4. Cellular defense mechanisms following nanomaterial exposure; A focus on oxidative stress and cytotoxicity.

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Conflict of Interest Statement: The authors are responsible for all content and views portrayed within this chapter. The authors declare no conflict of interest.

Acknowledgements: The authors would like to acknowledge all members of the *In Vitro* Toxicology group who contribute to the exhaustive scientific discussions.

1. Overview

There is increased public perception of nanotechnology due to its heightened production and application within everyday products. This inevitable level of exposure, whether it be accidental or intentional, is considered a potential hazard to human health, and thus pertinent understanding of this risk is necessary. Despite the overwhelming need to conceive the true interaction of nanoparticles (NPs) with the human body, and it's building blocks (*i.e.* tissues and more importantly, cells) and associated it with any negative effect, it is similarly important to note the impact that NPs can have upon the innate defence mechanisms of the cell. Thus, the purpose of this chapter is to consider oxidative stress in response to the NP-cell interaction, and how this can incite the different cellular defence mechanisms, and how this may relate to a hazardous response, or lack of.

2. Background

In regards to the potential and perceived hazard posed by NPs, that is concomitant in response to the significant increase in nanotechnology over the last three decades, the plethora of engineered nanomaterials (ENMs) now becoming available, understanding as to how nano-sized particles may impact upon human health has become a dominating area of research worldwide since the late 1990's (Stone *et al.* 2017). At this current moment if one were to conduct a literature search with the terms 'nanoparticle' and 'toxicology', they would have be rewarded with >10'000 manuscripts exhibiting these key-words. With the ever increasing number of articles associated with this key discipline within particle toxicology, the message relating to the potential hazard has become confused and convoluted as to what the risk is, and what biological mechanism is related to such a potential risk.

Whilst approaches constantly adapt to the increasing number and variety of ENMs produced for a plethora of different applications, the quantity of alternative physico-chemical characteristics, a key factor in the potential hazard of ENMs (Bouwmeester *et al.* 2011), is further increasing in number and type. Although it is well documented which characteristics influence ENM toxicity, as allude to above, the precise mechanism by which this observed toxicity occurs is not fully understood (Clift *et al.* 2011). Despite this, as a result of increased laboratory-based investigations that have been conducted over the last three decades (Stone

et al. 2017), a number of specific paradigms have been formulated in order to deduce and define the potential (human health) hazard posed by ENMs.

3. Paradigms in Particle Toxicology

Of the three specific paradigms, also known as theory's, the main one is the 'oxidative stress paradigm', which is discussed in the latter paragraphs. However, while the potential for ENMs to cause oxidative stress has been the basis for increased research since the advent of nanoparticle toxicology in the early 1990's (Ferin *et al.* 1992), two further paradigm's/theory's also exist; the fibre paradigm (Donaldson and Tran, 2004; Donaldson *et al,* 2010), and the theory of genotoxicity (Schins and Knaapen, 2007; Evans *et al.* 2017). Please refer to both Donaldson et al (2010) and Evans et al (2017) to understand both these respective theory's further.

As previously discussed in Clift and Rothen-Rutishauser (2013), although the oxidative stress paradigm can fit to any form or NP, as it has predominantly been focused upon through the assessment of the biological response to spherical, crystalline, and non-fibrous NPs. Furthermore, such NPs have been demonstrated to be able to cause a greater toxic response to cells, compared to their larger particle counterparts at the same mass dose (Oberdorster *et al*., 2005; 2007). Pertinently, this is in strong correlation to their ability to cause cellular oxidative stress.

The authors have previously discussed this concept within nanotoxicology (Evans *et al.* 2018). Yet, briefly, oxidative stress occurs when a greater number of oxidants than antioxidants are present within the cell, causing an oxidant/antioxidant imbalance. Increased oxidation can occur within cells, such as macrophages following activation. The activation of macrophage cells can cause the generation of the superoxide anion, which is readily converted into the hydroxyl radical ($^{\bullet}OH$) via the influence of superoxide dismutase. The presence of the $^{\bullet}OH$, as well as the superoxide anion, which are examples of reactive oxygen species (ROS), can thus cause increased oxidation within the cell because these molecules possess unpaired electrons and are highly unstable. Additionally, ROS can be produced via nicotinamide adenine

dinucleotide phosphate (NADPH) oxidase, which is the most common form of ROS found in cells and is usually produced when cells are performing the phagocytosis of xenobiotics. Therefore, this suggests that although cells purposefully clear hazardous particles from the tissue, the phagocytosing cells can unintentionally or intentionally produce ROS. In addition, the potential production of ROS following encapsulation of particles via phagocytosis further emphasizes the necessity to understand the specific uptake mechanism of NPs, in order to determine their potential route within the cell, and how their uptake may relate to their toxicity. For a succinct overview of cellular uptake mechanisms please refer to Connor and Schmid (2002), and further Unfried *et al.* (2007) as regards the specificity of NPs and their cellular uptake mechanisms. These, together with oxidative stress (*i.e.* oxidative burst) can act as an ideal cellular defence mechanism to any foreign substance, such as NPs are postulated (Stone *et al.* 2017).

