Nanomaterial-induced cell death in pulmonary and hepatic cells following exposure to three different metallic materials

Nanomaterial induced cell death in pulmonary and hepatic cells following exposure to three different metallic materials: the role of autophagy and apoptosis

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Abstract

Autophagy is the catabolic process involving the sequestration of the cytoplasm within double-membrane vesicles, which fuse with lysosomes to form autolysosomes in which autophagic targets are degraded. Since most endocytic routes of nanomaterial uptake converge upon the lysosome and the possibility that autophagy induction by NMs may be an attempt by the cell to self-preserve following the external challenge, this study investigated the role of autophagy following exposure to a panel of widely used metal-based NMs with high toxicity (Ag and ZnO) or low toxicity (TiO_2) in a pulmonary (A549) and hepatic (HepG2) cell line. The *in vitro* exposure to the Ag and ZnO NMs resulted in the induction of both apoptosis and autophagy pathways in both cell types. However, the progression of autophagy was blocked in the formation of the autolysosome, which coincided with morphologic changes in the actin cytoskeleton. This response was not observed following exposure to low-toxicity TiO_2 NMs. Overall; the results show that high toxicity NMs can cause a dysfunction in the autophagy pathway which is associated with apoptotic cell death.

Keywords

Nanomaterials, Cell death, Autophagy, Apoptosis, Hepatic cells, Pulmonary cells

Introduction

The rapid expansion of technological and commercial exploitation of molecular scale materials due to their unique properties has led to an escalating interest in the fields of nanoscience, nanotechnology and nanomedicine (Maynard et al. 2006). There are now over 1600 consumer products on the market with a claim of containing aspects of nanotechnology (CPI 2016). The widespread utilisation of engineered nanomaterials (NMs) suggests that exposure of humans and the environment is also likely to increase and since air pollution particles of similarly small dimensions are known to induce morbidity and mortality (Hoet et al. 2004; Nel et al. 2006), certain concerns about potential human health impacts associated with NM exposure have been raised (Nel et al. 2006; Papp et al. 2008).

The lungs and the gastrointestinal tract are primary exposure sites for NMs (Sadauskas et al. 2009; Geiser et al. 2010). However, it is now understood that a proportion of NMs administered via ingestion, inhalation and in particular intravenous injection reach secondary tissues including the liver (Geiser et al. 2010; Kermanizadeh et al. 2015). The liver is the most important multi-functional organ in the mammalian system identified as the metabolic centre of the body. It is responsible for the storage, synthesis, metabolism and redistribution of carbohydrates, fats and vitamins as well as detoxification and removal of gastrointestinal derived antigens and filtration of blood (Taub 2004).

Autophagy is the generic term for the catabolic degradation of cellular components, including organelles and macromolecules. Currently three different varieties of autophagy have been described which include microautophagy, chaperone-mediated autophagy and macroautophagy. Microautophagy involves the infolding of lysosomal membranes, resulting in the sequestration and degradation of cytosolic components. Chaperone-mediated autophagy is the selective translocation of altered cellular component proteins across the lysosomal membrane mediated via chaperone proteins, which allows their unfolding and eventual translocation into lysosomes. Finally macroautophagy, hereafter referred as autophagy (and

the focus of this study), is categorised by the formation of double membrane vesicles called autophagosomes, which sequester the cytoplasmic material destined to be degraded. The autophagosomes coalesce with lysosomes to form autolysosomes resulting in the degradation and recycling of the cargo and thus the maintenance of both nutrient and energy homeostasis (He et al. 2009).

In most cell types and under normal conditions, autophagy occurs at basal rate to maintain normal cellular homeostasis and elimination of misfolded proteins and damaged organelles. However, this process can be induced under stress conditions, such as oxidative stress, inflammation, metabolic stress (amino acid or growth factor deficiency), hypoxia, endoplasmic reticulum stress or an infection (He et al. 2009; Mizushima et al. 2008), with the overall aim of enhancing the chances of the cell to survive. Therefore, autophagic dysfunction is believed to play a pivotal role in a wide range of diseases including cancer, inflammatory and pulmonary diseases and aging (Levine et al. 2008; Mizushima et al. 2008).

Engineered nanomaterials are manufactured from a diverse group of substances with varying physicochemical characteristics, hence it is necessary that a range of materials are evaluated for a comprehensive toxicity profile. Here, autophagy was investigated following exposure to three widely utilised metallic NMs (Ag, ZnO and TiO_2). The Ag and ZnO are typically regarded as high-toxicity NMs, whereas TiO_2 typically is associated with low *in vitro* cytotoxicity as supported by a recent European collaborative project where these NMs were assessed (Kermanizadeh et al. 2016). Following an investigation of NM-induced autophagy in two cell types sourced from the lungs (A549) and the liver (HepG2); the hepatic cells were selected for a subsequent detailed and more comprehensive assessment of the recycling system (detailed in the methods section).

Methods

Nanomaterials

The NMs were purchased as stated: ZnO - BASF Z-Cote; uncoated, 100 nm; Ag - RAS GmbH; capped, < 20 nm. The TiO_2 sample was procured by the National Research Centre for the Working Environment (Copenhagen, Denmark) and produced from a neutrally charged rutile material (10 nm) as previously described (Kermanizadeh et al. 2013a).

Characterisation of the panel of engineered NMs

The NMs were characterised by a combination of analytical techniques in order to infer primary physical and chemical properties. The phase compositions and average crystallite sizes were determined from powder X-ray diffractograms obtained at room temperature (25°C) using a Bruker D8 Advanced diffractometer in reflection mode with Bragg-Brentano geometry. A sealed Cu X-ray tube was run at 40kV and 40 mA, wavelength Cu $K_{\alpha 1}$ 1.5406 Å from a primary beam Ge monochromator, fixed divergence slit 0.2°, step size 0.02, step time 1 s $step^{-1}$, linear PSD detector (Lynx-eye) with opening angle 3.3°. The sample holders used for the reflection data were either a standard sample holder containing an approximately 2 mm thick sample or a single Si sample holder. The Ag was measured as transmission in a capillary. The phases were identified by using the EVA 14.0 software from Bruker AXS. The ratios and size were calculated using Topas 4.1 from Bruker. The primary and aggregate size range, shape and crystal structure of the test materials were determined by 3010 transmission electron microscope operating at 300 kV (Jeol, Japan). The surface areas and pore volumes were obtained by nitrogen adsorption on a Micrometritics ASAP2000 Accelerated Surface Area and Porosimetry System at an adsorption temperature of -196°C, after pre-treating the sample under high vacuum at 300°C for 2 hr. In order to assess the dissolution of ZnO and Ag, the materials were diluted in both ultrapure and filtered

water and complete medium. The analysis was carried out using an atomic absorption spectrometer (Ag and Zn hollow cathode lamp) (Perkin Elmer AAnalyst 200, USA).

