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Research paper

A cross-species and model comparison of the acute toxicity of nanoparticles used in the pigment and ink industries

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ABSTRACT

A major user of nanoparticles (NPs) is the pigment and ink industry, where NPs are incorporated into numerous products (e.g. paints, food, plastics, printers, personal care products, and construction materials). Assessment of NP toxicity requires potential impacts on human health and the environment to be evaluated. In this study, we examined the toxicity of a range of NPs, of varied physico-chemical properties, used in the pigment and ink industries including silver (Ag), iron oxide (Fe_2O_3), titanium dioxide (TiO_2), aluminium oxide (Al_2O_3), zinc oxide (ZnO), cobalt aluminium oxide ($CoAl_2O_4$) and cadmium selenide/zinc sulphide (CdSe/ZnS) quantum dots (QDs). Acute toxicity exerted by this NP panel to mammalian cells in vitro (macrophages, hepatocytes and alveolar epithelial cells) and aquatic environmental organisms (*Raphidocelis subcapitata Daphnia magna*, *Lumbriculus variegatus*) was investigated. For mammalian cells, cytotoxicity was assessed 24 h post exposure, at concentrations ranging from 1 to 125 μg/ml using the LDH and WST-1 assays. The aquatic toxicity of the NP panel was assessed according to OECD protocols (201, 202, 315), up to 96 h post exposure. Rats were exposed to selected NPs via intratracheal instillation (62 μg) and the pulmonary inflammatory response quantified 24 h post exposure. This cross-species comparison revealed that Ag, QDs and ZnO NPs were consistently more toxic than the other NPs tested. By looking across mammalian and aquatic ecotoxicological models we obtained a better understanding of the sensitivity of each model, and thus which models should be prioritised for selection in the future when assessing the mammalian and ecotoxicity of NPs, and in particular when screening the toxicity of a panel of NPs. We recommend that macrophage and daphnia models are prioritised when assessing the mammalian toxicity and ecotoxicity of NPs, respectively, due to their increased sensitivity, compared to the other models tested. Of interest is that the in vitro and invertebrate models used were able to predict the toxic potency of the NPs in rodents, and thus our approach has the potential to enhance the implementation of the 3Rs principles in nanotoxicology and reduce reliance on rodent testing when assessing NP safety. By identifying hazardous NPs the data obtained from this study can feed into the selection of (low toxicity) NPs to use in products and will also contribute to the safe design of future generations of NPs used by the pigment and ink industries.

1. Introduction

A major user of nanoparticles (NPs) is the pigment and ink industry, where NPs are used in numerous products such as paints, food, plastics, paper, printers, dyes, personal care products (e.g. toothpaste, cosmetics), ceramics, and construction materials (e.g. Weir et al., 2012). A range of different NPs are exploited by these industries, for example, TiO_2 NPs are commonly used as white pigments in food, personal care products and paints (Weir et al., 2012), whilst iron oxide NPs can be used in the building and paper industries (Montes-Hernandez et al., 2006). The physico-chemical properties of NPs (e.g. particle size, chemical composition, morphology and surface charge) are able to influence their biological behaviour. A huge diversity of NPs are used by the pigment and inks industries, hence improving our understanding of the relationship between NP physico-chemical properties and their hazard potential will be critical to the safe and responsible development of

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nanotechnology. This includes decision making (e.g. selection of (low toxicity) NPs to use in products/applications), informing safety by design as well as supporting the development of evidence based legislation and risk management measures to protect human health and the environment from any potential risks of NPs.

The use of NPs by the pigment and ink industry means that humans may be exposed to these materials via inhalation, ingestion (via hand to mouth contact), and dermal routes in occupational, consumer and environmental settings during their production, use and disposal. Our study was focused on assessment of the hazards posed by NPs to human health in an occupational setting, and investigated the response of lung epithelial cells, macrophages and hepatocytes to NPs in vitro.

Assessment of the response of the lung is critical within NP safety assessments as exposure via inhalation is anticipated to be one of the primary routes of human exposure in an occupational setting. It is established that NPs can deposit in the alveolar region of the lung following pulmonary exposure (Oberdorster et al., 2002, Semmler-Behnke et al., 2008) and from there are able to translocate to other areas of the body. Thus, many studies have assessed the response of alveolar epithelial cells, and in particular the human A549 cell line, to NPs of varied physico-chemical characteristics such as ufCB, Ag, TiO2 (e.g. Geiser et al., 2005). Accordingly, we selected the A549 cell line to evaluate the toxicity of NPs used by the pigment and ink industries to the lung.

NPs have been observed to cross the epithelial barrier of the lung (e.g. Oberdorster et al., 2002), to reach the blood circulation. Translocation of NPs from the lung, and their accumulation NPs in secondary target sites suggests that there may be widely distributed toxic effects (Oberdorster et al., 2005). A major site of NP sequestration after intravenous injection (Ogawara et al., 1999; Semmler-Behnke et al., 2008), pulmonary exposure (Nemmar et al., 2002; Takenaka et al., 2001; Oberdorster et al., 2002; Semmler et al., 2004) or ingestion (Schleh et al., 2012; Jani et al., 1990) is the liver. The liver may therefore be a prime target organ for NPs, regardless of the route of exposure, and thus investigation of the hepatic response to NPs is relevant when performing safety assessments for NPs. In vitro studies have primarily assessed the response of hepatocytes when investigating the hepatotoxicity of NPs as hepatocytes represent the main cell population in the liver. Of interest is that the response of hepatocyte cell lines (e.g. C3A) has been found to be comparable to that of primary rat or human cells when NP toxicity has been assessed previously (e.g. Johnston et al., 2010; Kermanizadeh et al., 2013). Furthermore, the toxicity exhibited by Ag NPs to the liver in vivo has been observed to be similar to the response observed in vitro (C3A cell line) (Gaiser et al., 2013), which promotes the use of non-rodent, alternative models when assessing NP toxicity. Accordingly, the C3A hepatocyte cell line was selected for investigation of NP toxicity in this study.

