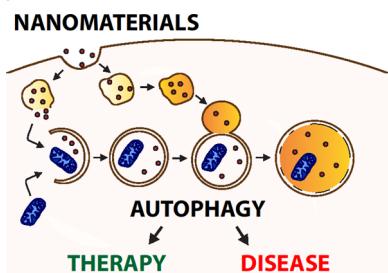


Exploiting Intrinsic Nanoparticle Toxicity: The Pros and Cons of Nanoparticle-Induced Autophagy in Biomedical Research

³ Karen Peynshaert,^{†,‡} Bella B. Manshian,[§] Freya Joris,^{†,‡} Kevin Braeckmans,^{†,‡} Stefaan C. De Smedt,^{*,†,||}
⁴ Jo Demeester,[†] and Stefaan J. Soenen^{*,†,§}

⁵ [†]Lab of General Biochemistry and Physical Pharmacy, Faculty of Pharmaceutical Sciences, [‡]Centre for Nano- and Biophotonics, and
⁶ Ghent Research Group on Nanomedicine, Ghent University, B9000 Ghent, Belgium

⁷ Biomedical MRI Unit/MoSAIC, Department of Imaging and Pathology, Faculty of Medicine, Catholic University of Leuven, B3000
⁸ Leuven, Belgium



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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 U.S. 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

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87 quantum dots,⁴ (c) the localized surface plasmon resonance
88 (LSPR) effect of silver or gold NPs,⁵ and (d) the high rigidity
89 of carbon nanotubes.⁶ Even though these materials were
90 initially developed for industrial use, the same properties have
91 also awoken the interest of the medical and biological
92 communities, since these properties can be harnessed to create
93 novel and powerful therapeutic and/or diagnostic tools. As
94 such, nanomedicine was born as the scientific discipline in
95 which these NMs are utilized for medical purposes. However,
96 this high interest in using NPs for medical applications and
97 their increasing use in various technological applications and
98 consumer goods (e.g., clothing and food products) has raised
99 high concerns on their possible impact on human and
100 environmental health. It is therefore of vital importance to
101 carefully characterize the toxicity of these NMs to enable the
102 field of nanotechnology to fulfill some of its truly exciting
103 possibilities in many aspects of human life. In view of this,
104 increasing production and use of NPs has led to the instillment
105 of another scientific discipline: nanotoxicology.⁷ Although the
106 area of nanotoxicology was initially a small niche within the
107 field of particle and fiber inhalation studies, the field has rapidly
108 expanded to become an important stand-alone scientific
109 discipline encompassing multiple domains such as *in vitro*, *in*
110 *vivo*, environmental, and human toxicology.

111 Recently, an increasing number of nanotoxicological studies
112 have reported the ability of various types of NPs to modulate
113 the cellular process of autophagy. Autophagy is an evolution-
114 narily conserved catabolic process by which endogenous (e.g.,
115 organelles) as well as foreign (e.g., pathogens) materials are
116 sequestered in double-membraned vesicles (i.e., the autophag-
117 gosomes) and degraded upon fusion of these autophagic
118 vesicles with lysosomes. This process is vital for cytoplasmic
119 quality control as it removes excessive or damaged components
120 such as organelles and aggregated proteins. Autophagy is
121 usually present at a basal level but can be induced as a
122 cytoprotective mechanism in the case of cell stress; for instance,
123 during starvation autophagy aids to overcome this food drop by
124 degrading and recycling less essential cytoplasmic material.⁸
125 Considering the importance of autophagy in cellular homeo-
126 stasis, it is not surprising that autophagy dysfunction has been
127 correlated with a variety of disorders including cancer and
128 neurodegenerative diseases.⁹ NPs have been found to be
129 capable of modulating autophagy, and it has been put forward
130 that autophagy induction may be a potential common cellular
131 reaction toward NMs as an attempt to eliminate the foreign
132 material.¹⁰ Therefore, the indirect effect of autophagy
133 modulation on potentially protecting the cells from NM-
134 induced damage is an interesting research topic that is in full
135 development. On the other hand, NMs able to modulate
136 autophagy have been proposed as potential agents against
137 autophagy-related diseases. Intriguingly, several studies have
138 shown that certain NPs are even able to selectively over-
139 stimulate autophagy in cancerous cells, leading to cell death
140 without significantly affecting the level of autophagy in
141 noncancerous cells.¹¹ This suggests that NPs can exhibit an
142 intrinsic toxicity toward cancer cells, which would be of high
143 therapeutic impact. For this reason, it is essential to study the
144 effect of NMs on autophagy both from a nanotoxicological as
145 well from a therapeutic viewpoint.

146 The current review will provide a clear overview of reports
147 on NM-mediated modulation of autophagy and will focus on
148 the importance of autophagy in both health and disease. We
149 will briefly discuss the different ways in which this process can

possibly be manipulated, and in the final part, we will examine
150 how and why NP-induced autophagy can be exploited as a
151 novel therapeutic tool.
152

2. NANOMEDICINE

Exploration of NMs in medical or pharmaceutical applications
153 is vastly increasing, where a lot of effort is being put in the
154 development and fine tuning of new materials.¹² Accordingly,
155 the number of NM-based agents currently undergoing clinical
156 trials is expanding.¹³ However, due to the pertaining
157 uncertainties concerning the potential danger of NMs and
158 the lack of appropriate legislation, the nanotechnology industry
159 is facing significant setbacks in their attempt to implement NMs
160 in a clinical setting.¹⁴ This clinical implementation differs
161 greatly between lipid- or polymer-based NMs and metallic
162 NMs. Soft NMs (lipid- or polymer-based) have been widely
163 applied in clinics for over a decade, and many new formulations
164 are currently undergoing clinical trials.¹⁵ Hard (often metal,
165 metal oxide, or ceramic) NPs, on the other hand, rarely see
166 their potential being translated into a clinical setting despite
167 their substantial scientific improvement.¹⁶ This section will
168 briefly describe the various types of NMs currently used in or
169 explored for clinical settings, mainly in the field of cancer
170 therapy. The principal focus will lie on introducing the different
171 types of materials, a short description of their most important
172 properties, and an overview of their current and potential future
173 applications.
174

2.1. Soft Nanomaterials

Soft NMs can generally be classified under lipid- and polymer-
175 based materials. The former are often organized into liposomal
176 structures, originally discovered by Bangham and colleagues in
177 1965.¹⁷ Liposomes are typically small (confined between 50
178 and 200 nm diameter) and present an inner aqueous
179 compartment that is separated from the outer aqueous
180 compartment by a single lipid bilayer. The variety of lipids
181 and potential incorporation of proteins or other lipophilic
182 compounds allows easy fine tuning of the chemical makeup of
183 liposomes and their surface chemistry, which enables further
184 modification of these vectors.¹⁸
185

For clinical use, a common surface molecule is poly(ethylene
186 glycol) (PEG) as it reduces opsonization of the liposomes by
187 immune components and proteins by which it increases blood
188 circulation time through impeding liposomal clearance by the
189 reticuloendothelial system.¹⁹ This antifouling effect stems from
190 the flexible nature of the PEG chains which, when the NPs are
191 in circulation, will constantly adopt different conformations and
192 thus prevent binding of any biomolecules. Additionally,
193 targeting moieties such as small molecules or antibodies against
194 a particular marker can be added to enhance delivery of the
195 liposomes to the desired (targeted) location.
196

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

Next to lipid-based formulations, polymer-based NMs are
207 also frequently employed as delivery systems for anticancer
208

Table 1. Overview of Soft and Hard NMs That Are Clinically Approved or in Clinical Trials

	soft NMs			hard NMs			other
	liposomes	polymers	other	IONPs	silver	gold	
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

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 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 copolymers in a size range from 20 to 100 nm. 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 anticancer 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, metal oxide, 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 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 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.

Metal oxide NPs are a broad collection of materials with many different applications. For example, titanium dioxide (TiO_2) 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; Fe_2O_3 or Fe_3O_4) 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.^{25–27}

Cerium oxide (CeO_2) 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 preclinical setting, CeO_2 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 and 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 α) using PEGylated Au NPs (Aurimmune; CytImmune Sciences, Rockville, MD),³¹ which were found to display a much higher tumor targeting efficacy and tumor toxicity than free TNF α .

Other interesting materials are colloidal semiconductor NPs (quantum dots (QDs)) that consist of a semiconductor core with a narrow band gap 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, use of heavy metals such as Cd $^{2+}$, a known toxicant, is severely impeding their progress into clinical applications. Novel formulations, including Cd $^{2+}$ -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 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

318 to study their interaction with biological specimens, being the
319 environment, product end users, or workers at a production
320 site. In this respect reports on how to analyze NP toxicity and
321 their possible interference with toxicological assays have been
322 published.³⁸ Simultaneously, several main focus points have
323 been put forward in order to meet the current shortcomings in
324 nanotoxicology. A major focus point should be proper
325 characterization of the chemical composition, colloidal stability,
326 and purity of the applied NPs. This is crucial in order to relate
327 any toxic effects to NP-related features and prevent, for
328 instance, "side effects" stemming from NP contaminants.³⁹ This
329 also allows one to reliably pinpoint potential common
330 mechanisms by which NPs affect biological samples, such as
331 induction of reactive oxygen species (ROS; see section 4.1 for
332 more details) or alteration of cell morphology.⁴⁰ Furthermore,
333 multiparametric methods for toxicological testing could provide
334 a more complete picture of toxic NP effects.^{40a} In combination
335 with new automated high-content machinery this could then
336 generate large databases that could be applied for computer-
337 driven modeling and predictive toxicology.⁴¹

338 Another main focus point in toxicology in general is the
339 attempt to bridge the *in vitro*–*in vivo* gap. Primary efforts to
340 address this issue are the use of novel *in vitro* models, such as
341 3D cell cultures and coculture models that better mimic the
342 human physiology.⁴² Moreover, there is a great need to
343 thoroughly investigate certain "uncharted" fields, such as (a)
344 the effect of dosing and NP sedimentation on cellular uptake
345 and toxicity,⁴³ (b) the effect of varying cellular microenvironments on the colloidal stability or degradation of NPs,⁴⁴ (c)
346 long-term effects of NPs as well as their degradation products
347 on cells or tissues, also in the form under which they will be
348 used, for instance, in electronic products, clothing, or gel-based
349 creams, (d) the need to link toxicity data with functional effects,
350 such as the toxicity of IONPs or QDs with their ability to
351 visualize cells by MRI or fluorescence microscopy, respectively.⁴⁵ While all these focus points are helping the field of
352 nanotoxicology to further develop into a strong and mature
353 scientific discipline, it is inherently faced with a few major
354 challenges. One challenge lies in the fact that toxicology in itself
355 is a bearer of bad news; therefore, most studies will report on
356 unwanted and dangerous effects creating a negative atmosphere
357 around the field. This hardens the publication of, for example,
358 unfavorable data or papers that present no effect by a certain
359 NM, while this is essential information for scientists and would
360 also allow the nanofield to advance faster.⁴⁶ Another challenge
361 lies in the need for new equipment and novel ways to properly
362 characterize the location, concentration, and chemical compo-
363 sition of NMs in a complex biological matrix, preferably in real
364 time at the highest sensitivity possible.⁴⁷ A last challenge is the
365 wide variety of parameters that can affect NP–cell interaction,
366 by which reliable and straightforward interpretation of observed
367 effects is hampered. Since there are too many parameters to be
368 tested, it is a near impossible task to conclusively say that a
369 certain NP under certain conditions has no effect at all. Because
370 of these challenges, there is a huge need for more data to be
371 generated and to attempt to define key mechanisms that allow a
372 rapid screening of NP toxicity. The most promising NPs can
373 then be subjected to more in-depth investigations. As
374 mentioned above, induction of ROS is one such key
375 mechanism to concentrate on. This review focuses on another
376 mechanism that has been associated with various types of NPs,
377 being the modulation of autophagy. In the next part we will

