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Ability of biopersistent/biodurable manufactured nanomaterials (MNs) to induce lysosomal membrane permeabilization (LMP) as a prediction of their long-term toxic effects

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or contact:

OECD Environment Directorate,

Environment, Health and Safety Division

2 rue André-Pascal

75775 Paris Cedex 16

France

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

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Foreword

The OECD has a key role in standardising methodologies for hazard testing and assessment and promoting best practices for the safe use of chemicals and the protection of human health and the environment. The OECD has established a number of programmes addressing different aspects of chemical safety enabling a sound harmonised approach for industrial chemical management. The Working Party on Manufactured Nanomaterials (WPMN) was established to ensure that the approaches for hazard, exposure and risk assessment for manufactured nanomaterials are properly integrated in the assessment of chemicals and aligned with the high quality, science-based and internationally harmonized tools developed by the OECD Chemicals Programme.

With this in mind, the WPMN launched the project *Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation*. The objective is to contribute to the future development and application of AOPs for MN regulatory decision making, by following the principles established by the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). The outcomes of the project are presented in three complementary documents addressing:

The scope of the project, its development and summary of the main conclusions. The document includes a methodology to identify, analyse and evaluate existing nanotoxicology literature with the objective to prioritize Key Events (KEs) relevant for MNs;

A case study focused on a specific Key Event (KE) in the inflammation pathway to analyse the empirical evidence and contribute to the development of a knowledge base to inform AOP development and assessment for MNs; and

The report from the OECD workshop *Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation*, which was organised in collaboration with the European Union (EU) Horizon 2020 projects *SmartNanoTox* and *Physiologically Anchored Tools for Realistic nanOmateriaL hazard aSsessment* (PATROLS). At this workshop, stakeholders had an opportunity to provide feedback on the methodology proposed, as well as on the case study, and to reach consensus on areas that could be further explored in the short, medium and long term.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

Executive Summary

This document describes the expanding knowledge on the implications and biological significance of lysosomal and autophagy dysfunction and the subsequent assembly and activation of inflammasome NLRP3 induced by biodurable manufactured nanomaterials.

Lysosomal membrane permeabilization (LMP) and lysosomal and autophagy dysfunction with inflammasome activation has a profound impact on cell homeostasis, resulting in manifold pathological situations, including infectious diseases, neurodegeneration, aging as well as fibrotic diseases and cancer.

Different types of biodurable nanomaterials are shown to cause LMP, affect autophagy and cause NLRP3 inflammasome activation. The compilation of the available information in this report will contribute to the development of test systems to predict the long-term toxicity and hence the safety of manufactured nanomaterials

Knowledge derived from the cellular and molecular processes underlying nanomaterial-induced toxic effects will also establish the scientific foundations for the risk assessment of manufactured anomaterials through alternative screening approaches to predict long-term effects.

Abbreviations

AgNPs	Silver nanoparticles
AO	Acridine orange
AOP	Adverse Outcome Pathway
AuNPs	Gold nanoparticles
C_{60} NPs	Fullerenes nanoparticles
CBNPs	Carbon black nanoparticles
CeO_2 NPs	Cerium nanoparticles
CMA	Chaperone-Mediated Autophagy
CNHs	Carbon nanohorns
CNTs	Carbon nanotubes
CuO NPs	Copper oxide nanoparticles
FeO NPs	Iron oxide nanoparticles
GOs	Graphene oxides
HCECs	Human cerebral endothelial cells
HeLa	Human cervix carcinoma cell line from **He**nrietta **La**cks
HepG2	Name of a human liver-derived hepatoma cell line (**Hep**atoma **G2**)
HUVECs	Human umbilical vein endothelial cells
IATA	Integrated approach to testing and assessment
ITS	Integrated testing strategy
KEs	key events
LLC-PK1	Name of a renal proximal tubule cell line (**L**illy **L**aboratories **c**ell-**p**rocine **ki**dney)
LMP	Lysosomal membrane permeabilization
LMR	Lysosomal membrane rupture
MH-S	Mouse alveolar macrophage cell line
HMGB1	High mobility group box-1
IL-1β	Interleukin-1β
IL-18	Interleukin-18

MIE	Molecular initiating event
MMPs	Matrix metalloproteases
MNs	Manufactured nanomaterials
MNPs	Magnetic nanoparticles
MOMP	Mitochondrial outer membrane permeabilization
MWCNTs	Multiwall carbon nanotubes
NDs	Nanodiamonds
Nd_2O_3 NPs	Neodymium nanoparticles
NLRP3	Inflammasome complex that belongs to the NOD-like receptor (NLR), an intracellular family of pathogen recognition molecules, that contain pyrin
NPs	Nanoparticles
PAMAM	Poly(amidoamine) dendrimers
PVPNO	Polyvinyl-pyridine-N-oxide
PS-NH_2 NPS	Amino-functionalized cationic polystyrene nanoparticles
QDs	Quantum dots
REO NPs	Rare earth oxide nanoparticles
SiO_2 NPs	Silicon dioxide nanoparticles
SWCNTs	Single wall carbon nanotubes
TiO_2 NPs	Titanium dioxide nanoparticles
TEM	Transmission electron microscopy
WO3/Pt NPs	Tungsten trioxide platinum nanoparticles
VO2NPs	Vanadium dioxide nanoparticle
ZnO NP	Zinc oxide nanoparticles

Introduction

1. Recent advances have been made in describing lysosomal and autophagy dysfunction as a mechanism in manufactured nanomaterials (MNs)-induced toxicity and pathology. It is therefore relevant to describe in more detail the potential mechanisms of MNs toxicity and specifically the implications and biological significance of MNs-induced lysosomal and autophagy dysfunction (Stern et al., 2012).

2. MNs can enter the cell through a variety of endocytic mechanisms and are mostly commonly sequestered in vesicular structures (endosomes, phagosomes) that merge with endogenous lysosomes. Within the endosome-lysosome/phagosome-lysosome, MNs may be degraded by different hydrolytic enzymes in an acidic environment. However, the biodurable MNs are resistant to this biodegradation. There is a growing body of evidence that biodurable MNs through their ability to induce lysosomal and autophagy dysfunction produce the accumulation of autophagic and lysosomal vacuoles and may thus lead to long term toxicological consequences.

3. One common form of phagolysosomal dysfunction that has been associated with MNs exposure is lysosomal membrane permeabilization (LMP) which is also recognized as a cell death mechanism that can result in mitochondrial outer membrane permeabilization (MOMP) (Bunderson-Schelvan et al., 2017). LMP also results in the assembly and activation of inflammasome NLRP3 and chronic inflammation producing pathological effects including fibrosis and cancer (Sayan and Mossman, 2016). Therefore, this project proposes to compile the available information on biopersistent / biodurable MNs regarding their ability to induce autophagy and lysosome dysfunction, LMP, and NLRP3 inflammasome activation which may in turn provide means to predict their long-term toxicity, pathogenicity and subsequently assess their long-term effects and hence their safety.

Scope

4. The scope of this document is to:

- Compile available information on biopersistent/biodurable MNs regarding their ability to induce autophagy and lysosome dysfunction, LMP, and NLRP3 inflammasome activation.
- Compile comprehensive available information on LMP as an initiating intracellular event that may differentiate between toxic biodurable and non-toxic MNs.
- Add on to dissolution rate studies, which only assess the stability of nanomaterials in relevant biological media without establishing mechanisms involved to aid in the prediction of their long-term effects.
- Identify different intervention strategies proposed in the literature which may allow prevention of the pathological consequences of exposure to biodurable toxic MNs.

5. The report will be for consideration by the OECD member countries to determine how to incorporate mechanistic biochemical information when screening of MNs for their toxicity and pathogenicity (long-term effects). The will inform the development of specific Adverse Outcome Pathways (AOP), their molecular initiating event (MIE) and subsequent key events (KEs). The report may also be helpful in the development of OECD Test Guidelines.

General introduction on lysosomal functioning and damage

6. There are several potential pathways that can be involved in nanomaterial cellular uptake. Physicochemical properties of nanoparticles such as size, shape, and surface (e.g., charge and coating), and cell type, all play a significant role in determining the predominant uptake pathway(s) (Hillaireau and Couvreur 2009, Kuhn et al. 2014, Stern et al. 2012, Tlotleng et al. 2016, Zaki and Tirelli 2010). However, once inside the cell, their effects could be shown to be dependent on intracellular events rather than on uptake *per se* (Hamilton et al. 2013a).