The hydrodynamic size distributions of the NMs dispersed in both appropriate complete cell culture media (supplemented with fetal bovine serum (FBS)) was determined in the 10-50 µg/ml concentration range by Nanoparticle Tracking Analysis (Nanosight LM20, USA) (Table 1).

Cell culture and NM treatment

The human hepatocellular carcinoma cell line (HepG2) (Sigma Aldrich, UK) were maintained in Minimum Essential Medium Eagle (MEM) with 10% FBS, 2 mM L-glutamine, 100 U/ml Penicillin/Streptomycin and 1% non-essential amino acids. The human lung epithelial cell line (A549) were sourced from ATCC (American Type Culture Collection, USA) and maintained in Ham's F-12 nutrient mix supplemented with 10% FBS, 2 mM L-glutamine and 100 U/ml Penicillin/Streptomycin. The cells were incubated and exposed to NMs at 37ºC and 5% CO_2. All experiments were conducted utilising cells between passage 3 and 20.

The Ag NMs were supplied in de-ionised water with a stabilizing agent (7% ammonium nitrate, 4% each of polyoxyethylene glycerol trioleate and 4% Tween 20). The TiO_2 and ZnO NMs were supplied as a dry powder. All NMs were dispersed utilising de-ionised water with 2% FBS before sonication for 16 min without pause following the protocol developed for EU funded project ENPRA (Jacobsen et al. 2010). Following the sonication, all samples were kept on ice before dilution in complete medium and utilised within 30 min. The same dispersion protocol was utilised prior to all experiments throughout.

WST-1 cell viability assay

The cells were seeded in 96 well plates (10^4 cells per well in 100 µl of cell culture medium) and incubated for 24 hr. The following day the cells were exposed to the NMs for 24 hr. The plates were washed twice with phosphate buffered saline (PBS), followed by the addition of 10 µl of the WST-1 cell proliferation reagent (Roche, USA) and 90 µl of fresh medium. The plates were then incubated for 1 hr. The supernatant was transferred to a fresh plate and the absorbance was measured by spectrophotometry at 4507/630 nm (the supernatants were transferred into fresh plates in order to decrease the potential interference of the NMs during the measurements). These experiments were repeated on five separate occasions. To examine the toxicity of NMs to the two cell types, concentrations between 0.31 and 156.25 µg/cm^2 were utilised (equivalent to 1-500 µg/ml).

Evaluation of gene expression levels

The cells were seeded in 6 well plates (10^6 cells per well in 2 ml of the cell culture medium) and incubated for 24 hr. The following day the cells were exposed to the NMs for 6 hr. Additionally, control cells were exposed to 100 nM of rapamycin (inducer of autophagy) (Sigma, UK) and bafilomycin (inhibitor of autophagy) (Sigma, UK) for 2 hr. The cells were washed with PBS and detached using a trypsin solution. The collected cells were centrifuged at 500 g for 5 min and supernatant discarded. The cells were lysed and RNA extracted and isolated using a Total RNA Isolation System (Promega, USA). The RNA concentration and purity was measured utilizing a BioPhotometer (Eppindorf, USA). The High Capacity cDNA RT kit (Applied Biosystems, UK) was used according to the protocol to transcribe RNA into cDNA. Following this step, equal quantities of cDNA from three treatments in the same group were pooled and 300 ng was used in RT reactions. PCRs were conducted in triplicate on a 7900 RT fast PCR system and SDS 2.4 software in 384 well plates (Applied Biosystems, USA), using TaqMan® kits with FAM (fluorescin) dye for 40 cycles. The following human primers were utilised: *LC3B*: Hs00797944_s1;

atg12: Hs01047860_g1; *atg3*: Hs00223937_m1; *atg4b*: Hs003670889_m1; *atg5*: Hs00169468_m1; *p62*: Hs02621445_s1 and housekeeping gene *18s*: Hs99999901_s1 (Applied Biosystems, USA).

Subsequent to the 6 hr analysis of gene expression levels described above a comprehensive time course (1, 2, 4, 6 and 12 hr) investigation of *LC3B*, *p62* and *atg12* levels was undertaking in HepG2 cells following exposure to the panel of NMs.

Western blotting

The expression of target proteins (LC3 and p62) was detected by western blot analysis. Following 4, 6 and 24 hr exposure of hepatocytes to the panel of NMs the cells were lysed with RIPA lysis buffer containing a complete protease inhibitor mixture and a protein phosphatase inhibitor (Sigma, UK). Next, 40 μg of protein from each sample was added to a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The electrophoresis productions were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-rad, USA), blocked with 5% bovine serum albumin and 0.1% Tween 20, incubated with primary anti-LC3B and anti-p62 antibodies (1:1000) (Abcam, UK) and anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000) (Abcam, UK), respectively. GADPH was used as an internal control (Abcam, UK). The proteins were visualized using the Clarity™ ECL Western substrate kit (Bio-Rad, USA) and an Amersham Imager 600 (GE Healthcare, UK). Finally, immunoblotting signals were quantitated using Image Studio 4.0 (LI-COR Biotechnology, USA).

Transmission electron microscopy (TEM)

The cells were seeded in 6 well plates in cell culture medium (10^6 cells per well) and incubated for 24 hr before exposure to the NMs for 6 hr (control cells were treated with fresh medium). The cells were thoroughly washed with fresh medium (x2) before being removed from the plastic and transferred to

BEEM capsules. The tubes were spun for 5 min at 1000 g, before the addition of 1 ml of 2.5% gluteraldehyde in PBS for 2 hr. The cells were then washed (x3) with PBS followed by the addition of 1 ml of 1% osmium tetroxide to the pellet for 10 min before being rinsed five times with PBS and two times with distilled water. The samples were dehydrated in 50%, 70%, 90% and 100% ethanol for 10 min each. After the removal of the ethanol a 50:50 mixture of resin and ethanol was added for 45 min before being replaced with 100% resin and incubated for 24 hr. The samples were embedded and cured before being fastened to a trimming block. An ultramicrotome was used to produce a final trapezoid shape of around 1 mm or less. The samples were cut into 50-100 nm sections and transferred onto TEM grids. The grids were coated with 5 nm of evaporated carbon prior to imaging to minimise charging. A FEI Tecnai TF20 FEGTEM microscope fitted with a Gatan Orius SC600 CCD camera and an Oxford Instrument 80 mm^2 X-Max energy dispersive X ray (EDX) detector was utilised to image the cells.

