whereas Hu et al. (2011) using the same cell line reported internalization and concentration-dependent toxicity of GO when fetal bovine serum (FBS), a frequently used component in cell culture medium, was employed at low concentration. Similarly, Horvath et al. (2013) also observed internalized GO and reactive oxygen species production in A549 cells. The ability of GO to cause plasma membrane damage, to appear free in the cytoplasm and to induce oxidative stress in a human hepatoma cell line (Hep G2) was revealed by Lammel et al. (2013). Nevertheless, in the fish liver-derived PLHC-1 cell line Lammel and Navas (2014) observed that internalized GO could appear free in the cytoplasm and disrupt mitochondrial membranes, what constitutes a mechanism contributing to the induction of oxidative stress.

Despite being the final destination of many contaminants, assessment of the effects of GRMs on aquatic ecosystems are limited to a small number of in vivo studies (Oberdörster, 2004; Oberdörster et al., 2006). Because there is a move towards alternative test methods to evaluate the toxicity of NMs in aquatic organisms, in vitro assays based on fish cell lines seem to be a promising tool (Lammel and Navas, 2014). They can be used as a preliminary assessment allowing ranking the NMs for further more elaborated and resource consuming higher tier in vivo tests (Bermiejo-Nogales et al., 2017a, 2017b). In particular cases, the information generated in the in vitro systems could be of value in itself and be used in a weight of evidence approach in the risk assessment of substances.

The main objective of the present work was to check the usefulness of a battery of in vitro cytotoxicity assays applied on the fish cells in order to generate preliminary ecotoxicity data of a number of GRMs used in a variety of intermediate industrial products. These data could be applied in different ways by manufacturers. For instance, for ranking these materials for further more complex in vivo tests or for selecting those materials with the lowest toxicity in further work framed in a SbD approach. In order to fully understand the obtained results, transmission electron microscopy (TEM) was used to visualize the interaction between GRMs and cells. Two different fish cell lines were used: Topminnow fish hepatoma cell line (PLHC-1), due to their key role played by liver in detoxification, and a carp leukocyte cell line (CLC), due to their contribution to NM clearance, have been chosen as the most appropriate fish cell models.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma Aldrich (Madrid, Spain) unless otherwise stated. Eagle’s Minimum Essential Medium (EMEM) with non-essential aminoacids, (NEA) and Na Pyruvate without l-Glutamine, EMEM with Earle’s Balanced Salt Solution without l-Glutamine, Penicillin/Streptomycin (P/S, 10,000 units penicillin and streptomycin per ml), l-Glutamine solution (200 mM), and 100x NEAA were obtained from Lonza (Barcelona, Spain). Serum-free/phenol red-free MEM was purchased from PAN Biotech (Aidenbach, Germany). AlamarBlue reagent and 5-Carboxyfluorescine diacetate-acetoxymethyl ester (CFDA-AM) were from Life Technologies (Madrid, Spain). Bovine serum albumin (BSA) and Ni(II) sulphate hexahydrate were from Merck (Darmstadt, Germany).

2.2. Nanomaterials

All NMs were synthesized and provided by Grupo Antolin Ingeniería, SA (Burgos, Spain). Grupo Antolin helical-ribbon carbon nanofibers (CNF) with graphitization degrees of 60% (GAtam), 70% (GAFG) and 90% (GANFg) were supplied as dry powders. GAgUA, consisted of a derivative of GANF supplied in deionized water (3 wt%). GAFG, GAtam and GAgUA contain residual Ni catalyst (10.5, 11.1 and 15.2%, respectively). Graphene oxide (GO) was derived from GANF by chemical oxidation and was supplied as powder (GRANPh®). As reported, GRANPh® consists of single or few layers of graphene sheets and exhibits lateral size mainly submicrometric.