7. There is ample evidence that nanoparticles enter the cell and localise in lysosomes. For example, silica, gold, iron oxide, polystyrene particles, and quantum dots have been detected in lysosomes (Al-Rawi et al. 2011, He et al. 2009, Silver and Ou 2005, Chithrani et al. 2006). Cellular excretion of NPs is estimated to be low, and therefore, accumulation of NPs in lysosomes and chronic impairment of lysosome function may occur (Fröhlich 2016). Non-biodegradable/biodurable NPs persist in cells and may cause cell damage and due to the localization of the NPs in lysosomes, it is recommended to investigate their effects on lysosomes (Mrakovcic et al. 2013).

Lysosomes and LMP

8. Lysosomes are organelles containing hydrolases responsible for the degradation of macromolecules delivered via the endocytic, phagocytic, and autophagic pathways (Luzio et al. 2014, Appelmans et al. 1955), but also are involved in many vital cellular processes, including plasma membrane repair and mediation of cell death (Kreuzaler et al. 2011, Appelmans et al. 1955, Appelqvist et al. 2013). Maintaining lysosomal membrane integrity and function is therefore crucial for cellular homeostasis (Wang et al. 2018a). Therefore, lysosomal membranes consisting of the lipid bilayer, like cell membranes, are important fundamental structures in biology and possess characteristic permeability, stability, and mechanical properties (Antunes et al. 2001). Disruption of lysosomal membrane integrity, and the release of lysosomal enzymes into the cytosol, can have grave cytotoxic consequences resulting in apoptotic, necrotic, or autophagy-dependent cell death (Johansson et al. 2010, Guicciardi et al. 2004, Kirkegaard and Jaattela 2009, Wan et al. 2013).

9. The involvement of lysosomes in most of these processes is now known to depend on the ability of lysosomes to move throughout the cytoplasm (Pu et al., 2016). The ability of lysosomes to move within the cytoplasm is crucial to many cellular functions, and perturbations of lysosome movement contribute to the pathogenesis of various diseases. For example, lysosome movement towards the cell periphery is also required for cancer growth, invasion and metastasis (Kroemer and Jaattela 2005, Kallunki et al. 2013) where a particularly notable change is a shift of the lysosome population from the central to the peripheral cytoplasm (Nishimura et al. 2002, Nishimura et al. 2003).

10. Lysosomes are also heterogeneous. The heterogeneity and the changes in amount and quality of lysosomes are indicated by co-staining with combinations of different lysosomal proteins, pH, and enzyme activity (Fröhlich 2017). When cells were incubated with LysoTracker, a fluorescent weak base

that accumulates in highly acidic organelles, it was found that most, but not all, lysosomes accumulated LysoTracker. However, it could be seen that a greater proportion of the most acidic (LysoTracker positive) lysosomes resided in the perinuclear region, whereas less LysoTracker accumulated in peripheral lysosomes (Johnson et al. 2016). The distribution and motility of lysosomes also change where upon cytosol acidification causes dispersal of the perinuclear lysosome population, whereas subsequent alkalinization returns them to their central location (Pu et al. 2016, Heuser 1989, Parton et al. 1991).

11. Lysosomal activity is essential to preserve cellular homeostasis. Structurally, lysosomes are single membrane bound organelles and their membrane contains specific sets of glycoproteins the functions of which are the protection of lysosomal membranes against autolysis.

12. LMP is any damage to the lysosomal membrane characterised by lysosomal size, number, pH, cellular positioning, motility and changes in lysosomal membrane properties that triggers the release of hydrolytic enzymes into the cytosol thus leading to apoptotic (Roberg et al. 2002, Bivik et al. 2006, Schestkowa et al. 2007) or necrotic cell death (Li et al. 2000). It has also been established that lysosomal destabilization triggers the mitochondrial pathway of apoptosis (Cesen et al. 2012, Repnik et al. 2012).

13. LMP allows the release of cathepsin B, an apoptotic mediator, into the cytoplasm, where it can initiate the intrinsic apoptotic pathway (Ben-Ari et al. 2005). Under normal physiological conditions, cathepsin B is sequestered into the lysosomes of intact cells to participate in normal turnover of proteins (Puissant et al. 2010). Once into the cytoplasm, cathepsin B can cleave and activate pro-apoptotic proteins, including Bid (Zhang et al. 2009), followed by engaging Matrix metalloproteases (MMPs) and release of cytochrome c (Laforge et al. 2013). Thereafter, the release of cytochrome c causes the activation of effector caspases and triggers a caspase-dependent apoptotic pathway (Jiang and Wang 2004).

LMP and inflammasome activation

14. LMP accompanied by cathepsin B release initiates NLRP3 inflammasome assembly and Caspase-1 activation leading to inflammation. Assembly of the NLRP3 inflammasome activates caspase-1 and mediates the processing and release of the cytokines interleukin, interleukin 1-beta (IL-1β) and Interleukin-18 (IL-18), and thereby serves a central role in the inflammatory response and in diverse human diseases including neurodegeneration, autoimmune diseases and cancer (Baroja-Mazo et al. 2014, Boya 2012).

Lysosomal and autophagy dysfunction

15. A process that is dependent on lysosome positioning is autophagy (Yang and Klionsky 2010, Pu et al. 2016). Autophagy, first described by Christian De Duve in 1963 (De Duve 1963), is a normal catabolic process that ensures the degradation and recycling of cellular components within the lysosomes (Ravikumar et al. 2010). It is serving as a protective mechanism that facilitates the degradation of superfluous or damaged cellular constituents (Nakatogawa et al. 2009, Wan et al. 2013). Autophagy consists of several sequential steps: formation of autophagosomes, fusion of autophagosomes with lysosomes, and degradation in the autolysosome. Autophagic flux is used to describe the whole dynamic process of autophagy (Klionsky 2007).

16. Of the more than 20 lysosomal membrane proteins identified, protein- 1 and-2 (LAMP1 and LAMP2) are the most abundant. LAMP proteins, but especially LAMP-2, are important regulators in successful maturation of both autophagosomes and phagosomes where an association could be

established between this protein and autophagy (Saftig et al. 2008, Ruivo et al. 2009). Mutual disruption of both LAMPs is associated with an increased accumulation of autophagic vacuoles, altered lysosomal appearance, and disturbed cholesterol metabolism, while protein degradation rates are not affected (Saftig et al 2008).

17. Macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) are three types of autophagy. All autophagy involves the lysosomal degradation step. (Nakatogawa et al. 2009, Tanida et al. 2004, Mijaljica et al. 2011)."

18. Autophagy dysfunction can result from lysosomal overload or alkalization, which prevents autophagosome-lysosome fusion (Stern et al. 2012). Many lysosomal diseases also show evidence of autophagy dysfunction, with blockade of autophagosome and lysosome fusion, and accumulation of autophagosomes and autophagy substrates (e.g., ubiquitinated protein aggregates (Settembre et al. 2008) and therefore has been recognised as an important lysosome-based pathway of cell death (Lin et al. 2013, Patel et al. 2012).

19. Lysosomal dysfunction resulting in an accumulation of unmetabolized substrates in the lysosome and lysosomal overload can have several possible deleterious consequences to the cell, including prevention of autophagosome-lysosome fusion through autophagy dysfunction. Lysosomal dysfunction has been associated with several disease states, including lysosomal storage disorders (Huang and Klionsky 2007, Ravikumar et al. 2010, Platt et al. 2018).

20. Autophagy dysfunction is defined as excessive autophagy induction or blockade of autophagy flux. Autophagy dysfunction is recognized as a potential mechanism of cell death, resulting in either apoptosis autophagy-dependent cell death (Kroemer and Jaattela 2005). Similar to lysosomal dysfunction, dysfunction of the autophagy pathway has also been linked to a variety of diseases (Ravikumar et al. 2010) including cancer development and progression (White and DiPaola 2009).

21. Figure 1: The interplay between LMP, autophagy induction and autophagy blockade where the biodurable NPs lead to LMP upon which both cathepsins and NPs are released. The released cathepsins result in autophagy induction via ROS generation and mitophagy whereas the released NPs are ubiquitinated, which also results in autophagy induction. The initial autophagy induction (induced by either cathepsins or ubiquitinated NPs) would eventually result in autophagy flux blockade as the resultant autophagosomes cannot fuse with lysosomes undergoing LMP. Thus, LMP initially induces autophagy flux, which then results in the blockade of autophagy flux due to LMP (Wang et al 2018b; Stern et al 2012).