Fluorescence microscopy

The cells were seeded in 8 well microscopy chambers (Ibidi, Germany) (10^4 cells per well in 200 µl of the cell culture medium) and incubated for 24 hr. The cells were exposed to the NMs (LC_{20}) for 6 hr. An additional control group included cells treated with 60 µM of Chloroquine (inducer of autophagy) (Life Technologies, USA). The cells were fixed with 3.7% formaldehyde for 15 min at room temperature before being permeabilized with 0.2% Triton X-100 in PBS for an additional 15 min. Both cell types were exposed to 0.5 µg/ml of a LC3B primary rabbit antibody (Life Technologies, USA) for 1 hr at room temperature. The cells were washed before being exposed to a chicken anti-rabbit secondary antibody with Alexa Fluor® conjugate (Life Technologies, USA) for 30 min. The cells were washed with PBS and counterstained with Hoechst 33342 (Life Technologies, USA) for 15 min in the dark before being observed under a Leica AF6000 inverted wide field fluorescence microscope (Leica, Germany).

Alteration of the structure of the cytoskeleton was assessed by staining actin filaments in HepG2 cells. The cells were seeded in 8 well microscopy chambers (10^4 cells per well in 200 µl of the cell culture medium) and incubated for 24 hr before being exposed to the NMs (LC_{20}) for 6 hr. Prior, to antibody treatment the cells were fixed and the non-specific binding sites were blocked with 10% FBS with 0.2% Triton X-100 in PBS for 1 hr. The hepatocytes were exposed 1 µg/ml of a globular Actin (G-actin) primary mouse antibody (Abcam, UK) overnight at 4°C. The cells were washed before exposure to a goat anti-mouse secondary antibody with a DyLight® conjugate (Abcam, UK) for 30 min. Next, the cells were washed with PBS and counterstained with Hoechst 33342 for 15 min in the dark before being observed under a fluorescence microscope.

In addition, the HepG2 cells were stained with CytoPainter Phalloiden (also known as filamentous (F-actin) iFlour kit (Abcam, UK) according to manufacturer's instructions.

Cathepsin B activity assay

The enzymatic activity of cathepsin B following 24 hr exposure of HepG2 cells to the panel of NMs was determined utilising cathepsin B activity fluorometric assay kit (Abcam, UK) according to the manufacturer's protocol. Briefly, cell lysates were centrifuged at 10000 g for 10 min at 4°C and the supernatant was used for the assay. The isolated protein was incubated at 37°C for 1 hr with 10 mM Ac-RR-AFC (substrate for cathepsin B). After the incubation, fluorescence was measured utilising a microplate reader (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Scientific, USA).

Caspase 3 activity assay

The cells were seeded in 6 well plates (10^6 cells per well) and incubated for 24 hr. The following day the cells were exposed to the NMs (or 6 µM of Camptothecin) (inducer of apoptosis) for 6 hr. At this juncture, caspase 3 enzyme activity was measured using a caspase 3 assay kit (Abcam, UK) according to the manufacturer's protocol. Briefly, the cells were re-suspended in 50 µl of chilled lysis buffer and incubated on ice for 10 min. The proteins were separated by centrifugation at 10000 g for 1 min at 4℃. The supernatant (cytosolic extract) was collected and protein concentration determined. Next, equal amount of protein from each sample was incubated with caspase 3 substrate (DEVD p-NA) for 2 hr at 37℃ and the absorbance measured.

Flow cytometry to detect apoptotic cells

The cells were seeded in 6 well plates (10^6 cells per well) and incubated for 24 hr. The following day the cells were exposed to the NMs for 24 hr. Additionally, control cells were exposed to 6 µM of Camptothecin (Sigma, UK) for 6 hr. The cells were detached using trypsin and re-suspended in 500 µl of binding buffer and stained with 5 µl of PE labelled Annexin-V. Next, 5 µl of 7-amino-actinomycin (7-AAD) was added to the samples after they were stained with Annexin-V. Following 15 min incubation at room temperature in the dark, the cells were analysed using an Accuri C6 flow cytometer (Becton Dickinson, USA).

Statistical analysis

The data is expressed as mean ± standard error of the mean (SEM) where appropriate. For statistical analysis, the results were compared to their corresponding control values using one-way ANOVA with

Tukey's multiple comparison. All statistical analysis was carried out utilizing Minitab 17. A p value of < 0.05 was considered to be significant.

Results

NM characterisation

The NMs were characterised by a combination of analytical techniques in order to infer their primary physical and chemical properties (including manufacturer's characterisation) which is useful for understanding their toxicological behaviour. A list of these properties is reproduced from previously published work and presented in Table 1 (Kermanizadeh et al. 2013a).

Identifying the soluble fraction of Ag and ZnO NMs in complete cell culture medium is crucial for the discrimination between NM-induced effects and the dissolved elements therein. Therefore, 24 hr dissolution of the ZnO and Ag was investigated in complete medium, with the data demonstrating that about 50% of the added ZnO NMs were dissolved in the cell culture medium after 24 hr. The amount of dissolved Ag was less than 1% by weight over the 24 hr period.

TABLE 1

NM-induced cytotoxicity

The cytotoxicity (or viability) was measured with an assay that determines the formation of a tetrazolium salt WST-1 to formazan in metabolically active cells. From the WST-1 data it was clearly evident that there was a concentration-dependent decrease in cell viability at 24 hr across the entire NM panel. The concentrations, which caused 20% or 50% lethality in the cell culture (LC_{20} or LC_{50}) were determined from concentration-response curves. The LC_{50} could only be determined after exposure to Ag and the ZnO NMs (Table 2). The TiO_2 NMs were considered to be relatively low toxicity materials as the LC_{50} was not reached after a 24 hr exposure to the cells at the range investigated. From this data the LC_{20} and

LC_{50} concentrations were chosen for the subsequent experiments. For the TiO_2 NM in which an LC_{50} was not obtained only the LC_{20} and the serial dilution above was utilised where stated.

TABLE 2

NM-induced autophagy

In order to investigate whether autophagy was induced post NM exposure (6 hr), the expression of a range of important genes in the cell recycling process was investigated. These genes included *LC3B, atg12, atg3, atg4b, atg5* and *p62*. The data demonstrated an up-regulation of *LC3B, atg4b, p62* and a down-regulation of *atg12* and *atg5* following exposure to the Ag and ZnO NMs in both cell types (Figure 1). Overall, the gene expression data suggested an induction of the autophagy process (formation of autophagosomes) following treatment with the highly toxic Ag and ZnO NMs. However, the data indicate that the process is blocked in the latter stages (i.e. the formation of autolysosome). In contrast, the described patterns were less obvious or entirely absent following exposure to the TiO_2 NMs.