380 discuss the process of autophagy, its involvement in cell death, 380
381 and the main ways to study this process. 381

3.2. Process of Autophagy

Autophagy is an evolutionarily conserved process that through 382
degradation of cytoplasmic material supports cell preservation 383
in response to various forms of stress. Since overstimulation of 384
this self-degradative process would lead to cell death, a complex 385
signaling network tightly balances the level of autophagy. The 386
present section will focus on the mechanistic steps of autophagy 387
as well as the most relevant triggers influencing this process. 388

Efficient degradation of intracellular constituents like 389
proteins is of vital importance for guarding cellular homeostasis 390
because of their role in the regulation of multiple crucial 391
pathways including signal transduction, cell cycle regulation, 392
and cytoplasmic quality control. This degradation can occur via 393
multiple cellular pathways including autophagy. Autophagy is a 394
collective term for various selective and nonselective processes 395
comprising microautophagy, chaperone-mediated autophagy, 396
and macroautophagy. Microautophagy involves formation of 397
lysosomal invaginations resulting in a direct and nonspecific 398
sequestration of cytosolic components following breakdown.⁴⁸ 399
In the case of chaperone-mediated autophagy, which is a highly 400
specific process, unfolded proteins are recognized and bound 401
by a chaperone complex, resulting in their translocation into the 402
lysosome lumen.⁴⁹ However, in most cases and also in this 403
paper, the designation "autophagy" refers to the process of 404
macroautophagy. 405

Macroautophagy, or simply autophagy, is a complex 406
multistep process which is coordinated by key proteins encoded 407
by autophagy-related genes, i.e., Atg genes. In brief, a part of the 408
cytoplasm is sequestered in typical double-membraned vesicles 409
(i.e., autophagosomes) that fuse with lysosomes to form 410
autolysosomes, a process termed autophagy flux. Next, the 411
delivered lysosomal enzymes break down the inner membrane 412
and cargo of the autolysosome (Figure 1).⁵⁰ 413 fl

The process begins with nucleation of a phagophore for 414
which the recruitment and functionality of a phosphatidylino- 415
sitol 3-kinase Class III (PI3KCIII) complex is essential. An 416
important component of this multiprotein complex is Beclin-1 417
(Atg6), which acts as a recruitment platform for other proteins 418
required for autophagosome formation.⁵¹ The activity of 419
Beclin-1 is blocked upon interaction with Bcl-2, an important 420
antiapoptotic protein.⁵² This interaction is an illustration of the 421
fine regulation of autophagy and the crosstalk between 422
autophagy and other important processes such as apoptosis. 423
After phagophore elongation and simultaneous sequestration of 424
cytoplasm, the vesicle closes to form the typical double- 425
membraned autophagosome, one of the key markers used in 426
autophagy research. For this maturation process, two ubiquitin- 427
like conjugation pathways are needed: (1) lipidation of 428
microtubule-associated protein 1 light chain 3 (LC3; Atg8) 429
by attachment of phosphatidylethanolamine (PE) and its 430
subsequent incorporation into the autophagic membrane, and 431
(2) attachment of the Atg12–Atg5–Atg16 conjugate to the 432
phagophore.⁵³ The degradative power of the autophagy 433
pathway is linked with the creation of an auto(phago)lysosome 434
by fusion of an autophagosome with a lysosome. This 435
autolysosome thus contains the digestive enzymes necessary 436
for efficient breakdown of the autophagosome and its content. 437
Finally, the content of the autophagosome and the inner 438
membrane will be degraded after which permeases can 439
transport the resulting molecules to the cytoplasm, hereby 440

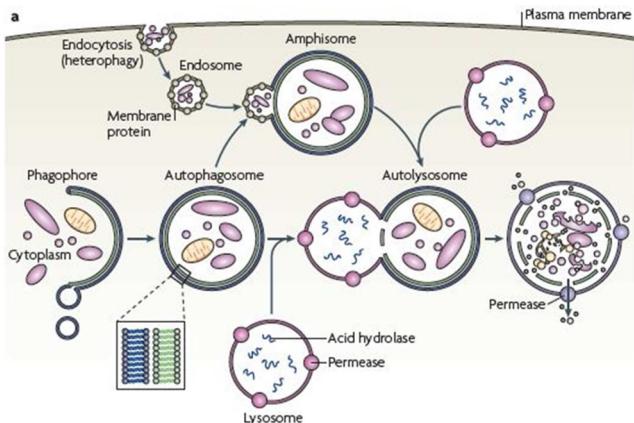


Figure 1. Overview of the mechanistic steps of autophagy. During autophagy a phagophore is created which elongates into a double-membraned autophagosome while sequestering cytoplasmic material. This autophagosome can next fuse with a lysosome, resulting in an autolysosome. Alternatively, an autophagosome 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.

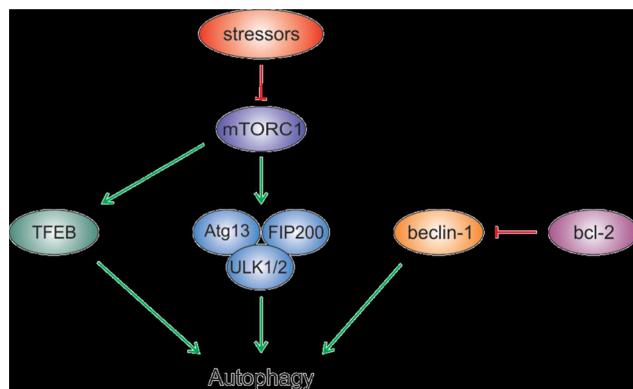


Figure 2. Regulation of autophagy. Regulation of autophagy is built around the central point mTOR. mTORC1 activates Atg proteins (like the Atg13-ULK1/2-FIP200 complex) necessary for autophagy upon inhibition by upstream stressor sensors (e.g., energy sensing). On the other hand, mTORC1 can indirectly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

3.3. Autophagy and Cell Death

The process of autophagy has generally been labeled as a pro-⁴⁷⁵ survival pathway; however, it has been argued that excessive⁴⁷⁶ self-digestion can result in autophagic cell death (ACD).⁶⁰⁴⁷⁷ Albeit not abundant, there are some studies that report on⁴⁷⁸ autophagy performing a pro-death role, as revised in a review⁴⁷⁹ by Shen et al.,⁶¹ which led to generation of the term ACD. On⁴⁸⁰ the other hand, ACD has long been relatively unexplored,⁴⁸¹ which resulted in different interpretations and considerable⁴⁸² misuse of the term. The process of cell death is indeed often⁴⁸³ very complex, and during its progress, damaged cells typically⁴⁸⁴ display various markers representative of different cell death⁴⁸⁵ pathways such as apoptosis and autophagy. Although⁴⁸⁶ morphological changes typical for autophagy can be observed,⁴⁸⁷ it usually remains unclear whether autophagy is a true killer, a⁴⁸⁸ mere side phenomenon accompanying death, or an unsuccessful⁴⁸⁹ attempt to save the cell.^{61,62} In order to overcome these⁴⁹⁰ issues, the definition of ACD has evolved from merely⁴⁹¹ comprising morphological changes (i.e., autophagic vacuoles)⁴⁹² to a list of clear biochemical requirements drafted by the⁴⁹³ Nomenclature Committee on Cell Death.⁶³ In line with this,⁴⁹⁴ Shen et al. argue the term ACD is only justified when the⁴⁹⁵ observations made meet the following standards: (1) cell death⁴⁹⁶ must be apoptosis independent, (2) autophagy inhibition,⁴⁹⁷ preferably by knockdown of a minimum of two relevant Atg⁴⁹⁸ genes,⁶³ prevents the observed cell death, and (3) an increase in⁴⁹⁹ autophagy flux is detected.⁶¹ Even though these requirements⁵⁰⁰ have been brought forward, the discussion remains if even⁵⁰¹ stricter terms should be met and if ACD is a real albeit rare⁵⁰² phenomenon or instead merely a misnomer.⁶⁴ Specialists agree⁵⁰³ that on top of the above-mentioned criteria, ACD must be a⁵⁰⁴ separate death pathway that stands alone, and cases by which⁵⁰⁵ autophagy promotes other cell death pathways (e.g., apoptosis)⁵⁰⁶ must be excluded.^{64a} As there is so much controversy⁵⁰⁷ surrounding ACD, the term should be applied with great care⁵⁰⁸ and, at a minimum, should only be used in compliance with the⁵⁰⁹ conditions advised by the Committee. In conclusion, it is⁵¹⁰ important for any researcher to understand that autophagy in⁵¹¹ itself can be either pro-survival or pro-death and that the co-⁵¹²

441 providing the necessary energy and/or building blocks for the
442 de novo synthesis of cellular components.⁵⁰

443 One key aspect of autophagy is the ability to engulf a portion
444 of the cytoplasm by which it selectively removes certain
445 proteins, pathogens, or complete organelles such as mitochondria.⁵¹ This cargo selectivity is mediated by the protein p62/
446 Sequestome-1 that, due to its multiple binding domains, can
447 create a direct link between LC3 and components targeted for
448 autophagy.⁵⁴ As will be discussed later, p62 is selectively
449 degraded by autophagy and therefore frequently used as a
450 marker for autophagy flux.⁵⁵

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

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

s₁₃ occurrence of cell death and autophagy does not automatically s₁₄ imply that autophagy in itself is leading to cell death. s₁₅ Interestingly, however, NPs have been introduced as potential s₁₆ inducers of autophagy as well as ACD as will be discussed s₁₇ further in this review.⁶⁵

s₁₈ Over the past decade it has gradually been revealed that s₁₉ autophagy and apoptosis are interconnected at several levels. s₂₀ Autophagy can influence apoptosis by direct interaction of s₂₁ autophagy proteins with the apoptotic machinery, by s₂₂ autophagic degradation of apoptotic factors, and/or by s₂₃ providing a platform for caspase activation. Likewise, apoptotic s₂₄ proteins can affect autophagy through interaction with and/or s₂₅ caspase-mediated cleavage of its proteins. It is thus proposed s₂₆ autophagy can induce cell death by (1) dismantling of the cell s₂₇ through self-digestion and (2) promotion of apoptosis.⁶⁶ The s₂₈ cellular decision to activate a certain cell death pathway is likely s₂₉ to depend on the cellular stresses involved; yet, given its role in s₃₀ damage control it is suggested that autophagy affects the onset s₃₁ of cell death. For example, when autophagy is dysfunctional or s₃₂ not able to restore the ATP level, apoptosis or necrosis is s₃₃ initiated.⁶⁷ It is important to note that cell death is a dynamic s₃₄ phenomenon, and multiple cell death types are often co- s₃₅ observed within the same cell.⁶⁸ As described in section 4, s₃₆ autophagy and apoptosis are often successively or simulta- s₃₇ neously detected upon treatment with NMs.