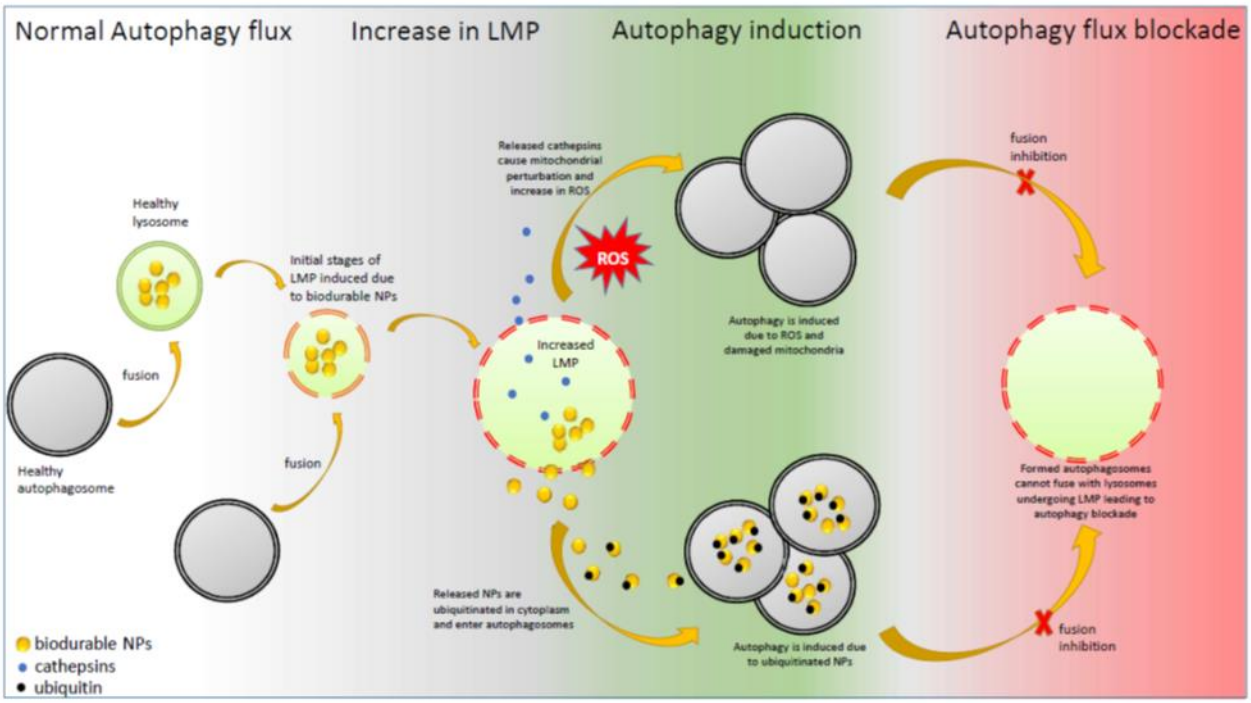

Figure 1: The interplay between LMP, autophagy induction and autophagy blockade (Wang et al 2018b; Stern et al 2012)

LMP and cell death

22. The degree of LMP will determine the fate of cells whether to undergo apoptotic or non-apoptotic death. LMP results in release of cathepsins, such as cathepsins B and D, initiates a cascade of cell signalling events leading to apoptotic cell death (Boya and Kroemer 2008, Aits and Jaattela 2013, Galluzzi et al. 2018, Johansson et al. 2010, Boya 2012).

23. Partial and selective LMP can result in two distinct outcomes for the cell: engagement of cell death pathways or cell survival mediated by the endolysosomal damage-response mechanisms. It was proposed that at least two factors affect the extent of LMP, and therefore influence the cell's response to a stimulus: the number and type of lysosomes affected, and the size of the pores created in the lysosomal membrane (Wang et al. 2018a). Cell death can be inhibited by blocking cathepsin activity with protease inhibitors or by increasing the activity of endogenous cathepsin inhibitors such as serpin and cystatins (Gomez-Sintes et al. 2016).

24. In lysosomal membrane rupture (LMR), membrane damage is severe and various degradative enzymes in the lysosomes, such as acidic hydrolases, are released to the cytoplasm where they degrade cytoplasmic structures, leading to necrosis (Wang et al. 2018a, Boya and Kroemer 2008, Kagedal et al. 2001, Antunes et al. 2001).

Biopersistent/Biodurable nanomaterials and disease

25. Biopersistence of mineral particles and fibres is defined as the extent to which they are able to resist chemical, physical, and other physiological clearance mechanisms in the body. Biodurability, defined as the ability to resist chemical/biochemical alteration, is a significant contributor to biopersistence (Utembe et al. 2015, OECD 2018).

26. Biopersistent/biodurable MNs may produce prolonged exposure conditions, which might produce persistent lysosomal membrane damage and promote disease (Stern et al. 2012, Bunderson-Schelvan et al. 2017). While the autophagy and endo-lysosomal pathways have the potential to influence the disposition of nanomaterials, there is also a growing body of literature suggesting that biopersistent nanomaterials can, in turn, negatively impact these pathways (Stern et al. 2012). Indeed, lysosomotropic agents, including particles, have been known to cause lysosomal dysfunction and associated toxicity for several decades (de Duve et al. 1974, Schneider et al. 1997, Villamil Giraldo et al. 2014).

Induction or protection of LMP and Inflammasome Activation by particles and fibres

Larger particles that induce LMP

27. Crystalline micro-sized particles were confirmed to induce LMP with subsequent NLRP3 inflammasome activation (Hornung et al. 2008, Biswas et al. 2014). The mechanisms involved in crystalline silica-induced LMP were shown to be partially dependent upon lysosomal cathepsins B and L and that lysosome acidification being a prerequisite for particle-induced LMP. The resultant leak of lysosome cathepsins was also indicated to be a primary regulator of ongoing NLRP3 inflammasome activity.

Induction or protection of LMP, autophagy, and NLPR3 inflammasome activation and nanomaterials

28. *LMP induced by nanomaterials*: Lysosomes are shown to be the main organelle to accumulate many types of MNs (Dong et al. 2013, Moore et al. 2009) which may cause lysosomal dysfunction (Stern et al. 2012) through LMP or LMR, based on the degree of membrane damage they produce. Indeed, LMP was proposed as a potential mechanism of nanomaterial-induced toxicity in human fibroblasts and macrophages, and was found to be associated with loss of mitochondrial membrane potential and apoptosis (Sohaebuddin et al. 2010, White and DiPaola 2009).

29. *Autophagy dysfunction*: Recently, autophagy dysfunction as emerging mechanisms by MNs was also proposed as a mechanism of their toxicity (Stern et al. 2012) and therefore understanding of MN-induced autophagy impairment could aid to fully understanding MNs toxicity (Cohignac et al. 2014).

30. Activation of autophagy can occur in the event of stress due to starvation, depletion of growth factors, endoplasmic reticulum stress, oxidative stress and infection (He and Klionsky 2009). While autophagy is generally considered a nonselective response to these cellular stress conditions, autophagy is also reported to be homeostatic and selective in the removal of damaged organelles, ubiquitinated proteins, and pathogens (Komatsu and Ichimura 2010). There is also evidence that autophagy can selectively compartmentalize nanomaterials. A variety of nanoparticles have also been shown to induce dysfunction of the autophagy pathway (Stern and Johnson 2008, Johnson-Lyles et al. 2010).

31. NLRP3 inflammasome activation and nanomaterials: The NLRP3 inflammasome, a multiprotein complexes formed of members of the nucleotide-binding domain and leucine-rich repeat (LRR)-

containing (NLR) family and the pyrin and HIN domain (PYHIN) family, activate caspase-1, which leads to the maturation of IL-1β and IL-18 (Davis et al. 2011) and the induction of pyroptosis, a form of cell death (Lamkanfi 2011). Multiple cellular signals have been proposed to trigger its activation, including K+ efflux, Ca2+ signalling, mitochondrial dysfunction, reactive oxygen species (ROS), and LMR. The importance of the NLRP3 inflammasome in immunity and human diseases has been well documented (He et al. 2016, Jo et al. 2016). Most importantly, NLRP3 activation by micro and nano-sized particles has been confirmed (Dostert et al. 2008, Sayan and Mossman 2016, Yazdi et al. 2010, Jessop et al. 2017, Hornung et al. 2008).

