**Exploiting intrinsic nanoparticle toxicity: the pros and cons of nanoparticle-induced autophagy in biomedical research.**

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**Table of contents.**

**1. Introduction4**

**2. Nanomedicine6**

2.1. Soft nanomaterials7

2.2. Hard nanomaterials8

**3. Nanotoxicology and the role of autophagy11**

3.1. Key focus points and challenges in the field of nanotoxicology……………………………..11

3.2. The process of autophagy…………………………………………………………………….13

3.3. Autophagy and cell death…………………………………………………………………….16

3.4. How to study autophagy……………………………………………………………………...18

3.5. Chemical modulation of autophagy………………………………………………………….21

**4. Nanomaterial-induced autophagy22**

4.1. Modulation of autophagy by soft particles22

4.1.1. Liposomes……………………………………………………………………………22

4.1.2. Polymeric nanomaterials…………………………………………………………….23

4.2. Modulation of autophagy by hard nanoparticles25

4.2.1. Gold nanoparticles…………………………………………………………………...25

4.2.2. Iron oxide nanoparticles……………………………………………………………..26

4.2.3. Quantum Dots……………………………………………………………………….26

4.2.4. Zinc oxide nanoparticles……………………………………………………………..27

4.2.5. Carbon-based nanomaterials………………………………………………………....28

4.2.6. Other hard nanomaterials…………………………………………………………….29

4.2.7. Physiological effects of nanoparticle-mediated autophagy modulation……………..30

4.3. Influence of nanoparticle characteristics on autophagy deregulation………………………..31

4.4. Mechanisms of autophagy induction by nanomaterials………………………………………33

**5. The dangers of autophagy modulation36**

5.1. Autophagy in neurodegenerative diseases37

5.2. Autophagy and cancer40

**6. The possibilities of nanomaterial-induced autophagy 42**

6.1. Selective destruction of cancer cells42

6.1.1. Selective autophagy in cancer cells………………………………………………….42

6.1.2. Cancer-specific induction of autophagy by nanomaterials..........................................43

6.1.3. The potential of nanomaterials in anticancer therapy..................................................48

6.2. Autophagy-mediated synergistic effect of nanoparticles and chemotherapeutics……………51

6.3. Enhanced tumor antigen presentation through nanoparticle-mediated autophagy…………...52

6.4. Autophagy induction as a self-protection process against nanotoxicity52

6.5. The potential of nanoparticles for neuropathological therapy54

**7. Conclusions and outlook 55**

**8. References57**

**9. Tables73**

**10. Figure captions81**

**1. Introduction**

In his 1959 keynote lecture, "There is plenty of room at the bottom", Richard Feynman introduced the concepts that would later form the basis of a new era of technology, being nanotechnology. After this lecture, it became increasingly clear that by miniaturization materials can acquire novel or altered properties that are quite unique. This discovery subsequently created an enormous industry based on the use of materials in the nanosize range which is expected to have a market value of 2.95 trillion US dollars by 2015.1 The official definition of a nanomaterial (NM) that has been put forward by the European Commission is the following: “A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range from 1 - 100 nm”.2 The majority of NMs exhibit unique physical, chemical and optical properties that have opened the door for many new technological applications. Examples of such interesting properties include *a)* the superparamagnetic nature of iron oxide nanoparticles (NPs),3 *b)* the very high fluorescent brightness and excellent photostability of colloidal quantum dots,4 *c)*the localized surface plasmon resonance (LSPR) effect of silver or gold NPs,5 and *d)* the high rigidity of carbon nanotubes.6 Even though these materials were initially developed for industrial use, the same properties have also awoken the interest of the medical and biological communities, since these properties can be harnessed to create novel and powerful therapeutic and/or diagnostic tools. As such, nanomedicine was born as the scientific discipline in which these NMs are utilized for medical purposes. However, this high interest in using NPs for medical applications and their increasing use in various technological applications and consumer goods (e.g. clothing and food products) has raised high concerns on their possible impact on human and environmental health. It is therefore of vital importance to carefully characterize the toxicity of these NMs to enable the field of nanotechnology to fulfill some of its truly exciting possibilities in many aspects of human life. In view of this, the increasing production and use of NPs has led to the instilment of another scientific discipline: nanotoxicology.7 Although the area of nanotoxicology was initially a small niche within the field of particle and fiber inhalation studies, the field has rapidly expanded to become an important stand-alone scientific discipline, encompassing multiple domains such as *in vitro*, *in vivo*, environmental and human toxicology.

Recently, an increasing number of nanotoxicological studies have reported the ability of various types of NPs to modulate the cellular process of autophagy. Autophagy is an evolutionarily conserved catabolic process by which endogenous (e.g. organelles) as well as foreign (e.g. pathogens) materials are sequestered in double-membraned vesicles (i.e. the autophagosomes) and degraded upon fusion of these autophagic vesicles with lysosomes. This process is vital for cytoplasmic quality control as it removes excessive or damaged components such as organelles and aggregated proteins. Autophagy is usually present at a basal level but can be induced as a cytoprotective mechanism in case of cell stress; for instance, during starvation autophagy aids to overcome this food drop by degrading and recycling less essential cytoplasmic material.8 Considering the importance of autophagy in cellular homeostasis, it is not surprising that autophagy dysfunction has been correlated with a variety of disorders including cancer and neurodegenerative diseases.9 NPs have been found to be capable of modulating autophagy, and it has been put forward that autophagy induction may be a potential common cellular reaction toward NMs as an attempt to eliminate the foreign material.10 Therefore, the indirect effect of autophagy modulation on potentially protecting the cells from NM-induced damage is an interesting research topic that is in full development. On the other hand, NMs able of modulating autophagy have been proposed as potential agents against autophagy-related diseases. Intriguingly, several studies have shown that certain NPs are even able of selectively overstimulating autophagy in cancerous cells, leading to cell death without significantly affecting the level of autophagy in non-cancerous cells.11 This suggests that NPs can exhibit an intrinsic toxicity toward cancer cells, which would be of high therapeutic impact. For this reason, it is essential to study the effect of NMs on autophagy both from a nanotoxicological as well as a therapeutic viewpoint.

The current review will provide a clear overviewof reports on NM-mediated modulation of autophagy and will focus on the importance of autophagy in both health and disease. We will briefly discuss the different ways in which this process can possibly be manipulated, and in the final part, we will examine how and why NP-induced autophagy can be exploited as a novel therapeutic tool.

**2. Nanomedicine**

The exploration of NMs in medical or pharmaceutical applications is vastly increasing, where a lot of effort is being put in the development and fine-tuning of new materials.12 Accordingly, the number of NM-based agents currently undergoing clinical trials is expanding.13 However, due to the pertaining uncertainties concerning the potential danger of NMs and the lack of appropriate legislation, the nanotechnology industry is facing significant setbacks in their attempt to implement NMs in a clinical setting.14 This clinical implementation differs greatly between lipid-or polymer based NMs and metallic NMs. Soft NMs (lipid- or polymer-based) have been widely applied in clinics for over a decade and many new formulations are currently undergoing clinical trials.15 Hard (often metal, metal oxide or ceramic) NPs on the other hand, rarely see their potential being translated into a clinical setting despite their substantial scientific improvement.16 This is primarily caused by concerns regarding the safety and appropriate regulations of these NPs. This section will briefly describe the various types of NMs currently used in or explored for clinical settings, mainly in the field of cancer therapy. The principal focus will lie on introducing the different types of materials, a short description of their most important properties and an overview of their current and potential future applications.

2.1. Soft nanomaterials

Soft NMs can generally be classified under lipid-and polymer-based materials. The former are often organized into liposomal structures, originally discovered by Bangham and colleagues in 1965.17 Liposomes are typically small (confined between 50 and 200 nm diameter) and present an inner aqueous compartment that is separated from the outer aqueous compartment by a single lipid bilayer. The variety of lipids and potential incorporation of proteins or other lipophilic compounds allows easy fine-tuning of the chemical make-up of liposomes and their surface chemistry, which enables further modification of these vectors.18

For clinical use, a common surface molecule is poly(ethylene glycol) (PEG) as it reduces opsonization of the liposomes by immune components and proteins by which it increases blood circulation time through impeding liposomal clearance by the reticuloendothelial system.19 This anti-fouling effect stems from the flexible nature of the PEG-chains which, when the NPs are in circulation, will constantly adopt different conformations and thus prevent binding of any biomolecules. Additionally, targeting moieties such as small molecules or antibodies against a particular marker can be added to enhance delivery of the liposomes to the desired (targeted) location.

In general, the main applications of liposomes lie in their use as vehicles for the enhanced delivery of potent anti-cancer agents since these can either be enclosed within the aqueous central cavity (for hydrophilic compounds) or embedded within the lipid layers (for hydrophobic compounds). As such, a great number of liposomal systems have been generated and put into clinical trials in combination with various therapeutic agents, mostly anti-cancer drugs (see **Table 1**).13, 15 A full overview of current clinical trials and clinically approved NMs can be found elsewhere.15

Next to lipid-based formulations, polymer-based NMs are also frequently employed as delivery systems for anti-cancer drugs. The advances in chemical research have led to the discovery of a wide variety of different polymers that offer many exciting possibilities for clinical applications, such as good blood circulation times, pH-dependent drug release profiles and the ease of further functionalization by chemical means, including the incorporation of targeting ligands or PEG chains.20 Both natural and synthetic polymers can be used for drug delivery, many of which have been optimized to present high levels of biocompatibility.20 Amphiphilic polymers are often used as polymeric micelles or block co-polymers in a size range from 20 up to 100nm. These dimensions allow leakage through damaged cancerous vessel walls, providing enhanced permeability without leakage from normal vessel walls.21 These vehicles are therefore ideal for the transport and delivery of more hydrophobic anti-cancer agents.20 Consequently, many of the clinically approved formulations therefore consist of polymeric micelles, either untargeted or bestowed with an antibody against a specific marker containing hydrophobic compounds such as Paclitaxel or Cisplatin.22

2.2. Hard nanomaterials

Hard NMs represent a wide variety of compounds, including metal, metaloxide, semiconductor, carbon and ceramic materials. These hard NMs are characterized by their unique properties in comparison with their bulk counterparts as a direct result of their small size.23 Further progress of NP synthesis and surface chemistry has enabled the development of NPs consisting of different materials, for example, gold-coated iron oxide NPs, thus creating theranostic tools in which both diagnosis and therapy can be applied by a single entity.24 Many chemical linking strategies are also available that allow the conjugation of chemical drugs, fluorophores or small compounds to these hard NMs in order to further enhance their application range. Given these exciting properties, hard NMs are currently receiving a lot of attention in view of possible biomedical applications.

Metaloxide NPs are a broad collection of materials with many different applications. For example, titanium dioxide (TiO2) NPs are often used in pharmaceutical tablets because of their whitening effect, zinc oxide (ZnO) NPs are commonly added to sunscreens for their high UV absorbance and iron oxide NPs (IONPs; Fe2O3 or Fe3O4) are often used in clinical settings as magnetic resonance imaging (MRI) contrast agents due to their superparamagnetic nature, resulting in high magnetic susceptibility without remnant magnetization.25,26, 27

Cerium oxide (CeO2) NPs are also gaining attention as they can possibly be implemented to scavenge oxide radicals and prevent radical-mediated cell damage owing to their potent antioxidant capacity.28 In a pre-clinical setting, CeO2 NPs have been shown to *a)* prevent the loss of dopaminergic neurons in the substantia nigra, a key factor in the onset of Parkinson's disease and *b)* promote the growth of novel dopaminergic neurons which also contributes to reduce the effects of Parkinson's disease.29

Metal NPs such as silver (Ag) or gold (Au) are increasingly being explored for clinical applications as delivery vehicles, diagnostic and/or therapeutic agents.16, 30 Both Ag and Au NPs can be made in a variety of shapes and sizes through various chemical synthesis routes. Silver NPs are widely used in consumer goods such as deodorants due to their potent antibacterial properties.30 Similarly, the same properties have led to their use in wound dressing. AuNPs are a leading example of theranostic agents. These NPs can, for instance, convert near infrared (NIR) light into heat, providing an interesting platform for triggered cancer therapy via hyperthermia.16 Additionally, Au NPs are efficient delivery vehicles as demonstrated in multiple clinical trials. A leading example is the delivery of tumor necrosis factor alpha (TNF) using PEGylated Au NPs (Aurimmune; CytImmune Sciences, Rockville, MD),31 who were found to display a much higher tumor targeting efficacy and tumor toxicity than free TNF.