2.3. NM dispersion and characterization

The initially received stock suspension of GAgUA was diluted in the corresponding cell culture medium containing 0.027% w/v BSA.
to a concentration of 200 μg ml⁻¹ and sonicated in a water bath (Elmasonic S 40 H, Elma, Germany) at 37 KHz for 10 min. The rest of the NMs (in powdered forms) was dispersed in Milli-Q water (18.2 MΩcm at 25 °C) at a concentration of 5 mg ml⁻¹ and sonicated for 20 min using a probe sonicator (2 mm horn, 80% continuous mode, Vibra-Cell™ VCX 130, Sonics, Newton, CT, USA). Following sonication, a solution of BSA (80 mg ml⁻¹) was added to the stock dispersion (5 mg ml⁻¹) and mixed thoroughly to improve NM stability. NM stock dispersions were diluted in the cell culture medium to a final concentration of 200 μg ml⁻¹, and vortexed briefly prior to sonication in a water bath (37 KHz) for 10 min. All suspensions of NMs were freshly prepared before each in vitro toxicity test.

Hydrodynamic diameters (HDD) of NMs were determined at time 0 in Milli-Q water suspensions (before adding BSA) and after 0, 24 and 72-h of incubation in cell culture media suspensions by means of dynamic light scattering (DLS) using a Zetasizer Nano Series (Malvern Instruments, UK). Four independent measurements were carried out with each measurement consisting of six runs. Due to the rapid sedimentation of NMs in Milli-Q water no reliable measurements were obtained. NM size was also assessed by means of TEM. For TEM images at 0 and 72-h of incubation, samples were prepared by placing a drop of suspensions on carbon-coated copper TEM grids and allowed to evaporate at room temperature before analysis. NMs were observed in a JEOL 1400 Plus TEM (JEOL Ltd., Japan).

2.4. Cell cultures

Nanomaterials cytotoxicity and interaction with cells were studied in vitro by means of two piscine cell lines: a topminnow fish hepatoma cell line (PLHC-1) and a carp leukocyte cell line (CLC). Both cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). PLHC-1 was maintained in EMEM (with NEAA and Na Pyruvate without l-Glutamine) supplemented with 1% l-glutamine, 1% P/S and 5% FBS at 30 °C, 5% CO₂. For cell treatments, medium was supplemented with 10% FBS. CLC was cultured in EMEM (with Earle’s Balanced Salt Solution without l-Glutamine) supplemented with 1% l-glutamine, 1% P/S, 1% NEAA and 10% FBS at 28 °C, 5% CO₂. According to Lammel and Navas (2014) the use of 10% FBS in medium would contribute positively to the stability of the NM suspensions. Both cell lines were subcultured twice a week using trypsin EDTA (in phosphate buffered saline).

PLHC-1 cells were seeded in flat-bottomed 96-well plates (Greiner Bio-One GmbH, Germany) at a density of 5 × 10⁴ cells ml⁻¹, whereas CLC cells were plated on poli-l-lysine coated flat-bottomed 96-well plates (Greiner Bio-One GmbH, Germany) at a density of 7.5 × 10⁴ cells ml⁻¹. Following 24 h incubation, confluent cells were exposed to a concentration range of 0–200 μg ml⁻¹ of NMs (dilution factor 2) in a 100 μl volume for 24 and 72 h. Although exposure concentrations may not represent the actual NMs (in powdered forms), was dispersed in Milli-Q water (33 MΩcm at 25 °C) at a concentration of 5 mg ml⁻¹, and sonicated in a water bath (37 KHz) for 10 min. All suspensions of NMs were freshly prepared before each in vitro toxicity test.

2.5. Cytotoxicity measurements

Cell viability was measured on the same set of cells according to a modified version (Lammel et al., 2013; Lammel and Navas, 2014) of a protocol described by Dayeh et al. (2013). After 24 or 72 h of exposure to NMs, medium was removed and cells were washed twice with PBS. Wells received 100 μl of 1.25% (v/v) AB and 4 μM CFDA-AM prepared in serum-free/phenol red-free MEM (containing 1% NEAA). Fluorescence was measured on a microplate reader (Tecan GENios, Männendorf, Switzerland) at a wavelength of 535/590 nm (excitation/emission) for AB, or at 485/535 nm for CFDA-AM after 30 min of incubation in the dark. Cells were washed with PBS and incubated with 100 μl of neutral red solution (33 μg ml⁻¹ in serum-free/phenol red-free MEM containing 1% NEAA) for 1 h in the dark. Following incubation, cells were rinsed with PBS and the retained dye was extracted with 100 μl of an acidified (1% glacial acetic acid) 50% ethanol/49% Milli-Q water solution (extraction solution). Thereafter, fluorescence was measured at 532/680 nm. The fluorescence values were corrected for the cell-free control results and normalized against the medium control values.