32. The extracellular release of high mobility group box-1 (HMGB1) alarmin also plays a pivotal role in inflammatory processes known to play a critical role in acute lung injury during the sterile inflammation that occurs with particle-induced exposures (Jessop and Holian 2015). HMGB1 is a non-histone nuclear protein that has a dual function. Inside the cell, HMGB1 binds DNA, regulating transcription and determining chromosomal architecture. Outside the cell, HMGB1 can serve as an alarmin to activate the innate system and mediate a wide range of physiological and pathological responses (Pisetsky et al. 2008). The extracellular release of high mobility group box-1 (HMGB1) alarmin also plays a pivotal role in inflammatory processes known to play a critical role in acute lung injury during the sterile inflammation that occurs with particle-induced exposures (Jessop and Holian 2015).

33. The release of high mobility group box-1 (HMGB1) plays a pivotal role in inflammatory processes known to play a critical role in acute lung injury during the sterile inflammation that occurs with particle-induced exposures and lysosome acidification is a prerequisite for particle-induced LMP, and the resultant leak of lysosome cathepsins is a primary regulator of ongoing NLRP3 inflammasome activity and release of HMGB1. (Jessop et al. 2017).

34. Autophagy and NRLP3 are assumed to interact both ways, in that not only NLRP3 affecting autophagy but also autophagy affecting NLRP3 as it could be shown that autophagy would negatively regulate NLRP3 inflammasome activation (Shi et al. 2012). The latter in turn, is shown to be associated with LMP, considered as a central pathway for activation of the NLRP3 inflammasome by inhaled particles (Jessop et al. 2017, Kroemer and Jaattela 2005).

Carbon based nanomaterials

35. Carbon nanomaterials come with different formations including carbon black nanoparticles (CBNPs), fullerenes, carbon nanotubes and fibres (Hurt et al. 2006).

Carbon black nanoparticles

36. The ability of CBNPs to activate the inflammasome (Caspase 1 and IL-1β Release) was shown *in vitro* in RAW264.7 cells (Reisetter et al. 2011) and produce persistent pulmonary inflammation in mice exposed via intratracheal instillation *in vivo* (Bourdon et al., 2012).

37. *In vivo* studies with nanomaterials could also present evidence of the involvement of LMP in producing for example, inflammation with persistent nanomaterials. For example, when C57BL/6 mice were intratracheally instilled with CBNPs. It was found that a single instillation could induce neutrophil influx as early as 4 h post-exposure and macrophages showed necrotic features and were characterized by LMR, cathepsin B release, reactive oxygen species generation, and reduced intracellular ATP level. Further analyses suggested that the resulting leakage of mtDNA from the necrotic cells activated neutrophils and triggered severe inflammation *in vivo*. It was therefore concluded that LMR and Cathepsin B release played a key role in CBNP-induced cellular toxicity (Yuan et al., 2020).

Fullerenes

38. Pristine fullerenes nanoparticles (C60 NPs) were shown to facilitate the formation of micropores in lipid membranes contributing to membrane leakage (Qiao et al. 2007). Similarly, fullerenol the hydroxylated fullerene induced cytotoxicity (mitochondrial depolarization and actin cytoskeleton disruption) was associated with increased autophagic vacuole accumulation in renal proximal tubule cells (LLC-PK1) (Johnson-Lyles et al. 2010), and in Human umbilical vein endothelial cells (HUVECs) with activation of an autophagic death pathway (Yamawaki and Iwai 2006). Similarly, the polyalkylsulfonated C60, or FC4S, a highly water-soluble caged fullerene derivative, when administered to Sprague-Dawley rats could form phagolysosomal and/or lysosomal inclusions in the cytoplasm of their renal tubular epithelium (Chen et al. 1998). Moreover, the accumulation of pristine fullerenes in isolated hepatopancreas cells and embryos of marine organism oysters, *Crassostrea virginica*, with subsequent lysosomal destabilization could be shown confirming that endocytotic and lysosomal pathways may be major targets of fullerenes and other nanoparticles and thus contributing to their long-term, chronic effects to humans as well as ecosystem health (Ringwood et al. 2009).

39. In contrast, the conversion of hydrophobic fullerenes to amphipathic fullerenes through surface functionalization with malonic acid (C60(C(COOH)2)2) could be shown to stabilize lysosomal membranes and inhibit tumour necrosis factor alpha-initiated cellular apoptosis. This could be explained with the notion that these nanoparticles once inside the acidic environment of lysosomes, form aggregates that could be dispersed and enable the single amphipathic (C60(C(COOH)2)2) to insert themselves into the lysosomal membranes. With this insertion, these nanoparticles could further stabilize the membranes and decrease the release of cathepsins from lysosomes, leading to the inhibition of tumour necrosis factor-induced apoptosis and through upregulation of expression of Hsp 70 (Li et al. 2011).

40. As some fullerene (C60) nanoparticles and their derivatives have been considered as important candidates for biomedical applications (Liang et al. 2010, Bakry et al. 2007) it was proposed that by understanding the mechanisms by which C60(C(COOH)2)2 affects lysosome integrity may provide new possibilities for developing potential drugs that modulate lysosomal stability (Li et al. 2011).

Carbon Nanotubes

41. Different studies have shown the ability of carbon nanotubes (CNTs) to induce LMP and apoptotic cell death in various cell types. The effects of different physicochemical properties were therefore, investigated on their ability to produce LMP in these cells (Wan et al. 2013, Yang et al. 2014).

42. The effect of stiffness of CNTs on intracellular behaviour was investigated where it could be shown that stiff nanotubes beyond a critical length were compressed by lysosomal membranes causing persistent tip contact with the inner membrane leaflet, leading to lipid extraction, lysosomal permeabilization, release of cathepsin B into the cytoplasm, and cell death. However, most importantly, it could be proposed that this process may be used to distinguish pathogenic from biocompatible varieties of these MNs leading to a predictive pathogenicity classification that distinguishes toxic from biocompatible nanomaterials based on their geometry and stiffness (Zhu et al. 2016).

43. Effects of diameter and length as well as impurities and surface modification with carboxyl functional groups of multiwall carbon nanotubes (MWCNTs) was also investigated on inflammasome activation and cytotoxicity in THP-1 cells (monocytes that can be differentiated to macrophages) and primary alveolar macrophages from C57BL/6 mice. Purification had little effect on the original MWCNT, but functionalization with the -COOH groups completely eliminated bioactivity in THP-1 cells. Similar results were obtained using alveolar macrophages isolated from C57BL/6 mice (Hamilton et al. 2013a, Hamilton et al. 2013b). More recent comparative toxicity study with acid-purified MWCNTs and concentrated acid-functionalized MWCNTs-COOH has confirmed the observation with -COOH

functionalized MWCNT yielding less cytotoxicity and release of inflammatory mediators (Sweeney et al. 2016).

44. In contrast, acid functionalization of single wall carbon nanotubes (SWCNTs) resulted in their accumulation in murine peritoneal macrophage lysosomes, leading to lysosome membrane destabilization and autophagosome accumulation in macrophages, indicating reduced autophagic degradation. The autophagosome accumulation may also represent a blockage on autophagosomal maturation and degradation. From these results, it was concluded that -COOH functionalised SWCNTs were able to induce autophagosome accumulation, decreased autophagic degradation and lysosomal impairment (Wan et al. 2013).

45. *In vivo* studies using raw, purified, −COOH-terminated raw MWCNT, and −COOH-terminated purified MWCNT demonstrated that all three original MWCNT caused similar inflammatory responses. However, consistent with the in vitro results, increasing width or length of MWCNT caused more severe pathology with the longest MWCNT causing the most severe inflammation and the same two larger MWCNT were retained more in the lung at 7 days (Hamilton et al. 2013a).