Next, a time-course gene analysis of *LC3B, p62* and *atg12* following exposure of HepG2 cells to the panel of NMs was carried out (Figure 2). The data clearly demonstrated a time-dependent positive trend for *LC3B* and *p62* gene expressions following exposure to the ZnO and Ag NMs. There was no significant change following exposure to the TiO_2 NM.

FIGURE 1

FIGURE 2

Protein expression of LC3-II and p62 in HepG2 cells

To confirm the above observed findings the effects of NM exposure on autophagy in HepG2 cells was investigated by evaluating the level of LC3-II and p62 via analysis by the western blotting assay (Figure 3). The data clearly demonstrated that exposure to Ag and ZnO NMs increased the levels of LC3-II in a time-dependent manner. Furthermore an accumulation of p62 was noted following exposure to the ZnO and Ag NMs and was most noticeable at the later time-points (protein involved in the formation of autophagosomes and constitutively degraded by the autophagic pathway - an increase in the level of p62 reflects an impaired autophagic flux).

FIGURE 3

Internalization of NMs and observation of autophagic ultrastructural features

Figure 4 depicts electron microscopy images of HepG2 cells after 6 hr exposure to Ag, ZnO and TiO_2 NMs. The electron micrographs clearly demonstrated double membrane autophagosome-like structures which were formed for sequestration of intracellular components following exposure. These structures were significantly more quantifiable in the cells exposed to the Ag and ZnO NMs (Figure 4a and b). The TiO_2 NMs were easily detected in single membrane vesicles (Figure 4c). However, the number of detectable autophagosomes was less visible in these cells. The uptake of the ZnO and Ag NMs by the hepatocytes was confirmed via EDX - although this was more difficult for the ZnO NMs due their high solubility (Supplementary figure 1).

FIGURE 4

Visualisation of the autophagosomes and cytoskeleton alterations

The LC3 protein plays a critical role in autophagy. Under normal circumstances, this protein resides in the cytosol, but following cleavage and lipidation with phosphatidylethanolamine, the LC3 associates with the phagophore. This localization was used as a general marker for the autophagosome formation. The confocal immunofluorescence photomicrographs of NM exposed A549 and HepG2 cells stained for LC3 protein (green) and with Hoechst (blue) for nucleic acids are shown in Figure 5. It should be stated that in these pictures, it is not possible to distinguish between LC3-I and LC3-II due to the fact that the antibody recognises both types of the protein. The exposure of the cells to the LC_{20} concentrations of the Ag and ZnO NMs resulted in formation of autophagosomes. However this was not the case or significantly less evident following treatment with TiO_2 NMs (Figure 5). Furthermore, in order to quantify the numbers of autophagosome structures, a total of three pictures per treatment were analysed in a blinded fashion on three subsequent days, with the slides being allocated a rank from 1 to 15 (pictures for each cell type ranked from lowest number of autophagosomes to the highest) with the data presented in Table 3. Finally, it should be clearly stated that there was no significant difference in the cell numbers between the different treatments of NMs in these experiments.

The involvement of the cytoskeleton in the blockage of autophagy in the ZnO and Ag NM treated cells was assessed by staining actin filaments/fibres in HepG2 cells (network of elongated protein polymer filaments that support cell shape, compartmentalization and intracellular trafficking or even whole-cell movement) (Figure 6). The immunofluorescence analysis of the HepG2 cells showed very little variation in the F-actin network between controls, rapamycin and NM treated cells, suggesting that these filaments are not visibly involved in autophagy or any potential NM-induced impairment in HepG2 cells (Figure 6f-h). Interestingly, however, a number of alterations in the G-actin networks was observed following exposure to the ZnO and Ag NMs (most evident for the Ag NMs) (Figure 6c and d). The G-actin network

appeared to be more condensed/bundled (green colour in the images) as compared to the controls and TiO_2 NM-exposed cells.

FIGURE 5

TABLE 3

FIGURE 6

NM-induced apoptosis

The suspected dysfunction of the autophagy pathway following exposure to ZnO and Ag NMs might be linked to apoptotic/necrotic cell death. Flow cytometry was performed in order to determine the effects of NM cell damage (24 hr exposure) in terms of apoptosis and/or necrosis (Annexin V and 7-AAD staining) (Figure 7). From the data it was evident that exposure to the Ag and ZnO NMs (in which a dysfunction of autophagy flux is shown) apoptosis is the main mechanism of cell death.

FIGURE 7

Cathepsin B activity

Autophagy and apoptosis are both well-controlled biological processes instrumental during development, homeostasis and disease. It is believed that interactions among components of the two pathways indicate at an intricate cross-talk which can be induced by the same stimuli (Nikoletopoulou et al. 2013). Additionally, an increasing body of evidence has emerged, which demonstrates that autophagy and apoptosis can cooperate or antagonize each other hence influencing the eventual fate of the cell. In order,

to investigate the cellular pathways that might be involved in NM-induced cell death, cathepsin B activity was investigated to provide an insight into the mechanism underlying the potential interplay between autophagy and apoptosis following exposure to the panel of NMs in the HepG2 cells (Figure 8). The data did not show a significant change in cathepsin B activity above the negative control for any of the NM treatments, which suggests that, despite varying levels of cell death autophagy levels are fairly consistent across all treatments.

FIGURE 8

Caspase 3 activity

Caspase 3 expression was monitored in HepG2 cells following exposure to LC_{20} and LC_{50} concentrations of the Ag and ZnO NMs and the LC_{20} of the TiO_2 NMs for 24 hr (Figure 9). It was noted that the caspase 3 expressions corresponded well with the apoptosis data (Figure 7) confirming caspase dependent apoptotic cell death following exposure to these particular NMs.

FIGURE 9

Discussion

The expansion of the field of nanotechnology can have great industrial and medical advances. However an increasing body of evidence suggests that exposure to certain NMs is linked to adverse effects in exposed individuals. Numerous *in vitro* and *in vivo* studies have demonstrated that NM exposure can alter cellular viability by inducing membrane, mitochondrial or genetic damages resulting in apoptosis (Belade et al. 2015; Duan et al. 2015; Muller et al. 2005; Ravichandran et al. 2011; Thit et al 2015). In keeping with earlier studies in other cell lines, the Ag and ZnO NMs were substantially more cytotoxic than TiO_2 (Kermanizadeh et al 2012; 2013b; Lai et al. 2015).