Other interesting materials are colloidal semiconductor NPs (quantum dots (QDs)), that consist of a semiconductor core with a narrow bandgap made up of elements from groups 12 and 16 (CdSe, CdTe, ZnS) or groups 13 and 15 (InP, GaN). For imaging purposes, these QDs offer many possibilities as their size-dependent broad excitation spectra and narrow emission spectra enable efficient multiplexing.32 However, the use of heavy metals such as Cd2+, a known toxicant, is severely impeding their progress into clinical applications. Novel formulations, including Cd2+-free QDs are being considered, but more research is needed before these materials can be used for clinical trials. Similar problems are associated with carbon-based materials. Although they exhibit a high potential as potent delivery vehicles or as links to promote neuronal communication, carbon-based materials are relatively hard to produce in a 100% purified manner and often contain certain levels of contaminants that could induce toxic effects.33

Ceramic NPs, such as silica NPs, receive a lot of interest since they can be bestowed with chemical compounds such as drugs or organic fluorophores, making them suitable for both therapy and diagnosis.34 Recently, researchers at Cornell University have developed C-Dots (Cornell dots), which are small (8 nm diameter) silica NPs containing several molecules of organic fluorophore.35 These particles have been shown to display excellent optical properties compared to free unbound organic dyes, resulting in high levels of fluorescence intensity and high photostability.36 In mice, these small NPs, when PEGylated, were found to be small enough to have long blood circulation times while finally being cleared from the body through the kidneys. Using C-Dots that have been conjugated to tumor-targeting molecules, similar studies will now be performed in a clinical setting on melanoma patients to verify the safety and efficacy of these C-Dots in detecting tumor sites.37

**3. Nanotoxicology and the role of autophagy**

3.1. Key focus points and challenges in the field of nanotoxicology

As mentioned in section 1, the field of nanotoxicology is relatively new and progressing rapidly in order to keep up with the rapid pace at which nanotechnology itself is advancing. A lot of attention is being paid to the detection of NMs and ways to study their interaction with biological specimens, being the environment, product end-users or workers at a production site. In this respect reports on how to analyze NP toxicity and their possible interference with toxicological assays have been published.38 Simultaneously, several main focus points have been put forward in order to meet the current shortcomings in nanotoxicology. A major focus point should be the proper characterization of the chemical composition, colloidal stability and purity of the applied NPs. This is crucial in order to relate any toxic effects to NP-related features and prevent, for instance, ‘side-effects’ stemming from NP contaminants.39 This also allows to reliably pinpoint potential common mechanisms by which NPs affect biological samples, such as the induction of reactive oxygen species (ROS; see section 4.1 for more details) or alteration of cell morphology.40 Furthermore, multiparametric methods for toxicological testing could provide a more complete picture of toxic NP effects.40a In combination with new automated high-content machinery this could then generate large databases that could be applied for computer-driven modeling and predictive toxicology.41

Another main focus point in toxicology in general is the attempt to bridge the *in vitro* – *in vivo* gap. Primary efforts to address this issue are the use of novel *in vitro* models, such as 3D cell cultures and co-culture models that better mimic the human physiology.42 Moreover, there is a great need to thoroughly investigate certain “uncharted” fields, such as: *a*) the effect of dosing and NP sedimentation on cellular uptake and toxicity,43 *b*) the effect of varying cellular microenvironments on the colloidal stability or degradation of NPs,44 *c*) long-term effects of NPs as well as their degradation products on cells or tissues, also in the form under which they will be used, for instance in electronic products, clothing or gel-based creams, *d*) the need to link toxicity data with functional effects, such as the toxicity of IONPs or QDs with their ability to visualize cells by MRI or fluorescence microscopy, respectively.45 While all these focus points are helping the field of nanotoxicology to further develop into a strong and mature scientific discipline, it is inherently faced with a few major challenges. One challenge lies in the fact that toxicology in itself is a bearer of bad news and therefore, most studies will report on unwanted and dangerous effects creating a negative atmosphere around the field. This hardens the publication of for example, unfavorable data or papers that present no effect by a certain NM, while this is essential information for scientists and this would also allow the nanofield to advance faster.46 Another challenge lies in the need for new equipment and novel ways to properly characterize the location, concentration and chemical composition of NMs in a complex biological matrix, preferably in real-time at the highest sensitivity possible.47 A last challenge is the wide variety of parameters that can affect NP-cell interaction, by which reliable and straightforward interpretation of observed effects is hampered*.* Since there are too many parameters to be tested, it is a near impossible task to conclusively say that a certain NP under certain conditions has no effect at all. Because of these challenges, there is a huge need for more data to be generated and to attempt to define key mechanisms that allow a rapid screening of NP toxicity. The most promising NPs can then be subjected to more in-depth investigations. As mentioned above, the induction of ROS is one such key mechanism to concentrate on. This review focuses on another mechanism that has been associated with various types of NPs, being the modulation of autophagy. In the next part we will discuss the process of autophagy, its involvement in cell death and the main ways to study this process.

3.2. The process of autophagy

Autophagy is an evolutionarily conserved process that through the degradation of cytoplasmic material supports cell preservation in response to various forms of stress. Since overstimulation of this self-degradative process would lead to cell death, a complex signaling network tightly balances the level of autophagy. The present section will focus on the mechanistic steps of autophagy as well as the most relevant triggers influencing this process.

# The efficient degradation of intracellular constituents like proteins is of vital importance for guarding cellular homeostasis because of their role in the regulation of multiple crucial pathways including signal transduction, cell cycle regulation and cytoplasmic quality control. This degradation can occur via multiple cellular pathways including autophagy. Autophagy is a collective term for various selective and non-selective processes comprising microautophagy, chaperone-mediated autophagy and macroautophagy. Microautophagy involves the formation of lysosomal invaginations resulting in a direct and non-specific sequestration of cytosolic components following breakdown.48 In case of chaperone-mediated autophagy, which is a highly specific process, unfolded proteins are recognized and bound by a chaperone complex, resulting in their translocation into the lysosome lumen.49 However, in most cases and also in this paper, the designation ‘autophagy’ refers to the process of macroautophagy.

Macroautophagy, or simply autophagy, is a complex multistep process which is coordinated by key proteins encoded by autophagy-related genes, i.e. Atg genes. In brief, a part of the cytoplasm is sequestered in typical double-membraned vesicles (i.e. autophagosomes) that fuse with lysosomes to form autolysosomes, a process termed autophagy flux. Next, the delivered lysosomal enzymes break down the inner membrane and cargo of the autolysosome (**Figure 1**).50

The process begins with the nucleation of a phagophore for which the recruitment and functionality of a phosphatidylinositol 3-kinase Class III (PI3KCIII) complex is essential. An important component of this multiprotein complex is Beclin-1 (Atg6), which acts as a recruitment platform for other proteins required for autophagosome formation.51 The activity of Beclin-1 is blocked upon interaction with Bcl-2, an important anti-apoptotic protein.52 This interaction is an illustration of the fine regulation of autophagy and the crosstalk between autophagy and other important processes such as apoptosis. After phagophore elongation and simultaneous sequestration of cytoplasm, the vesicle closes to form the typical double-membraned autophagosome, one of the key markers used in autophagy research. For this maturation process, two ubiquitin-like conjugation pathways are needed: *1)* the lipidation of microtubule-associated protein 1 light chain 3 (LC3; Atg8) by the attachment of phosphatidylethanolamine (PE) and its subsequent incorporation into the autophagic membrane, and *2)* The attachment of the Atg12-Atg5-Atg16 conjugate to the phagophore.53 The degradative power of the autophagy pathway is linked with the creation of an auto(phago)lysosome by the fusion of an autophagosome with a lysosome. This autolysosome thus contains the digestive enzymes necessary for efficient breakdown of the autophagosome and its content. Finally, the content of the autophagosome and the inner membrane will be degraded after which permeases can transport the resulting molecules to the cytoplasm, hereby providing the necessary energy and/or building blocks for the *de novo* synthesis of cellular components.50

One key aspect of autophagy is the ability to engulf a portion of the cytoplasm by which it selectively removes certain proteins, pathogens or complete organelles such as mitochondria. This cargo selectivity is mediated by the protein p62/Sequestome-1 that, due to its multiple binding domains, can create a direct link between LC3 and components targeted for autophagy.54 As will be discussed later, p62 is selectively degraded by autophagy and therefore frequently used as a marker for autophagy flux.55

The most reported and actively studied pathway known to coordinate the level of autophagy is focused around the convergence point mammalian target of rapamycin complex 1 (mTORC1), a serine/threonine kinase that inhibits autophagy upon activation by phosphorylation. The phosphorylation status of mTORC1 depends on the action of multiple upstream mediators of which the activity is driven by the nutrient level and energy status of the cell (**Figure 2**).56

Besides performing its housekeeper functions at a basal level, autophagy also serves as a cytoprotective pathway upon activation by triggers that represent a certain form of stress. For example, nutrient starvation leads to autophagy upregulation, promoting the breakdown of less essential cellular components to macromolecules ready to be recycled.57 Invading pathogens can also stimulate autophagy, as a part of the cellular defense, leading to their selective removal.58 Additionally, oxidative stress, by formation of reactive oxygen species (ROS), can also give rise to autophagy, hereby promoting the degradation of the ROS-damaged organelles, typically mitochondria. As mitochondria are the predominant sources of ROS and are also sensitive to ROS-induced damage, they are seen as the main regulators of ROS-induced autophagy.59

3.3. Autophagy and cell death

The process of autophagy has generally been labeled as a pro-survival pathway, however, it has been argued that excessive self-digestion can result in autophagic cell death (ACD).60 Albeit not abundant, there are some studies that report on autophagy performing a pro-death role - as revised in a review by Shen *et al*., 61 which has led to the generation of the term ACD. On the other hand, ACD has long been relatively unexplored, which resulted in different interpretations and considerable misuse of the term. The process of cell death is indeed often very complex and during its progress, damaged cells typically display various markers representative of different cell death pathways such as apoptosis and autophagy. Although morphological changes typical for autophagy can be observed, it usually remains unclear whether autophagy is a true killer, a mere side phenomenon accompanying death or rather an unsuccessful attempt to save the cell.61-62 In order to overcome these issues, the definition of ACD has evolved from merely comprising morphological changes (i.e. autophagic vacuoles) to a list of clear biochemical requirements drafted by the Nomenclature Committee on Cell Death.63 In line with this, Shen *et al.* argue the term ACD is only justified when the observations made meet the following standards: *1)* cell death must be apoptosis-independent, *2)* autophagy inhibition, preferably by the knockdown of minimum two relevant Atg genes,63 prevents the observed cell death and *3)* an increase in autophagy flux is detected.61 Even though these requirements have been brought forward, the discussion remains if even stricter terms should be met and if ACD is a real albeit rare phenomenon or instead merely a misnomer.64 Specialists agree that on top of the above-mentioned criteria, ACD must be a separate death pathway that stands alone, and cases by which autophagy promotes other cell death pathways (e.g. apoptosis) must be excluded.64a As there is so much controversy surrounding ACD, the term should be applied with great care and, at a minimum, should only be used in compliance with the conditions advised by the Committee. In conclusion, it is important for any researcher to understand that autophagy in itself can either be pro-survival or pro-death and that the co-occurrence of cell death and autophagy does not automatically imply that autophagy in itself is leading to cell death.. Interestingly, however, NPs have been introduced as potential inducers of autophagy as well as ACD as will be discussed further in this review.65

Over the last decade it has gradually been revealed that autophagy and apoptosis are interconnected at several levels. Autophagy can influence apoptosis by direct interaction of autophagy proteins with the apoptotic machinery, by autophagic degradation of apoptotic factors and/or by providing a platform for caspase activation. Likewise, apoptotic proteins can affect autophagy through interaction with and/or caspase-mediated cleavage of its proteins. It is thus proposed autophagy can induce cell death by *1)* the dismantling of the cell through self-digestion and *2)* the promotion of apoptosis.66 The cellular decision to activate a certain cell death pathway is likely to depend on the cellular stresses involved; yet, given its role in damage control it is suggested that autophagy affects the onset of cell death. For example, when autophagy is dysfunctional or not able to restore the ATP level, apoptosis or necrosis is initiated.67 It is important to note that cell death is a dynamic phenomenon and multiple cell death types are often co-observed within the same cell.68 As described in section 4 autophagy and apoptosis are often successively or simultaneously detected upon treatment with NMs.

3.4 How to study autophagy

As a result of the increasing interest in autophagy, multiple methods have been developed in the past years to detect autophagic markers of which the most widely examined are LC3, p62 and key proteins of the autophagic machinery. The following section presents a short overview of the most extensively used methods to detect these markers.