2.6. Cytotoxicity of Ni residue in CNFs

Since GANF, GATam and GaAqua contain Ni as a catalyst in the synthesis process of CNFs by Grupo Antolin, additional experiments were carried out to assess the contribution of Ni residues to the toxicity of CNFs. The possible cytotoxic effect of Ni was determined by exposing cells to Ni(II) sulphate hexahydrate. The range of Ni exposure concentrations (equivalent to Ni(II) up to 57.2 μg ml⁻¹) covered the estimated amounts of Ni in CNFs (highest concentration estimated up to 28.2 μg ml⁻¹). Given that the exact form of Ni mobilized from the CNFs is unknown, the next step was to expose the cells to the leachate of CNFs. For this, 10 ml of GaAqua (CNF already dispersed in deionized water) was centrifuged at 5000 g for 30 min at room temperature to remove any CNFs from the supernatant. GANF and GATam were dispersed separately in Milli-Q water (5 mg ml⁻¹), sonicated (under the same conditions as described above) and centrifuged (5000 g for 30 min at room temperature). The absence of CNFs in the supernatants was confirmed by DLS. CNF-free supernatants of the three CNFs were prepared for cytotoxicity assessments in the same way as described for each NM stock suspension (e.g. dilution in cell culture media).

2.7. Interference assessment

Before starting any experiment, potential interferences of NM dispersions with cytotoxicity assays were tested in the presence of cells to simulate a more realistic assay scenario. For that, cells were seeded and exposed to NMs in the same way as for the toxicity tests. Fluorescence readings of exposed cells were taken at the same wavelengths used for the three assays before and after washing the cells twice with PBS. Measurements were repeated following the addition of serum-free/phenol red-free MEM (containing 1% NEAA) to the cells. Next, cells were incubated in the dark under exposure conditions with the conversion products of AB (0.1 and 1 μM of resorufin) and CFDA-AM (0.4 and 4 μM of 5-carboxyfluorescein) and fluorescence was read at time 0 and after 30 min. After the washing step, neutral red (33 μg ml⁻¹) prepared in extraction solution was added to the cells and fluorescence was determined at time 0 and after 60 min at the corresponding wavelengths.

2.8. Internalization of NMs

Transmission electron microscopy was used to investigate possible internalization of the NMs in the cells. For that, cells were seeded on poly-l-lysine coated cover slips in a 24-well plate and were exposed to three different concentrations of each NM for 72 h. Exposure concentrations were chosen according to the outcomes of
cytotoxicity tests (non-toxic, low toxic and relatively toxic). Sample preparation for TEM analysis was carried out as described by Lammel et al. (2013) and included washing steps (Millonig phosphate buffer, pH 7.3), primary fixation (4% paraformaldehyde/2.5% glutaraldehyde), post fixation (1% osmium tetroxide), gradual dehydration steps (30–100% acetone), embedding (gradual infiltration with Spurr’s resin) and a polymerisation step (65 °C, 48 h). Ultrathin sections were stained in uranyl acetate and lead citrate and viewed in a JOEL 1010 JEM TEM (JEOL Ltd, Tokyo, Japan).

2.9. Statistical analyses

All data are expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using Sigma Plot (version 12.5, Systat Software, Inc., Chicago, IL, USA). The program checks automatically for homogeneity of variance. The normality of the distribution was confirmed with Shapiro-Wilk test. Data were parametric, thus experimental results were compared to their corresponding control values using one-way repeated measures analysis of variance (p < 0.05) followed by a post hoc Dunnett’s test.

The concentration required to cause 10 and 50% of inhibition with respect to the control (IC10 and IC50, respectively) was calculated by fitting a four parameter logistic model $y = \frac{max/[1 + (x/IC_{50})]^b]}{min}$ + min, (where max is the maximal response observed, b is the slope of the curve, and min is the minimal response).