Autophagy is a complex biological process and requires the execution of an elaborate pathway of signals and steps in the process of self-digestion that not only provides nutrients to maintain vital cellular functions during periods of stress, but also facilitates the disposal of superfluous or damaged organelles, misfolded proteins and invading microorganisms. Autophagy occurs at low basal levels in virtually all cells, but is rapidly up-regulated when cells need to generate intracellular nutrients and energy. In addition, the recycling process is up-regulated when cells are preparing to undergo structural remodelling or during oxidative stress, infection, or protein aggregate accumulation (Dretic et al. 2013). The mechanisms by which NMs affect the autophagy pathway are not clearly understood. The data from this study suggest an impairment and blockage of autophagy following exposure of HepG2 and A549 cells to Ag and ZnO NMs, whereas this was not the case following exposure to the TiO_2 NMs. This highlights the importance of the need for the distinction between different NMs for an accurate interpretation of NM-induced effects. Additionally, the observed impairment of autophagy was similar between the two cell types sourced from differing organs.

The intracellular autophagy mechanism can be segregated into several steps which include initiation, autophagosome formation, the fusion of the autophagosome-lysosome and the degradation and recycling

of the cargo. It is believed that about 30 proteins are involved in the regulation of this process (Dunlop et al. 2014; Feng et al. 2015; Russell et al. 2014; Waide et al. 2016). Once autophagy is initiated, the phagophore elongates, leading to the formation of a double membrane vesicle referred to as the autophagosome. The ubiquitin-like conjugation of atg12 to atg5 by atg7 and atg10 are crucial for the elongation of pre-autophagosomal structure. The atg12-atg5 conjugate further interacts with a coiled-coil protein atg16, which links the atg12-atg5-atg16 complex into a tetramer (Tang et al. 2015). This macro-complex will dissociate from the mature autophagosome. The second conjugation system leads to the formation of the LC3-PE (microtubule associated-protein 1 light chain 3-phosphatidylethanolamine) conjugate. In the LC3 conjugation system, the LC3 is first processed by a cysteine protease, atg4, exposing a C-terminal glycine residue. Three human LC3 isoforms (LC3A, LC3B and LC3C), undergo post-translational modification during the autophagy process. The cleavage of LC3 following synthesis results in the formation of the cytosolic LC3-I. The LC3-I is converted to LC3-II through lipidation involving atg7 and atg3 allowing LC3 to become associated with the autophagy vesicles (Noda et al. 2015; Tang et al. 2015; Waide et al. 2016). The data (western blot and gene expression) in this study clearly demonstrate a time-dependent accumulation of LC3-II following exposure to the ZnO and Ag NMs, which indicates that the induction of the autophagic cascade following exposure to these NMs. These findings were further corroborated with visualisation of the autophagosomes utilising fluorescent microscopy and transmission electron microscopy in the ZnO and Ag NM treated cells. At this point it is worth mentioning that in hindsight the selection of HepG2 cells was not the best choice for imaging purposes. This is due to the fact that by nature hepatocytes grow as clusters (on top of one other) which is not optimal for imaging (fluorescent or confocal microscopy).

In the final stages of autophagy, the mature autophagosomes fuse with lysosomes to form autolysosomes. After the fusion with the lysosome, the inner membrane of the autophagosome and the cytoplasmic material sequestered in autophagosomes are degraded by lysosomal acid hydrolases including cathepsins B, D and L (Furuta et al. 2010). This results in the degradation of the proteins which are then recycled

back to the cytosol. The data in this study showed that cathepsin B activity in HepG2 cells remained similar to the negative control after exposure to all NMs which suggests that the autophagy cascade is impaired during the formation of the autolysosome.

The impairment of the autolysosome formation following exposure to the Ag and ZnO NMs was further substantiated by the accumulation of p62 following exposure of these two material. The levels of p62 are elevated if mammalian cells are deficient in autophagy (Rusten et al. 2010). Under normal circumstances the p62 protein recognizes toxic cellular waste, which is then scavenged and degraded by autophagy.

The dysfunction of the autophagy pathway and its link to apoptotic/necrotic cell death was investigated in HepG2 cells. The data from flow cytometry and caspase 3 analysis showed that apoptosis was the main mechanism of cell death following exposure to the NMs. The observations indicate an association between stalled autophagy and apoptosis, although it cannot be ruled out that it is parallel, yet independent, mechanisms of action that leads to cell death. Nevertheless, autophagy and apoptosis are both tightly regulated biological processes that are instrumental in the development, homeostasis and disease (Nikoletopoulou et al. 2013).

In this study the exposure of the HepG2 and A549 cells to the ZnO and Ag NMs resulted in either an impairment of the autophagosome-lysosome fusion and/or a defect in normal lysosomal function, whereas exposure of the cells to the TiO_2 NMs did not induce or impair autophagy within the cells at the tested concentrations. One possible explanation for the blockage in the autophagy mechanism is a NM-induced interference of cellular cytoskeleton dynamics (Cohignac et al. 2014). It is believed that an efficient protein trafficking during autophagosome formation is mediated by cytoskeletal networks. Microtubules are involved in autophagy in higher eukaryotes. In mammalian cells, autophagosomes are formed at random locations in the cell, but transported directionally toward the nucleus after completion. In this study a number of alterations in the G-actin networks was observed following exposure to the ZnO and

Ag NMs. Previous data suggests that autophagosomes associate with microtubule tracks and fuse with endosomes or lysosomes, with this process driven by the dynein motor (Monastyrska et al. 2009). After passing the cell membrane, NMs could interact with the proteins of the cytoskeleton, affect their functions and then potentially lead to an impairment of the autophagy process. As described previously the suspected NM-induced blockage in autophagy in this study is during the final phase where a fusion of the autophagosome with lysosomes is required. It has been demonstrated previously that certain NMs are capable of lysosomal perturbation (i.e. Cho et al. 2011; Hussain et al. 2010; Sohaebuddin et al. 2010). Therefore, this could be a potential contributor to NM-mediated defects of the autophagy pathway.