3.4. How to Study Autophagy

s₃₈ As a result of the increasing interest in autophagy, multiple s₃₉ methods have been developed in the past years to detect s₄₀ autophagic markers of which the most widely examined are s₄₁ LC3, p62, and key proteins of the autophagic machinery. The s₄₂ following section presents a short overview of the most s₄₃ extensively used methods to detect these markers.

s₄₄ Initially, transmission electron microscopy (TEM) used to be s₄₅ the only key technique to detect autophagy, and it still remains s₄₆ an important method today as it can provide highly detailed s₄₇ ultrastructural information, e.g., autophagosomal cargo and s₄₈ potential inclusion of NPs (Figure 3).⁶⁹ TEM allows detection s₄₉ of the distinct steps of the process based on the specific s₅₀ morphology of the autophagic organelles; yet, since autophagy s₅₁ is a dynamic process identifying these structures can be s₅₂ troublesome and thus requires special expertise.⁷⁰ Newer s₅₃ techniques such as immunoelectron microscopy by means of s₅₄ LC3 immunogold labeling or correlative light and electron s₅₅ microscopy (CLEM) can conveniently help to avoid mis- s₅₆ identification of autophagic structures. Nonetheless, as the s₅₇ instruments are expensive and sample preparation is technically s₅₈ demanding, epifluorescence or confocal microscopy might be s₅₉ practically preferred.⁶⁹ The latter can be used to visualize s₆₀ autophagy with dyes such as monodansylcadaverine (MDC), an s₆₁ autofluorescent compound that selectively accumulates in s₆₂ autophagic vacuoles presumably as a result of ion-trapping s₆₃ and/or interaction with membrane lipids.⁷¹ Upon autophagy s₆₄ induction the number of MDC-positive vesicles substantially s₆₅ increases. As a result, changes in autophagic activity can be s₆₆ measured by evaluating the number of cells that show MDC- s₆₇ labeled vesicles or the number of positive vesicles per cell.⁷² s₆₈ Nevertheless, use of MDC as a specific autophagic dye remains s₆₉ a matter of debate as some studies are in favor of its s₇₀ selectivity,⁷³ while others argue that its staining does not differ s₇₁ from other commonly used acidotropic dyes.⁷⁴

s₇₂ To date, LC3, which is selectively incorporated into s₇₃ autophagic membranes, has been the most specific and

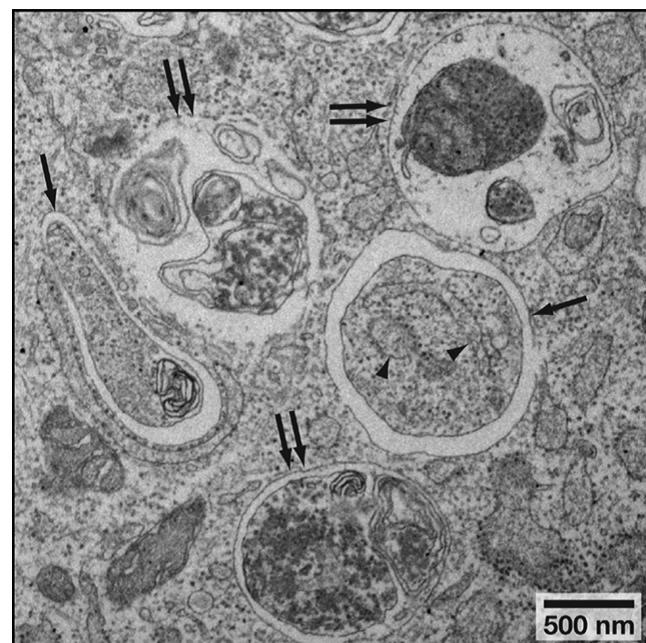


Figure 3. EM image of starved mouse fibroblasts. Arrows indicate double-membraned autophagosomes, and double arrows indicate autolysosomes/amphisomes. Arrowheads designate endoplasmic reticulum debris as autophagosomal cargo. Reprinted with permission from ref 78b. Copyright 2010 Elsevier.

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

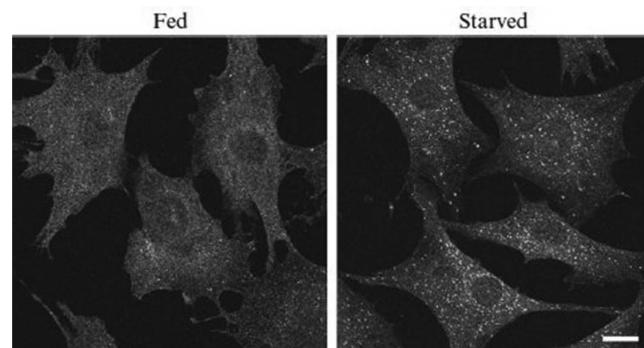


Figure 4. Immunofluorescent labeling of endogenous LC3 in fed and starved mouse fibroblast cells. Reprinted with permission from ref 77b. Copyright 2009 Elsevier.

591 accumulation and therefore do not allow one to differentiate
 592 between the induction of autophagy or an impaired autophagy
 593 flux, which both result in higher numbers of autophagosomes.
 594 An impaired autophagy flux can readily be determined by the
 595 above-mentioned LC3 Western Blot experiment in the
 596 presence of lysosomal protease inhibitors (e.g., pepstatin A)
 597 or buffers (e.g., chloroquine, ammonium chloride) that inhibit
 598 LC3-II degradation. If the amount of LC3-II remains the same
 599 in the presence and absence of the inhibitor, it can be
 600 concluded that the increased level of LC3-II is likely to result
 601 from an autophagy flux blockage (Figure 5).^{76a} Autophagy flux

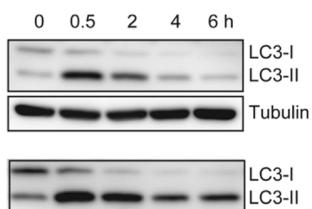


Figure 5. Western blot evaluating LC3-I conversion in starved mouse fibroblasts in the absence (upper part) and presence (bottom part) of lysosomal protease inhibitors (E64d and pepstatin A). Reprinted with permission from ref 76a. Copyright 2007 Landes Bioscience.

602 can also be evaluated based on other Western Blot experiments
 603 such as GFP-LC3 cleavage. Since GFP is relatively resistant to
 604 lysosomal hydrolysis the amount of free GFP can be detected
 605 as a measure of autophagosome degradation.⁷⁸ In addition to
 606 LC3, an increase in the level of undegraded p62 can also
 607 indicate a potential blockage of autophagy flux.^{55a}

608 The turnover of autophagosomes into autolysosomes can be
 609 visualized by means of autophagosome–lysosome colocaliza-
 610 tion studies using LC3 labeling and lysosomal markers (e.g.,
 611 LAMP-1 or Lysotracker).^{77a} Likewise, this maturation can be
 612 assessed microscopically as well as via flow cytometry using, for
 613 instance, an mRFP-GFP tandem fluorescent-tagged LC3
 614 (tfLC3). As GFP is pH sensitive and more prone to quenching
 615 than mRFP, the various autophagic vesicles can be
 616 distinguished through their different fluorescence signal;
 617 autophagosomes will show both signals (i.e., yellow), while
 618 after lysosomal fusion GFP is quenched, and thus autolyso-
 619 somes can be identified as mRFP-positive vesicles.^{77a,79} Flow
 620 cytometry can also be applied to determine the total
 621 fluorescence intensity of GFP-LC3 as a measure of autophagic
 622 activity.⁸⁰

623 Turning now to evaluation of signaling events involved in
 624 autophagy, studies can focus on assessing the cellular level and/
 625 or activity of TFEB, which is a transcription factor that upon
 626 activation translocates to the nucleus to interact with lysosomal-
 627 and autophagy-related genes. This nuclear shift can be
 628 visualized using labeled antibodies or quantified by Western
 629 Blotting after nuclear and cytosolic fractionation of the
 630 sample.⁸¹ A more autophagy-specific approach would be to
 631 evaluate the activity of mTOR and its interacting proteins. This
 632 is usually examined to study the effect of various stimuli
 633 (including NPs) on the status of autophagy and/or the
 634 mechanism behind an observed induction or inhibition. Since
 635 the activity of these proteins depends on their phosphorylation
 636 status, Western Blotting by means of phospho-specific
 637 antibodies is the favored method. In this way the activity of
 638 mTOR can be measured directly by analyzing its phosphor-

639 ylation level or indirectly by determining the activity of its
 640 substrates (e.g., p70S6K) or upstream mediators (e.g., Akt).⁸²

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

3.5. Chemical Modulation of Autophagy

When evaluating the influence of NPs on autophagy it is
 649 essential to include controls that help to reliably verify and
 650 interpret observed changes in autophagic activity. Here, we will
 651 give a summary of the multiple known autophagy-modulating
 652 chemicals and conditions widely used in autophagy research.
 653

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

3-Methyladenine (3-MA) and Wortmannin are both known
 663 to negatively regulate autophagy through inhibition of PI3K⁸⁷
 664 and are thus commonly used to identify the role of autophagy
 665 in NP-induced cell death. However, also in this case the
 666 experimental conditions should be well optimized because, for
 667 example, prolonged periods of their administration can lead to
 668 enhancement of autophagy flux being the exact opposite of
 669 what we aim for.⁸⁷ Furthermore, chemicals that influence
 670 lysosomal activity by alkalization of its acidic lumen (e.g.,
 671 chloroquine)⁸⁸ or inhibition of lysosomal enzymes (e.g.,
 672 pepstatin A) can inhibit lysosomal fusion and/or block the
 673 breakdown of the sequestered cargo in the autolysosome.
 674