46. Effect of composition and size on different cell types was investigated. Different diameters of MWCNTs (< 8 nm, 20-30 nm, > 50 nm; but same length 0.5-2 μm) were tested for their effect on LMP in 3T3 fibroblasts, RAW 264.7 macrophages, and telomerase-immortalized (hT) bronchiolar epithelial cells. It was found that 3T3 cells treated with MWCNT <8 nm caused pronounced LMP as evidenced by the release of lysosomal contents into the cytoplasm. Minor lysosomal damage could also be seen in some 3T3 cells treated with MWCNT 20-30 nm and >50 nm. On the other hand, no detectable destabilization of lysosomal membranes could be observed in hT or RAW cells when exposed to MWCNT <8 nm and 20-30 nm, and minor lysosomal damage in hT or RAW cells were noted when exposed to MWCNT >50 nm. It was therefore concluded that the induction of LMP in 3T3, but not in hT bronchiolar epithelial cells and RAW macrophages explains the toxicity induced by <8 nm MWCNTs in 3T3 fibroblats compared to the other two cell types (Sohaebuddin et al. 2010).

47. These findings support earlier observations that a high degree of lysosomal membrane destabilization early after nanoparticle exposure may lead to excessive leakage of lysosomal contents into the cytoplasm causing cell death (Kagedal et al. 2001, Guicciardi et al. 2004, Jaattela 2004).

48. The effects of morphology of carbon nanotubes has also been studied. The toxicity of carbon nanohorns (CNHs), a typical type of carbon nanotubule, was tested using RAW264.7 murine macrophages where it could be shown that they preferentially localized in lysosomes leading to the destabilization of their membranes by LMR or LMP leading to apoptotic, as well as necrotic, cell death with low levels of cytokines (Tahara et al. 2012). These observations were confirmed later by other authors with CNHs using the same macrophage cell lines with the suggestion that LMP may be the primary reason for their toxicity (Yang et al. 2014). In contrast, in a more recent study, it was reported that CNHs were prone to acellular myeloperoxidase enzymatic oxidation and to cellular degradation by two types of macrophage cell lines, RAW 264.7 and THP-1 with no release of inflammatory markers such as pro-inflammatory cytokines interleukin 6 and tumour necrosis factor α induction (Zhang et al. 2015).

49. More recently, it was reported that CNTs, but not spherical NPs produced both blockage of autophagic flux and lysosomal dysfunction and therefore are suggested as the underlying mechanisms responsible for the CNT-induced toxicity (Cohignac et al. 2018). Moreover, the release of HMGB1 alarmin was also shown to be involved in multi-walled carbon nanotube (MWCNT)-induced inflammation in vivo (Jessop and Holian 2015).

Graphene oxide

50. Graphene oxides (GOs) were also shown to accumulate in macrophage lysosomes, leading to lysosome membrane destabilization, autophagosome accumulation leading to reduced autophagic degradation. These GOs were more potent than -COOH functionalized SWCNTs in producing these effects (Wan et al. 2013).

51. The effect of surface chemistry by varying the degrees of oxidation of grapheme nanosheets in their interaction with model lipid bilayers have been examined. It has been reported that graphene nanosheets tend to pierce the cellular membrane and to take a configuration vertical to the membrane which increased with degree of oxidization. These findings imply that graphene nanosheets with larger size and higher oxidization degree may lead to greater cytotoxicity (Mao et al. 2014).

Prickly nanodiamonds

52. Lack of toxicity of nanodiamonds (NDs) was attributed to their ability to enter the cell through endocytosis, escape endosomes through rupturing of their membranes followed by stable residence in cytoplasm. The NDs were seldom found to co-localize with lysosomes and therefore NDs caused little LMP and they have shown low cytotoxicity (Chu et al. 2014, Chu et al. 2015).

Carbon based polymeric nanomaterials

Amino-Functionalized Polystyrene Nanoparticles

53. RAW 264.7 macrophages (Abelson leukemia virus transformed cell line derived from BALB/c mice), have previously been shown to be susceptible to caspase activation and apoptosis after polystyrene nanoparticle uptake (Xia et al. 2008). Similar effects could also be observed by amino-functionalized cationic polystyrene nanoparticles (PS-NH2 NPs) of ∼100 nm in diameter, but not by carboxyl- or nonfunctionalized particles, in human macrophages. This was explained by the ability of PS-NH2 to cause proton accumulation in lysosomes leading to lysosomal destabilization, release of cathepsin B, and damage of the mitochondrial membrane (Lunov et al. 2011).

Cationic poly(amidoamine) dendrimer

54. Poly(amidoamine) (PAMAM) dendrimers carrying different amounts of surface amino groups have been shown to enter the KB, a subline of the cervical carcinoma (HeLa) cells, and accumulate in lysosomal compartment where they increased the lysosomal pH and cytotoxicity as a function of the number of surface amino groups on the dendrimers. In contrast, PAMAM dendrimers that were surface-neutralized by acetylation of >80% of the surface amino groups did not show any cytotoxicity. It was concluded that PAMAM dendrimers are endocytosed into the KB cells through a lysosomal pathway, leading to lysosomal alkalinization and induction of mitochondria-mediated apoptosis (Thomas et al. 2009).

Metal and metal oxide nanomaterials

55. Within this category of NPs, it should be noted that there are those with higher dissolution kinetics and those with lower dissolution kinetics.

Cerium oxide

56. It was demonstrated that oxides of rare earth element cerium nanoparticles ((CeO_2) NPs)-induced autophagy and that this induction may act as a pro-death mechanism leading to increased cytotoxicity of human monocytes (Hussain and Garantziotis 2013). It was also shown that cationic CeO_2 NPs and polystyrene particles induced disruption of lysosomes (Bexiga et al. 2011). Moreover, it was also demonstrated that surface charge affected entry and subcellular localization of ceria NPs in normal and cancerous cells. NP carrying positive and neutral charges entered the cells, but the former localised in lysosomes producing toxicity and the latter in the cytoplasm producing no toxicity. In contrast NP carrying a negative charge did not enter the cell and no toxicity was observed. With these results, it was concluded that differential surface-charge-dependent localization of nanoceria in the lysosomes in normal and cancer cells played a critical role in the toxicity profile of ceria nanoparticles (Asati et al. 2010).

57. The surface oxidation state (Ce^{3+}/Ce^{4+} ratio) of cerium oxide was also shown to be important in their catalytic and other activities. For example, CeO_2 with a higher Ce^{3+}/Ce^{4+} ratio or oxygen vacancy are shown to be protective against diseases associated with oxidative stress or inflammation because of their superior SOD mimetic activity while CeO_2 with lower Ce^{3+}/Ce^{4+} ratio exhibited anticancer and antibacterial activities due to their higher CAT-mimetic activity (Gupta et al. 2016).

58. Additional work described the mechanisms involved in CeO_2 NPs induced toxicity and inflammatory processes through the involvement of lysosomes as an upstream trigger in initiating a pro-inflammatory response. CeO_2 NPs were shown capable of activating the NLRP3 inflammasome and inducing IL-1β production. Moreover, these nanoparticles were also shown to be capable of disrupting autophagosome fusion with lysosomes and thus disruption of autophagic flux thereby disrupting homeostatic regulation of activated NLRP3 complexes leading to the accumulation of the inflammasomes, and resulting in robust and sustained IL-1β production (Li et al. 2014).

Neodymium oxide

59. Induction of autophagy has also been observed following treatment with several other rare earth oxides such as Neodymium nanoparticles (Nd_2O_3 NPs) in non-small cell lung cancer NCI-H460 cells at micromolar equivalent concentration range though extensive autophagy in the cytoplasm of the cells (Chen et al. 2005) and gadolinium oxide in HeLa cells (Yu et al. 2009).

Zinc oxide nanoparticles

60. Zinc oxide nanoparticles (ZnO NP) are shown to cause lysosomal membrane destabilization in THP-1 cells in culture while their intratracheal instillation has caused pulmonary fibrosis. Similar effects could be observed with dissolved Zn^{2+}. It was therefore proposed that lysosomes were destabilized by a mechanism, which seemed likely to involve dissolution of ZnO NP in the acidic environment of lysosomes, resulting in subsequent loss of lysosomal integrity induced by ZnO NP accompanied by cell death. Thus, the Zn^{2+} released from the phagolysosomes of dead or damaged cells was the source of the Zn^{2+} after ZnO NP uptake in the lung and therefore the rapid, pH-dependent dissolution of ZnO NP inside of phagosomes may be the main cause of ZnO NP-induced diverse progressive severe lung injuries (Cho et al. 2011, Vandebriel and De Jong 2012). Moreover, exposure HeLa cells to bare and

coated nano-sized ZnO resulted in enhancement of autophagosome formation, but also in blockage of the autophagic flux (Popp and Segatori 2019).