3. Results and discussion

3.1. Characterization of NMs

Given that DLS assumes a spherical or near-spherical particle shape, obtained readouts for the fibers or platelets used in the present work should be interpreted with caution. HDD of NMs suspended in cell culture media over 72 h are summarized in Table 1. Changes in hydrodynamic sizes did not follow a specific trend over a period of 72 h in any of the cell culture media. In both media, DLS measurements indicate the presence of two size populations, with a main particle size ranging from 256 to 673 nm and a smaller proportion of large (or aggregated/agglomerated) particles ranging from 3870 to 4970 nm.

Size estimation by TEM is in accordance with the size measurements of the NM dispersion by DLS. TEM images (Fig. 1) revealed no remarkable differences in size or shapes for each NM between Milli-Q water or cell culture media dispersion. Similarly, no differences were observed for each NM at different exposure durations (0 and 72 h). Due to the morphology of these NMs it is difficult to draw conclusions on their agglomeration/aggregation states. Overall, CNFs consist of a mixture of individually dispersed and bundled nanofibers (Fig. 1A–D). TEM images at higher magnification revealed the internal structure of CNFs comprised of conical graphene layers (arranged as stacked cups) (Fig. 1B). GaqUA was found to be up to a few microns in length, what could be related with the different sonication intensity (bath sonication instead of probe sonication) employed (Fig. 1D). TEM images of the GO dispersion confirmed the presence of single or few layer graphene sheets (Fig. 1E). Surface area of GO sheets varied between one and a few μm² and some wrinkles as well as foldings on the edge can be observed at several places (Fig. 1F).

Toxicity tests require homogeneous samples. In order to obtain homogeneous dispersions of GRMs it was necessary to use dispersants and apply mechanical energy. Nevertheless, the dispersion of GRMs without influencing their toxicological behaviour is one of the major challenges in in vitro NM toxicology. For instance,

Table 1. Changes in hydrodynamic sizes did not follow a specific trend over a period of 72 h in any of the cell culture media. In both media, DLS measurements indicate the presence of two size populations, with a main particle size ranging from 256 to 673 nm and a smaller proportion of large (or aggregated/agglomerated) particles ranging from 3870 to 4970 nm.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>PHC-1 medium</th>
<th>Average HDD (%)</th>
<th>CLC medium</th>
<th>Average HDD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>z-ave (nm)</td>
<td>PdI</td>
<td>z-ave (nm)</td>
<td>PdI</td>
</tr>
<tr>
<td>GANF</td>
<td>371.1 ± 9.2</td>
<td>0.3</td>
<td>453.3 ± 12.7 (95)</td>
<td>0.4</td>
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<tr>
<td>24</td>
<td>363.1 ± 34.7</td>
<td>0.3</td>
<td>4654.9 ± 1163.5 (5)</td>
<td>0.4</td>
</tr>
<tr>
<td>72</td>
<td>486.3 ± 16.6</td>
<td>0.4</td>
<td>499.4 ± 31.6 (93)</td>
<td>0.4</td>
</tr>
<tr>
<td>GANFg</td>
<td>311.6 ± 8.5</td>
<td>0.3</td>
<td>362.0 ± 15.9 (95)</td>
<td>0.3</td>
</tr>
<tr>
<td>24</td>
<td>335.0 ± 6.6</td>
<td>0.4</td>
<td>374.6 ± 16.7 (91)</td>
<td>0.3</td>
</tr>
<tr>
<td>72</td>
<td>313.5 ± 5.4</td>
<td>0.3</td>
<td>361.1 ± 10.5 (94)</td>
<td>0.3</td>
</tr>
<tr>
<td>GAtam</td>
<td>306.9 ± 23.3</td>
<td>0.3</td>
<td>447.3 ± 180.4 (6)</td>
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<td>24</td>
<td>430.5 ± 29.5</td>
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<td>481.0 ± 23.8 (93)</td>
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<td>72</td>
<td>399.1 ± 32.9</td>
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<td>435.2 ± 23.1 (92)</td>
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<td>GaQUA</td>
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<td>617.8 ± 43.4 (86)</td>
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<td>24</td>
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<td>660.7 ± 70.5 (93)</td>
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<tr>
<td>72</td>
<td>665.1 ± 58.8</td>
<td>0.5</td>
<td>617.8 ± 74.3 (72)</td>
<td>0.4</td>
</tr>
<tr>
<td>GpAnPH</td>
<td>329.3 ± 24.2</td>
<td>0.3</td>
<td>435.0 ± 56.4 (95)</td>
<td>0.3</td>
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<td>0.4</td>
<td>406.5 ± 32.7 (94)</td>
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<tr>
<td>72</td>
<td>353.3 ± 8.1</td>
<td>0.4</td>
<td>367.7 ± 17.7 (87)</td>
<td>0.3</td>
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</tbody>
</table>
the presence of proteins in cell culture medium can mitigate the cytotoxicity of carbon-based NMs by protein corona formation as has been shown by Hu et al. (2011). Apart from breaking up aggregates, sonication may cause fractionation or introduce defects on the material surface or edges, thereby modifying the interaction with biological systems. As an example, length-dependent toxicity of multi-walled CNTs in macrophage models was observed by Boyles et al. (2015), who reported frustrated phagocytosis and production of pro-inflammatory products in response to long fibre (>20 μm), but lack of such effects when exposed to shorter fibres. Besides the length, fibre morphology (e.g. straight, tangled) and aggregation status are important factors in the cytotoxicity as revealed by Brown et al. (2007).