Macrophages represent the major cell type of the immune system responsible for the clearance of NPs from the lungs and other tissues (e.g. liver) (Geiser et al., 2008; Semmler-Behnke et al., 2007; Ogawara et al., 1999). Similarly, phagocytosis of NPs by macrophages in vitro has been observed for many NP types (e.g. Gehr et al., 2011), Furthermore, Kupffer cells (resident liver macrophages) have been observed to play a central role in the liver's response to Ag NPs in vivo, following intravenous administration (Kermanizadeh et al., 2014). Interestingly, it has been observed that macrophage responses to NPs in vitro can predict the pulmonary toxicity of NPs in rodents following inhalation (e.g. Wiemann et al., 2016). Thus assessment of the macrophage response is prudent when investigating the response of the lung and liver to NPs. A huge variety of cell types have been used to investigate to the response of macrophages to NPs in vitro, including cell lines (e.g. THP-1, J774, MM6, RAW264.7, NR8383), primary human rat or mouse macrophages (derived from blood, the lungs or the peritoneum). We selected the murine J774 macrophage-like cell line as we have previously demonstrated that this cell type can provide a comparable response to that of primary macrophages (e.g. Brown et al., 2004).

Comparison of NP toxicity across the different cell types was assessed via investigation of the impact of NPs on cell viability. This approach enabled ranking of the toxicity of a panel of different NPs across cell models, to identify differences in cell sensitivity and to rank NP toxicity. We compared two assays which measure cell viability/cytotoxicity via different approaches; the WST-1 assay which assesses mitochondrial function as an indicator of cell viability, and the lactate dehydrogenase (LDH) assay which measures release of LDH from cells to assess plasma membrane integrity, and is indicative of cell death. The sensitivity of each cytotoxicity/viability assay was compared in order to identify those potentially useful for identifying hazardous materials when screening NP toxicity using in vitro models in the future.

In vitro cell based models, representing different target sites, are commonly used to screen NP toxicity in order to decrease the cost and increase the efficiency of testing, and to better align toxicology testing with the 3Rs principles of scientific research (replacement, refinement and reduction of animal use). However it is necessary to consider whether in vitro models are able to predict the in vivo response. An infiltration of neutrophils into the exposure site (e.g. lung) is commonly used as an indicator of the acute toxicity of NPs in vivo (e.g. Gosens et al., 2015; Landsiedel et al., 2014; Poland et al., 2008; Brown et al., 2001). Therefore, we assessed the ability of selected NPs to stimulate an acute pulmonary inflammatory response in rats following intratracheal instillation in our study. The toxic potency of the NPs observed in vivo will be compared to that observed in vitro in order to identify if in vitro models provide a good prediction of NP toxicity.

The production, use and disposal of NPs are likely to lead to their release into the environment (e.g. via wastewater) (Nowack et al., 2012). In parallel to assessing the impacts of NPs on human health it is therefore essential to evaluate the ecotoxicity of NPs. Measurement and modelling studies have analysed and predicted the release levels and fate of NPs into different environmental compartments (e.g. Mueller and Nowack, 2008, Gottschalk et al., 2009, Johnson et al., 2011, reviewed in Gottschalk et al., 2013). Evaluation of the aquatic (freshwater and marine) and terrestrial toxicity of NPs is typically evaluated using model environmental organisms, following OECD protocols.

R. subcapitata, *D. magna*, and *L. variegatus* were selected to assess NP toxicity to aquatic (freshwater) organisms as these have been commonly used to assess the aquatic toxicity of chemicals and NPs previously (e.g. O'Rourke et al., 2015; Sohn et al., 2015; Khan et al., 2015; Li et al., 2014; Wang et al., 2014). *Raphidocelis subcapitata* is a freshwater microalga, and toxicity to this organism is typically assessed via assessment of growth rate inhibition (via measurement of optical density) (Van Hoecke et al., 2008). *Lumbriculus variegatus* (California blackworm), is a freshwater dwelling oligochaete which is widespread throughout Europe and North America. It is common in shallow waters, and can burrow into the sediment. *L. variegatus* is often used as a test organism for toxicants applied in water or via sediment (e.g. Pakarinen et al., 2011). We tested acute toxicity of NPs to *L. variegatus* via the water column without addition of sediment to have a simple model in which NPs are easily quantifiable and detectable and to facilitate characterisation of the NPs. Toxicity to this organism is typically assessed via investigation of mortality and behaviour (Rajala et al., 2016). *Daphnia magna* are crustaceans that reside in the water column, and toxicity to this organism is typically assessed via investigation of immobility, and impacts on reproduction (OECD Guidelines, 1984).

The panel of NPs selected for investigation in this study were; silver (Ag), iron oxide (Fe_2O_3), titanium dioxide (TiO_2), aluminium oxide (Al_2O_3), zinc oxide (ZnO), cobalt aluminium oxide ($CoAl_2O_4$) and cadmium selenide/zinc sulphide (CdSe/ZnS) quantum dots (QDs). The aim of the study was to perform a cross species comparison of the toxicity of this panel of NPs to identify the sensitivity of different mammalian (cell lines and rodents) and environmental models to NPs. In addition, the obtained data were used to compare and rank NP toxicity in order to identify hazardous NPs, whose surface will be modified with the aim of reducing their toxicity. The toxicity of these