NANOMATERIALS AS NANOCOMPOSITE FILLERS: CYTOTOXICITY AT DIFFERENT STAGES OF THEIR LIFE CYCLE

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ABSTRACT

MWCNT and SiO_2 NP are widely used as nanofillers in polymeric matrices for different applications. We have studied the cytotoxicity of these NM at the different stages of the life cycle in A549 and HepG2 cell lines.

To evaluate NM (MWCNT and SiO_2 NP) at the different stages of the life cycle, these NM were recovered from the polymer matrix by different processes, either by calcination of the polymer nanocomposite or by dissolution followed by filtration or centrifugation. These processes induced surface modification on the NM that were reflected in their toxicological profile. In the case of MWCNT calcination processes leaded to higher cytotoxicity and for hydrophilic SiO_2 NP after the have been recovered by calcination they are not cytotoxic anymore. Surface chemistry is driving the cytotoxic effects of NM along their life cycle.

Keywords: MWCNT, SiO_2, cytotoxicity, life cycle, nanocomposite

1. INTRODUCTION

Since it was discovered that nanomaterials could provide new and enhanced properties to already existing materials, nanotechnologies have been growing exponentially. Nanotechnology is expected to reach an impact of one trillion dollars at 2015 [1].

Nanomaterials are used for the development of nanocomposites [2] to improve the properties of polymers. Carbon nanotubes are widely used in car industry, cycling, army, electronics, nanomedicine, etcetera because they improve the mechanical, thermal and electrical properties of polymers. SiO_2 NP are extensively used in electronics, glass industry, optical fibers, ceramics, additive, as enhancers of lithium ion batteries, as reinforcers, as flame retardants, as additive in paints, pharmaceuticals and cosmetics, and in microelectronics [3].

Therefore, it is important to evaluate the potential impact of these nanomaterials on human health and in the environment. It has been demonstrated that some nanomaterials present (eco)toxicological effects when *in vitro* or *in vivo* studies were performed [4-11].

During the life cycle of nanomaterials, they can suffer physicochemical changes that can be reflected as changes in their (eco)toxicological profile. Our studies were then focused on the evaluation of the physico-chemical properties of different NP (with different chemical nature: Composition, size and shape and surface properties) along their life cycle as well as their release and potential impact to human health and environment. The work presented here will be focused on the cytotoxicity studies of these NM when they are included in polymer matrices (PA6) at different life cycle stages: Before they are included in the polymeric matrix (raw NM), after manufacturing of NC, and when NM are recovered for recycling purposes (end of life stage) after they have been aged (accelerated ageing simulating external use of polymer NC). In order to evaluate the cytotoxicity of the NM recovered from aged polymer nanocomposites, three different methods were studied: Calcination, chemical dissolution followed either by filtration or by centrifugation. The toxicological data presented here showed that surface chemistry modifications occurring during the life cycle due to different processes play an important role in the toxicological profile of a NM.

2. MATERIALS AND METHODS

MWCNT were obtained from Nanocyl (Belgium) whereas non-functionalized SiO_2 (hydrophilic) NP and octyl-modified SiO_2 (hydrophobic) NP were obtained from PlasmaChem (Germany). Table 1 summarizes the physicochemical properties given by the suppliers.

Additional physicochemical characterization was performed with dried powders of NM and NM dispersed in MilliQ water or cell culture medium.

2.1 Physicochemical characterization of NM

TGA was performed by using Sympatec System Particle Technology Helos / BR (FKV, Italy). Additionally, surface area and porosity of NP was measured by a multi-point isotherm BET assay (Nova 2200e series, Quantachrome, Germany). FTIR (IR Affinity-1 8400, Shimadzu) and TEM (JEM-2011, Jeol) were also carried out.

2.2 NM dispersions

Nanomaterial dispersions were prepared at concentrations of 1 mg/mL by mixing the NP in powder form with the cell culture media (Dubelcco's Modified Eagle's Medium –DMEM- high glucose) (Sigma-Aldrich, Switzerland) or MQ water. For the hydrophobic SiO_2NP, the addition of polyvinylpyrrolidone (PVP) at 1% and 5% for cell media and water, respectively, was needed. Afterwards, the sample was sonicated (SONICS VCX 750,

Spain) applying a power of 400 W for 15 minutes without pauses for 25 mL dispersions. Dispersions were kept cold during the sonication process by using a water-ice bath around the solution flask.