Titanium dioxide nanoparticles

61. Various cell types in the toxicity of lysosomal damage by titanium dioxide nanoparticles (TiO_2 NPs) (100% anatase) including mouse fibroblast L929 cells (Jin et al. 2008) and autophagic response in HeLa cells (Popp et al. 2018), were investigated. Lysosomal membrane destabilization was also observed by TiO_2 nanoparticles (99.7% pure anatase) in A SV40 large T- antigen transformed bronchial epithelial cell line (16HBE14o-) and Normal Human Bronchial Epithelial Cells (NHBE) leading to their apoptotic cell death (Hussain et al. 2010). In contrast, when exposed to TiO_2 nanoparticles (99% pure Anatase) apoptotic cell death could not be observed in 3T3 fibroblasts that were telomerase immortalized (hT) and RAW 264.7 macrophages (Sohaebuddin et al. 2010). TiO_2 nanoparticles (Aeroxide P25-80% anatase, 20 % rutile) were also shown to accumulate in HCECs (human cerebral endothelial cells) and induce autophagy (Kenzaoui et al. 2012). Reduction in lysosomal integrity by TiO_2 (75% rutile and 25% anatase) could also be observed in environmental organisms including rainbow trout (*Oncorhyncus mykiss*) (Vevers and Jha 2008) and in adult eastern oysters, *Crassostrea virginica* (Johnson et al. 2015).

62. In addition to cell type, it has also been shown that the shape (wire, fibre, belt or tube) and length of TiO_2 (pure anatase) affects the lysosomal destabilisation and NLRP3 inflammasome activation and release of inflammatory cytokines through a cathepsin B-mediated mechanism. TiO_2 nanomaterials in a fibre structure of greater than 15 µm were highly toxic and initiated an inflammatory response by alveolar macrophages (Hamilton et al. 2009).

Silica nanoparticles

63. Earlier studies have shown that crystalline silica particles exert toxicity on lysosomal membrane following their phagocytosis by mouse alveolar macrophage cell line (MH-S) leading to their apoptotic death (Thibodeau et al. 2004, Allison et al. 1966). This was later confirmed with crystalline silica where phagolysosome acidification was required for silica and engineered nanoparticle-induced lysosome membrane permeabilization and resultant NLRP3 inflammasome activity (Hamilton et al., 2017). Similarly, amorphous silica nanoparticles (SiO_2 NPs) could induce autophagy dysfunction via lysosomal impairment and inhibition of autophagosome degradation in hepatocytes (Wang et al., 2017a). And in HeLa cells (Schutz et al. 2016). Amorphous SiO_2 NPs may also cause lysosomal overload in mouse lung epithelial (FE1) cells (Decan et al., 2016) where accumulated SiNPs have been reported to cause destabilization of lysosomes and permeabilization of lysosomal membranes in mouse bone marrow-derived macrophages (Kusaka et al., 2014) and also 3T3 fibroblasts, but not in in telomerase-immortalized human bronchiolar epithelial cells (hT) and RAW 264.7 macrophages (Sohaebuddin et al. 2010).

64. Upon coating with aluminium or polyvinyl-pyridine-N-oxide (PVPNO) of crystalline silica also protected the lysosomes from particle-induced damage to their membranes and cell death in macrophages (Allison et al. 1966). Surface modification modifications of amorphous SiO2 NPs could also reduce activation of pro-inflammatory complex NLRP3 inflammasome in THP-1 cells (Morishige et al., 2010).

Copper oxide

65. Exposure of Human adenocarcinoma A549 cells to copper oxide nanoparticles (CuO NPs) produced high toxicity and also could induce the autophagic biomarker LC3-II indicating that the cytoxicity of CuO NPs may involve the autophagic pathway in these cells (Sun et al. 2012). Moreover,

exposure to CuO NPs could induce agglomeration of lysosomes and subsequent cellular damage, which lead to cell death in HUVEC cells (Zhang et al., 2018).

Iron oxide

66. Treatment of human umbilical vein endothelial cells with nanoscale iron oxide nanoparticles (FeO NPs) resulted in autophagy dysfunction (Wu et al. 2010). It could also be seen that FeO NPs induced significant autophagic effect when they were aggregated (Huang et al. 2015). It was also hypothesized that the shear forces created by the generation of oscillatory torques of magnetic nanoparticles (MNPs) bound to the lysosomal membranes would lead to membrane permeabilization, causing extravasation of lysosomal content inducing apoptosis (Zhang et al. 2014) or MNPs rotation under a weak alternating magnetic field as a unique method for specific mechanical lysis of cells (Vegerhof et al. 2016).

Vanadium dioxide nanoparticle

67. Vanadium dioxide nanoparticles (VO_2NPs) are known to be amphoteric oxides, which can act as either an acid or base in a reaction and therefore their transformation may be possible in acidic conditions. The toxicity of the pristine and acid transformed VO_2NPs were studied with epithelial cells and macrophages where it was found that both pristine/untransformed and transformed VO_2NPs displayed no obvious toxicity to epithelial cells, while the acidic transformed VO_2NPs could rapidly induce macrophage cell death. Upon further investigation it was observed that transformed VO_2NPs could cause LMP at an early stage, and therefore it was proposed that LMP could be considered as an early and sensitive end point for nanotoxicological study (Xu et al. 2015).

68. In contrast, it was shown that paramontroseite VO_2 nanocrystals (P–VO2) could induce cyto-protective, rather than death-promoting, autophagy in cultured HeLa cells. P–VO_2 with simultaneous up-regulation of heme oxygenase-1 (HO-1), a cellular protein with a demonstrated role in protecting HeLa cells against death under stress situations (Zhou et al. 2013).

WO3/Pt nanoparticles

69. It is shown that during visible light exposure, but not in darkness, tungsten trioxide platinum nanoparticles (WO3/Pt NPs) could trigger lysosomal membrane disruption (Clark and Petty 2016).

Silver nanoparticles

70. Exposure of human liver derived hepatoma (HepG2) cells to subcytotoxic concentrations of silver nanoparticles (AgNPs) (≤10 µg/ml) caused LMP following their accumulation, leading to activation of NLRP3- inflammasome (caspase-1, IL-1β), disruption of the autophagy-lysosomal fusion and finally cell apoptosis. This process was size dependent where 10-nm AgNPs showed the highest cellular responses compared with 50- and 100-nm AgNPs based on equal mass dosimetry (Mishra et al. 2016). In fact many earlier studies have confirmed defective autophagy and a defect in autophagosome–lysosome fusion through interference of AgNPs with ubiquitination (Mao et al. 2016), perturbation of lysosomal pH by AgNP in A549 cells (Yang et al. 2012), and LMP (Miyayama and Matsuoka 2016).

71. Similar observations of lysosomal destabilisation were made by AgNP in hepatopancreas cells from adult oysters (Crassostrea virginica) (Ringwood et al. 2010), and in newly-fertilized oyster embryos in hepatopancreas tissues of adult oysters (Ringwood et al. 2010; McCarthy et al. 2013; Edge et al. 2012).

Gold nanoparticles

72. Exposure of MRC-5 human lung fibroblasts in vitro to gold nanoparticles (AuNPs) was shown to induce autophagy concomitant with oxidative stress and also accumulation of autophagosomes (Li et al. 2010). To elucidate the processes involved in the AuNP-induced autophagosomes accumulation, it was shown that AuNPs could indeed induce the processing of LC3, an autophagosome marker protein while blocking the degradation of the autophagy substrate p62 indicating that autophagosome accumulation resulted from blockade of autophagy flux, rather than from the induction of autophagy. Results obtained from this study also confirmed the accumulation of AuNPs in lysosomes causing the impairment of lysosome degradation capacity through alkalinization of lysosomal pH (Ma et al. 2011). The effect of shape of gold nanoparticles on lysosomal dysfunction has also been presented (Zhou et al. 2018).

73. Such lysosomal membrane instability was also measured in haemolymph in Mytilus edulis by AuNP where it was considered to be mechanism of toxicity (Tedesco et al. 2010).