A general issue seen with these chemical modulators,
 675 especially with those just described, is their lack of specificity
 676 for autophagy. Indeed, they influence other cellular processes
 677 that can indirectly affect autophagy. It is therefore recom-
 678 mended to combine chemical modulation with other
 679 approaches such as genetic inhibition or functional knockdown
 680 of relevant Atg genes.^{78b,89}

In conclusion, to study the complex and dynamic process of
 682 autophagy a number of different assays and detection
 683 techniques are required to accurately and reliably link certain
 684 effects to the modulation of autophagy. It is however necessary
 685 to comprehend that assays based on autophagosome detection
 686 not necessarily provide information on the status of autophagy
 687 flux. Also, since each of the above-described methods has its
 688 advantages and flaws, it is strongly advised to combine several
 689 complementary assays. For a more extensive overview of the
 690 various applied methods and accompanying guidelines we
 691 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
 693 metallic NP-induced autophagy, evidence for polymeric and
 694 lipid particles remains rather limited. However, several reports
 695

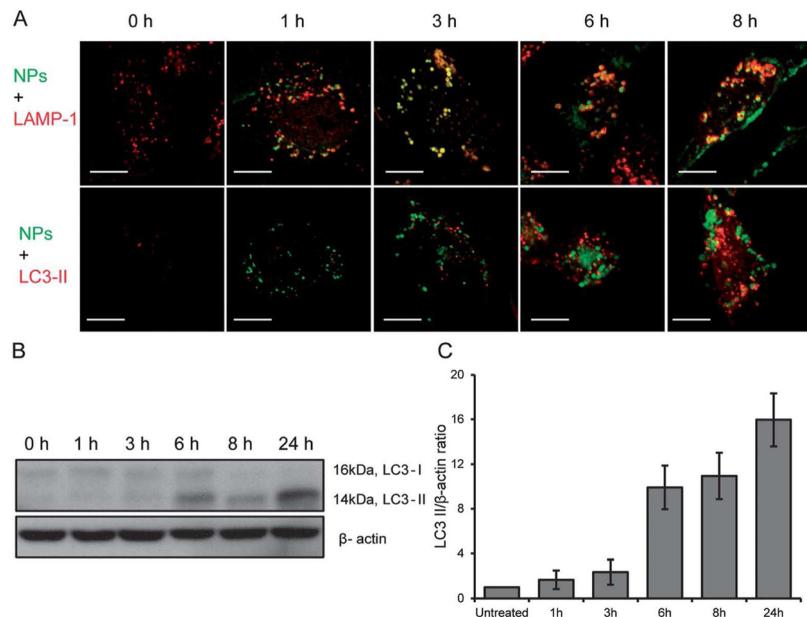


Figure 6. Polystyrene NP treatment modulates autophagy in human brain astrocytoma cells. (A) LAMP-1 immunostaining of lysosomes shows increasing NP-lysosome colocalization as well as lysosomal enlargement with time. (Bottom) Level of autophagosomes is enhanced upon NP treatment, although only a few NPs 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 6 h time point. Reprinted with permission from ref 96. Copyright 2013 The Royal Society of Chemistry.

696 have been generated, indicating that also these materials are
697 capable of modulating autophagy.

698 **4.1.1. Modulation of Autophagy by Liposomes.**
699 Induction of autophagy has been observed in HeLa cells
700 upon treatment with dioleoyltrimethylammonium propane
701 (DOTAP), a cationic lipid commonly used as a transfection
702 agent. The results not only suggest that DOTAP enhances
703 autophagosome formation but also demonstrate that the
704 induction is mTOR independent. The autophagy activation
705 may be caused by the fact that DOTAP is a synthetic lipid and
706 the cell undergoes problems degrading it. As a result, the cell
707 aims to increase its total degradative capacity, that is, by the
708 induction of autophagy. Naturally, the fact that transfection
709 agents as such are able to enhance autophagy casts doubt on
710 observations made in transfected cells, particularly if autophagy
711 is the process examined. At the same time, this implies that
712 inhibition of autophagy might aid to improve transfection
713 efficiency.⁹⁰ The latter hypothesis was corroborated by Roberts
714 et al., who found that cationic liposomes were delivered to the
715 autophagic pathway through endosome–autophagosome fu-
716 sion, indicating a cellular attempt to eliminate the foreign
717 material. In addition, gene delivery and expression was
718 remarkably enhanced in autophagy-defective Atg5^{-/-} cells.⁹¹
719 Interestingly, treatment with uncharged lipids (i.e., dioleoyl-
720 phosphatidylethanolamine; DOPE) failed to alter autophagy,⁹⁰
721 a finding that is in contrast with the ability of neutral PEGylated
722 C6-ceramide nanoliposomes to activate fully functional
723 autophagy in liver HepG2 cells.⁹²

724 **4.1.2. Modulation of Autophagy by Polymeric NPs.**
725 Autophagy modulation was also observed upon treatment of
726 macrophages with positively charged polymer (Eudragit RS)
727 particles. In this case, TEM showed significant localization of
728 particles inside or in contact with mitochondria. Furthermore,
729 substantial signals of oxidative stress were observed. The
730 authors thus propose that the cell aims to remove the damaged

mitochondria by triggering autophagy followed by apoptosis- 731
independent cell death.⁹³ 732

Cationic polyamidoamine (PAMAM) dendrimers of multiple 733 generations (G3–G8) have been proposed to induce 734 autophagy in A549 lung cancer cells with involvement of the 735 mTOR pathway. However, it was not specified if the increased 736 level of LC3, assessed by microscopy and Western Blotting, was 737 caused by an enhanced on rate or decreased off rate of 738 autophagosomes; therefore, an autophagy blockage cannot be 739 excluded.⁹⁴ As PAMAM dendrimers have been reported to 740 cause lysosomal alkalinization,⁹⁵ potentially resulting in 741 lysosomal impairment, a blockage of flux is rather likely. On 742 the other hand, it has been put forth that these dendrimers can 743 affect mTOR activity during their endocytic uptake; the 744 observed autophagy alteration could thus be a combination of 745 multiple effects. Intriguingly, comparable with the conflicting 746 findings obtained with differently charged lipids, anionic G5.S 747 PAMAM particles did not affect autophagic activity. Together, 748 these data suggest that charge may have an impact on the 749 autophagy-inducing potential of NPs; however, without any 750 uptake comparison between the various particles it cannot be 751 excluded that this is merely because of differences in uptake 752 efficiency.⁹⁶ 753

An elegant study by Wang et al. described a time-resolved 754 analysis of the effect of amine-conjugated polystyrene (PS) NPs 755 on lysosomal health (Figure 6). They displayed flow cytometry 756 data revealing two populations based on scatter plots and 757 LysoTracker fluorescence intensities. The authors hypothesized 758 that the high-intensity population exhibit enlarged lysosomes, 759 while the low-intensity population represents cells with 760 damaged (burst) lysosomes. In the latter, population-enhanced 761 ROS and compromised mitochondrial membrane potential 762 (MMP) was indeed detected, which is in line with their theory 763 of lysosomal cathepsin release upon NP-inflicted lysosomal 764 damage. The mechanism underlying this damage was reported 765 to be degradation of the protein layer surrounding the particles 766

767 after endocytosis and concurrent damage to the lysosomal
 768 membrane. Furthermore, an accumulation of autophagosomes
 769 was observed, and although no flux experiments were
 770 performed, the well-established link between lysosomal impairment
 771 and autophagy dysfunction suggests a flux blockage is
 772 probable. On the other hand, autophagy served to boost
 773 survival since cotreatment with 3-MA decreased ATP content
 774 and enhanced caspase activation. However, multiple apoptotic
 775 markers were detected after NP exposure, suggesting autophagy
 776 was unable to restore the disturbed homeostasis (Figure 7).⁹⁶

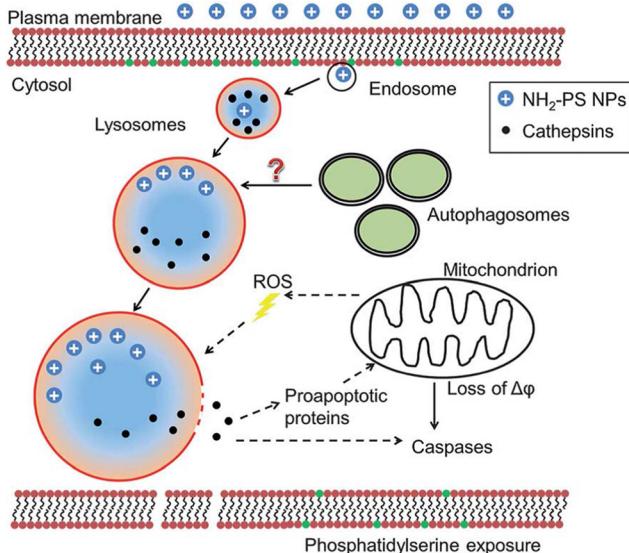


Figure 7. Overview of the cellular effects induced by polystyrene NPs. NPs enter the cell via endocytosis and end up in lysosomes leading to their enlargement. NP accumulation within these vesicles can result in lysosomal burst by which proteases can be released into the cytoplasm. These enzymes can 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 96. Copyright 2013 The Royal Society of Chemistry.

4.2. Modulation of Autophagy by Hard Nanoparticles

777 **4.2.1. Gold Nanoparticles.** As mentioned in section 4.1,
 778 there is an increasing amount of literature providing evidence
 779 for autophagy modulation by hard NPs.⁹⁷ As a leading example,
 780 several research groups have shown alterations in autophagic
 781 activity upon treatment with different types of Au NPs. Ma et
 782 al. demonstrated an Au NP-mediated mTOR-independent
 783 accumulation of autophagosomes owing to a blockage of
 784 autophagosome degradation. The lysosomal impairment
 785 elicited by these gold particles, demonstrated by lysosomal
 786 enlargement and alkalinization, probably accounts for this
 787 blockage. Interestingly, in line with the observed size-depend-
 788 ent uptake, larger particles (50 nm) were more potent
 789 autophagy flux disruptors compared to their smaller equivalents
 790 (10 and 25 nm). Next to size, preliminary results further
 791 uncovered a potential charge-dependent autophagy response
 792 with more autophagosome accumulation upon treatment with
 793 positively charged NP compared to equally sized negative
 794 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.⁸¹³ 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 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,¹⁰³ yet this needs to be further demonstrated.