Initially, transmission electron microscopy (TEM) used to be the only key technique to detect autophagy, and it still remains an important method today as it can provide highly detailed ultrastructural information, e.g. autophagosomal cargo and potential inclusion of NPs (**Figure 3**).69 TEM allows detection of the distinct steps of the process based on the specific morphology of the autophagic organelles; yet, since autophagy is a dynamic process identifying these structures can be troublesome and thus requires special expertise.70 Newer techniques such as immunoelectron microscopy by means of LC3 immunogold labelling or Correlative Light and Electron Microscopy (CLEM) can conveniently help to avoid misidentification of autophagic structures. Nonetheless, as the instruments are expensive and the sample preparation is technically demanding, epifluorescence or confocal microscopy might be practically preferred.69 The latter can be used to visualize autophagy with dyes such as monodansylcadaverine (MDC), an autofluorescent compound that selectively accumulates in autophagic vacuoles presumably as a result of ion-trapping and/or interaction with membrane lipids.71 Upon autophagy induction the number of MDC-positive vesicles substantially increases. As a result, changes in autophagic activity can be measured by evaluating the number of cells that show MDC-labeled vesicles or the number of positive vesicles per cell.72 Nevertheless, the use of MDC as a specific autophagic dye remains a matter of debate as some studies are in favor of its selectivity,73 while others argue that its staining does not differ from other commonly used acidotropic dyes.74

To date, LC3, which is selectively incorporated into autophagic membranes, has been the most specific and therefore also the most analyzed autophagy marker. Still, whereas it is well established that autophagosomes can be selectively detected by LC3 immunofluorescence or GFP-LC3 transfection, it is necessary to remain cautious since LC3 has also been observed on other vesicles such as phagosomes and macropinosomes.75 As the conjugation of PE to LC3-I (forming LC3-II) during autophagy is accompanied by a mobility shift, the amount of autophagosomes – which corresponds to the ratio of LC3-II/LC3-I - is also easily quantifiable by Western Blotting.76 Accordingly, GFP-LC3 is normally diffusely scattered throughout the cytoplasm while upon autophagy activation autophagosomes can be identified as distinct puncta; an increased number of punctate structures per cell thus correlates with autophagy induction or decreased autophagosomal off-rate (**Figure 4**).76b, 77

We must emphasize that the previously described methods are based on static measurements of autophagosome accumulation and therefore do not allow to differentiate between the induction of autophagy or an impaired autophagy flux, which both result in higher numbers of autophagosomes. An impaired autophagy flux can readily be determined by the above-mentioned LC3 Western Blot experiment in the presence of lysosomal protease inhibitors (e.g. pepstatin A) or buffers (e.g. chloroquine, ammonium chloride) that inhibit LC3-II degradation. If the amount of LC3-II remains the same in the presence and absence of the inhibitor, it can be concluded that the increased level of LC3-II is likely to result from an autophagy flux blockage (**Figure 5**).76a Autophagy flux can also be evaluated based on other Western Blot experiments such as GFP-LC3 cleavage. Since GFP is relatively resistant to lysosomal hydrolysis the amount of free GFP can be detected as a measure of autophagosome degradation.78 In addition to LC3, an increase in the level of undegraded p62 can also indicate a potential blockage of autophagy flux.55a

The turnover of autophagosomes into autolysosomes can be visualized by means of autophagosome-lysosome colocalization studies using LC3-labeling and lysosomal markers (e.g. LAMP-1 or LysoTracker®).77a Likewise, this maturation can be assessed microscopically as well as via flow cytometry using for instance, an mRFP-GFP tandem fluorescent-tagged LC3 (tfLC3). As GFP is pH-sensitive and more prone to quenching than mRFP, the various autophagic vesicles can be distinguished through their different fluorescence signal; autophagosomes will show both signals (i.e. yellow) while after lysosomal fusion GFP is quenched and thus autolysosomes can be identified asmRFP positive vesicles.77a, 79 Flow cytometry can also be applied to determine the total fluorescence intensity of GFP-LC3 as a measure of autophagic activity.80

Turning now to evaluation of signaling events involved in autophagy, studies can focus on assessing the cellular level and/or activity of TFEB, which is a transcription factor that upon activation translocates to the nucleus to interact with lysosomal and autophagy related genes. This nuclear shift can be visualized using labeled antibodies or quantified by Western Blotting after nuclear and cytosolic fractionation of the sample.81 A more autophagy-specific approach would be to evaluate the activity of mTOR and its interacting proteins. This is usually examined to study the effect of various stimuli (including NPs) on the status of autophagy and/or the mechanism behind an observed induction or inhibition. Since the activity of these proteins depends on their phosphorylation status, Western Blotting by means of phospho-specific antibodies is the favored method. In this way the activity of mTOR can be measured directly by analyzing its phosphorylation level or indirectly by determining the activity of its substrates (e.g. p70S6K) or upstream mediators (e.g. Akt).82

Whereas *in vitro* detection of autophagy has seen a positive progress over the past years, *in vivo* methods are not yet thoroughly developed. As a consequence *in vivo* monitoring of autophagy remains limited, albeit there are some methods being suggested such as imaging tissue samples of transgenic mice expressing fluorescently tagged LC3.83 Finally, NMs (i.e. QDs) have also been proposed as tools to detect changes in autophagy.84

3.5 Chemical modulation of autophagy

When evaluating the influence of NPs on autophagy it is essential to include controls that help to reliably verify and interpret observed changes in autophagic activity. Here, we will give a summary of the multiple known autophagy-modulating chemicals and conditions widely used in autophagy research.

The most extensively applied chemical inducer is rapamycin, which directly inhibits mTORC1 and thus stimulates autophagy.85 Still, it is essential to note that the working dose should be cautiously selected since rapamycin at relatively high dosage can inhibit autophagy flux and hence lead to misinterpretation.86 More natural stimuli of autophagy are serum starvation and amino acid deprivation, which can be used as a positive control in studies with the aim of identifying autophagy inducers (e.g. NPs).78b

3-Methyladenine (3-MA) and Wortmannin are both known to negatively regulate autophagy through inhibition of PI3K,87 and are thus commonly used to identify the role of autophagy in NP-induced cell death. However, also in this case the experimental conditions should be well optimized because for example prolonged periods of their administration can lead to enhancement of autophagy flux being the exact opposite of what we aim for.87 Furthermore, chemicals that influence lysosomal activity by alkalinization of its acidic lumen (e.g. chloroquine)88 or inhibition of lysosomal enzymes (e.g. pepstatin A) can inhibit lysosomal fusion and/or block the breakdown of the sequestered cargo in the autolysosome.

A general issue seen with these chemical modulators, especially with those just described, is their lack of specificity for autophagy. Indeed, they influence other cellular processes that can indirectly affect autophagy. It is therefore recommended to combine chemical modulation with other approaches such as genetic inhibition or functional knockdown of relevant Atg genes.78b, 89

In conclusion, to study the complex and dynamic process of autophagy a number of different assays and detection techniques are required to accurately and reliably link certain effects to the modulation of autophagy. It is however necessary to comprehend that assays based on autophagosome detection not necessarily provide information on the status of autophagy flux. Also, since each of the above-described methods has its advantages and flaws, it is strongly advised to combine several complementary assays. For a more extensive overview of the various applied methods and accompanying guidelines we therefore refer the reader to other recent reviews.77a, 78b

**4. Nanomaterial-induced autophagy**

4.1 Modulation of autophagy by soft particles

Compared to the substantial amount of literature describing metallic NP-induced autophagy, the evidence for polymeric and lipid particles remains rather limited. However, several reports have been generated, indicating that also these materials are capable of modulating autophagy.

4.1.1. Modulation of autophagy by liposomes.

Induction of autophagy has been observed in HeLa cells upon treatment with dioleoyltrimethylammonium propane (DOTAP), a cationic lipid commonly used as a transfection agent. The results not only suggest that DOTAP enhances autophagosome formation but also demonstrate that the induction is mTOR-independent. The autophagy activation may be caused by the fact that DOTAP is a synthetic lipid and the cell undergoes problems degrading it. As a result, the cell aims to increase its total degradative capacity, that is by the induction of autophagy. Naturally, the fact that transfection agents as such are able of enhancing autophagy casts doubt on observations made in transfected cells, particularly if autophagy is the process examined. At the same time, this implies that inhibition of autophagy might aid to improve transfection efficiency.90 The latter hypothesis was corroborated by Roberts *et al.* who found that cationic liposomes were delivered to the autophagic pathway through endosome-autophagosome fusion, indicating a cellular attempt to eliminate the foreign material. In addition, gene delivery and expression was remarkably enhanced in autophagy-defective Atg5-/- cells.91 Interestingly, treatment with uncharged lipids (i.e. dioleoylphosphatidylethanolamine; DOPE) failed to alter autophagy.90 A finding that is in contrast with the ability of neutral PEGylated C6-ceramide nanoliposomes to activate fully functional autophagy in liver HepG2 cells.92

4.1.2. Modulation of autophagy by polymeric NPs

Autophagy modulation was also observed upon treatment of macrophages with positively charged polymer (Eudragit RS) particles. In this case TEM showed significant localization of particles inside or in contact with mitochondria. Furthermore, substantial signals of oxidative stress were observed. The authors thus propose that the cell aims to remove the damaged mitochondria by triggering autophagy followed by apoptosis-independent cell death.93

Cationic polyamidoamine (PAMAM) dendrimers of multiple generations (G3 to G8) have been proposed to induce autophagy in A549 lung cancer cells with involvement of the mTOR pathway. However, it was not specified if the increased level of LC3, assessed by microscopy and Western Blotting, was caused by an enhanced on-rate or decreased off-rate of autophagosomes, therefore, an autophagy blockage cannot be excluded.94 As PAMAM dendrimers have been reported to cause lysosomal alkalinization,95 potentially resulting in lysosomal impairment, a blockage of flux is rather likely. On the other hand it has been put forth that these dendrimers can affect mTOR activity during their endocytic uptake; the observed autophagy alteration could thus be a combination of multiple effects. Intriguingly, comparable with the conflicting findings obtained with differently charged lipids, anionic G5.5 PAMAM particles did not affect autophagic activity. Together, these data suggest that charge may have an impact on the autophagy-inducing potential of NPs, however, without any uptake comparison between the various particles it cannot be excluded that this is merely because of differences in uptake efficiency.

An elegant study by Wang *et al.* described a time resolved analysis of the effect of amine-conjugated polystyrene (PS) NPs on lysosomal health (**Figure 6**). They displayed flow cytometry data revealing two populations based on scatter plots and LysoTracker® fluorescence intensities. The authors hypothesized that the high intensity population exhibit enlarged lysosomes while the low intensity population represents cells with damaged (burst) lysosomes. In the latter population enhanced ROS and compromised mitochondrial membrane potential (MMP) was indeed detected, which is in line with their theory of lysosomal cathepsin release upon NP-inflicted lysosomal damage. The mechanism underlying this damage was reported to be the degradation of the protein layer surrounding the particles after endocytosis and concurrent damage to the lysosomal membrane. Furthermore, an accumulation of autophagosomes was observed and although no flux experiments were performed, the well-established link between lysosomal impairment and autophagy dysfunction suggests a flux blockage is probable. On the other hand, autophagy served to boost survival since co-treatment with 3-MA decreased ATP content and enhanced caspase activation. However, multiple apoptotic markers were detected after NP exposure, suggesting autophagy was unable to restore the disturbed homeostasis (**Figure 7**).96

4.2. Modulation of autophagy by hard nanoparticles

4.2.1. Gold Nanoparticles

As mentioned in section 4.1, there is an increasing amount of literature providing evidence for autophagy modulation by hard NPs.97 As a leading example, several research groups have shown alterations in autophagic activity upon treatment with different types of Au NPs. Ma *et al.* demonstrated an Au NP-mediated mTOR-independent accumulation of autophagosomes owing to a blockage of autophagosome degradation. The lysosomal impairment elicited by these gold particles, demonstrated by lysosomal enlargement and alkalinization, probably accounts for this blockage. Interestingly, in line with the observed size-dependent uptake, larger particles (50nm) were more potent autophagy flux disruptors compared to their smaller equivalents (10 and 25nm). Next to size preliminary results further uncovered a potential charge-dependent autophagy response with more autophagosome accumulation upon treatment with positively charged NP compared to equally sized negative ones.98

The ability of Au NPs decorated with Simian Virus 40 (SV40) peptides to modulate autophagy was proven to be dependent on their potential to block nucleocytoplasmic shuttling. As for cells treated with NPs incapable of blocking this transfer, no indication of autophagy was observed.99 Several groups reported on autophagy stimulation triggered by oxidative stress induced by the NPs. FBS-coated Au NPs generated significant signs of oxidative stress in human lung fibroblasts, a probable cause of the simultaneously observed autophagosome accumulation.100 Oxidative stress was also detected in oral cancer cells in the presence of iron core-gold shell particles (Fe@Au), although this was not the primary cause of NP toxicity. Notably, these particles provoked different levels of autophagy in cancerous and benign cells which further led to the hypothesis that the concurrently observed selective growth inhibition is caused by a different reaction of the cancerous and healthy mitochondria toward the induced stress.11a NPs of comparable composition, i.e. gold-coated iron oxide NPs, activated autophagy in multiple cell types. Moreover, upon conjugation to anti-EGFR antibodies, autophagy upregulation by these particles was successfully limited to EGFR positive cells.101

4.2.2. Iron oxide nanoparticles

Analogous to the Fe@Au NPs, bare IONPs selectively induced cytotoxicity in lung cancer cells while causing a minor decrease in cell viability in normal lung fibroblasts. The origin of this differential toxicity was suggested to be oxidative stress and subsequent autophagy upregulation via the AMPK-Akt-mTOR pathway, which was supported by the observed mitochondrial damage and ATP depletion.11b Bare IONPs also provoked oxidative stress in macrophages and human cerebral endothelial cells, although the by the authors proposed autophagy induction conflicts with the increased and unchanged level of p62, respectively.102 Finally, IONPs were suggested to induce autophagy in other cell types yet this was, in our opinion, insufficiently demonstrated.103

4.2.3. Quantum Dots

QDs of multiple compositions have been repeatedly advanced as autophagy activators. Again, the stimulation of autophagy is regularly put forth as a response to oxidative stress, although, at the same time there are discrepant results between different studies.81b, 104As an illustration COOH-functionalized CdSe/ZnS QDs were able to provoke ROS-dependent LC3-II accumulation, also, a ROS scavenger as well as 3-MA enhanced cell death indicating autophagy serves as a protective mechanism against QD cytotoxicity.105 The latter finding is in contrast with a study conducted with similar COOH-conjugated CdTe QDs by which 3-MA could reduce NP-induced apoptosis, suggestive of a pro-death role for autophagy.104d Secondly, despite the observed QD – autophagosome colocalization and increase in LC3-II in both studies, Stern *et al.* did not detect significant oxidative stress nor apoptosis,106 while treatment of human glioblastoma cells with photoexcited graphene QDs resulted in both substantial ROS as well as apoptosis **(Figure 8)**.104b

These graphene QDs along with streptavidine-coated core-shell ones were found to induce autophagy rather than impair autophagy flux. It was further noted that the induction of autophagy by streptavidin-coated QDs could be abrogated by antioxidant treatment.104c For graphene QDs, ROS-dependency was also suggested, yet this was not experimentally demonstrated.104b

Neibert *et al*. showed that treatment with uncapped CdTe QDs significantly activated TFEB, which they suggest is a cellular attempt to support the lysosomal and autophagic system that aims to remove the damaged cytoplasmic material generated by QD treatment.81b Remarkably, Seleverstov *et al.* further reported that smaller QDs modulated autophagy more extensively than their larger counterparts.104a Another example of size-dependent autophagy alteration was reported with neodymium oxide particles with non-nanoscale particles being less effective.107 In contrast to this finding, micro-sized polystyrene particles did induce authentic autophagy.108 Together, these studies imply that the extent of autophagy activation may depend on particle size. This size-dependency is however likely to be explained by dissimilarities in uptake levels of the particles and the different surface area to volume ratio of the various nano-sized materials.