Quantum dots

74. Quantum dots (QDs), could be shown to produce size dependent autophagic response in human mesenchymal stem cells (Seleverstov et al. 2006) and in porcine kidney cells (Stern et al. 2008) through swelling of lysosomes linked to morphological alterations (Funnell and Maysinger 2006). It was also concluded that QD cytotoxicity in porcine kidney cells through autophagy was dependent upon properties of the particle as a whole (CdSe), and not exclusively the metal core materials (Cd) and that Cd-dependent mechanisms alone were not responsible for the cytotoxicity and thus confirming that mechanisms involving the intact QD such as autophagy were also important (Stern et al. 2008).

Mechanism involved in nanomaterials induced lysosomal damage

75. Several mechanisms have been proposed through which MNs could induce lysosomal damage. These have included release of toxic ions from ZnO and CuO NPs (Vandebriel and De Jong 2012), the swelling of lysosomes and morphological alteration by QDs (Funnell and Maysinger 2006), and charge where cationic CeO2 NPs and polystyrene particles could induce disruption of lysosomes (Bexiga et al. 2011) or by direct action on lysosomal membranes to increase permeability by MWCNTs (Sohaebuddin et al. 2010).

LMP, Inflammasome Activity and Adverse health outcome

76. As several degradation pathways including Autophagic pathways. efflux of molecules from endo/autolysosomes. endosomal and autophagosomal pathways, terminate in the lysosome, lysosomal dysfunction has a profound impact on cell homeostasis, resulting in manifold pathological situations, including infectious diseases, neurodegeneration, aging as well as fibrotic diseases and cancer.

LMP and Inflammasome activation, autophagy and inflammation and fibrosis

77. With long-term exposures to crystalline silica and asbestos, LMP with subsequent cathepsin B release and inflammasome activation have been suggested as primary effects that may eventually lead to some form of lung pathology such as fibrosis (Biswas et al. 2014, Franchi et al. 2009, Dostert et al. 2008, Cassel et al. 2008, Hornung et al. 2008). A possibility may therefore exist that similar mechanisms may apply with nanomaterials that are shown to cause LMP and also produce inflammasome activation (Hamilton et al. 2009, Stern et al. 2012, Franchi et al. 2009, Girtsman et al. 2014).

78. Indeed, results presented recently with MWCNTs are consistent with LMP being a key regulator in this MN toxicity and lung inflammation (Hamilton et al. 2018). Silicon-based quantum dots (Si/SiO_2 QDs) were also shown to generate inflammation in lung cells and in addition cause an imbalance in extracellular matrix turnover through a differential regulation of MMPs and tissue inhibitor of metalloproteinase-1 protein expression (Stan et al. 2015). The role of MMPs in interstitial fibrosis is well known in fibrosis - the common end stage of several interstitial lung diseases - is thought to reflect an aberrant wound healing response followed by injury triggered by different cellular processes and agents including asbestos and crystalline silica, a common feature chronic inflammation that causes continual and repeated injury to the alveoli over a prolonged period (O'Connor and FitzGerald 1994).

79. The relationship between MNs-induced inflammation and fibrosis was reviewed (Wang et al., 2017b) with recent studies to suggest that many MNs could activate the NLRP3 inflammasome, which plays an important role in the generation of chronic granulomatous inflammation and fibrosis in the lung (Wang et al., 2012). Subsequently a predictive paradigm that link lysosome injury and cytokine production to the pathogenesis of pulmonary fibrosis was proposed (Wang et al., 2015).

LMP, Inflammasome activation, autophagy, inflammation and cancer

80. MNs-induced inflammation is one of the most studied areas of concern because of the known role for inflammation in many, if not most, chronic, and degenerative health diseases (Sethi et al., 2012, Kundu and Surh 2008). Inflammation resulting from permeabilization of the lysosomal (alternatively referred to as late endosome) membrane followed by activation of the NLRP3 inflammasome appears to play a critical role (Bunderson-Schelvan et al. 2016). Some particles initiate acute inflammation by

destabilizing the lysosomal membrane, which releases the catalytic enzyme contents of the lysosome, triggering inflammatory signalling and potentially cell death (Bunderson-Schelvan et al. 2017). This acute inflammation might become sustained and result in a chronic inflammatory condition that manifests in various pathologies depending upon the inflammatory area (Bunderson-Schelvan et al. 2016).

81. Similar to lysosomal dysfunction, dysfunction of the autophagy pathway has also been linked to a variety of diseases (Ravikumar et al. 2010). There is evidence that autophagy dysfunction plays a role in both cancer development and progression possibly by allowing the accumulation of damaged organelles, such as mitochondria, that can then induce oxidative stress, inflammation, and DNA damage. (White and DiPaola 2009). There is also data to suggest that in cancer once established, autophagy may play a pro-survival role, allowing tumours to grow under nutrient deprived conditions or survive chemotherapy-induced stress (Livesey et al. 2009). Some nanoparticles have also recently been shown to potentiate the cytotoxicity of chemotherapeutics by disrupting autophagy (Cordani and Somoza 2019) and by injuring lysosomal membrane structures (Zhang et al., 2014) Subsequently by targeted crystallization of mixed-charge nanoparticles in lysosomes could induce selective death of cancer cells (Borkowska et al., 2020). A recent review has describet the relationship between nanomaterial induced LMP, Inflammasome activation, and autophagy and their importance in cancerous cell death (Gulumian and Andraos 2018).

Tests and cell types that can be used to assess lysosome morphology and function

82. Several reviews on methods to evaluate lysosome morphology, function, and LMP have been published summarising different approaches (Kroemer and Jaattela 2005, Repnik et al. 2016, Wang et al. 2018a, Aits et al. 2015a).

Assessment of morphological changes using microscopy technologies

83. Microscopic techniques are frequently used for the assessment of NPs because they allow the correlation of intracellular localization and cellular effects. Morphological changes of lysosomes can be performed by image analysis of fluorescence-labelled lysosomes and can be identified by transmission electron microscopy (TEM). Cells transduced/transfected with fluorescent protein-LAMP-1 constructs can also be used for these studies. By using LAMP-1 transfected cells, it was found that small 20 nm carboxyl-functionalized polystyrene particles were preferentially located in the perinuclear region, whereas 200 nm particles were detected to a greater extent in the cellular periphery (Fröhlich 2017).

84. Using Fluorescence microscopy, the extent of LMP can be estimated using dextrans of different molecular weights (10-250 kDa). Smaller FITCdextran molecules are released into the cytosol in response to apoptosis-associated LMP, while larger dextran molecules are retained (Bidere et al. 2003). One drawback of this method is that normal lysosomal function can be affected by the obligatory preloading of dextrans into lysosomes by endocytosis. Moreover, fluorescence-labeled dextran requires active uptake for the labeling (Vogel et al. 2001).

Assessment of lysosomal membrane integrity

85. The cellular distribution of pH-dependent lysosomotropic (acidotropic) dyes (e.g., acridine orange (AO) and neutral red), and lysosomotropic particles (e.g., gold-coupled albumin and fluorescence-labelled dextran) can be used as a measure of pH.

86. AO is a metachromatic fluorophore and a lysosomotropic base that diffuses into cells and accumulates in lysosomes by proton trapping. It fluoresces red (upon excitation with blue light) at high concentrations when it accumulates in the lysosome. In response to LMP, AO dissipates throughout the cytosol and fluoresces green (Zdolsek et al. 1990). Using AO, the LMP caused by TiO2 and SiO2 nanoparticles, and MWCNTs can be assessed (Sohaebuddin et al. 2010, Thibodeau et al. 2004).

87. Neutral red is weakly cationic and readily crosses lipid membranes in a non-ionized form at neutral pH. Inside the low pH environment of the lysosome, it becomes charged and accumulates.

Neutral red assay for lysosomal pH can therefore be implemented by measuring quantitatively the uptake and retention of the dye (Repetto et al. 2008).

88. Most recently, new lysosome-targeting fluorescent pH probes were developed namely, (CQ-Lyso) based on the chromenoquinoline chromorphore (Liu et al. 2017) and RML with methylcarbitol unit as the lysosome-targeting group to rhodamine B, which is highly sensitive to pH changes (Wang et al. 2020).

Lysosomal function

89. Information on lysosomal function can be obtained by detection of enzymatic activity. Lysosomal function assessment that can be determined by changes in the expression of lysosome-related genes or proteins and fluorescent substrates or fluorescent dyes, which indicate changes in enzyme activities or pH (Fröhlich 2017).