4.2.3. Quantum Dots. QDs of multiple compositions have been repeatedly put forward as autophagy activators. Again, 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,104} As 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} Second, despite the observed QD–autophago- some 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

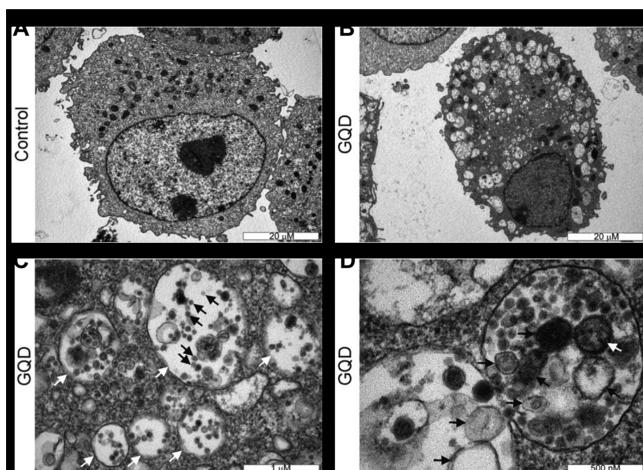


Figure 8. TEM analysis of U251 cells after GQD exposure and blue light irradiation. (A) Cells are treated with a control solution or with GQDs and irradiation treatment (B–D). (C) QDs (black arrows) are engulfed by vesicles (white arrows). (D) NPs are enclosed within autophagic vesicles together with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

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, microsized 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 nanosized materials.⁸⁷⁰

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

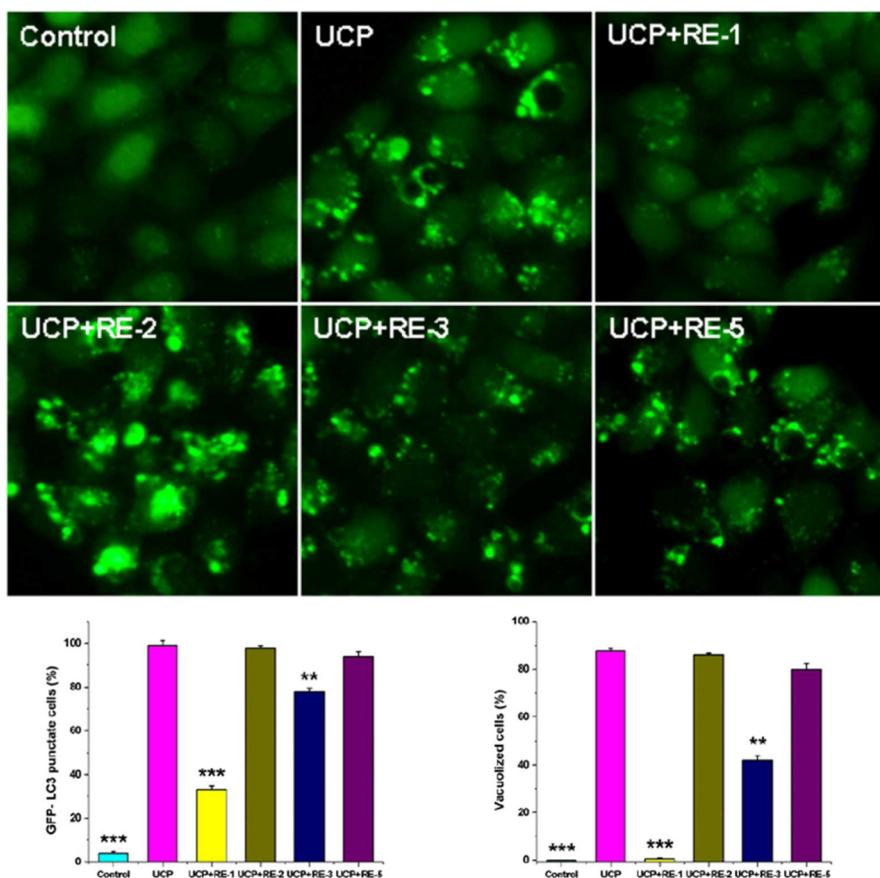


Figure 9. Effect of coating with RE-1 variants on UCP-induced GFP-LC3 dot formation. HeLa cells expressing GFP-LC3 were treated for 24 h with uncoated UCP (control) or UCP coated with peptide RE-1 and three of its variants. Lower panel depicts the percentage of GFP-LC3 punctate cells and vacuolized cells. 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.

Table 2. Summary of NP-Mediated Autophagy Alteration

		NP	AP induction ^a	AP flux blockage ^a	ROS dependent ^b	mTOR dependent ^b	cell type	ref
soft nanoparticles	lipids	cationic lipids	X	X (delayed or no fusion with lysosomes)	/	no	human cervical cancer (HeLa) cells; Mouse embryonic fibroblasts (MEF) cells	90
		cationic liposomes and polyplexes	X	/	/	/	Chinese hamster ovary cells (CHO); MEF cells; HeLa; primary mouse skin and dendritic cells; primate Vero cells	91
		PEGylated Ceramideno-nanoparticles	X	/	/	/	HepG2 human liver cancer cells; LS174T human colon cancer cells	92
other	Eudragit RS	suggested	suggested	/	/	/	NR8383 rat macrophages	93
	PAMAM	suggested	suggested	/	/	yes	A549 human lung cancer cells; BalB/c mice	94
	AuNP	SV40-conjugated PEGylated AuNP	suggested	X	/	no	normal rat kidney epithelial cells (NRK)	98
hard nanoparticles	gold NPs	FBS-coated AuNP	suggested	/	suggested	/	HeLa cells; human cervical cancer (SiHa) cells	99
	Au-coated	Fe@Au	suggested	/	no	/	MRC-5 human lung fibroblasts	100
		EGFR-targeted Au-coated IONP	X	/	/	/	OECM1 human oral cancer cell line	11a, 134
iron oxide NPs	bare IONP	suggested	suggested	yes	yes	yes	IMR-90 human normal lung fibroblasts; A549 human lung cancer cells	11b
	bare IONP	bare IONP	suggested	suggested	suggested	/	RAW264.7 mouse macrophages	102a
	bare and PVA-coated IONP	bare and PVA-coated IONP	suggested	suggested	suggested	/	Human cerebral endothelial cells	102b
quantum dots	PLL-stabilized IONP	suggested	suggested	/	/	/	L929 mouse fibroblast cells	103a
	photoexcited graphene QDs	X	suggested	suggested	/	/	ECV-304 human endothelial cell line	103b
	uncapped CdTe QD	suggested	suggested	/	/	/	U251 human glioblastoma cell line	104b
	QDot	suggested	suggested	/	/	/	PC12 undifferentiated rat pheochromocytoma cells	81b
	PEGylated QDs	X	suggested	/	/	/	human mesenchymal stem cells	104a
	streptavidin-QDs	suggested	suggested	/	/	/	LLC-PK1 porcine renal proximal tubule cells	105
	COOH-QDs	suggested	suggested	yes	/	/	HeLa cells; primary neuronal hippocampus cells; Wistar rats	104c
	COOH-CdTeQDs	suggested	suggested	yes	/	/	RAG mouse renal adenocarcinoma cell line	105
zinc oxide NPs	ZnO nanorods	suggested	suggested	/	/	/	HepG2 human liver cancer cells	104d
	ZnO nanorods	suggested	suggested	yes	/	/	JB6 Cl 41–5a mouse skin epidermal normal cells	109
silica NPs	SiO ₂	suggested	suggested	suggested	/	/	FaDu head and neck squamous cell carcinoma	110
rare-earth-based NPs	SiO ₂ (multiple shapes)	suggested	suggested	/	/	/	A549 human lung cancer cells; RAW264.7 mouse macrophages	118a
	neodymium oxide	suggested	suggested	/	/	/	NCLH460 nonsmall cell lung cancer cells	117
	lanthanide-based UCP	X	suggested	suggested	/	/	HeLa cells; Balb/c mice	107
	CeO ₂	suggested	suggested	/	/	/	primary human peripheral blood monocytes	119e
	Sm ₂ O ₃	X	suggested	/	/	/	HeLa cells	119c
	Gd ₂ O ₃	X	suggested	/	/	/	HeLa cells	119c
	Tb ₂ O ₃	X	suggested	/	/	/	HeLa cells	119c
	Eu ₂ O ₃	X	suggested	/	/	/	HeLa cells; PC12 rat pheochromocytoma cells; neuro 2a mouse neuroblasts	119d
	Eu(OH) ₃ nanorods	X	suggested	/	/	/	HeLa cells; MEF cells	119a
	Y ₂ O ₃	X	suggested	/	/	/	HeLa cells	124b

Table 2. continued

	NP	AP induction ^a	AP flux blockage ^a	ROS dependent ^b	mTOR dependent ^b	cell type	ref
carbon NTs	Yb ₂ O ₃ COOH-SWCNT MWCNT	X suggested suggested	/	/	/	HeLa cells; MEF cells A549 human lung cancer cells BEAS-2B human lung epithelium cells	119a 112 124f
graphene oxide NPs	acid functionalized-SWCNT graphene oxide	X X suggested X suggested	X X /	/	/	primary murine peritoneal macrophages primary murine peritoneal macrophages	113 113
fullerenes	graphene oxide C ₆₀ C ₆₀	suggested X suggested	/	/	/	RAW264.7 mouse macrophages	115
C ₆₀ (Nd)	X	autolysosome accumulation suggested	yes, not only	/	/	HeLa cells; MCF-7 human breast cancer cells HeLa cells; MEF cells; MCF-7 human breast cancer cells	116d 116a 116c
fullerenol	fullerenol PEG-C ₆₀ derivatives	suggested, low concentration suggested suggested	suggested, high concentration	no	/	LLC-PK1 porcine renal proximal tubule cells	116b
copper oxide NPs	CuO	X	/	/	/	HUVEC human umbilical vein endothelial cells	116e
silver NPs	CuO Ag nanowires Ag NP	X X X X TiO ₂ TiO ₂ MnO	suggested suggested suggested suggested suggested suggested X	suggested /	/	mouse neuroblastoma neuro-2A cells A549 human lung cancer cells; H1650 human nonsmall cell lung cancer cells; CNE-2Z human nasopharyngeal cancer cells	116f 118b
titanium dioxide NPs	Mn NP α -Al ₂ O ₃ Al ₂ O ₃	/	/	/	/	MCF7 human breast cancer cells	123
manganese NPs	amine-modified polystyrene beads polystyrene beads (μ m size) P-V ₂ O ₅ crystals Ni–Co NPs Pd NP	X X X X X	suggested suggested suggested suggested suggested	/	/	AS49 lung cancer cells THP-1 human monocyteic cell line Sprague–Dawley rats H4 human glioblastoma cells human primary epidermal keratinocytes human cerebral endothelial cells HeLa cells; HepG2 human liver cancer cells; HaCat immortalized human keratinocyte cells; COS-7 monkey fibroblast cell line	122 124d 125 111 124c 102b 78a
alumina NPs	Pd NP	/	/	/	/	N27 dopaminergic cell line; primary mesencephalic neuronal cells C57BL/6 mice; dendritic cells	162 124a
other	/	/	/	/	/	HeLa cells; Wistar Rats	151e
Polystyrene particles	/	/	/	/	/	132IIN1 human brain astrocytoma cells	96
						peripheral blood mononuclear cells (PMBC)	124g

^aThe rise in the number of autophagosomes can stem from either a direct upregulation of autophagy or a halt in autophagy (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"). ^bMany 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 proven to be dependent on ROS and/or mediated by the mTOR pathway. Where applicable this has been indicated in the table, 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").