4.2.4. Zinc oxide nanoparticles

A ROS-dependent rise in autophagosomes was detected upon treatment of normal skin cells with rod-shaped ZnO NPs. Indeed, treatment with ROS-scavengers resulted in a decrease in autophagic vacuoles and markers (e.g. Atg5). ZnO toxicity was also associated with mitochondrial damage and ATP depletion potentially resulting in the observed autophagy modulation.109 A study on the involvement of autophagy in the photocatalytic toxicity of ZnO nanorods showed that the degree of stress induced by ZnO NP toxicity influenced the role of autophagy in cell death. When cellular (oxidative) stress was limited, i.e. by exposure to NPs or UVA-1 treatment, autophagy prevented cell death. Instead, cell death by combination treatment could be partially aborted by 3-MA, indicating autophagy was at least partly responsible for the observed cytotoxicity.110 In contrast to the two previous studies, cytotoxic spherical ZnO NP failed to elicit autophagy in human colon cancer cells, suggestive of a shape-dependent effect, or discrepancies due to intrinsic differences in the various cell types used in the respective studies.111

4.2.5. Carbon-based nanomaterials

Various types of carbon-based NPs have been shown to alter autophagy. For instance, carboxyl functionalized carbon nanotubes (CNT) affected autophagy in an mTOR dependent manner in A549 cells, conversely, differently functionalized particles (with poly(m-aminobenzenesulphonic acid or PEG) did not. Even so potential differences in uptake by the different CNTs were not examined, this does suggest that surface group characteristics may influence the impact of NPs on autophagy modulation. Interestingly, besides restoring cell viability *in vitro,* pre-treatment with 3-MA partly abrogated NP-mediated lung inflammation in mice indicating a role for autophagy in lung toxicity.112

Furthermore, a detailed study of Wan *et al.* demonstrated that acid-functionalized SWCNT and graphene oxides caused autophagosome accumulation by compromised autophagy flux in primary murine peritoneal macrophages. The underlying mechanism was clarified by means of LysoTracker staining and FITC-dextran labeling of NP treated cells, which revealed decreased lysosomal quantity and lysosomal membrane damage, respectively;113 surely, as described above, it is well established lysosomal health strongly influences autophagy.114 It is noteworthy that, despite their comparable chemical composition and surface functionalization, CNT and graphene oxides affected autophagy to a different extent. This suggests physical characteristics might also influence autophagy modulation, yet a more thorough study is necessary to substantiate this.113 The effect of graphene oxides on macrophages was evaluated by Chen *et al.,* who demonstrated for the first time that NPs could trigger autophagy with the involvement of TLR signaling. Indeed, silencing of e.g. TLR4 and TLR9 reduced the abundance of LC3 puncta and beclin-1 aggregates as assessed by immunofluorescence microscopy.115

Fullerenes have been correlated with autophagy induction as well as dysfunction.113 For example, fullerene (C60) and Neodymium functionalized fullerenes (C60(Nd)) are presented as autophagy inducers,116c, d while Johnson-Lyles *et al.* suggest fullenerol NP can perturb autophagy at high concentration. They hypothesize the observed NP-mediated cytoskeleton disruption results in autophagy dysfunction and consequently ATP depletion.116b

4.2.6. Other hard nanomaterials

A study describing the response of A549 cells and macrophages toward diversely shaped silica (SiO2) NP treatment reported that the cell type but not the geometry of the particles shaped this response.117 Interestingly, a total of three studies carried out in A549 cells describe the cytotoxicity of SiO2 NPs as apoptosis-independent, although autophagy was only observed in two of those studies.117, 118

A great variety of rare-earth element based NPs were described to induce authentic autophagy in HeLa cells.116 Among these studies there was a remarkable example of how surface group characteristics can influence the autophagy-inducing potential of a NP. This was presented by Zhang *et al.,* who were able to adapt the level of induction upon treatment with lanthanide-based upconversion NPs by coating them with different peptides (**Figure 9**). By affecting the sedimentation and cellular interaction of the NPs, this peptide coating allowed for autophagic tuning.120

Not only surface characteristics but also chemical composition has been brought forward as a way of tuning autophagy. Treatment of HeLa cells with Nickel-Cobalt NPs with different molar concentrations of both components revealed that the higher the Ni component, the more potent the impact on cytotoxicity and autophagy. Moreover, autophagy was involved in NP-mediated toxicity since treatment with 3-MA significantly restored cell viability.121

Several groups have also reported on cellular responses to copper oxide (CuO) NPs. In respiratory cell types the by CuO elicited autophagy served as a pro-death mechanism and apoptosis was not apparent.118b, 122 Instead, in breast cancer cells autophagy aimed to protect the cell and its inhibition triggered apoptosis.123 Finally, there is even more literature available on various types of NPs that are able to alter autophagy in a variety of cell types including titanium dioxide (TiO2), silver nanowires and palladium particles (**Table 2**).124 However, this review intends to summarize the most relevant findings and mainly focuses on the impact of physicochemical parameters on autophagy modulation besides looking into common effects or discrepancies found in literature. In section 6.2 we will focus more thoroughly on the proposed mechanisms lying at the root of the observed cancer cell selectivity of certain NPs.

4.2.7. Physiological effects of nanoparticle-mediated autophagy modulation

An *in vivo* study in rats aiming to identify the role of autophagy in AgNP-mediated hepatotoxicity elegantly characterized the connection between changes in autophagy, apoptosis and ATP depletion (**Figure 10**). After one day of AgNP administration the authors observed a considerable decrease in ATP levels in the liver, likely because of damage to hepatic mitochondria, while at the same time autophagic markers were significantly increased. This autophagy induction is probably an attempt to remove these dysfunctional organelles and restore the reduced energy levels. However, since autophagy failed to compensate for this energy drop, the level of autophagy decreased in time in favor of apoptosis - which became the predominant mechanism.125 These data reveal the complex interplay between the different pathways and highlight the important protective role of autophagy. In cultured cells, the interrelationship between the different factors is altered due to the lack of complex animal physiology. *In vitro*, high levels of autophagy may therefore persist longer, which can then result in ACD.

4.3. Influence of nanoparticle characteristics on autophagy deregulation

Table 2 provides a full overview of the different types of NMs that have been described to be associated with autophagy deregulation. Still, the wide variety in NMs and different experimental setups hinders a clear understanding of how NPs can result in autophagy induction. At the same time, the notion that such widely differing NMs can elicit similar effects on autophagy does suggest that autophagy might be a general response toward NPs. It is however noteworthy that multiple examples have led to the conclusion that the precise composition of NPs does play a role in the extent of autophagy and its final outcome, as for instance shown for P-VO2 and Y2O3 NPs, where the former resulted in pro-survival autophagy and the latter in pro-death autophagy.124b

In order to understand the impact of the various NP-associated parameters on autophagy, it is essential to conduct in-depth studies that focus on the autophagy-modulating potential of a small set of NPs that differ from each other in only a single physicochemical property.126 This is undoubtedly a challenging task, as altering one property (e.g. surface charge) more than often simultaneously influences several other factors (e.g. hydrodynamic size, colloidal stability),127 by which our ability to link these parameters with the observed cellular effects is limited. Next to the need of a systematic experimental setup, the latter issue underscores the importance of ongoing research on controllable synthesis of NPs as well as the relevance of extensive NP characterization.47

Throughout the previous sections several NP properties were put forth as probable influencing factors on of NP-mediated autophagy deregulation, that is, size, shape, surface group and charge, and chemical composition. For most NPs, the extent of autophagy is presumably determined by a complex interplay of these different parameters. Based on the available data on the mechanisms by which NPs can induce autophagy, any change in NP physicochemistry that affects at least one of the following aspects is likely to alter its influence on autophagy: *1)* Autophagy has been associated with oxidative stress, therefore, the capability of NPs to generate this type of toxicity can be linked to the level of autophagy. As metal-oxide particles or heavy metal-containing NPs are generally more prone to inducing oxidative stress,128 those may have high autophagy-modulating properties. *2)* Autophagy alterations have been associated with lysosomal dysfunction and a decrease in cellular degradative capacity. For this reason, non-degradable NPs (for instance Au NPs) or formulations containing compounds that cannot be efficiently metabolized (e.g. cationic lipids) that are taken up by endocytosis at high doses are more likely to result in autophagy induction. *3)* Autophagy is a cellular response to the NP-induced damage and is consequently closely related to intracellular NP levels. In line with autophagy modulation, the uptake efficiency of NPs is influenced by the following parameters: *a)* In terms of surface coating, positively charged ones will generally promote NP uptake while PEG functionalization will hinder NP-cell interaction and thus impede cellular uptake.127, 129 Logically, surface coating can influence colloidal stability, as formation of larger aggregates will also limit internalization.130 NP coating can also have an impact on the ROS-inducing ability of the NPs thus controlling their ROS-mediated autophagy modulation. *b)* Along with surface coating, NP size also plays a complex role in the deregulation of autophagy. It is postulated that NPs of 40-50 nm result in optimal cellular uptake efficiency, however, despite their limited internalization, larger NPs may have a greater effect on the lysosomal degradative potential than a larger number of smaller particles, which can be more readily degraded. Alternatively, the total surface area of all internalized NPs combined will be substantially higher for small NPs than for larger ones owing to their higher surface over volume ratio.131 This higher surface area can then result in raised levels of oxidative stress that can in turn influence autophagy. This aspect of size versus surface area clearly illustrates that it is often hard to predict the impact of NP modification on NP-cell interaction.

In conclusion, it is clear that as autophagy can be modified by various mechanisms, most parameters can affect autophagy by multiple ways. More detailed comparative studies are therefore required to shed more light on the intricate interplay between the various NP properties and the manner by which they influence autophagy.

4.4. Mechanisms of autophagy induction by nanomaterials

There is an increasing amount of literature discussing NP-mediated effects on autophagy. Indeed, NPs in general have been suggested as a new class of autophagy activators affecting it through various pathways such as oxidative stress.10 Generation of ROS has been described to be one of the main causes of cytotoxicity for a wide variety of NPs and is thus proposed as a potential universal byproduct of NP exposure.40a, 132 NPs can provoke oxidative stress through multiple interactions, being: *1)* Interaction of NPs or intrinsically formed ROS species with mitochondria can induce mitochondrial membrane damage, resulting in the disruption of the MMP and respiratory chain. As the latter is one of the main sites for the generation of ROS, any perturbation of this electron transport chain can result in increased ROS production. Damaged mitochondria can next directly stimulate autophagy in an attempt of the cell to remove the dysfunctional organelles in order to preserve cytoplasmic homeostasis.105, 109, 133 *2)* Direct interaction with cytoplasmic enzymes that act in maintaining cellular redox potential. *3)* Interaction of NPs with cell surface receptors leading to the activation of intracellular signaling cascades that induce the formation of ROS.40a Furthermore, degradation of the NP coating and core in the lysosomal environment can directly induce ROS by means of any byproducts created or the presence of a bare (reactive) NP surface in an acidic environment. Besides direct ROS generation NPs can release redox active metal ions (e.g. Fe2+) that participate in ROS-generating reactions (e.g. Fenton reaction).132, 134

This ROS formation can next damage the entire cytoplasmic environment including organelles, proteins and lipids. As a result, autophagy will be activated to attempt to restore this stressful situation by removal of the respective components. It is important to note that not only the secondary effects of ROS (e.g. mitochondrial damage) but also increased levels of ROS as such are able of tuning the level of autophagy by altering the activity of different intracellular signaling molecules.59, 133, 135 Evidence is accumulating on the role of ROS on autophagy, whereby autophagy has been shown to be regulated by different types of ROS, and ROS-mediated autophagy is involved in various pathologies, including cancer.136

Since the majority of NMs enter the cell through endocytosis, the lysosomes are also frequently a target for their toxicity. NMs can cause lysosomal dysfunction by alkalinization of its lumen, NP overload or cytoskeleton disruption.96, 138 This dysfunction can indirectly upregulate autophagy as a mechanism for the cell to compensate for insufficient degradative capacity. The signaling link between lysosomal sensing of stress and autophagy is effected by Transcription Factor EB (TFEB), a main regulator of the Lysosomal Expression and Regulation (CLEAR) network.137 Upon starvation and lysosomal stress TFEB will detach from the sensing machinery present on the lysosome and translocate to the nucleus, where it will aid to the transcription of lysosomal and autophagic genes.81

Lysosomal dysfunction by the presence of non-degradable NPs can have wide-ranging consequences. Indeed, next to their pivotal role in the degradation and recycling of macromolecules, lysosomes are also involved in essential cellular processes such as plasma membrane recycling and cell death. It is therefore not surprising that, comparable with autophagy, disruption of lysosomal health is associated with a wide variety of pathologies.139 Alternatively, it has been argued that autophagy assists in preserving genomic stability by the regulation of cell fate after DNA damage and even plays a role in micronuclei degradation,140 which are both processes that have been shown to be modulated directly and/or indirectly by cell-internalized NPs.141 NPs can also directly influence the mTOR pathway or gene expression of relevant autophagy genes.136 It has further been hypothesized that NPs can be directed toward autophagic degradation in a manner similar to pathogens and cytoplasmic material. In practice, this involves NP ubiquitination and binding of p62 that finally links the NPs to the autophagic machinery.79b, 142 Accordingly, autophagy induction might be a way to try to eliminate these foreign particles.