Previous studies have demonstrated that NMs can both induce or block autophagy, which results in a similar phenotype but completely different biological consequences. As some examples, in a recent study exposure of A549 cells to iron oxide NMs (30-65 nm) resulted in induction of autophagy, which correlated with reactive oxygen species (ROS) production and mitochondrial damage (Khan et al. 2012). Similarly, the exposure of A549 cells to copper oxide NMs (< 50 nm) resulted in the induction of autophagic cell death and the use of the autophagy inhibitors significantly improved cell survival (Sun et al. 2012). In addition, exposure of macrophages to ZnO NMs (50 nm) increased the number of autophagosomes and autophagy marker proteins. The ZnO NMs also activated the cleavage of apoptosis markers caspases 3, 8 and 9 (Roy et al. 2014). The exposure of HepG2 cells to silica NMs (~ 60 nm) also induced autophagy in a concentration-dependent manner which correlated with elevated intracellular ROS levels (Yu et al. 2014). In another study exposure of mouse fibroblasts to Ag NMs (~ 30 nm) resulted in an induction of ROS production, up-regulation of heme oxygenase 1, apoptosis and autophagy Lee et al. 2014). Conversely, a 24 hr exposure of RAW264.7 cells to magnetic iron oxide NMs (~ 30 nm) led to mitochondrial damage and generation of ROS, which preceded apoptotic cell death. Furthermore, the authors suggested that the blockage of autolysosome formation may accelerate apoptotic cell death (Park et al. 2014). Additionally, a 24 hr exposure of human umbilical vein endothelial cells to ferric oxide NMs (15 nm) was associated with increased levels of nitric oxide, IL-1β and TNF-α. Moreover, the authors

noted an increased accumulation of autophagosomes and LC3-II in the cells through both autophagy induction and the blockade of autophagy process (Zhang et al. 2016). As highlighted, it is essential to state the absolute need for careful examination and distinction between the "upstream induction" of autophagosome formation and the "downstream blockage" of fusion between autophagosome and lysosome.

Although a number of studies have previously described Ag and ZnO NM induced autophagy, our experiments offers many new insights adding to the current literature available as well as certain aspects that have been investigated for the first time: a) This is a scarce study in which three materials and two cell lines are investigated side by side in order to determine specific NM and cell responses. b) The principal body of literature has only investigated the induction of autophagy and not the potential of NM-induced blockage. c) To best of our knowledge this is the first study in which the role of cytoskeleton has been investigated in a NM and autophagy context. d) Here we explore the role of cathepsins and caspases in NM induced cell death/autophagy which again are rarely investigated.

Conclusion

Based on new understandings of the physiological functions of autophagy it is clear that both basal levels and stress-induced increases in autophagy are very important in promoting mammalian health. In this study we show that a dysfunction of this autophagic pathway contributes to apoptotic death in two cell types sourced from different organs following exposure to ZnO and Ag NMs but not TiO_2 NMs. It is therefore important to perform comprehensive analysis of the whole autophagic process which has a tremendous potential to aid in our understanding of nanotechnology risks, and design of safer nanomaterials and nanomedicines.

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Declaration of interest

The authors declare that there are no competing interests.

References

Belade E, Chrusciel S, Armand L, Simon-Deckers A, Bussy C, Caramelle P, Gagliolo JM, Boyer L, Lanone S, Pairon JC, Kermanizadeh A, Boczkowski J. 2015. The role of p53 in lung macrophages following exposure to a panel of manufactured nanomaterials. Archives of Toxicology 89: 1543-1556.

Cho WS, Duffin R, Howie SE, Scotton CJ, Wallace WA, Macnee W, Bradley M, Megson IL, Donaldson K. 2011. Progressive severe lung injury by zinc oxide nanoparticles; the role of Zn^{2+} dissolution inside lysosomes. Particle and Fibre Toxicology 8: 27.

Cohignac V, Landry MJ, Boczkowski J, Lanone S. 2014. Autophagy as a possible underlying mechanism of nanomaterial toxicity. Nanomaterials 4: 548-582.

CPI home page - http://www.nanotechproject.org/cpi/ - accessed on 21-3-2016.

Dretic V, Saitoh T, Akira S. 2013. Autophagy in infection, inflammation and immunity. Nature Reviews Immunology 13: 722-737.

Duan WX, He MD, Mao L, Qian FH, Li YM, Pi HF, Liu C, Chen CH, Lu YH, Cao ZW, Zhang L, Yu ZP, Zhou Z. (2015). NiO nanoparticles induce apoptosis through repressing SIRT1 in human bronchial epithelial cells. Toxicology and Applied Pharmacology 286: 80-91.

Dunlop EA, Tee AR. 2014. mTOR and autophagy: A dynamic relationship governed by nutrients and energy. Seminars in Cell and Developmental Biology 36: 121-129.

Feng YC, Yao ZY, Klionsky DJ. 2015. How to control self-digestion: transcriptional, post-transcriptional and post-translational regulation of autophagy. Trends in Biology 25: 354-363.

Furuta N, Fujita N, Noda T, Yoshimori T, Amano A. 2010. Combinational soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins VAMP8 and Vti1b mediate fusion of antimicrobial and canonical autophagosomes with lysosomes. Molecular Biology of the Cell 21: 1001-1010.

Geiser M, Kreyling WG. 2010. Deposition and biokenetics of inhaled nanoparticles. Particle and Fibre Toxicology 7: 2.

He C, Klionsky DJ. 2009. Regulation mechanism and signalling pathways of autophagy. Annual Review of Genetics 43: 67-93.

Hoet PHM, Hohlfeld IB, Salata O. 2004. Nanoparticles - known and unknown health risks. Journal of Nanobiotechnology 2: 12-27.

Hussain S, Thomassen LC, Ferecatu I, Borot MC, Andreau K, Maretens JA, Fleury J, Baeza-Squiban A, Marano F, Boland S. 2010. Carbon black and titanium dioxide elicit distinct apoptotic pathways in bronchial epithelial cells. Particle and Fibre Toxicology 7: 10.

Jacobsen NR, Pojano G, Wallin H, Jensen KA. 2010. Nanomaterial dispersion protocol for toxicological studies in ENPRA. Internal ENPRA Project Report. The National Research Centre for the Working Environment. Available on request.

Kermanizadeh A, Balharry D, Wallin H, Loft S, Møller P. 2015. Nanomaterial translocation - the biokinetics, tissue accumulation, toxicity and fate of materials in secondary organs - a review. Critical Reviews in Toxicology 45: 837-872.

Kermanizadeh A, Gaiser BK, Hutchison GR, Stone V. 2012. An *in vitro* liver model - assessing oxidative stress and genotoxicity following exposure of hepatocytes to a panel of engineered nanoparticles. Particle and Fibre Toxicology 9: 28.

Kermanizadeh A, Gosens I, MacCalman L, Johnston H, Danielsen PH, Jacobsen NR, Lenz AG, Fernandes T, Schins RPF, Cassee FR, Wallin H, Kreyling W, Stoeger T, Loft S, Møller P, Tran L, Stone V. 2016. A multi-laboratory toxicological assessment of a panel of ten engineered nanomaterials to human health - ENPRA project - the highlights, limitations and the current and future challenges. Toxicology and Environmental Health - Part B 19: 1-28.