3.2. Cytotoxicity of GRMs

Interference of NMs with AB assay reagents in the medium (higher than 20% with respect to controls) limited the use of higher concentrations for the cytotoxicity assessment (Figs. 2 and 3 and Table 2). In general, GRMs used in this work caused limited cytotoxicity to PLHC-1 cells so that a concentration-dependent decrease in cell viability following 24 or 72 h of exposure was observed only in some cases (Fig. 2). For these cells, IC50 values were possible to determine only for GAtam after 72 h exposure (AB assay) and for GRAnPH (AB and NR assays) after 24 and 72 h exposure, although these inhibitory concentrations were greater than 100 μg ml−1 (Table 2). IC10 values were also derived. However, the error associated with some of these data was too large so that these values
should be taken with caution. GANF caused a cytotoxic effect preferentially through alteration of cell membrane integrity (CFDA-AM assay), exhibiting the highest toxicity among the tested materials. GANFg caused also alterations on cell membrane integrity, but a strong effect was also observed at the lysosomal level (NR assay) both at 24 h and at 72 h. On its hand, GAtam caused disruption of the plasma membrane and lysosomal membrane as well after 72 h. Similarly, GAqUA also caused alterations at the plasma membrane integrity and lysosomal levels. However, the strongest effect of GAqUA was detected with the AB assay indicating reduced metabolic activity. GRAnPH® affected cell viability at the three different levels tested (metabolic activity, plasma membrane integrity and lysosome functionality) at both time points.

Similarly to the PLHC-1 cells, no clear inhibition pattern was observed in CLC cells exposed to NMs (Fig. 3). Overall, cytotoxicity was dependent on the measured endpoints and longer exposure periods led to an increased toxicity. 50% of inhibition of cell viability was reached following exposure to GAtam and GAqUA, whereas for the other NMs, IC50 values were higher than 100 µg ml⁻¹ (Table 2). GANF affected the plasma membrane integrity after 24 h and this effect was more remarkable after 72 h of exposure (CFDA-AM assay). GANFg was also able to alter plasma membrane, but only
after 72 h of exposure. GAtam not only caused an impairment in the plasma membrane integrity, but also in the metabolic activity (AB assay). Among the four CNFs, only GAqUA was able to alter lysosomal function in CLC cells at 72 h (NR assay). Moreover, for this NM reduced cell viability was observed using AB and CFDA-AM assays after 72 h of exposure. GRAnPH® had an impact on metabolic activity and membrane integrity already in cells after 24 h of exposure.