The above-described pathways of NP-mediated changes in autophagy are schematically depicted in **Figure 11**.

**5. The dangers of autophagy modulation**

The above-described modulation of autophagy by NMs could possibly be exploited in numerous applications. Nonetheless, as with every modulation of a tightly regulated process, there are some potential dangers to keep in mind. It is important to recall that autophagy is a crucial pathway for the clearance or turnover of unwanted (e.g. aggregation-prone proteins, mitochondria, pathogens) or superfluous cytoplasmic material and is therefore of vital importance for maintaining cellular homeostasis. For example, the removal of damaged or toxic cytoplasmic material can prevent genomic instability. Consequently, insufficient or defective autophagy hinders this housekeeping role, a condition that is argued to lie at the origin of multiple pathologies including neurodegenerative and non-neurodegenerative diseases.143 The following sections will focus on the role of autophagy in neuropathologies and cancer, although autophagy alterations have been associated with a variety of other diseases such as myopathies,144 auto-immune diseases,145 and metabolic diseases.9 Unfortunately, this may also imply that NPs capable of disrupting autophagy may result in or contribute to the development of these pathologies. NPs can impede autophagy by for instance directly damaging the autophagosomal and/or lysosomal compartment through ROS,96 by blocking autophagosomal-lysosomal fusion by affecting lysosomal activity (*via* e.g. alkalinization)98 or perturbing the cytoskeleton.146

In context of disease, it is yet again pivotal to note the complexity of the autophagic process, and that the impact of autophagy deregulation on cell or animal physiology can vary and is even hard to predict. Therefore, it is important to comprehend the precise mechanisms involved and to discover the most optimal ways of controlling the outcome of any alteration in autophagy regulation.

For example, pro-survival autophagy can have beneficial effects on both healthy cells (decreased nanotoxicity or enhanced immune reactivity by improved presentation of antigens in dendritic cells)105, 147 and diseased cells (ameliorated clearance of dysfunctional organelles or protein aggregates in myopathies or neurodegenerative diseases). Pro-death autophagy can furthermore result in direct clearance of tumor cells or sensitize them toward chemotherapy. The following sections provide a brief overview of the thus far uncovered links between autophagy and several pathologies and discuss the dangerous ambiguity in final cellular or physiological outcome that can result from autophagy deregulation.

5.1. Autophagy in neurodegenerative diseases

Two theories form the basis of the potential link between NMs and neurodegenerative diseases. Firstly, epidemiological studies have revealed that exposure of humans to polluted air, which contains several types of NPs, is highly associated with the prevalence of neuropathologies including Alzheimer’s (AD) and Parkinson’s (PD) disease.148 However, in order for the NMs to be able to affect the brain, they must be capable of passing the blood-brain barrier (BBB). Even so an intact BBB seems effective in preventing the translocation of NPs to the brain as demonstrated for a variety of engineered metal NPs of multiple sizes,149 several groups working on brain drug delivery by NMs report that they can be specifically designed for this translocation by carefully controlling the architecture and physicochemical properties of the NPs. Successful strategies include: *a)* the limitation of NP size below 100 nm, *b)* positive surface charges to enhance electrostatic interaction with endothelial cells lining the BBB, and *c)* the use of surfactants, growth factors or small molecules such as insulin or transferrin that can bind transport molecules naturally present on the BBB.150 The notion that specific NPs can cross the BBB implies that it is plausible that also other engineered NPs can enter the central nervous system while this is intrinsically not desirable. In this regard, several studies have indicated that different types of systemically administered NMs damage the BBB, inducing leakage and higher permeability toward proteins and the NPs themselves, finally resulting in neurotoxicity.151 It is furthermore postulated that upon inhalation or nasal installation the nanoscale size permits NPs to bypass the BBB by nose-to-brain transport via olfactory nerves after which they can penetrate further into the brain.149, 152 Next to the olfactory nerve, it has also been suggested that a similar NP translocation mechanism can take place via sensory nerve endings.153 Together, the above-mentioned findings substantiate that the proposed correlation between NP-containing pollution and neuropathologies is indeed plausible.

Secondly, increasing evidence shows that autophagy alterations may lie at the root of neurodegenerative diseases.154 This evidence includes an observed accumulation of autophagic vesicles in the brains of AD, PD as well as Huntington’s disease (HD) patients.155 Moreover, neurodegeneration and an elevated level of protein aggregation was detected in mice with neuron-specific knockdown of key autophagy proteins (i.e. Atg5, Atg7 and beclin-1).156 It is further argued that autophagy is likely to be responsible for the removal of htt, -synuclein and -amyloid, as such a reduced level of autophagy would result in an accumulation of these harmful proteins that form the basis of respectively HD, PD and AD.155 In summary, these observations present autophagy as a protective mechanism against the accumulation of toxic protein aggregates and therefore also against the diseases to which they give rise; logically autophagy malfunctions may then result in neuropathology. The sensitivity of neurons toward autophagy deregulation is not that surprising, as it is plausible that autophagy may be even more essential in quiescent cells where unwanted cytoplasmic material cannot be diluted by cell division.154, 157

At first sight the different pathologies arise from a similar malfunction, being autophagy deregulation. However, there are distinct autophagic impairments for each disorder demonstrating the complexity of the autophagic process as well as the respective disorders. For example, in HD the predominant issue seems failed cargo recognition,154, 158 while in AD and PD lysosomal abnormalities hinder efficient autophagosome maturation.155, 159 Strikingly, there is evidence that -amyloid, the protein involved in the onset of AD, is formed in autophagic vacuoles which questions the protective role of autophagy.160 For a full scope of the impact of specific autophagic defects on the pathogenesis of these diseases we refer to a recent review of Nixon.154

As the link between some types of NMs and autophagy is well accepted, these findings support the hypothesis of neurotoxic danger associated with NM-mediated autophagy modulation.161 This hypothesis was substantiated by several studies concerning NP-autophagy interactions in neurons.104c, 151e, 162 Chen *et al.* observed brain accumulation of alumina NPs after their administration in the cerebral circulation of mice. There the NPs reduced ATP content, diminished tight junction protein expression and enhanced BBB permeability. Furthermore, treatment of human cerebral microvascular endothelial cells with nano alumina showed mitochondrial damage as well as autophagy modulation.151e Another group reported that CdSe/ZnS QDs induced autophagy-dependent synaptic dysfunction in mouse brains after intrahippocampal infusion.104c

These studies indicate that NP-mediated autophagy modulation can potentially pose a risk for neurological health and caution is advised. However, the involvement and nature of autophagy deregulation in the pathogenesis of the above-described diseases needs further investigation as to be able to draw reliable conclusions regarding the real neurological dangers of NMs and to eventually efficiently target autophagy as a therapeutic strategy. Clearly, there is a great need to delineate the molecular mechanisms by which the various NMs influence autophagy as distinct alterations in the autophagic pathway may give lead to a different extent and/or type of toxicity.

5.2. Autophagy and cancer

Autophagy is a hot topic in cancer research, where it has been associated with tumor suppression as well as with promotion of tumor survival and tumorigenesis. It is crucial to note that depending on the context autophagy impairment or upregulation may have distinct consequences and accordingly, therapeutic strategies targeting autophagy should be adapted to its role at a particular tumor stage and/or cancer type.8, 163 In general the role of autophagy in cancer is usually described in terms of two cancer stages: tumor initiation and progression of actual tumors (**Figure 12**).

In an early stage autophagy mainly acts as a tumor suppressor preventing carcinogenesis.164 This function is logically based on its role in cellular homeostasis and damage control during stress. Indeed, autophagy inhibition by chloroquine in rats subjected to hepatocarcinogenesis increased ROS, DNA damage, genomic instability, cell proliferation and expression of inflammatory mediators (e.g. TNFα and IL-6); all of which are conditions that promote tumor formation. The protective role of autophagy against tumor development was further substantiated by the notion that only 30% of the rats with fully functional autophagy developed liver tumors compared to 90% in chloroquine treated rats.165 Furthermore, it is argued that upregulation of p62, a protein degraded by autophagy, can activate Nrf2 proangiogenic signaling as well as proinflammatory NFκB signaling thus forming a tumor creating environment.166 Based on these observations, it is not surprising that autophagy induction has been brought forward as a way of preventing carcinogenesis.164, 167

Similar to the cytoprotective function in benign cells, which serves to our advantage, in established tumors autophagy might serve as a cancer survival pathway aiding the cells to overcome several stressors such as hypoxia, starvation or even chemotherapy.164b, 168 Multiple studies have indeed described autophagy induction in various cell types in response to the oxidative and metabolic stress induced by anti-cancer therapy finally resulting in therapy resistance.169 In many cases autophagy-mediated enhanced survival is likely to be based on its ability to maintain a healthy mitochondria pool and its support of the energy balance of the cancer cells.164b, 170 Moreover, several cancer cell types (e.g. RAS-activated) are highly dependent on autophagy and a further induction of this pathway may inadvertently promote tumor growth.171 By assisting cancer cell health autophagy might even support metastatic attempts by for instance, preventing anoikis (detachment-mediated cell death).164a, 172 In this stage induction of autophagy would therefore not have the desired therapeutic effect, but rather pose serious dangers on tumor progression. However, even so in this context autophagy inhibition is presented as a way to combat cancer and chemoresistance,170, 172 in the context of apoptosis resistance, eradication of cancer is also being evaluated by the induction of ACD by overstimulation of autophagy, as discussed in section 6.

It is noteworthy that autophagy in cancer cells not only influences the respective tumor cells but also various cell types in the tumor microenvironment, for instance immune cells. Recent evidence proposed that autophagy can serve as a secretion system for several immunological relevant factors (e.g. IL-1β) and thus can modulate the microenvironment and at the same time the immunogenicity of the tumor.164a, 173 One of these studies demonstrated that only cancers exhibiting functional autophagy attracted immune cells, which indicates that autophagy is indispensable for creating an anti-tumor immune response. This was based on the observation that autophagy is responsible for the pre-mortem production and subsequent release of ATP, a feature of immunogenic cell death.174 Yet again the role of autophagy seems ambiguous since it has also been associated with immune evasion by preventing the tumor infiltration of immune cells through decreased production of chemokines.164a

Given the complexity of the autophagy process and its involvement in tumor suppression as well as promotion, the process must be tightly controlled in order to rule out the possible dangers of autophagy modulation.164bAccordingly, more research is necessary to further elucidate the effect of autophagy on cancer before we can safely use it as an anti-cancer target. For a detailed overview of the knowledge of this dual role for autophagy we refer the readers to a recent review by Maes *et al*. 164a

**6. The possibilities of nanomaterial-induced autophagy**

6.1. Selective destruction of cancer cells

6.1.1. Selective autophagy in cancer cells

Autophagy induction is currently receiving a lot of interest as a potential tool in cancer therapy.175 One key aspect in this research is the selective induction of autophagy and the concurrent selective destruction of cancer cells with minimal effects on non-cancerous cells. It has been proposed that this selectivity would be based on a different sensitivity of cancerous and benign cells toward oxidative stress, as reported by Chen *et al*.. They detected significant cell death and and autophagy activation upon treatment of HeLa, HEK and U87 cancer cells with H2O2 and 2-methoxyestradiol (2-ME). Cytotoxicity was furthermore dependent on autophagy since functional knockdown of several relevant autophagy genes considerably improved viability. Additionally, stable overexpression of superoxide dismutase-2 (SOD2) efficiently diminished ROS species and autophagy in HeLa indicating ROS are a primary trigger of autophagy. In contrast to cancerous cells, treatment of primary mouse astrocytes with H2O2 and 2-ME evoked no substantial autophagy activation as examined by TEM, GFP-LC3 dot formation and LC3-I conversion.176