888 effect, or discrepancies due to intrinsic differences in the various
889 cell types used in the respective studies.¹¹¹

890 **4.2.5. Carbon-Based Nanomaterials.** Various types of
891 carbon-based NPs have been shown to alter autophagy. For
892 instance, carboxyl-functionalized carbon nanotubes (CNT)
893 affected autophagy in an mTOR-dependent manner in A549
894 cells; conversely, differently functionalized particles (with
895 poly(*m*-aminobenzenesulfonic acid or PEG) did not. Even so,
896 potential differences in uptake by the different CNTs were not
897 examined; this does suggest that surface group characteristics
898 may influence the impact of NPs on autophagy modulation.
899 Interestingly, besides restoring cell viability *in vitro*, pretreatment
900 with 3-MA partly abrogated NP-mediated lung
901 inflammation in mice indicating a role for autophagy in lung
902 toxicity.¹¹²

903 Furthermore, a detailed study of Wan et al. demonstrated
904 that acid-functionalized SWCNT and graphene oxides caused
905 autophagosome accumulation by compromised autophagy flux
906 in primary murine peritoneal macrophages. The underlying
907 mechanism was clarified by means of Lysotracker staining and
908 FITC-dextran labeling of NP-treated cells, which revealed
909 decreased lysosomal quantity and lysosomal membrane
910 damage, respectively;¹¹³ surely, as described above, it is well
911 established that lysosomal health strongly influences autoph-
912 agy.¹¹⁴ It is noteworthy that, despite their comparable chemical
913 composition and surface functionalization, CNT and graphene
914 oxides affected autophagy to a different extent. This suggests
915 physical characteristics might also influence autophagy
916 modulation, yet a more thorough study is necessary to
917 substantiate this.¹¹³ The effect of graphene oxides on
918 macrophages was evaluated by Chen et al., who demonstrated
919 for the first time that NPs could trigger autophagy with the
920 involvement of TLR signaling. Indeed, silencing of, e.g., TLR4
921 and TLR9 reduced the abundance of LC3 puncta and beclin-1
922 aggregates as assessed by immunofluorescence microscopy.¹¹⁵
923 Fullerenes have been correlated with autophagy induction as
924 well as dysfunction.¹¹³ For example, fullerene- (C₆₀) and
925 neodymium-functionalized fullerenes (C₆₀(Nd)) are presented
926 as autophagy inducers,^{116c,d} while Johnson-Lyles et al. suggest
927 fullerenol NP can perturb autophagy at high concentration.
928 They hypothesize the observed NP-mediated cytoskeleton
929 disruption results in autophagy dysfunction and consequently
930 ATP depletion.^{116b}

931 **4.2.6. Other Hard Nanomaterials.** A study describing the
932 response of A549 cells and macrophages toward diversely
933 shaped silica (SiO₂) NP treatment reported that the cell type
934 but not the geometry of the particles shaped this response.¹¹⁷
935 Interestingly, a total of three studies carried out in A549 cells
936 describe the cytotoxicity of SiO₂ NPs as apoptosis independent,
937 although autophagy was only observed in two of those
938 studies.^{117,118}

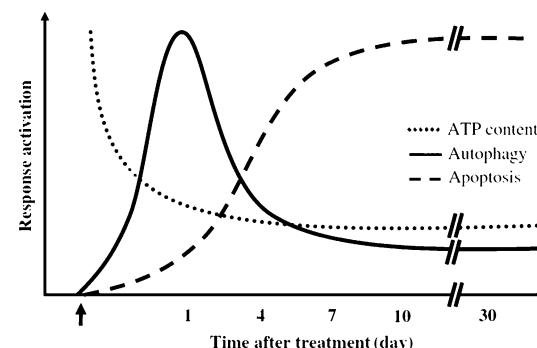
939 A great variety of rare-earth-element-based NPs were
940 described to induce authentic autophagy in HeLa cells.¹¹⁶
941 Among these studies there was a remarkable example of how
942 surface group characteristics can influence the autophagy-
943 inducing potential of a NP. This was presented by Zhang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

949 Not only surface characteristics but also chemical composi-
950 tion has been brought forward as a way of tuning autophagy.

951 Treatment of HeLa cells with nickel–cobalt NPs with different
952 molar concentrations of both components revealed that the
953 higher the Ni component, the more potent the impact on
954 cytotoxicity and autophagy is. Moreover, autophagy was
955 involved in NP-mediated toxicity since treatment with 3-MA
956 significantly restored cell viability.¹²¹

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

973 **4.2.7. Physiological Effects of Nanoparticle-Mediated**
974 **Autophagy Modulation.** An *in vivo* study in rats aiming to
975 identify the role of autophagy in AgNP-mediated hepatotoxicity
976 elegantly characterized the connection between changes in
977 autophagy, apoptosis, and ATP depletion (Figure 10). After 1
978 f10



979 **Figure 10.** Correlation among the level of ATP content, autophagy,
980 and apoptosis in rat livers after Ag NP treatment. Reprinted with
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982 day of AgNP administration the authors observed a
983 considerable decrease in ATP levels in the liver, likely because
984 of damage to hepatic mitochondria, while at the same time
985 autophagic markers were significantly increased. This autoph-
986 agy induction is probably an attempt to remove these
987 dysfunctional organelles and restore the reduced energy levels.
988 However, since autophagy failed to compensate for this energy
989 drop, the level of autophagy decreased in time in favor of
990 apoptosis, which became the predominant mechanism.¹²⁵
991 These data reveal the complex interplay between the different
992 pathways and highlight the important protective role of
993 autophagy. In cultured cells, the interrelationship between the
994 different factors is altered due to the lack of complex animal
995 physiology. In *vitro*, high levels of autophagy may therefore
996 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. On the basis of 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, nondegradable 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 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. (1) Interaction of NPs or intrinsically formed ROS species with mitochondria can induce mitochondrial membrane damage, resulting in disruption of the MMP and respiratory chain. As the latter is one of the main sites for 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 activation of intracellular signaling cascades that induce 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 to tune 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.^{96,137} This dysfunction can indirectly upregulate

autophagy as a mechanism for the cell to compensate for insufficient degradative capacity.^{81b} 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.¹³⁸ 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 the transcription of lysosomal and autophagic genes.⁸¹

Lysosomal dysfunction by the presence of nondegradable NPs can have wide-ranging consequences. Indeed, next to their pivotal role in the degradation and recycling of macro-molecules, 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.

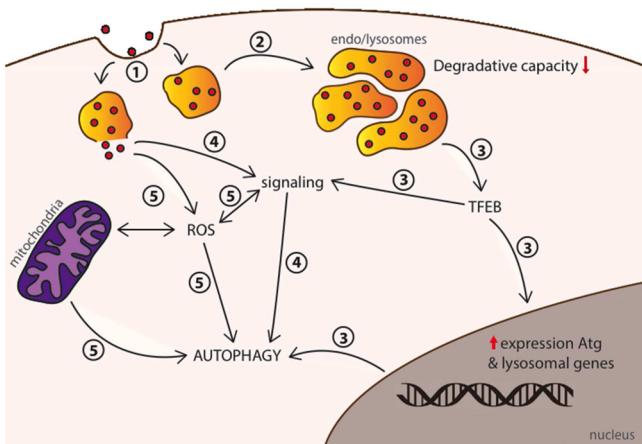


Figure 11. Schematic overview of NP-associated autophagy induction pathways. NPs will mostly enter the cell via endocytosis, by which they are enclosed in endocytic vesicles (1); they can then move further down the endocytic pathway and end up in late endosomes and/or lysosomes (2). Multiple NPs have been 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 formation of ROS (5). ROS are important regulators of autophagy that can affect its machinery and signaling and/or lead to mitochondrial damage, thus triggering autophagy (5).

5. 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 therefore of vital importance for maintaining cellular homeostasis. For example, 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 autoimmune diseases,¹⁴⁵ and metabolic diseases.³ Unfortunately, this may also imply that NPs capable of disrupting autophagy may result in or contribute to 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 by perturbing the cytoskeleton.¹⁴⁶

In the 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 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. First, 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 though 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) limitation of NP size below 100 nm, (b) positive surface charges to enhance electrostatic interaction

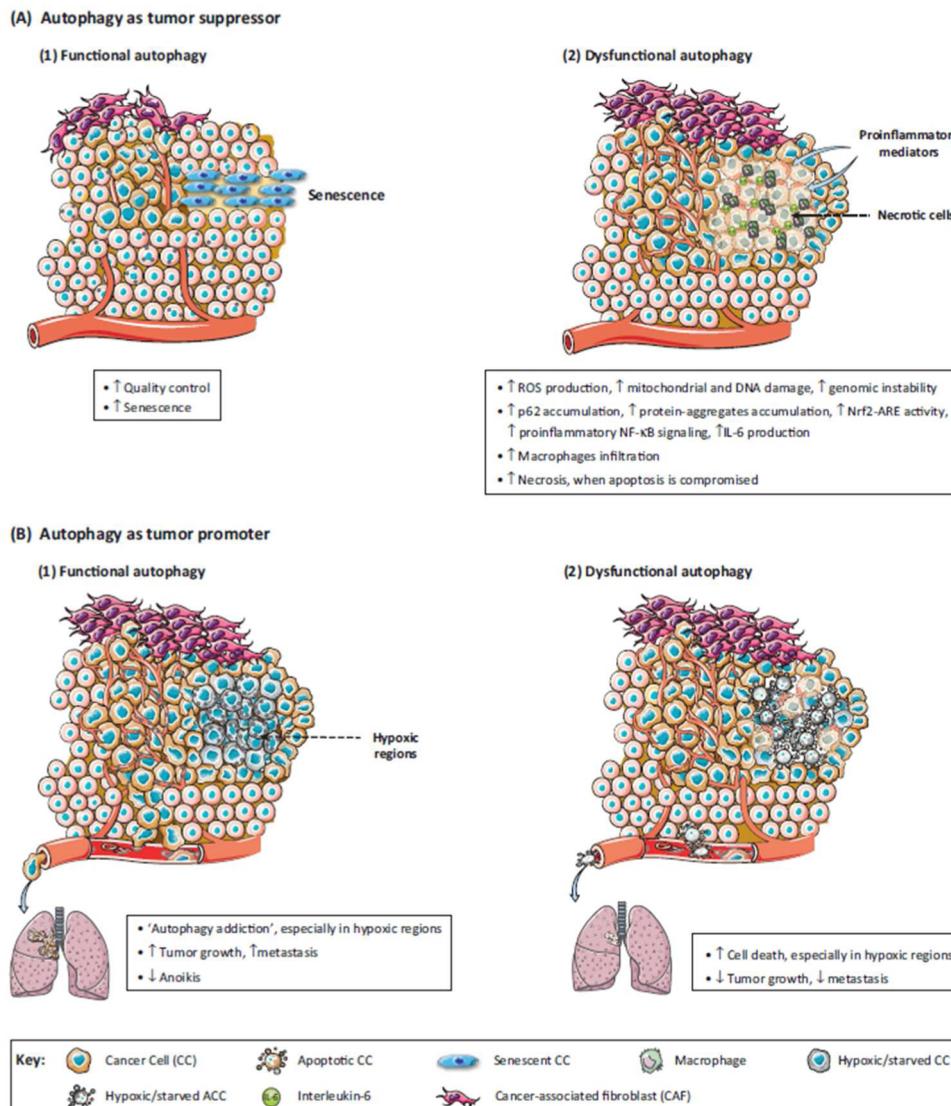


Figure 12. Overview of the mechanisms underlying the ambiguous role of autophagy in cancer. (A) Autophagy as tumor suppressor. (1) Autophagy maintains cellular homeostasis, thus preventing tumorigenesis. (2) Autophagy dysfunction creates a tumor-promoting environment through multiple mechanisms such as genomic instability and p62 accumulation. (B) Autophagy as tumor promoter. (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.

with endothelial cells lining the BBB, and (c) 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 further 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 neuro-pathologies is indeed plausible.