In vitro toxicological testing, which can also be based on fish cell lines, has been proposed as a promising method to screen the ecotoxicity of NMs (Boyles et al., 2015; Fernandez-Cruz et al., 2013). These methodologies offer a cost and resource effective alternative that allows generating important toxicological information in short periods of time. In addition they permit to avoid some animal tests or reduce the total number of animals used in such tests favouring the 3Rs concept what has evident ethical implications. These methodologies also provide essential information about the interaction and possible effects of NMs at cellular level.

The information produced with in vitro assays can serve, for instance, for a first ranking of NMs that can be used in grouping exercises or simply in the selection of NMs for further more resource consuming higher tier toxicity tests. In the present work...
we tried to rank the NMs used in order to check if such an approach would be suitable for application in broader and more complex sets of GRMs. For most of the assays and materials used IC50 was not reached, or was higher than 100 μg ml⁻¹ after 72 h exposure (except for GAtam and GAqUA in CLC cells). Therefore, in order to establish toxicity ranking for the tested NMs, the lowest IC10 values from the different assays (AB, CFDA-AM and NR) were taken into account. In PLHC-1 cells, GANF and GAqUA revealed enhanced toxicity compared to GANFg, GAtam and GRAnPH®. In CLC cells, again GANF and GAqUA exhibited, together with GAtam, higher toxicity than GANFg and GRAnPH®. Therefore GANF as powder or in aqueous suspension (GAqUA) exhibited the highest toxicity, followed by GAtam, and GANFg and GRAnPH® appeared in the group of the lowest toxicity. It seems, therefore, that fibers have a higher toxicity than graphene, and that the highest degree of graphitization (GANFg, 90%; GANF 70%; GAtam 60%) in fibers is also associated with lower toxicity.

In vitro assays show also some limitations that can be exacerbated when these approaches are applied to NMs. An important shortcoming that must always be taken into consideration is the potential interference between the NMs and cytotoxicity assay reagents. Such interactions have been reported for a variety of carbon-based NMs, in particular, when employing the AB and Neutral Red assays (Casey et al., 2007; Monteira-Riviere et al., 2009). In the present study we thoroughly tested the possible interference of NMs with the assays at different levels: due to own fluorescence of materials, due to fluorescence quenching or due to possible direct interference (for instance through adsorption) with assay products. We observed fluorescence quenching by GRMs at higher concentrations only in the case of AB assay. Therefore these results were interpreted with caution.

Among the various mechanisms proposed to explain the cytotoxic effects induced by GRMs are the physical damage of cell membrane (Hu et al., 2011) and the effect of metal impurities (Kagan et al., 2006). In general, GRMs tested in the present study affected the plasma membrane integrity (CFDA-AM assay) of both cell types, being this effect slightly more pronounced in CLC cells (Table 2). 72 h IC50 values in the CFDA-AM assay indicate that GAqUA and GAtam altered the cell membrane of CLC cells at a larger scale in comparison to GANF, GANFg and GRAnPH®. To further investigate whether NMs with lower IC50 values in the CFDA-AM assay caused more pronounced disruption of plasma membrane integrity, TEM images of exposed cells have been analysed. Micrographs, however, did not reveal visible changes/differences in the membrane structure of cells exposed to the different NMs tested.

3.3. Cytotoxicity of Ni residue in CNFs

Ni(II) sulphate decreased the viability of PLHC-1 cells (>20%) in terms of metabolic activity, plasma membrane integrity and lysosomal function at a concentration equivalent to 28.6 μg ml⁻¹ of Ni(II) and higher (Supplementary Fig. 1). In the case of CLC cells, more than 20% decrease in metabolic activity and plasma membrane integrity was observed at a concentration equivalent to 7.1 μg ml⁻¹ of Ni(II) and higher, whereas the amount of Ni(II) affecting lysosome functionality (more than 20% decline in cell viability) is equivalent to 28.6 μg ml⁻¹ and higher (Supplementary Fig. 1).

CNF-free supernatant of GAqUA did not cause cytotoxicity to either cell lines. Nor did the supernatants of GANF and GAtam to PLHC-1 cells. In the case of CLC cells only the supernatant of 200 μg ml⁻¹ GAtam provoked a decrease in cell viability according to the CFDA (approx. 38% decrease) and the AB (approx. 58% decrease) assays (Fig. S1).