Kermanizadeh A, Pojana G, Gaiser BK, Birkedal R, Bilaničová D, Wallin H, Jensen KA, Sellergren B, Hutchison GR, Marcomini A, Stone V. 2013a. *In vitro* assessment of engineered nanomaterials using C3A cells: Cytotoxicity, pro-inflammatory cytokines and function markers. Nanotoxicology 7: 301-313.

Kermanizadeh A, Vranic S, Boland S, Moreau K, Squiban AB, Gaiser BK., Andrzejczuk LA, Stone V. 2013b. An *in vitro* assessment of panel of engineered nanomaterials using a human renal cell line: Cytotoxicity, pro-inflammatory response, oxidative stress and genotoxicity. BMC Nephrology 14: 96.

Khan AI, Mohammad A, Patil G, Naqvi SAH, Chauhan LKS, Ahmad I. 2012. Induction of ROS, mitochondrial damage and autophagy in lung epithelial cancer cells by iron oxide nanoparticles. Biomaterials 33: 1477-1488.

Lai XF, Wei YF, Zhao H, Chen SN, Bu X, Lu F, Qu DD, Yao LB, Zheng JY, Zhang J. 2015. The effect of Fe_2O_3 and ZnO nanoparticles on cytotoxicity and glucose metabolism in lung epithelial cells. Journal of Applied Toxicology 35: 651-664.

Lee YH, Cheng FY, Chiu HW, Tsai JC, Fang CY, Chen CW, Wang YJ. 2014. Cytotoxicity, oxidative stress, apoptosis and autophagic effects of silver nanoparticles in mouse embryonic fibroblasts. Biomaterials 35: 4706-4715.

Levine B, Kroemer G. 2008. Autophagy in the pathogenesis of disease. Cell 132: 27-42.

Maynard AD, Aitkin RJ, Butz T, Colvin V, Donaldson K, Oberdorster G, Philbert MA, Ryan J, Seaton A, Stone V, Tinkle S, Tran L, Walker NJ, Warheit DB. 2006. Safe handling of nanotechnology. Nature 444: 267-269.

Monastyrska I, Rieter E, Klionsky DJ, Reggiori F. 2009. Multiple roles of the cytoskeleton in autophagy. Biological Reviews of the Cambridge Philosophical Society 84: 431-438.

Mizushima N, Levine B, Cuervo AM, Klionsky DJ. 2008. Autophagy fights disease through cellular self-digestion. Nature 451: 1069-1075.

Muller J, Huaux F, Moreau N, Misson P, Heilier JF, Delos M, Fonseca A, Nagy JB, Lison D. 2005. Respiratory toxicity of multi-wall carbon nanotubes. Toxicology and Applied Pharmacology 207: 221-231.

Nel A, Xia T, Madler L, Li N. 2006.Toxic potential of materials at the nanolevel. Science 311: 622-627.

Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N. 2013. Crosstalk between apoptosis, necrosis and autophagy. Biochmica et Biophysica Acta 1883: 3448-3459.

Noda NN, Inagaki F. 2015. Mechanisms of autophagy. Annual Review of Biophysics 44: 101-122.

Papp T, Schiffmann D, Weiss D, Castronova V, Vallyathan V, Rahman Q. 2008. Human health implications of nanomaterial exposure. Nanotoxicology 2: 9-27.

Park EK, Choi DH, Kim Y, Lee EW, Song J, Cho MH, Kim JH, Kim SW. 2014. Magnetic iron oxide nanoparticles induce autophagy preceding apoptosis through mitochondrial damage and ER stress in RAW264.7 cells. Toxicology in Vitro 28: 1402-1412.

Ravichandran P, Baluchamy S, Gopikrishnan R, Biradar S, Ramesh V, Goornavar V, Thomas R, Wilson BL, Jeffers R, Hall JC, Ramash GT. 2011. Pulmonary biocompatibility assessment of inhaled single-wall and multiwall carbon nanotubes in BALB/c mice. Journal of Biological Chemistry 286: 29725-29733.

Roy R, Singh SK, Das CM, Tripathi A, Dwivedi PD. 2014. Zinc oxide nanoparticles induce apoptosis by enhancement of autophagy via PI3K/Akt/mTOR inhibition. Toxicology Letters 227: 29-40.

Russell RC, Yuan HX, Guan KL. 2014. Autophagy regulation by nutrient signalling. Cell Research 24: 42-57.

Rusten TE, Stenmark H. 2010. p62, an autophagy hero or culprit? Nature Cell Biology 12: 207-209.

Sadauskas E, Jacobson NR, Danscher G, Soltenberg M, Larsen A, Kreyling W, Wallin H. 2009. Bio-disruption of gold nanoparticles in mouse lung following intratracheal instillation. Chemistry Central Journal 3: 16-23.

Semmler-Behnke M, Kreyling W, Lipka J, Fertsch S, Wenk A, Takeneka S, Schmid G, Brandau W. 2008. Bio-distribution of 1.4 and 18 nm gold particles in rats. Small 12: 2108-2111.

Sohaebuddin SK, Thevenot PT, Baker D, Eaton JW, Tang L. 2010. Nanomaterial cytotoxicity is composition, size, and cell type dependent. Particle and Fibre Toxicology 7: 22.

Sun T, Yan Y, Zhao Y, Guo F, Jiang C. 2012. Copper oxide nanoparticles induce autophagic cell death in A549 cells. Plos One 7: e43442.

Tang J, Di J, Cao H, Bai J, Zheng J. 2015. p53-mediated autophagic regulation: A prospective strategy for cancer therapy. *Cancer Letters* 363: 101-107.

Taub R. 2004. Liver regeneration: from myth to metabolism. Nature Reviews Molecular Cell Biology 5: 836-847.

Thit A, Selck H, Bjerregaard HF. 2015. Toxic mechanisms of copper oxide nanoparticles in epithelial kidney cells. Toxicology in Vitro 29: 1053-1059.

Waide EH. et al. 2016. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 12: 1-222.

Yu Y, Duan J, Yu Y, Li Y, Liu X, Zhou X, Ho KF, Tian L, Sun Z. 2014. Silica nanoparticles induce autophagy and autophagic cell death in HepG2 cells triggered by reactive oxygen. Journal of Hazardous Materials 270: 176-186.