Ph-Ch properties	MWCNT	Hydrophilic SiO_2	Hydrophobic SiO_2
Diameter	9,5 nm	7-14 nm	7-14 nm
Length	1,5 µm	---	---
Purity	90 % (10% metal oxide)	99,8 %	99,8 %
Surface area	250-300 m^2/g	200 m^2/g	150 m^2/g
Density	---	0,048 g/cm^3	0,050 g/cm^3

Table 1: Physicochemical properties given by the suppliers

2.3 Physicochemical characterization of dispersed NM

Once the sonication was done, the physicochemical characterizations were performed at 0 and 24 hours after sonication. Dynamic light scattering (DLS) and ζ-potential (Zetasizer nano series Nano-ZS, Malvern, UK), ultraviolet-visible spectrometry (UV-Vis) (UV-2450, Shimadzu, Japan) and visual characterization were carried out to evaluate dispersibility and stability of the colloidal solutions.

NM shape, dimensions and dispersibility were studied by high-resolution transmission electron microscopy (HR-TEM), with samples prepared on cooper TEM grids just after sonication.

2.4 Recovery of NM from aged NC

The three types of NM were extracted from PA6 NC by calcination (410 ºC, 3.5 hours). MWCNT were only possible to extract by this method. PA6 and raw NM were also calcinated as controls. Both NC containing SiO_2 NP were chemically dissolved with formic acid (1:6; w:w) at 60 ºC for 24 hours. After this, the solution containing hydrophilic SiO_2 NP were filtrated with glass microfiber filters and polymer NC containing hydrophobic SiO_2 NP were washed and centrifuged at 10000 xG several times. Aged NC were treated in the same manner to extract the NM.

2.5 Cell cultures

Human alveolar adenocarcinoma cells (A549) and liver hepatocellular cells (HepG2) were cultured under controlled conditions (37 ºC, 5% CO_2) in DMEM high glucose (Sigma-Aldrich) supplemented with 10 % (v/v) FBS (PAA Laboratories, GE Healthcare, UK), 100 U/mL penicillin / streptomycin (Lonza, Switzerland). Once reached the 80% of confluence A549 and HepG2 cells were harvested and seeded onto 75 cm^2 flasks once or twice a week, respectively.

2.6 Cell viability and apoptosis assays

Cells were seeded in 96-well plates (MicrotestTM 96, Becton Dickinson, Meylan Cedex, France) at 3000 cells / well and incubated at 37 ºC and 5% CO_2 for 24 hours prior to be exposed to serial concentrations of each NM. Once exposed, they were kept in the same conditions for 72 hours. After this time, AlamarBlue® assay was performed according to the supplier protocol (Invitrogen, UK). For apoptosis, caspase 3/7 activity was evaluated using the caspase-glo 3/7 assay (Promega, USA) at 72 exposition hours.

2.7 Data processing and statistical analysis

Data were processed and analyzed by the GraphPad Prism software (GraphPad software, USA). The IC_{50} value was calculated and Student's t-test was used to compare the means of two samples. One-way ANOVA (analysis of variance) was employed to evaluate differences of caspase 3/7 activity (log transformed) among groups. If ANOVA rejected a multisample hypothesis of equal means, Dunnett's multiple comparison tests were undertaken to evaluate the significance of difference between the control and exposed groups.

3. RESULTS

3.1 Physicochemical characterization

MWCNT dispersions were not evaluated by DLS because the complexity of the dispersion but the ζ-potential was calculated. In contrast, both types of SiO_2 NP were analyzed with both techniques. Results are summarized in Table 2 and Table 3.

Only hydrophilic SiO_2 NP were stable according to the DLS and the Z-potential.

UV-visible spectra demonstrated that MWCNT dispersions maintained the particles with Plasmon resonance in suspension along the time as well as the hydrophilic SiO_2 NP. Contrary, hydrophobic SiO_2 NP precipitated during the characterization.

The ICP-MS highlighted the presence of low concentrations of Al and Fe in the MWCNT coming from the catalysts used during the synthesis. BET analyses presented a surface area of 377, 191 and 135 m^2/g with a porosity of 4.3, 0.1 and 0.06 cm^3/g and size pores of 45, 9 and 9 nm for MWCNT, hydrophilic SiO_2 NP and hydrophobic SiO_2 NP, respectively. The rest of techniques used to complete the characterization of raw NM confirmed the surface chemistry (TGA and FTIR) of the different NM as well as their morphology and size (TEM).

Sample Name	Z-Ave	Peak 1 Mean Int	Peak 2 Mean Int	Peak 3 Mean Int
	d. nm	d. nm	d. nm	d. nm
SiO_2-0h	155	176	---	---
SiO_2-24h	160	181	---	---
Octyl-SiO_2-0h	2114	4363	613	---
Octyl-SiO_2-24h	780	1572	278	6,3

Table 2: DLS results of hydrophilic SiO_2 NP and hydrophobic SiO_2 NP (Octyl- SiO_2 NP) at t= 0h and t= 24h

Sample Name	Z-Ave (mV)	Mobility (µmcm/Vs)	Conductivity (mS/cm)
MWCNT-0h	-23,4	-1,833	0,189
MWCNT-24h	-17,2	-1,348	0,195
SiO_2-0h	-42,1	-3,301	0,640
SiO_2-24h	-36,0	-2,823	0,618
Oc-SiO_2-0h	5,2	0,409	0,065
Oc-SiO_2-24h	12,1	0,946	0,060

Table 3: ζ-potential results of the three NM studied at t = 0 and t = 24 h. MWCNT were dispersed with FBS (0.6 mg/mL) and diluted 1/50 to facilitate the analysis.