This selective autophagy modulation could be of high clinical importance, since a lack of selectivity and thus unwanted side effects is a major issue of current anti-cancer therapies. The various papers reporting differential toxicity of NPs between cancer cells and normal cells do not describe a fully elucidated mechanism, however, it is noteworthy that ROS are repeatedly suggested as a prime cause. This is in line with the following observations: *1)* oxidative stress is one of the most acknowledged toxicological effects associated with cellular NP exposure 40a, 132 *2)* cancer cells are known to have higher basal ROS levels than healthy cells,177 and *3)* it is established that ROS serve as important regulators of autophagy.59, 178

6.1.2. Cancer-specific induction of autophagy by nanomaterials

As a leading example Khan *et al*. showed that bare IONPs elicited a significant level of ROS in A549 cells, which subsequently resulted in a cascade of responses.11b They suggest that oxidative stress provokes a decrease in MMP following ATP depletion and autophagy stimulation. Their proposed mechanism is indeed plausible, as it is mechanistically known that either mitochondrial damage or ATP depletion can elicit autophagy activation.179 Cancer-selective induction of autophagy was based on the fact that the observed increase in autophagy and concurrent cytotoxicity was only significant in A549 lung cancer cells and not in normal lung fibroblasts. Interestingly, it seems autophagy indeed played a pro-death role since its inhibition by 3-MA nearly fully restored cellular viability.11b

Similar cancer cell selectivity was observed *in vitro* and *in vivo* upon treatment of oral cancer versus normal cells with gold-coated iron NPs (Fe@Au). It was specified that only the iron constituent of the particles, and more specifically its reduced form, exhibited significant toxicity. The authors therefore suggest that differences in the cellular microenvironment between malignant and nonmalignant cells (for instance, the lower pH that typically accompanies tumors) may influence iron oxidation status and at the same time its toxicity.134 The effect of NP exposure on mitochondrial health was further examined in a follow-up study,11a which revealed that Fe@Au treatment permanently reduced MMP in cancerous cells while in normal cells mitochondrial recovery was observed after 4 hours (**Figure 13**). Furthermore, the activity of the mitochondrial respiratory chain was substantially diminished in cancerous cells while no deterioration in activity was detected in non-cancerous cells. These findings suggest that these NPs selectively affect cancerous mitochondria, which is a logic target for oxidation-prone NPs. In this instance Fe@Au caused considerable ROS in cancerous cells, although co-incubation with ROS scavengers did not protect the cells from NP-mediated toxicity while 3-MA did. These data suggest that autophagy itself and not ROS is the main provoker of cytotoxicity and besides that the two mechanisms are not linked in this particular case. As several signs of autophagy alterations were observed in cancerous cells, it is proposed that, apart from ROS induction, the observed differential toxicity of NPs between cancerous and non-cancerous cells may be a result of mitochondria-mediated autophagy (**Figure 14**). Unfortunately, in the present study the level of autophagy was not specified for non-cancerous cells, which makes it difficult to draw a reliable conclusion regarding the precise mechanism and the extent of the NP-cell type selective effects. Next to this cancer-selective effect there was another noteworthy observation: the mechanism of NP-induced toxicity varied with NP dosage. While autophagy seemed predominant at concentrations slightly above the IC50, further increases in NP concentration partly abolished autophagy.11a In addition to selectivity toward cancer cells, Fe@Au NPs provoked varying degrees of cytotoxicity in different cancer cell types. The relatively high sensitivity of OECM1 and Caco-2 cells compared to other colorectal cancer cells was shown to originate from their different uptake profile of iron and gold. Furthermore, control experiments with purely Fe NPs revealed that the resistance toward Fe@Au NPs was based on the ability of the cell to cope with iron; Fe@Au sensitive cell types were less resistant to Fe NPs. In line with these findings, 3-MA was only able to restore viability in cells exhibiting loss of MMP being OECM1 and Caco-2. Surprisingly, the gold layer, initially applied to augment the biocompatibility of Fe NPs, was essential for toxicity in the more resistant colorectal cells (e.g. HT-29).180

Similar to the Fe@Au NPs, Harhaji *et al.* observed a dose-dependent involvement of ROS-independent autophagy upon treatment of rat glioma cells with low dosed fullerene particles resulting in higher levels of autophagy activation.116a Also, primary rat astrocytes were more resistant to NP treatment than their malignant equivalents. Admittedly, autophagy was only examined by means of acridine orange staining of autophagic vacuoles, which is not regarded as a reliable method nor does it allow quantitative analysis.116a

In terms of cancer treatment, the ability of various types of NPs to selectively induce autophagy in cancer cells, has led to the thought of utilizing this property to sensitize cancer cells for common chemotherapeutics, making their combined application far more effective. Preliminary results have indicated that several types of fullerenes preferentially induce autophagy-mediated chemosensitization in cancer cells compared to normal cells. This was established by Zhang *et al*. who detected less autophagy stimulation in primary MEF cells upon treatment with underivatized fullerenes (nC60). Accordingly, the chemosensitization effect was less effective in primary MEF cells compared to their immortalized counterparts (**Figure 15**). In this case, ROS-scavengers were able to reduce NP-mediated autophagy stimulation and the chemosensitization effect in HeLa, suggesting that ROS are crucial for the onset of the detected autophagy. However, ROS and other oxidative stress associated effects (e.g. mitochondrial damage) were not compared between the cell types, therefore the influence of ROS on the differential level of autophagy and toxicity cannot be determined. The authors did hint at other potential influencing factors such as dissimilarities in cell adhesion and growth characteristics between the two cell types.181 Neodymium-derivatized fullerene particles (C60(Nd)) were even more efficient in chemosensitized cancer killing.119a Again, primary MEF cells exhibited less autophagy and were more resistant to the NP-mediated chemosensitization compared to their immortalized equivalents. Strikingly, ROS-scavengers were not able to diminish the provoked autophagy stimulation nor the chemosensitization effect of C60(Nd) NPs while an autophagy inhibitor did decline chemosensitization. This suggests that the C60(Nd) elicited chemosensitization is likely to be dependent on autophagy, although the stimulation of the latter may not be primarily mediated by ROS. The authors further suggest that the lysosomes are unable of digesting these NPs, which would thus result in a blockage after autophagosome-lysosome fusion. In contrast with NP treatment, rapamycin, a commonly used autophagy inductor, did not provoke any chemosensitization but partly improved cancer cell survival.119a This discovery could be of great importance since this may imply that various autophagy stimulators can induce mechanistically and/or signaling-wise different types of autophagy. More importantly, this emphasizes the clinical relevance of delineating the mechanism by which autophagy inducers are able to elicit their effect since it seems that depending on the precise mechanism of autophagy induction, either pro- or anti-survival effects can be obtained, which can then either be used to our advantage in medicinal treatment of cancer, or may inadvertently impede cancer therapy. Finally, when looking for discrepancies of effects in different cell types it is important to evaluate all the parameters in both cell types to truly define the differences, particularly uptake, ROS and autophagic markers.

Albeit the selectivity of NP-induced autophagy toward cancer cells remains currently elusive, we propose that this is a combined effect of three different mechanisms. First, as described previously, NP-induced autophagy is closely linked to the intracellular NP levels. Many cancer cell types are known to have a high endocytic capacity resulting in much higher levels of NP internalization than other primary cell types.182 Second, it is established that cancer cells have an elevated metabolism as they require higher levels of ATP to maintain their rapid proliferation. Any mitochondrial damage might therefore result in autophagy induction more rapidly than would be the case for non-cancerous cells. Third, cancer cells have a deregulated pro-and anti-apoptotic balance in such a way that anti-apoptotic signaling is increased in order to promote cell survival and fast proliferation.183 Therefore, given their higher levels of anti-apoptotic signaling, NP-mediated cell damage might end in apoptosis to a lesser extent. As such, cancer cells are more likely to undergo NP-induced autophagy that, if persistent, can finally result in ACD. An intriguing question that thus far remains unsolved is the effect of the differences in tumor microenvironment compared to normal cellular microenvironments. Given the raised metabolism of cancer cells, tumors typically display a low pH in their immediate surroundings.184 Although it is possible that this will influence the behavior of the NPs (e.g. colloidal stability), this aspect has not received much attention to date. We hypothesize that the low pH may affect the NP coating and can result in particles with a partially damaged surface coating. These NPs can then result in higher levels of ROS, which would stimulate autophagy activation. Alternatively, particles with damaged coatings may be more prone to aggregation, which would impede their cellular uptake and reduce autophagy levels. It is clear that more *in vivo* studies are required to fully understand the complexity of all these various factors and how they contribute to the observed selectivity of autophagy induction in cancer cells.

6.1.3. The potential of nanoparticles in anticancer therapy.

ACD is undoubtedly an interesting target and therefore receives a lot of attention in anticancer research. This increasing interest in autophagy is amongst other factors built on the fact that cell death resistance remains one of the primary hallmarks of cancer and at the same time one of the major barriers to overcome. Indeed, cancer cells have developed an abundance of strategies that eventually result in apoptosis evasion,185 and which induces resistance toward anticancer therapy including chemo-and radiotherapy. An extensive study of Shimizu *et al.* demonstrated that apoptosis-inducing agents (e.g. etoposide) could induce cell death in MEF cells even despite their resistance to apoptosis (by Bax-/- and Bak-/- knockdown). Further investigation by means of chemical and genetic inhibition of autophagy clarified the cell death pathway to be autophagic cell death.186 Another example is given by Moretti *et al.* who detected autophagic cell death as the main death pathway in radiation treated apoptosis defective MEF cells.187 These are no isolated results; multiple studies show that autophagic cell death can be induced in apoptosis defective models corroborating the theory that autophagy induction is a promising new strategy for the eradication of resistant cancer.188

It is clear that the modulation of autophagy by new and conventional drugs is an intriguing subject yet the question remains why NPs in particular are such appealing candidates compared to more common autophagy promoting drugs, such as doxorubicin or paclitaxel? As already extensively discussed in section 2 nanotherapy offers multiple advantages compared to conventional drugs. These advantages are due to specific physicochemical properties attributed to NPs such as their small size and high surface area over volume ratio. However, one must keep in mind that these properties also usually contribute to NP toxicity. The small size of the NPs is associated with new functionally attractive physical properties (e.g. surface plasmon resonance for Au NPs, superparamagnetism for IONPs), which has enabled an evolution of innovative techniques and therapies.189 By taking advantage of their optical properties and ability for therapeutic functionalization, diagnosis and treatment can be combined into a single entity, permitting, for instance, image-based drug delivery.190 The high surface over volume ratio also provides a large platform permitting a high payload of potentially multiple ligands (e.g. drugs). Furthermore, their size allows the particles to penetrate deeply in tissues, a feature exploited in the Enhanced Permeability and Retention (EPR) effect, a phenomenon also referred to as ‘passive targeting’ since it allows particles to accumulate at tumor sites. This accumulation is established by enhanced angiogenesis in tumor tissue, which results in leaky vasculature of low quality, moreover, the lymph drainage in these tissues is frequently inefficient. In other words, NPs can readily penetrate tumor tissue by its leaky vasculature while their removal is relatively low; logically this selectively generates high local concentrations of NPs in cancerous tissue allowing efficient killing.191 In addition, NPs can be designed to acquire long blood circulation times which further enhances their tumor uptake.192 Passive targeting can efficiently be combined with active targeting by for example the binding of cancer specific receptor ligands or antibodies on the NP surface thus increasing selectivity and undesired toxicity toward non-cancerous cells.193

Another feature of NMs is their ability to bypass multidrug resistance (MDR). MDR represents a primary obstacle in anti-cancer therapy diminishing the potency of standard chemotherapeutics like paclitaxel. The most common mechanism of resistance is the overexpression of P-glycoprotein (Pgp), an efflux pump that transports the chemotherapeutic agent out of the cell in such way that its efficacy is dramatically reduced.194 However, several studies report on efficient MDR cancer killing by NMs. This is likely owing to the fact that NMs exceed the size limit of the transporter, thus preventing their efflux.195 Examples of NPs bypassing this resistance include Ag NPs, IONPs and phosphatidylserine-containing nanocarriers.195, 196

In conclusion, the above-listed benefits combined with the selective modulation of autophagy by certain NPs can be highly advantageous for anti-cancer applications.