Second, 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, α -synuclein, and β -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 neuro-pathology. 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 to be failed cargo recognition,^{154,158} while in AD and PD lysosomal abnormalities hinder efficient autophagosome maturation.^{155,159} Strikingly, there is evidence that β -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 neuro-nano materials.^{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 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., TNF α 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 NF κ B signaling, thus forming a tumor-creating environment.¹⁶⁶ On the basis of 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 anticancer 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 influences not only 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-1 β) 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 antitumor immune response. This was based on the observation that autophagy is responsible for the premortem 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.^{164b} Accordingly, more research is necessary to further elucidate the effect of autophagy on cancer before we can safely use it as an anticancer 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}

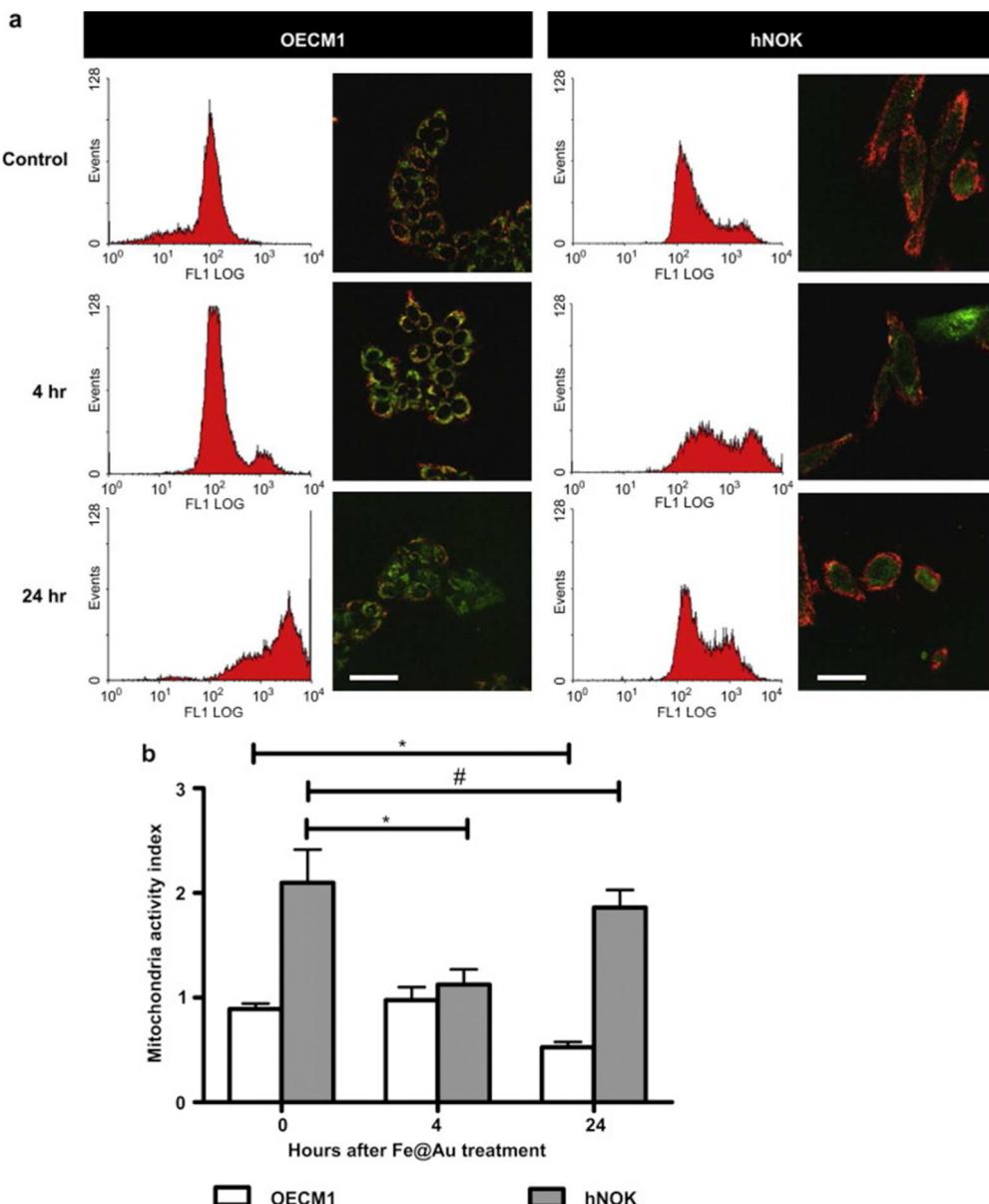


Figure 13. Fe@Au treatment elicited irreparable loss of mitochondrial membrane potential in cancerous cells (OECM1) but not in benign control cells (hNOK). (a) Both cell types were stained for JC-1 to evaluate the mitochondrial membrane potential by flow cytometry in the green channel (FL1) and by confocal microscopy. (b) 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 4 h time point is largely restored after 24 h (*, $p < 0.001$, paired FT-test; #, $p > 0.05$, paired FT-test). Reprinted with permission from ref 11a. Copyright 2011 Elsevier.

6. 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 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.¹³⁷⁸ 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 anticancer 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 h (Figure 13). Furthermore, the activity of the mitochondrial respiratory chain was substantially diminished in cancerous cells, while no deterioration in activity was detected in noncancerous 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 coincubation 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 noncancerous cells may be a result of mitochondria-mediated autophagy (Figure 14). Unfortunately, in the present study the level of autophagy was not specified for noncancerous 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

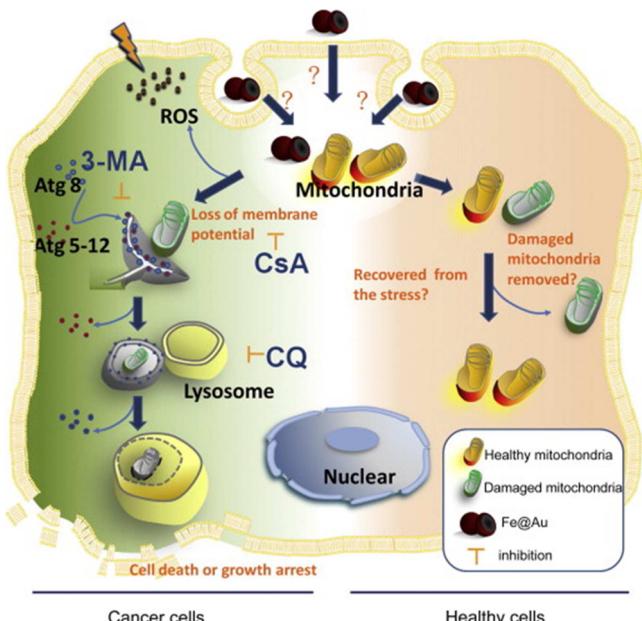


Figure 14. Fe@Au elicited selective cytotoxicity toward cancer cells through mitochondria-mediated autophagy. Fe@Au caused significant mitochondrial damage within 4 h in both cell types. Later, mitochondrial activity in healthy cells was restored, while the cancer cells could not recover from this damage.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

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

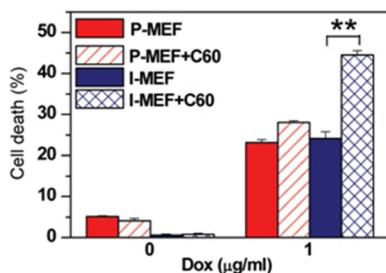


Figure 15. Fullerene-mediated chemosensitization in immortalized (I-MEF) but not primary MEF (P-MEF) cells. Both cell types were treated for 24 h with Doxorubicin, with or without a 24 h pretreatment with low-dose Nano-C60. Cell death was evaluated by Hoechst 33342/PI staining and calculated as the percentage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

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 stimulation of the latter may not be primarily mediated by ROS. The authors further suggest that the lysosomes are unable to digest these NPs, which would thus result in a blockage after autophago-some–lysosome fusion. In contrast with NP treatment, rapamycin, a commonly used autophagy inducer, 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 antisurvival effects can be obtained, which then either can 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 noncancerous cells. Third, cancer cells have a deregulated pro- and antiapoptotic 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 antiapoptotic 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.

In brief, the above-described studies propose that certain types of NPs are able to selectively induce autophagy in cancerous cell types while eliciting less or no significant autophagy stimulation in nonmalignant cells. More importantly, this different level in NP-mediated autophagy results in cancer cell-specific cytotoxicity. This intrinsic selectivity may therapeutically be highly advantageous since this may reduce collateral damage as observed with current anticancer therapies; moreover, this may simplify drug delivery by limiting the need for active targeting or enhance the effect of current chemo-therapeutics. No general conclusive mechanism has been reported, yet ROS have repeatedly appeared crucial for triggering autophagy and/or the detected cytotoxicity in cancer cells. There is a great need for a more thorough comparison between cell types, as, for instance, the uptake profile of NPs for the various cell types was only evaluated for Fe@Au NPs.^{134,180} To truly examine the autophagy-stimulating potential of a NP it would be valuable to experimentally determine this at similar intracellular concentrations to cancel out potential differences in NP uptake efficiency. Furthermore, the number of cancerous as well as benign cell types examined should be extended to find out if the promising results can be extrapolated and considered as a general effect prior to embarking on *in vivo* studies.