4. Cellular Defence Mechanisms in Mammailan Cells

Whilst oxidative stress has been well studied in terms of the mechanics of the toxicological response to ENMs, it is a common misconception that it is merely only a negative aspect within mammalian cells, and simply associated with a hazard effect response. In terms of the cellular defence of any cell type within the human body, there are a number of able defence mechnanisms, of which oxidative stress is one. Such defence mechanisms include specific active (and passive) uptake mechanisms (please refer to Conor and Schmid (2002) for a detailed review of these). Yet, a side-effect of this the two major forms of ‘cell-eating’, or scenario is also the (pro-)inflammatory response, which is another defence mechanism of the human body to any foreign body invasion (including ENMs). All of the defence mechanisms that mammalian cells have, it is their ability to engage the redox action that creates an imbalance between the cells antioxidant defence system, and the oxidants present in the cell/tissue.

5. Oxidative Stress, Antioxidants and Reactive Oxygen Species

As noted above, the major mechanism by which nanomaterials (NMs) are considered to induce cellular toxicity is via oxidative stress, which refers to a cellular redox imbalance as a result of increased intracellular highly Reactive Oxygen Species (ROS). The term ROS encompasses a number of molecules and free radicals derived from oxygen including primary ROS - H_2O_2, $O2^-$ and secondary ROS - OH• (Gamaley and Klyubin, 1999). During normal cellular function ROS are produced as by-products of metabolism. For example, a one electron gain by the oxygen molecule (O_2) results in the formation of the superoxide free ion $O_2^{\bullet}$. This reduction happens frequently during numerous biological processes such as the electron transfer chain within the mitochondria; as several components of complexes I, II and III express thermodynamic properties required for the reduction of O_2 to O_2^- (Cadenas et al., 1977). Other cellular source of O_2^- include the microsomal transfer chain via NADPH-cytochrome P_{450} and NADH-cytochrome b_5 reductase activities, the respiratory burst action of phagocytic cells, peroxisomal beta-oxidation and Fenton reactions (Poljsak et al., 2013). At low levels ROS may act as 'redox messengers' in intracellular signalling (Circu and Aw, 2010). This is achieved by the activation of Redox sensitive transcription factors include AP-1, p53 and NF-κB which regulate pro-inflammatory cytokine expression, cell differentiation and apoptosis (Burton and Jauniaux, 2011). This signalling maybe utilised during the initiation of an inflammatory response with in a tissue for example.

Due the fact that ROS are a natural cellular occurrence due to normal processes, a homeostasis is maintained by a series of antioxidant proteins. The main class of this antioxidants is superoxide dismutases (SOD) including Cu-Zn-SOD (SOD1) and Mn-SOD (SOD2). Both SOD1 and SOD2 catalyse the conversion of O_2• to the less reactive H_2O_2 which can subsequently be converted to H_2O by catalse and glutathione (GSH) (Zhang et al., 2016):

$$\mathbf{2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2}$$

$$\mathbf{2H_2O_2 \rightarrow O_2 + 2H_2O}$$

The role of antioxidants is critical to maintaining cellular health, if an imbalance occurs between the levels of ROS and antioxidants, indiscriminate damage may be inflicted on a range of biological molecules. This include lipid peroxidation where ROS attack

polyunsaturated fatty acids within the cell membrane, this results in the formation of a peroxyl-fatty acid radical and a subsequent chain reaction of membrane damage (Vasilaki and McMillan, 2011). Lipid peroxidation can ultimately lead to impaired cellular functioning and cell rupture. Furthermore oxidative damage to the mitochondrial membrane can result in electron chain dysfunction and subsequently cell death (Manke et al., 2013). ROS can also promote protein oxidation resulting in fragmentation at amino acid residues, protein cross links and oxidation of the amino acid chains resulting in loss of function (Dalle-Donne et al., 2003). The ability of ROS to cause protein damage has the potential to impact a multitude of cellular functions in addition to the risk of a build-up of malformed protein within the cell. In addition to protein oxidation and lipid peroxidation a key risk is ROS-induced DNA damage which is typified by single and double stranded DNA breaks, base modification (e.g. DNA adducted formation and DNA cross linkage (Singh et al., 2009).

6. NMs and oxidative stress

A number of NMs have been shown to be inducers of oxidative stress, in particular metal oxide nanoparticles which may release ions capable of inducing the formation of the highly reactive hydroxyl radical (•OH) by conversion of H_2O_2 by Fenton chemistry.