6.2. Autophagy-mediated synergistic effect of nanoparticles and chemotherapeutics

A synergistic anti-cancer effect was observed *in vitro* and *in vivo* with autophagy-inducing PEGylated nanoliposomal C6-ceramide in combination with the autophagy maturation inhibitor vinblastine. The enhanced cancer killing was proven to be apoptosis-mediated and dependent on autophagy, demonstrated by augmented caspase activity and neutralization of the effect by beclin-1 knockdown, respectively.92 We hypothesize that this type of co-treatment can give lead to enhanced sequestration of cytoplasmic components while the autophagosomal cargo cannot be degraded owing to impaired lysosomal functionality or autophagosome-lysosome fusion. This can thus result in a high abundance of dysfunctional vesicles following disruption of cell homeostasis and concurrently cell death. The successful combination of an autophagy inductor and inhibitor was already proven earlier, although only with chemical agents.197 autophagy disruptorsstimulating

Lu *et al.* evaluated the cytotoxic effect of manganese oxide (MnO) NP and doxorubicin co-treatment on several cancer cell types (including HeLa). This combination treatment proved to be highly efficient *in vivo* since it doubled the reduction in tumor weight compared to NP or doxorubicin treatment alone. Moreover, a control experiment with 3-MA showed that this synergistic effect was also dependent on autophagy.78a These studies highlight the potential of autophagy in the elimination of resistant cancer. This effect is however dual and more research is required to better control the outcome of autophagy induction. Chemotherapeutic agents can result in cell stress, such as damaged mitochondria or DNA damage, which in itself can result in autophagy. When the level of autophagy is rather low, this will mainly have a protective effect where the damaged parts will be isolated and destroyed after which the cells can recover. Still, if autophagy is strongly induced, this can result in ACD as the cell will degrade itself next to substantially loweringits ATP levels. Therefore, the induction of autophagy together with the addition of chemotherapeutic agents can have a synergistic effect, as both will result in autophagy upregulation, which can then result in ACD or stimulate apoptosis when ATP levels drop too low.

The above-listed studies clearly underscore the potential of autophagy in overcoming chemoresistance, and emphasize the relevance of investigating autophagy as an alternative cell death pathway.

6.3. Enhanced tumor antigen presentation through nanoparticle-mediated autophagy

Next to the ability of NPs to eliminate cancer cells, a recent study described the use of alpha-alumina (α-Al2O3) NPs to enhance cross-presentation of exogenous antigens and thus promote an anti-cancer immune response. Ovalbumine-conjugated NPs were phagocytosed in dendritic cells after which they presented the antigens to OVA-specific CD8+ T cells through an autophagy-dependent mechanism. This cross-presentation led to the activation and proliferation of cytotoxic T cells; an effect that was efficiently extrapolated to the *in vivo* situation seeing that vaccination with these NPs successfully eliminated established tumors. Interestingly, this principle was less potent with other NPs (e.g. TiO2) both *in vitro* as well as *in vivo*. The present study implies that NPs can aid to improve therapeutic cancer vaccination through autophagy modulation.124a

6.4. Autophagy induction as a self-protection process against nanotoxicity

As previously mentioned, the process of autophagy is very complex and its modulation can have widely varying outcomes, such as inducing cell death. Alternatively, induction of autophagy can result in a cytoprotective mechanism by which cells respond to external stress signals, such as serum starvation. It has been hypothesized that NM-induced autophagy may have a similar effect, so that the overall cytotoxicity of the particles could be reduced upon induction of autophagy. A few recent studies have supported this hypothesis, hinting at the importance of autophagy induction in mediating cellular responses to NM-induced stress. Neibert and Maysinger showed that exposure of rat pheochromocytoma cells (PC12) to CdTe QDs resulted in a substantial increase in intracellular antioxidant levels, enlargement of the entire lysosomal compartment and activation of TFEB.81b The authors argued that these processes aim to protect the cell as a response to the damage exerted by the QDs. In a similar study, Luo *et al*. exposed mouse renal adenocarcinoma cells to QDs, revealing clear induction of oxidative stress, autophagy and cell death. When cells were co-treated with an autophagy inhibitor (3-MA), this resulted in a significant rise in cell death (**Figure 16**). Remarkably, when the cells were co-treated with an antioxidant agent, the level of autophagy decreased and the level of cell death again increased. These data reveal the importance of NP-induced oxidative stress and the associated induction of autophagy as a survival mechanism by which the cell tries to combat NP-induced damage.105

Zhou *et al.* have put forward a new theory on the precise mechanism underlying the cytoprotective effects of autophagy upon NP-induced damage.124b The authors observed that upon treatment of HeLa cells with paramontroseite VO2 nanocrystals (P-VO2) or with Y2O3 NP, autophagy levels were upregulated compared to untreated control cells. Interestingly, in the case of P-VO2 NPs, this resulted in far less cell death than was the case for the Y2O3 NPs. The authors were able to link this difference to the expression of heme oxygenase-1 (HO-1), a cytosolic protein that has been found to play major roles in overcoming cellular oxidative stress. The expression of HO-1 was found to be dependent on autophagy levels, but interestingly, HO-1 expression was only upregulated by P-VO2 NPs and not by Y2O3 NP, for reasons that are yet unclear. This finding does explain the difference between the cytoprotective role of autophagy for P-VO2 NPs and the pro-death role of autophagy for Y2O3 NPs (**Figure 17**).124b

Altogether, these studies indicate that autophagy induction can be beneficial for cell labeling studies or can be one way of protecting ourselves against exposure to nanosized materials.

6.5. The potential of nanoparticles for neuropathological therapy

As discussed in section 5, autophagy deficiency is argued to lie at the root of the most common neurodegenerative diseases, making autophagy a potential key target for their treatment; for example, autophagy activation is likely to prevent the accumulation of toxic protein aggregates.198 At the same time NPs able of penetrating the brain and perturbing autophagy are proposed as a potential danger.161 However, their ability to cross the blood brain barrier (BBB) also makes them interesting vectors and/or therapeutic agents for combating brain diseases. Since autophagy stimulation has now been linked with multiple NPs, this suggests they might be able to prevent or help to combat the disease depending on the precise conditions of the pathology. This hypothesis has been supported by a recent study of Lee *et al*. who evaluated the potential of fullerene-mediated autophagy stimulation as a treatment for Alzheimer’s disease. The authors observed that treatment of neuroblast cells with fullerene-based NMs was able to reduce -amyloid based toxicity, an effect that was partly abrogated by co-treatment with 3-methyladenine. This indicates the elicited autophagy stimulation acts as a cytoprotective process and accordingly these NPs – and other NMs able of stimulating autophagy - may have therapeutic potential.116f Enhanced clearance of htt protein by autophagy was observed upon treatment of PC12 and Neuro 2a cells with europium hydroxide nanorods, indicating that NP-mediated autophagy induction can be exploited to accelerate the removal of harmful protein aggregates.119d It is noteworthy that more NMs are put forward as potential therapeutic vectors for the treatment of neurodegenerative diseases.199

**7. Conclusions and outlook**

This review presents an overview of the most relevant reports on NP-mediated autophagy alterations and their impact on nanotoxicology and nanomedicine. In general, the field of nanotechnology is greatly expanding, increasing the public’s exposure to NMs at a fast pace. Since many of these (in)organic NPs have been proposed to be capable of altering autophagy, and since autophagy dysfunction itself is associated with multiple pathologies (e.g. neurodegenerative diseases), it is of vital medical and toxicological importance to determine the effect of NPs on autophagy and its consequences. To efficiently address this potential danger more in-depth research is necessary to determine the role of autophagy in these pathologies and at the same time the mechanisms by which NPs are able of altering this process. Although, most studies report on autophagy induction, they often do not prove the actual induction and any observed effects may also be explained by other processes, such as a reduced turnover of autophagosomes. In general, the wide variety of NPs (e.g. differences in size or coating) and used cell types makes it difficult to draw any broad conclusions. Several papers do hint at possible physicochemical parameters influencing the autophagy-modulating effect, but there is still a great need for more systematic studies that will aid the design of NPs that do not affect autophagy at all or can be tuned to induce autophagy to our advantage.

There is growing evidence that NMs are able to activate autophagy in a whole variety of cell types, which suggest a probable common cellular response toward NP uptake. More importantly, NPs have shown intrinsic selectivity in inducing autophagy in cancer cells, resulting in their selective destruction compared to non-cancerous cell types. These exciting findings indicate that NMs have huge potential as anti-cancer therapeutics. The many advantages of NPs (e.g. active and passive targeting) in combination with this selective toxicity is indeed very promising and warrants further investigation. However, as the outcome of autophagy induction in cancerous tissue may differ depending on multiple factors, the dubious role of autophagy in cancer must first be further elucidated in order to safely turn these promising findings into practice. In conclusion, the findings summarized in this review indicate a bright future for NMs in cancer therapy, although more research on the autophagic process as well as NP-mediated autophagy modulation is needed to define the true danger and benefit of NPs.

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**9. Tables**

**Table 1**.Overview of soft and hard NMs that are clinically approved or in clinical trials.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | **Soft NMs** |  |  |  | **Hard NMs** |  |
|  | **Liposomes** | **Polymers** | **Other** | **IONPs** | **Silver** | **Gold** | **Other** |
| **Refs** | 13, 15 | 20-22 | 13 | 25-27 | 30 | 31 | 13 |
| **Clinical use** | 10 | 14 | 8 | 4 | 0 | 1 | 0 |
| **Phase III** | 3 | 8 | 2 | 2 | 1 | 0 | 0 |
| **Phase II** | 18 | 21 | 9 | 1 | 2 | 1 | 0 |
| **Phase I** | 19 | 11 | 10 | 1 | 0 | 1 | 1 |

**Table 2:** Summary of NP-mediated autophagy alteration.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **NP** | | | ***a*AP induction** | ***a*AP flux blockage** | ***b*ROS dependent** | ***b*mTOR dependent** | **Cell type** | **ref** |
| **Soft nanoparticles** | lipids | Cationic lipids | X |  | / | no | Human cervical cancer (HeLa) cells; Mouse embryonic fibroblasts (MEF) cells | 90 |
| Cationic liposomes and polyplexes |  | X (delayed or no fusion with lysosomes) | / | / | Chinese Hamster Ovary Cells (CHO); MEF cells; HeLa; primary mouse skin and dendritic cells; primate Vero cells | 91 |
| PEGylated C6Ceramidenanoliposomes | X |  | / | / | HepG2 human liver cancer cells; LS174T human colon cancer cells | 92 |
| other | Eudragit RS | suggested |  | / | / | NR8383 rat macrophages | 93 |
| PAMAM | suggested |  | / | yes | A549 human lung cancer cells; BALB/c mice | 94 |
| **Hard nnanoparticles** | Gold NPs | AuNP |  | X | / | no | Normal rat kidney epithelial cells (NRK) | 98 |
| SV40-conjugated PEGylated AuNP | suggested |  | / | / | HeLa cells; human cervical cancer (SiHa) cells | 99 |
| FBS-coated AuNP | suggested | / | suggested | / | MRC-5 human lung fibroblasts | 100 |
| Au-coated | Fe@Au | suggested | / | No | / | OECM1 human oral cancer cell line | 11a, 134 |
| EGFR-targeted Au-coated IONP | X |  | / | / | Non-small lung cancer cells | 101 |
| Iron oxide NPs | Bare IONP | suggested |  | yes | yes | IMR-90 human normal lung fibroblasts; A549 human lung cancer cells | 11b |
| Bare IONP | suggested |  | suggested | / | RAW264.7 mouse macrophages | 102a |
| Bare IONP | suggested |  | suggested | / | Human cerebral endothelial cells | 102b |
| Bare and PVA coated IONP | suggested |  | / | / | L929 mouse fibroblast cells | 103a |
| PLL-stabilized IONP | suggested |  | suggested | / | ECV-304 human endothelial cell line | 103b |
| Quantum Dots | Photoexcited Graphene QDs | X |  | suggested | / | U251 human glioblastoma cell line | 104b |
| Uncapped CdTe QD | suggested |  | / | / | PC12 undifferentiated rat pheochromocytoma cells | 81b |
| QDot® | suggested |  | / | / | Human mesenchymal stem cells | 104a |
| PEGylatedQDs | suggested |  | / | / | LLC-PK1 porcine renal proximal tubule cells | 105 |
| Streptavidin-QDs | X |  | yes | / | HeLa cells; primary neuronal hippocampus cells; Wistar rats | 104c |
| COOH-QDs | suggested |  | yes | / | RAG mouse renal adenocarcinoma cell line | 105 |
| COOH-CdTe QDs | suggested |  | / | / | HepG2 human liver cancer cells | 104d |
| Zinc Oxide NPs | ZnO nanorods | suggested |  | yes | / | JB6 Cl 41-5a mouse skin epidermal normal cells | 109 |
| ZnO nanorods | suggested |  | suggested | / | FaDu head and neck squamous cell carcinoma | 110 |
| Silica NPs | SiO2 | suggested |  | / | / | A549 human lung cancer cells | 118a |
| SiO2 (multiple shapes) | suggested |  | / | / | A549 human lung cancer cells, RAW264.7 mouse macrophages | 117 |
| Rare earth based NPs | Neodymium oxide | suggested |  | / | / | NCI-H460 non-small cell lung cancer cells | 107 |
| Lanthanide- based UCP | X |  | / | / | HeLa cells; Balb/c mice | 119b |
| Ce02 | suggested |  | / | / | Primary human peripheral blood monocytes | 119e |
| Sm2O3 | X |  | / | / | HeLa cells | 119c |
| Gd2O3 | X |  | / | / | HeLa cells | 119c |
| Tb2O3 | X |  | / | / | HeLa cells | 119c |
| Eu2O3 | X |  | / | / | HeLa cells | 119c |
| Eu(OH)3 nanorods | X |  | / | / | HeLa cells; PC12 rat pheochromocytoma cells; Neuro 2a mouse neuroblasts | 119d |
| Y2O3 | X |  | / | / | HeLa cells; MEF cells | 119a |
| Y2O3 | suggested |  | / | / | HeLa cells | 124b |
| Yb2O2 | X |  | / | / | HeLa cells; MEF cells | 119a |
| Carbon nanotubes | COOH-SWCNT | suggested |  | / | yes | A549 human lung cancer cells | 112 |
| MWCNT | suggested |  | / | no | BEAS-2B human lung epithelium cells | 124f |
| Acid functionalized-SWCNT |  | X | / | / | Primary murine peritoneal macrophages | 113 |
| Grapheme oxide NPs | Graphene oxide |  | X | / | / | Primary murine peritoneal macrophages | 113 |
| Graphene oxide | suggested |  | / | / | RAW264.7 mouse macrophages | 115 |
| fullerenes | C60 | X |  | yes | / | HeLa cells; MCF-7 human breast cancer cells; primary MEF cells | 116d |
| C60 | suggested |  | no | / | C6 rat glioma cell line; U251 human glioma cell line; rat primary astrocytes | 116a |
| C60(Nd) | X | autolysosome accumulation suggested | yes, not only | / | HeLa cells; MEF cells; MCF-7 human breast cancer cells | 116c |
| Fullerenol | suggested, low concentration | suggested, high concentration | no | / | LLC-PK1 porcine renal proximal tubule cells | 116b |
| Fullerenol | suggested |  | / | / | HUVEC human umbilical vain endothelial cells | 116e |
| PEG-C60 derivatives | suggested |  | suggested | / | Mouse neuroblastoma Neuro-2A cells | 116f |
| Copper oxide NPs | CuO | X |  | / | / | A549 human lung cancer cells; H1650 human non-small cell lung cancer cells; CNE-2Z human nasopharyngeal cancer cells | 118b |
| CuO | X |  | yes | / | MCF7 human breast cancer cells | 123 |
| CuO | suggested |  | suggested | / | A549 lung cancer cells | 122 |
| Silver NPs | Ag nanowires | suggested |  | / | / | THP-1 human monocytic cell line | 124d |
| Ag NP | suggested |  | / | / | Sprague Dawley rats | 125 |
| Titanium dioxide NPs | TiO2 | X |  | no | / | H4 human glioblastoma cells | 111 |
| TiO2 | suggested |  | / | / | Human primary epidermal keratinocytes | 124c |
| TiO2 | suggested |  | suggested | / | Human cerebral endothelial cells | 102b |
| Manganese NPs | MnO | X |  | / | / | HeLa cells; HepG2 human liver cancer cells; HaCat immortalized human keratinocyte cells; COS-7 monkey fibroblast cell line | 78a |
| Mn NP | suggested |  | / | / | N27 dopaminergic cell line; primary mesencephalic neuronal cells | 162 |
| Alumina NPs | α-Al2O3 | suggested |  | / | / | C57BL/6 mice; dendritic cells | 124a |
| Al2O3 | suggested |  | / | / | HeLa cells; Wistar Rats | 151e |
| Polystyrene particles | Amine-modified polystyrene beads |  | suggested | / | / | 1321N1 human brain astrocytoma cells | 96 |
| Polystyrene beads (µm size) | X |  | / | / | HeLa cells; MEF cells | 108 |
| other | P-VO2 crystals | suggested |  | / | / | HeLa cells | 124b |
| Ni-Co NPs | suggested |  | / | / | HeLa cells | 121 |
| Pd NP | suggested |  | suggested | / | Peripheral blood mononuclear cells (PMBC) | 124g |
|  |  |  |  |  |  |  |