Lysosomal proteins (e.g., immunostaining of lysosomal hydrolases)

90. The release into the cytosol of lysosomal enzymes such as cathepsins B and D proteins can be visualized (Minchew and Didenko 2017). Cathepsins confined within intact lysosomes are visualized as a punctate pattern of intense fluorescence and can be co-stained with antibodies against LAMP-1 or LAMP-2. By contrast, cathepsins released during LMP produce a diffuse fluorescence pattern throughout the cell (Boya et al. 2003). Cytosolic release of cathepsins can also be detected by Western blot of the cytosolic fractions after cell fractionation (Wang et al. 2018b) or by using cathepsin-specific substrates (eg, Magic Red) and a fluorescence plate reader. Fluorescence microscopy can also be used to count cathepsin positive cells in tissue sections (Gabande-Rodriguez et al. 2014).

Lysosomal galectin and ESCRT puncta assays

91. A highly sensitive method for detecting LMP is the recently developed lysosomal galectin puncta assay (Aits et al. 2015b). The cytosolic galectins can translocate to the membrane of leaky lysosomes where Galectins 1 and 3 are considered as the most relevant since they are widely expressed, rapidly translocate, and also the corresponding high-affinity antibodies are available. This assay is proposed to detect LMP much earlier than methods that monitor cathepsin release (Aits et al. 2015b) and also distinguishes the subset of damaged lysosomes from other intact lysosomes within the same cell (Pagliero et al. 2016). The ESCRT puncta assay was also developed to detect small lysosomal membrane ruptures where it was shown that ESCRT proteins are recruited to damaged lysosomes much earlier than galectins, and that the ESCRT machinery functions independently of lysophagy (Skowyra et al. 2018).

Enzyme activity

92. Activity measurement of acid phosphatases, β-glucuronidase, and β-hexosaminidase, which are released from damaged lysosomes, can be used as marker for lysosome function For example, the substrate SulfGreen is metabolized by all lysosomal sulfatases. for instance, CV-(RR)2 for CatB and MR-(FR)2 for cathepsin L indicate protease activity (Fröhlich et al. 2012). Using this assay, silica and engineered nanoparticle-induced LMP can be assessed (Jessop et al. 2017).

Interference of nanomaterials in test systems

93. **Lysosome markers**, such as gold-coupled albumin and fluorescence-labeled dextran, use active uptake for the labeling (Vogel et al. 2001). Since NPs may interfere with active uptake routes,

these markers are less suitable for NP studies. Information on lysosome function can be obtained by detection of enzymatic activity or pH-dependent dyes (Fröhlich 2017).

94. **TEM** can identify morphological changes, and a panel of vital stains allows the determination of intralysosomal pH and activity of lysosomal enzymes. While TEM analysis is time-consuming and lacks physiological information, immunocytochemical staining combined with vital stain is a good option to study lysosome function. In combination with fluorescent (-labeled) NPs, uptake and localization in different parts of the endosomal-lysosomal system can be shown.

95. **Acridine orange** has been used for many years to visualize organelles with acidic pH. The dye stains lysosomes in green and red fluorescence at low extracellular concentrations (2.6 µM). When the concentration of the dye in the staining solution is higher (26–37 µM), stacks can be formed and lysosomes show red fluorescence. Unfortunately, the red stacks bleach very fast and only the monomeric (green) form remains (Pierzynska-Mach et al. 2014). In addition to that, AO is phototoxic and induces burst of dye-loaded vesicles (Fröhlich 2017).

96. **LysoTracker™ Red DND-99** a lipophilic amine with logP 2.10 and pKa 7.5, is another marker for accumulation in lysosomes (Duvvuri and Krise 2005). Although it is not markedly phototoxic, its usefulness as lysosome marker is limited by bleaching. Membrane permeant dyes, such as LysoTracker™ and LysoSensor™ probes, label lysosomes in living cells (Chazotte 2011a). They are more selective than the classical neutral red and AO dyes. The compound commercialized as Lyso-ID® is a cationic amphiphilic tracer that accumulates in acidic organelles. It can be used as an indication for lysosome size and number and is used in drug screening for lysosomal damage (Coleman et al. 2010). Increased staining indicates swelling of lysosomes and increase in lysosome number. This increase is seen as adaptation to insufficient intracellular degradation capacity.

97. **Neutral red** can be used as indicator for functional lysosomes (Chazotte 2011b) but is less sensitive than fluorescent dyes and more often used as viability screening test but would not provide information on uptake or intracellular interaction.

98. **Enzyme activity assay** of acid phosphatases, β-glucuronidase, and β-hexosaminidase, which have been released from lysosomes, can be used as marker for lysosome function but needs isolation of the organelles. NPs that were located outside the lysosomes can get access to the assay compounds during the isolation procedure and cause artificial effects. Such interference occurred when cathepsin B activity of cells, exposed to polystyrene particles, was detected in homogenates (Fröhlich et al. 2012). *In situ* assays, where cells are not homogenized, can avoid this problem because only NPs located inside lysosomes get access to the substrate.

99. Based on the existing literature, lysosomes may be acutely damaged by high concentrations of nondegradable and biodurable MNs upon prolonged exposure (Fröhlich 2017). Tests that assess this damage may therefore assist in identifying such nondegradable and biodurable MNs. Among those identified, TEM can detect morphological changes, and a panel of vital stains allows the determination of intralysosomal pH and activity of lysosomal enzymes. While TEM analysis is time-consuming and lacks physiological information, immunocytochemical staining combined with cellular life stains is a good option to study lysosome function. In combination with fluorescent (-labelled) NPs, uptake, and localization in different parts of the endosomal-lysosomal system can be shown.

Screening of biodurable MNs for their toxicity

100. The OECD WPMN programme has successfully verified the testing methods used on Manufactured Nanomaterials. These tests are however conducted over short-term exposure periods to predict the acute short-term toxicity of MNs. Most nanomaterials are however biodurable and may accumulate in cells. To address this problem, culture systems have been developed that allow the evaluation of cellular effects over long-term exposure (Mrakovcic et al. 2013, Mrakovcic et al. 2014).

101. It is also proposed that another option to identify toxicity upon prolonged cellular contact to MNs is the study of organelles that are likely targets for damage by NPs. Most biodurable nanomaterials are delivered through uptake routes to lysosomes leading to their accumulation leading to lysosome dysfunction and cell damage. The option to identify toxicity upon prolonged contact will be the study of organelles such as lysosomes that are likely targets for damage by NPs. These in turn, may be used for screening of nanomaterials (Fröhlich 2017). A long persistence of nanomaterials in tissues has also been reported *in vivo* (Geraets et al. 2014, Geraets et al. 2012, Lee et al. 2018) where one of the plausible explanations for MNs-induced lysosomal dysfunction were stated to be bio-persistence (Fan et al., 2018).

102. Not all assays, however, are suitable for the assessment of NPs because colorimetric, fluorescent, and luminescent assays are prone to interference with NPs (Fröhlich et al. 2010). Interference is said to cause false-positive and false-negative results. For example, when chromophores are implemented for enzymatic activity and other assays, they may lead to over- or under estimation depending on the concentration of the nanomaterials tested and therefore it is proposed that the data should be compared with microscopical observation. Moreover, it is recommended to conduct parallel assessments by several techniques (plate reader and microscopy) and to switch to another detection method (fluorescence instead of absorbance) to help avoid false conclusions due to interference (Fröhlich et al. 2010, Fröhlich 2017; Jessop et al. 2017).

Conclusions

103. The overview provided in the present document regarding the central role played by LMP triggered by MNs both *in vitro* and *in vivo* emphasises the critical role of LMP in MNs toxicity and the useful implementation of LMP in the safety assessments conducted through alternative testing strategies. Cellular and molecular processes underlying MN-induced toxic effects will therefore provide the needed scientific information and facilitate the establishment of a knowledge-based foundation for their risk assessment. Test systems identified may therefore be implemented as reliable screening approaches to assess MNs hazards.

104. The central role played by LMP triggered by MNs may also be considered in informing specific Adverse Outcome Pathways (AOP), their molecular initiating events (MIE) and subsequent key events (KEs) and thus be considered as the basis for an integrated approach to testing and assessment (IATA) or an integrated testing strategy (ITS).

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