H_2O_2 is not reactive as it has no unpaired electrons but it is however a mediator in the formation of secondary ROS in the form of hydroxyl radicals (•OH). This •OH formation can be initiated via transition metal ion promoted Fenton chemistry (Valko et al., 2004, Valko et al., 2006)

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)} + {}^{\bullet}OH + OH^-$$

(M represents transition metal)

Transition metal based NM's such as iron, copper, nickel, cobalt, and zinc may therefore release ions that can take part in the Fenton reaction promoting an increase in intracellular •OH formation. This free radical presents a significant risk for DNA damage as •OH is capable of attacking the DNA backbone and nucleotide bases promoting the formation of DNA lesions. More than 20 oxidative base lesions have been identified, the most notable being 8-

hydroxygyanine (8-OH-dG) which frequently miss-pairs with thymine resulting double stranded breaks and point mutations (Cooke et al., 2003).

A number of studies have identified transition metal based NM's as inducers of oxidative stress. For example, copper oxide promoted increased micronucleus formation in the Neuro-2A cell line as a resulted of oxidative damage measured by the formation of malondialedhyde (MDA) (Perreault et al., 2012). Moreover, significant MDA formation has been exhibited in the brains of Wister rats following treatment with gold (Au) nanoparticles (NPs) (Siddiqi et al., 2012). Perhaps the mostly widely studied transition metal NM is silver (Ag) due to it antimicrobial properties. Indeed, Ag NPs have been shown to induce ROS formation in lung epithelial cells (A549) as measured by the 2'-7'-Dichlorodihydrofluorescein diacetate (DCFDA) assay (Foldbjerg et al., 2011). Similarly, when tested in HepG2 cells Ag NPs promoted increased ROS production (quantified by DCFDA) promoting downstream double stranded DNA breaks (Kim et al., 2009).

NM oxidative stress potential not limited to those comprised of transition metals, a number of NMs have been shown to catalyse ROS production at their surface in aqueous suspension including silica and carbon nanotubes (Magdolenova et al., 2014). This is likely due to immobilised free bonds of the atoms located on the NM surface. Quartz NPs for instance have been associated with the generation of ROS due to the presence of surface bound SiO• and SiO_2• (Huang et al., 2010). Furthermore, the quantum confinement effect of quantum dots modulates their ability to accept and donate electric charge and potentially enable them to catalyse ROS formation (Abdal Dayem et al., 2017).

7. NM induced immune response and oxidative stress

If a NM is capable of promoting an immune response *in vivo* this may result in the formation of ROS by the cellular components of the immune system. NMs have indeed been shown to be capable of triggering ROS production in activated phagocytes (macrophages and neutrophils) in the form of a NADPH mediated respiratory burst (Trouiller et al., 2009, Tulinska et al., 2015, Sun et al., 2011). If this respiratory burst is maintained downstream oxidative damage may be promoted in other cell types within the NM exposed tissue. ROS themselves are in fact mediators in the activation and recruitment of other immune cells, by

promoting inflammatory cytokine production via activation of the transcriptional regulatory factor NF-κB (Mitra and Abraham, 2006). A vicious circle of chronic inflammation inducing downstream genotoxicity is therefore a possible scenario upon NM exposure (Evans et al., 2017).

8. ROS and cytotoxicity

Due to the ability of ROS to mediate redox sensitive transcription factors its excessive presence in with in the cell can cause activation of apoptosis. This can be initiated by the upregulation of the tumour suppressor protein p53 which one cell stress is low can induce cell cycle arrest and DNA repair (Kaminskyy and Zhivotovsky, 2014). At high levels of cell stress however p53 can down regulate pro-survival factors, upregulate apoptotic factors and induction of the caspase cascade (Redza-Dutordoir and Averill-Bates, 2016). Due to the association of the upregulation of TNFα and ROS there is also evidence of linkage between ROS and apoptosis initiated by the extrinsic pathway (Vandenabeele et al., 2010).

9. Summary

The field of nanoparticle toxicology is a complex discipline that incorporates a plethora of different disciplines. It allows for the gaining of novel understanding towards an aspect that is vital regarding human long-term health effects. To date, there has been limited indication that nanomaterials are able to affect long-term human health, but this is due to a lack of research into this area and also the model systems to study it. Instead acute effects have been focussed upon, that have shown that commonly, realistic exposure concentrations/doses used in studies indicate that cellular machinery is often impeded, most notably by mechanisms associated with an oxidative stress response. Whilst oxidative stress is normal, it occurs within every organ/tissue/cell routinely, excess oxidative stress (commonly caused through reactive oxygen/nitrogen species) is a negative cellular response that can have both hazardous acute and chronic effects (*e.g.* inflammatory response), and so is essential to maintain in regards to the ENM-cell interaction.

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