A number of studies have discussed the role of metal impurities in CNT-induced toxicity, but their contributions to the overall toxicity remain unclear. Although metal catalysts entrapped at the extremities of tubular-shaped carbon NMs would not be expected to interact with cells, when they come into contact with biological fluids, they may become biologically active (Ge et al., 2012). Moreover, ultrasonication of carbon NM suspensions may release metals from the carbon encapsulation making them more bioavailable and therefore, more toxic to cells, as proposed by Lam et al. (2006).
Fig. 4. Selected TEM images of non-exposed (A) and 72-h GRM-exposed (B–H) PLHC-1 cells. B) Intracellular GANF (at 25 μg ml⁻¹) localized in membrane-bound vesicle. C) Internalization process of GANF (at 200 μg ml⁻¹). Arrows denote the membrane pit engulfing GANF. D) GANFg (at 200 μg ml⁻¹) in the cytoplasmic vesicle preserves the stacked-cup structure. E) GAtam (at 25 μg ml⁻¹) encapsulated in numerous vesicles. F) Internalized GAtam (at 200 μg ml⁻¹) concentrated in cytoplasmic vesicles. G) Intracellular GRAnPh® sheets present sharp-bended structure in the cytoplasm (at 50 μg ml⁻¹). H) Interaction of GRAnPh® (at 100 μg ml⁻¹) with plasma membrane. n nucleus, pm plasma membrane.
as well due to the acidic environment encountered in organelles (e.g., lysosomes). Further work will be necessary to quantify the extent of Ni mobilized from CNFs and to determine its contribution to their toxicity.

3.4. Intracellular presence of GRMs

In addition to the observations regarding cell membrane damage, TEM has also been used to investigate the internalization of GRMs in both cell lines. Graphene-related nanomaterials were observed in the inner of PLHC-1 cells after 72 h of exposure at concentrations ranging from 3.1 to 200 μg ml⁻¹ (Fig. 4). Based on TEM observation, CNFs were found exclusively in intracellular vesicles and their uptake was not limited to short fibers, but also to fibers reaching a length of up to 1 μm (Fig. 4B–E). Micrographs at higher magnification revealed that CNFs preserved the stacked-cup structure enclosed in the vesicles (Fig. 4D). Internalized GO was found accumulated in the intracellular vesicles as well as freely within the cytoplasm. Micro-sized GO sheets within the cells exhibited sharp-bended structure (Fig. 4G). TEM images also

![Figure 5](image_url)

**Fig. 5.** Selected TEM images of non-exposed (A) and 72-h GRM-exposed (B–H) CLC cells. B) Intracellular GANF (at 25 μg ml⁻¹) localized in membrane-bound vesicle. C) Cell debris (associated to cell death) observed following exposure to 200 μg ml⁻¹ GANF. D) GANFs (at 3.1 μg ml⁻¹) enclosed in cytoplasmic vesicles. Arrowheads indicate several mitochondria in the cytoplasm. E) Internalized GANFs (at 200 μg ml⁻¹) concentrated in cytoplasmic vesicles. F) Cellular uptake of GAtam of various sizes (at 25 μg ml⁻¹). Shorter nanofibers encapsulated in membrane-bound vesicles (white arrow) and longer nanofibers encountered free in the cytoplasm (black arrows). G) Intracellular location of GRAnPH® (at 50 μg ml⁻¹) in membrane-surrounded vesicles (white arrows) or throughout the cytoplasm (black arrows). H) Intracellular GRAnPH® sheets (at 100 μg ml⁻¹) present sharp bends (black arrows) and wrinkles (white arrow). n nucleus, pm plasma membrane.
captured the initial interactions between GRMs and the cell membrane (Fig. 4CH), however no further evidence for loss of membrane integrity was found.