**Table footnote**. *a*The rise in number of autophagosomes can stem from either a direct upregulation of autophagy or a halt in autophagy flux (blockage). Although it is critical for our understanding of these complex interactions which mechanism is predominant, this has thus far not received much attention. Where most papers only observe a rise in the number of autophagosomes, this is often labeled as autophagy upregulation. In the present table, we try to provide a full overview of the state of the art in this field and indicate whether it has been clearly proven whether autophagy has been directly upregulated or autophagy flux has been reduced (“X”) or whether this remains unclear (“suggested”).

*b*Many different types of NMs have been associated with either autophagy induction or autophagy blockage. In various studies the precise mechanisms underlying the observed autophagy alterations have been further examined and have proven to be dependent on ROS and/or mediated by the mTOR pathway. Where applicable this has been indicated in the Table above, where ROS and/or mTOR dependency (“yes”) or independency (“no”) is proven, not investigated (“/”) or when no direct link between ROS or mTOR has been proven but indirect data has been obtained (“suggested”).

**10. Figure captions**

**Figure 1:** Overview of the mechanistic steps of autophagy. During autophagy a phagophore is created which elongates into a double-membraned autophagosome while sequestering cytoplasmic material. This autophagosome can next fuse with a lysosome resulting in an autolysosome. Alternatively, an autophagosome can form an amphisome by fusion with an endosome, by which newly ingested material can be targeted for degradation (a process termed heterophagy). The enzymes present in the autolysosome lumen eventually degrade the inner membrane and autophagic cargo, thus providing macromolecules that can be transported into the cytosol via permeases. Reprinted with permission from ref.50. Copyright 2007 Nature Publishing Group.

**Figure 2:** Regulation of autophagy.The regulation of autophagy is built around the central point mTOR. mTORC1 activates Atg proteins (like the Atg13-ULK1/2-FIP200 complex) necessary for autophagy upon inhibition by upstream stressor sensors (e.g. energy sensing). On the other hand mTORC1 can indirectly stimulate autophagy via TFEB, a transcription factor that upon activation translocates to the nucleus where it promotes the transcription of lysosomal and autophagic genes. The anti-apoptotic protein Bcl-2 is an important inhibitor of autophagy through its suppressive interaction with Beclin-1 (Atg6), an essential protein for efficient autophagy.

**Figure 3:** EM image of starved mouse fibroblasts. Arrows indicate double-membraned autophagosomes, and double arrows indicate autolysosomes/amphisomes. Arrowheads designate endoplasmic reticulum debris as autophagosomal cargo. Reprinted with permission from ref.78b. Copyright 2010 Elsevier.

**Figure 4:** Immunofluorescent labeling of endogenous LC3 in fed and starved mouse fibroblast cells. Reprinted with permission from ref.77b. Copyright 2009 Elsevier.

**Figure 5:** Western blot evaluating LC3-I conversion in starved mouse fibroblasts in the absence (upper part) and presence (bottom part) of lysosomal protease inhibitors (E64d and pepstatin A). Reprinted with permission from ref.76a. Copyright 2007 Landes Bioscience.

**Figure 6:** Polystyrene NP treatment modulates autophagy in human brain astrocytoma cells. A) LAMP-1 immunostaining of lysosomes shows increasing NP-lysosome colocalization as well as lysosomal enlargement with time. The bottom panel shows the level of autophagosomes is enhanced upon NP treatment, although only few NPs colocalize with these vesicles. B) Western Blot analysis of LC3 shows an increase in LC3-I conversion with exposure time. C) Western Blot quantification by densitometry further demonstrates that the LC3-II/β-actin ratio increases with exposure time starting from the 6h time point. Reprinted with permission from ref.96. Copyright 2013 The Royal Society of Chemistry.

**Figure 7**: Overview of the cellular effects induced by polystyrene NPs. NPs enter the cell via endocytosis and end up in lysosomes leading to their enlargement. NP accumulation within these vesicles can result in lysosomal burst by which proteases can be released into the cytoplasm. These enzymes can next activate apoptotic factors inducing multiple downstream effects such as, mitochondrial damage, ROS formation, caspase 3/7 activation and phosphatidylserine exposure. Finally, apoptotic cell death occurs. Next to apoptotic markers, a rise in autophagosomes is observed, which is likely owing to the lysosomal impairment. Reprinted with permission from ref.96b. Copyright 2013 The Royal Society of Chemistry.

**Figure 8**: TEM analysis of U251 cells after GQD exposure and blue light irradiation. A) Cells are treated with a control solution, or with GQDs and irradiation treatment (B-D). C) Shows that the QDs (black arrows) are engulfed by vesicles (white arrows). D) Shows that these NPs are enclosed within autophagic vesicles together with cytoplasmic components, such as mitochondria (white arrow). Reprinted with permission from ref.104b. Copyright 2012 Elsevier.

**Figure 9**: Effect of coating with RE-1 variants on UCP-induced GFP-LC3 dot formation. HeLa cells expressing GFP-LC3 were treated for 24 h with uncoated UCP (control) or UCP coated with peptide RE-1 and three of its variants. The lower panel depicts the percentage of GFP-LC3 punctate cells and vacuolized cells. Mean SEM, *n*=3, \*\*\*P<0.005, \*\*P<0.01 compared to the “UCP” group. Reprinted with permission from ref.119b. Copyright 2012 Nature Publishing Group.

**Figure 10:** Correlation among the level of ATP content, autophagy and apoptosis in rat livers after Ag NP treatment. Reprinted with permission from ref.125. Copyright 2013 Biomed Central.

**Figure 11:** Schematic overview of NP-associated autophagy induction pathways. NPs will mostly enter the cell via endocytosis, by which they are enclosed in endocytic vesicles (1); they can then move further down the endocytic pathway and end up in late endosomes and/or lysosomes (2). Multiple NPs have shown to affect endo-and lysosomal homeostasis, which can then potentially result in their diminished degradative capacity. This reduction can activate TFEB, which will translocate to the nucleus and upregulate the expression of Atg and lysosomal genes. Besides, TFEB interacts directly with mTOR. (3) NMs can also directly influence autophagy-associated signaling pathways (4). Many NMs are known to enhance oxidative stress through the formation of ROS (5). ROS are important regulators of autophagy that can affect its machinery, signaling and/or lead to mitochondrial damage thus triggering autophagy (5).

**Figure 12:** Overview of the mechanisms underlying the ambiguous role of autophagy in cancer. (A) Autophagy as tumor suppressor. (1) Autophagy maintains cellular homeostasis thus preventing tumorigenesis. (2) Autophagy dysfunction creates a tumor-promoting environment through multiple mechanisms such as genomic instability and p62 accumulation. (B) Autophagy as tumor promotor. (1) By aiding cancer cells to overcome stressful conditions (for example, hypoxia) and preventing anoikis, autophagy may serve as a cancer survival pathway. (2) Accordingly, inhibition or dysfunction of autophagy can enhance cancer cell death and reduce tumor growth and metastasis. Reprinted with permission from ref.164a. Copyright 2013 Elsevier.

**Figure 13**: Fe@Au treatment elicited irreparable loss of mitochondrial membrane-potential in cancerous cells (OECM1), but not in benign control cells (hNOK). (a) Both cell types were stained for JC-1 to evaluate the mitochondrial membrane-potential by flow cytometry in the green channel (FL1) and by confocal microscopy. (b) Shows the mitochondrial activity, as determined by calculating the red to green ratio through JC-1 staining as measured by confocal microscopy. Both flow cytometry and confocal microscopy show that for the OECM1 cells mitochondrial activity decreases with time while for hNOK cells the reduction in mitochondrial activity observed at the 4h time point is largely restored after 24 hours. (∗, p < 0.001, paired FT-test; #, p > 0.05, paired FT-test). Reprinted with permission from ref. 11a. Copyright 2011 Elsevier.

**Figure 14**: Fe@Au elicited selective cytotoxicity toward cancer cells through mitochondria-mediated autophagy. Fe@Au caused significant mitochondrial damage within 4 h in both cell types. Later on, mitochondrial activity in healthy cells was restored, while the cancer cells could not recover from this damage. Accordingly, autophagy was triggered in cancerous cells by which cell growth was inhibited.Reprinted with permission from ref. 11a. Copyright 2011 Elsevier.

**Figure 15**: Fullerene-mediated chemosensitization in immortalized (I-MEF) but not primary MEF (P-MEF) cells. Both cell types were treated for 24 hours with Doxorubicin, with or without a 24 hour pre-treatment with low-dose Nano-C60. Cell death was evaluated by Hoechst 33342/PI staining and calculated as the percentage of PI-positive cells. (Mean ± SEM, *n* = 3, \*\*p < 0.01.) Reprinted with permission from ref.124d. Copyright 2009 Landes Bioscience.

**Figure 16**: 3-MA enhances QD-induced cytotoxicity in mouse renal adenocarcinoma cells. 3-MA (10 mM) augments QD-induced cytotoxicity at 6 h (A) and 24 h (B) as determined by the MTT assay. This indicates that autophagy attempts to reduce QD-induced toxicity. Mean ± S.D. *n*=4. (\*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001, compared with the untreated control).Reprinted with permission from ref. 105. Copyright 2013 American Chemical Society.

**Figure 17**: Overview of the cellular effects induced by P-VO2 and other nanocrystals. P-VO2 NPs trigger autophagy, which subsequently leads to HO-1 overexpression and cell survival. Accordingly, inhibition of autophagy via 3-MA treatment aborts this overexpression thus resulting in cell death. A chemical activator (Hemin) and inhibitor (ZnPP) of HO-1 expression lead to cell survival and cell death, respectively. Autophagy modulation elicited by other NPs also leads to cytotoxicity, indicating autophagy can play diverse roles in cell death depending on its original trigger. Reprinted with permission from ref.124b. Copyright 2013 IOP Publishing.