3.2 Cytotoxicity and apoptosis

Cytotoxic effects of MWCNT, on the cell lines studied, were not found until higher doses (100 µg/mL). However, when MWCNT were recovered from polymeric matrices by calcination processes, recovered MWCNT resulted to be cytotoxic for both cell lines (Table 4).

Hydrophilic SiO_2 NP were cytotoxic for cells tested (Table 4). However, when these are recovered by calcination from polymer NC after manufacturing process and after being aged did not showed any cytotoxicity. Contrary, octyl modified SiO_2 NP were not cytotoxic in any case (Table 4).

In order to investigate the cytotoxic pathways of hydrophilic SiO_2 NP, caspase 3/7 activity was measured in both cell lines. In both cases largest concentrations evoked an increase of this activity indicating apoptotic cell death. Because this is a luminescence assay, MWCNT could not be tested.

4. DISCUSSION

Nanoparticle physico-chemical properties change along their life cycle. The impact of nanomaterials released at different stages of the life cycle will be dependent on their physico-chemical properties at each stage. Nanomaterials widely used as nanofillers of polymer nanocomposites, such as MWCNT and SiO_2 NP, were monitored to evaluate their toxicological profile at different stages of their life cycle.

We have demonstrated that MWCNT with low metal impurities are not cytotoxic up to 100 µg/mL. However, their calcination changed their surface chemistry and consequently their toxicity (IC_{50} values of 40 µg/mL) for both cell lines. It has been described that calcination of MWCNT can lead to the oxidative cutting of nanotubes by the catalytic action of Fe impurities [12]. The difference between calcinated raw MWCNT and calcinated NC might be due to a protection effect of the polymer. The lack of cytotoxic effects in both cell lines when exposed to MWCNT extracted from aged NC might be due to a higher protective effect of the polymer to the effects of calcination as the time pass.

NM	A549	HepG2
MWCNT	< 20%	< 20%
Aged MWCNT	< 20%	< 20%
Calcinated MWCNT-Control	40 µg/mL	40 µg/mL
MWCNT recovered after manufacturing of NC (Calcination)	< 20%	90 µg/mL
MWCNT recovered from aged NC (calcination)	< 20%	< 20%
Calcinated PA6-Control	< 20%	< 20%
Calcinated Hydrophilic SiO_2	< 20%	< 20%
Hydrophilic SiO_2 NP recovered after manufacturing of NC (calcination)	< 20%	< 20%
Hydrophilic SiO_2 recovered from aged NC (calcination)	< 20%	< 20%
Hydrophilic SiO_2 NP	459 µg/mL	139 µg/mL
Aged Hydrophilic SiO_2 NP	< 20%	< 20%
Calcinated Hydrophobic SiO_2 NP	< 20%	< 20%
Hydrophobic SiO_2 NP recovered after manufacturing of NC (calcination)	< 20%	< 20%
Hydrophobic SiO_2 NP recovered from aged NC (calcination)	< 20%	< 20%
Hydrophobic SiO_2 NP	< 20%	< 20%
Aged Hydrophobic SiO_2 NP	< 20%	< 20%
Hydrophilic SiO_2 NP recovered after manufacturing of NC (filtration)	37%	329 µg/mL
Hydrophobic SiO_2 NP recovered after manufacturing of NC (centrifugation)	< 20%	< 20%

Table 4: Cell viability percentages respect the control and IC_{50} values for A549 and HepG2 cells exposed to the different NM.

Hydrophilic SiO_2 NP presented cytotoxic effects and these were through the caspase 3/7 apoptosis pathway.

Nonetheless, when these NP suffered an aggressive process (such as, calcination), they change their surface chemistry. The amorphous nature of SiO_2 NP could facilitate the changes in the surface chemistry of the NP along their life cycle. Zhang et al. (2012) [13] reported that amorphous silica NP contains siloxane (ΞSi-O-SiΞ) chains. However, the surface of these NP is covered by silanol groups (ΞSi-OH). These hydroxyl groups can lead to the formation of radicals, and, thus, exercise their toxic effects. Zhang et al. (2012) [13] demonstrated that calcination eradicated the surface hydroxyl groups and the radicals as well. In this work the changes in physico-chemical properties is demonstrated to be highly related to NM toxicological properties. Therefore, the monitoring of physico-chemical properties along the life cycle of NM when they are included in conventional materials it is really important to understand their toxicological impact in human health and environment.

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