All GRMs have been visualised in the inner of CLC cells after 72 h of exposure to different concentrations (Fig. 5). In general, CNFs were localized in intracellular vesicles (Fig. 5B–E). CLC cells were able to internalize larger CNFs (>500 nm in length) as well, however they were found free without being surrounded by any membrane structure in the cytoplasm (Fig. 5F). Exposure to CNFs at a concentration of 200 μg ml⁻¹ resulted not only in higher degree of NM internalization, but in cell death as well (Fig. 5C). GO platelets were found either in the vesicles or free in the cytoplasm of CLC cells (Fig. 5G and H). Interestingly, no GRMs were found attached to the outer surface of the cells and plasma membrane was apparently intact.

Although CNFs and GO have similar chemical composition, their shapes differ considerably, and therefore their interaction with cells are expected to be distinct (Zhang et al., 2010). While the shape of CNFs (“nano-needles”) clearly promote their penetration through the cell membrane piercing it, experimental and simulated studies have demonstrated that GO initiated cellular uptake at the corners or edges of the sheets in order to overcome high energy barriers (Li et al., 2013). Kucki et al. (2017) found folded and sharp–ended structures of internalized GO in undifferentiated human enterocytes, probably as a consequence of strong interaction with the cell membrane during the cellular uptake process. In line with these findings, TEM images of the present study showed certain deformations of the intracellular GO in both cell types. Thus, in addition to the lateral dimension, flexibility of GO sheets seems to be an important factor for the cellular uptake.

Different mechanisms by which GRMs enter the cell have been proposed, including energy-independent diffusion (Raffa et al., 2008) and energy-dependent endocytosis (Porter et al., 2007). In the present study, no specific markers or inhibitors were applied to elucidate the cellular uptake pathways, however, micrographs in one case captured evidence for CNFs engulfment through the formation of invagination on plasma membrane of PLHC-1 cells (Fig. 4c). Their intracellular localization in membrane-enclosed compartments also suggests an endocytic process of uptake. As reported by various studies, intracellular localization is dependent on the properties of NMs and cell types chosen. For instance, Horváth et al. (2013) using human lung epithelial cells (A549) and mouse peritoneal macrophages showed that GO was exclusively accumulated in membrane-confinned vesicles (i.e. phagocytic vesicles, endolysosomes), whereas Lammel et al. (2013) observed either freely-localized GO in the cytosol or enclosed in intracellular vesicles of human hepatocellular carcinoma cells. It is important to note that different intracellular localization of NMs may lead to different intracellular fate and toxicity. The material that has been taken up in membrane-bound structures may be less vulnerable to the cells than those encountered free in the cytoplasm. Indeed, Lammel and Navas (2014) demonstrated that accumulated GO in the cytosol of fish hepatoma cells (PLHC-1) that were not enclosed by membrane physically interacted with mitochondria and nucleus. On the contrary, in the present study, despite the GRMs which are not membrane bound, no evidence of such interactions with intracellular organelles has been observed. The study of the process of GRMs, in particular platelet-like GRMs as for instance GO, would need further in depth investigation to fully understand the process of GRM penetration into cells but the presence of platelets free in the cytoplasm has evident implications in relation to the accidental internalization of accompanying substances (pollutants present in the environment) or to the application of this kind of GRMs for the transport of products of interest to the inner of cells.

4. Conclusions

Both cell lines used in the present work have shown their suitability for the initial study of the cytotoxicity of GRMs. NMs used in the present study affected to different degrees the metabolic activity, membrane integrity and lysosomal function of fish hepatoma cells and macrophages. Apparently, CNFs in both cell lines exhibited greater toxicity than GO. Moreover, in CNFs higher degree of graphitization related to lower toxicity. Manufacturers can benefit from these results by identifying those materials with the lowest toxicity and therefore modifying the synthesis to generate safer products. Carbon nanofibers were taken up into vesicles of PLHC-1 cells in a size-independent manner, whereas in CLC, longer CNFs were encountered free in the cytoplasm and only the shorter CNFs were localized in membrane-bound compartments. Graphene oxide sheets were present within vesicles as well as free in the cytoplasm of both cell types. This information provides an insight into the intracellular fate of these GRMs and opens door to possible safe and sustainable use of NMs as carriers of other substances.

Conflicts of interest

There are no conflicts to declare.

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Appendix A. Supplementary data

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References


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