Zhang L, Wang XQ, Miao YM, Chen ZQ, Qiang PF, Cui LQ, Jing H, Guo YQ. 2016. Magnetic ferroferric oxide nanoparticles induce vascular endothelial cell dysfunction and inflammation by disturbing autophagy. Journal of Hazardous Materials 304: 186-195.

NM type	XRD size (nm)	TEM size (nm)	Surface area (BET) [m^2/g]	Size in complete MEM (mean - nm)	Size in complete F-12 (mean - nm)
ZnO	70 to > 100	20-250 / 50-350	14	100.6	81.8
Ag	7 14 <18	8-47 (average: 17.5)	NA	59.2	47.6
TiO_2	10	80-400	84	160.6	149.3

Table 1. The main physical and chemical properties of tested NMs (adapted and reproduced from Kermanizadeh et al. 2013a)
BET - Brunauer-Emmett-Teller adsorption eruption
F-12 - Ham's F-12 cell culture medium
MEM - Minimum Essential Eagle cell culture medium
NA - Not Assessed
XRD - X ray diffraction of tested NMs

	HepG2 - LC_{20} ($\mu g/cm^2$)	**HepG2 - LC_{50} ($\mu g/cm^2$)**	**A549 - LC_{20} ($\mu g/cm^2$)**	**A549 - LC_{50} ($\mu g/cm^2$)**
ZnO	1.25	2.5	0.62	2.5
Ag	2.5	5	0.62	2.5
TiO_2	39	not reached	78	not reached

Table 2. WST-1 cytotoxicity following 24 hr exposure of HepG2 hepatocytes and A549 epithelial cells to ZnO - 100 nm, Ag - < 20 nm and TiO_2 - positively charged rutile 10 nm NMs (n=5). The LC_{20} and LC_{50} concentrations are referred to a "low" and "high" in the figures, respectively. The "high" concentration of TiO_2 NMs is 78 and 156 $\mu g/cm^2$ in HepG2 and A549 cells, respectively.

A549	**Average**	SEM	**HepG2**	**Average**	SEM
Cont	**3.8**	0	Cont	**3.7**	0
Pos cont	**13.4 ***	0.9	Pos cont	**11.1 ***	2.3
Ag	**10.8 ***	1.3	Ag	**11.7 ***	1.3
ZnO	**8.1 ***	1.6	ZnO	**9.9 ***	1.2
TiO_2	**3.8**	0	TiO_2	**3.7**	0

Table 3. The quantification of LCB staining in A549 and HepG2 cells exposed to Chloroquine (Pos cont) and the three NMs at LC_{20} concentrations for 6 hr. Significance indicated by *= $p<0.05$ compared to non-exposed control (cont).

Figure legends

Figure 1. The expression levels of autophagy related genes **a)** *LC3B*, **b)** *atg4b*, **c)** *atg3*, **d)** *atg12*, **e)** *atg5* and **f)** *p62* following 6 hr exposure of HepG2 and A549 cell to NMs. The cells were exposed to two concentrations of each NM as highlighted in Table 2 and described as the low and high concentrations. The values represent mean ± SEM (n=3), statistical significance indicated by * = $p<0.05$ and ** = $p<0.005$ compared to the non-exposed control (cont).

Figure 2. The expression levels of autophagy related genes **a)** *LC3B*, **b)** *p62* and **c)** *atg12* following 1, 2, 4, 6 and 12 hr exposure of HepG2 cell to the panel of NMs. The cells were exposed to two concentrations of each NM as highlighted in Table 2 and described as the low and high concentrations. Values represent mean ± SEM (n=3), significance indicated by * = $p<0.05$ and ** = $p<0.005$ compared to the negative control.

Figure 3. Protein level changes based on Western blot analysis. GADPH was used as a loading control. The values represent means ± SEM (n = 3), significance indicated by * $p < 0.05$ and ** $p < 0.005$ compared to negative control at each time point **a)** LC3-II and **b)** p62 in HepG2 cells after treatment with the LC_{20} concentrations of the panel of NMs for 4, 6 and 24 hr.

Figure 4. Autophagic ultra-structural features in HepG2 cells exposed to LC_{20} concentration of engineered NMs for 6 hr **a)** ZnO NMs **b)** Ag NMs and **c)** TiO_2 NMs. The red arrows point at examples of autophagosome-like vacuoles. The red boxes surround TiO_2 NMs engulfed by the HepG2 cells.

Figure 5. The formation of autophagosomes monitored in A549 and HepG2 cells stained with anti-LC3B and counter stained with Hoechst dye. The cells were exposed to the NMs (LC_{20}) or 60 µM of Chloroquine for 6 hr. **a)** A549 - negative control, **b)** A549 - Chloroquine, **c)** A549 - ZnO NMs, **d)** A549 - Ag NMs, **e)** A549 - TiO_2 NMs, **f)** HepG2 - negative control, **g)** HepG2 - Chloroquine, **h)** HepG2 - ZnO NMs, **i)** HepG2 - Ag NMs and **j)** HepG2 - TiO_2 NMs (n=3 - three independent experiments).

Figure 6. The actin participation in the autophagic pathway in HepG2 cells following 6 hr exposure to the panel of NMs (LC_{20}) or 100 nM of rapamycin. The cells were counter stained with Hoechst dye. **a)** G-actin - negative control, **b)** G-actin - rapamycin, **c)** G-actin - Ag NM, **d)** G-actin - ZnO NM, **e)** G-actin - TiO_2 NMs, **f)** F-actin - negative control, **g)** F-actin – rapamycin and **h)** F-actin - Ag NM (n=4 - four independent experiments).

Figure 7. Representative flow cytometry plots of analysis of apoptosis in HepG2 cells **a)** HepG2 - negative control, **b)** HepG2 - positive control - Camptothecin, **c)** HepG2 - Ag LC_{50} and **d)** Flow cytometry analysis of apoptosis in A549 and HepG2 cells treated with the panel of engineered NMs for 24 hr. The data is presented as mean ± SEM (n=3).

Figure 8. Cathepsin B activity in HepG2 cells following a 24 hr exposure to LC_{20} or LC_{50} concentrations of the Ag and ZnO NMs and LC_{20} of TiO_2 NMs. The data is presented as mean ± SEM (n=3).

Figure 9. The effect of NM exposure (or positive control - Camptothecin (Cam)) on caspase 3 activity The data is presented as mean ± SEM (n=3), significance indicated by ** = $p<0.005$ compared to non-exposed control (cont).

Supplementary figure legend

Supplementary figure 1. NMs detected inside hepatocytes using EDX - **a)** TiO_2 and **b)** Ag with X-ray energy in keV shown on the scale bar.