6.1.3. 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 among 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 evasion¹⁸⁵ and which induces resistance toward anticancer

1596 therapy including chemo- and radiotherapy. An extensive study
1597 of Shimizu et al. demonstrated that apoptosis-inducing agents
1598 (e.g., etoposide) could induce cell death in MEF cells even
1599 despite their resistance to apoptosis (by $Bax^{-/-}$ and $Bak^{-/-}$
1600 knockdown). Further investigation by means of chemical and
1601 genetic inhibition of autophagy clarified the cell death pathway
1602 to be autophagic cell death.¹⁸⁶ Another example is given by
1603 Moretti et al., who detected autophagic cell death as the main
1604 death pathway in radiation-treated apoptosis defective MEF
1605 cells.¹⁸⁷ These are no isolated results; multiple studies show
1606 that autophagic cell death can be induced in apoptosis defective
1607 models, corroborating the theory that autophagy induction is a
1608 promising new strategy for eradication of resistant cancer.¹⁸⁸

1609 It is clear that the modulation of autophagy by new and
1610 conventional drugs is an intriguing subject, yet the question
1611 remains why NPs in particular are such appealing candidates
1612 compared to more common autophagy promoting drugs, such
1613 as doxorubicin or paclitaxel? As already extensively discussed in
1614 section 2, nanotherapy offers multiple advantages compared to
1615 conventional drugs. These advantages are due to specific
1616 physicochemical properties attributed to NPs such as their
1617 small size and high surface area over volume ratio. However,
1618 one must keep in mind that these properties also usually
1619 contribute to NP toxicity. The small size of the NPs is
1620 associated with new functionally attractive physical properties
1621 (e.g., surface plasmon resonance for Au NPs, superparamag-
1622 netism for IONPs), which has enabled an evolution of
1623 innovative techniques and therapies.¹⁸⁹ By taking advantage
1624 of their optical properties and ability for therapeutic
1625 functionalization, diagnosis and treatment can be combined
1626 into a single entity, permitting, for instance, image-based drug
1627 delivery.¹⁹⁰ The high surface over volume ratio also provides a
1628 large platform, permitting a high payload of potentially multiple
1629 ligands (e.g., drugs). Furthermore, their size allows the particles
1630 to penetrate deeply in tissues, a feature exploited in the
1631 enhanced permeability and retention (EPR) effect, a phenom-
1632 enon also referred to as “passive targeting” since it allows
1633 particles to accumulate at tumor sites. This accumulation is
1634 established by enhanced angiogenesis in tumor tissue, which
1635 results in leaky vasculature of low quality; moreover, the lymph
1636 drainage in these tissues is frequently inefficient. In other
1637 words, NPs can readily penetrate tumor tissue by its leaky
1638 vasculature while their removal is relatively low; logically this
1639 selectively generates high local concentrations of NPs in
1640 cancerous tissue allowing efficient killing.¹⁹¹ In addition, NPs
1641 can be designed to acquire long blood circulation times which
1642 further enhances their tumor uptake.¹⁹² Passive targeting can
1643 efficiently be combined with active targeting by, for example,
1644 the binding of cancer specific receptor ligands or antibodies on
1645 the NP surface, thus increasing selectivity and undesired
1646 toxicity toward noncancerous cells.¹⁹³

1647 Another feature of NMs is their ability to bypass multidrug
1648 resistance (MDR). MDR represents a primary obstacle in
1649 anticancer therapy, diminishing the potency of standard
1650 chemotherapeutics like paclitaxel. The most common mecha-
1651 nism of resistance is the overexpression of P-glycoprotein
1652 (Pgp), an efflux pump that transports the chemotherapeutic
1653 agent out of the cell in such way that its efficacy is dramatically
1654 reduced.¹⁹⁴ However, several studies report on efficient MDR
1655 cancer killing by NMs. This is likely owing to the fact that NMs
1656 exceed the size limit of the transporter, thus preventing their
1657 efflux.¹⁹⁵ Examples of NPs bypassing this resistance include Ag

NPs, IONPs, and phosphatidylserine-containing nanocar-
1658 riers.^{195,196}

In conclusion, the above-listed benefits combined with
1660 selective modulation of autophagy by certain NPs can be
1661 highly advantageous for anticancer applications.

6.2. Autophagy-Mediated Synergistic Effect of Nanoparticles and Chemotherapeutics

A synergistic anticancer effect was observed in vitro and in vivo
1664 with autophagy-inducing PEGylated nanoliposomal C6-ceram-
1665 ide in combination with the autophagy maturation inhibitor
1666 vinblastine. The enhanced cancer killing was proven to be
1667 apoptosis mediated and dependent on autophagy, demon-
1668 strated by augmented caspase activity and neutralization of the
1669 effect by beclin-1 knockdown, respectively.⁹² We hypothesize
1670 that this type of cotreatment can give lead to enhanced
1671 sequestration of cytoplasmic components, while the autoph-
1672 gosomal cargo cannot be degraded owing to impaired
1673 lysosomal functionality or autophagosome–lysosome fusion.
1674 This can thus result in a high abundance of dysfunctional
1675 vesicles following disruption of cell homeostasis and con-
1676 currently cell death. The successful combination of an
1677 autophagy inducer and inhibitor was already proven earlier,
1678 although only with chemical agents.¹⁹⁷ Besides cotreatment of
1679 NP with autophagy disruptors, NPs can be combined with
1680 chemicals that are able of stimulating autophagy flux.

Lu et al. evaluated the cytotoxic effect of manganese oxide
1682 (MnO) NP and doxorubicin cotreatment on several cancer cell
1683 types (including HeLa). This combination treatment proved to
1684 be highly efficient in vivo since it doubled the reduction in
1685 tumor weight compared to NP or doxorubicin treatment alone.
1686 Moreover, a control experiment with 3-MA showed that this
1687 synergistic effect was also dependent on autophagy.^{78a} These
1688 studies highlight the potential of autophagy in the elimination
1689 of resistant cancer. This effect is however dual, and more
1690 research is required to better control the outcome of autophagy
1691 induction. Chemotherapeutic agents can result in cell stress,
1692 such as damaged mitochondria or DNA damage, which in itself
1693 can result in autophagy. When the level of autophagy is rather
1694 low, this will mainly have a protective effect where the damaged
1695 parts will be isolated and destroyed after which the cells can
1696 recover. Still, if autophagy is strongly induced, this can result in
1697 ACD as the cell will degrade itself next to substantially lowering
1698 its ATP levels. Therefore, induction of autophagy together with
1699 addition of chemotherapeutic agents can have a synergistic
1700 effect, as both will result in autophagy upregulation, which can
1701 then result in ACD or stimulate apoptosis when ATP levels
1702 drop too low.

The above-listed studies clearly underscore the potential of
1704 autophagy in overcoming chemoresistance and emphasize the
1705 relevance of investigating autophagy as an alternative cell death
1706 pathway.

6.3. Enhanced Tumor Antigen Presentation through Nanoparticle-Mediated Autophagy

Next to the ability of NPs to eliminate cancer cells, a recent
1709 study described the use of α -alumina ($\alpha\text{-Al}_2\text{O}_3$) NPs to
1710 enhance cross-presentation of exogenous antigens and thus
1711 promote an anticancer immune response. Ovalbumine-
1712 conjugated NPs were phagocytosed in dendritic cells after
1713 which they presented the antigens to OVA-specific CD8⁺ T
1714 cells through an autophagy-dependent mechanism. This cross-
1715 presentation led to the activation and proliferation of cytotoxic
1716 T cells, an effect that was efficiently extrapolated to the *in vivo*

1718 situation seeing that vaccination with these NPs successfully
 1719 eliminated established tumors. Interestingly, this principle was
 1720 less potent with other NPs (e.g., TiO_2) both *in vitro* as well as
 1721 *in vivo*. The present study implies that NPs can aid to improve
 1722 therapeutic cancer vaccination through autophagy modulation
 1723 n.^{124a}

6.4. Autophagy Induction as a Self-Protection Process 1724 against Nanotoxicity

1725 As previously mentioned, the process of autophagy is very
 1726 complex and its modulation can have widely varying outcomes,
 1727 such as inducing cell death. Alternatively, induction of
 1728 autophagy can result in a cytoprotective mechanism by which
 1729 cells respond to external stress signals, such as serum starvation.
 1730 It has been hypothesized that NM-induced autophagy may have
 1731 a similar effect, so that the overall cytotoxicity of the particles
 1732 could be reduced upon induction of autophagy. A few recent
 1733 studies have supported this hypothesis, hinting at the
 1734 importance of autophagy induction in mediating cellular
 1735 responses to NM-induced stress. Neibert and Maysinger
 1736 showed that exposure of rat pheochromocytoma cells (PC12)
 1737 to CdTe QDs resulted in a substantial increase in intracellular
 1738 antioxidant levels, enlargement of the entire lysosomal
 1739 compartment, and activation of TFEB.^{81b} The authors argued
 1740 that these processes aim to protect the cell as a response to the
 1741 damage exerted by the QDs. In a similar study, Luo et al.
 1742 exposed mouse renal adenocarcinoma cells to QDs, revealing
 1743 clear induction of oxidative stress, autophagy, and cell death.
 1744 When cells were cotreated with an autophagy inhibitor (3-MA),
 1745 this resulted in a significant rise in cell death (Figure 16).
 1746 Remarkably, when the cells were cotreated with an antioxidant

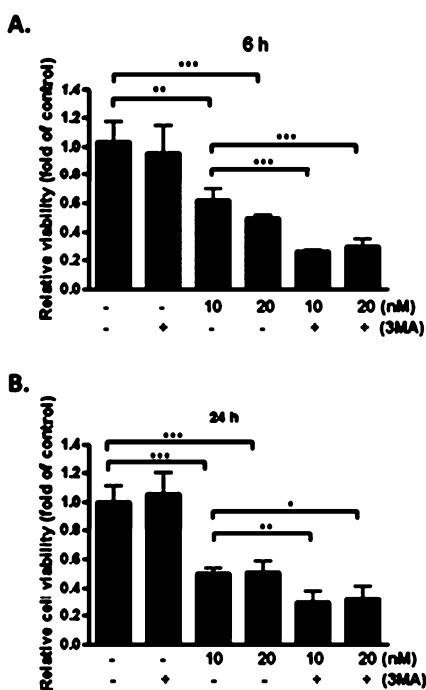


Figure 16. 3-MA enhances QD-induced cytotoxicity in mouse renal adenocarcinoma cells. 3-MA (10 mM) augments QD-induced cytotoxicity at 6 (A) and 24 h (B) as determined by the MTT assay. This indicates that autophagy attempts to reduce QD-induced toxicity. Mean \pm SD $n = 4$ (*, $p < 0.05$; **, $p <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

1747 agent, the level of autophagy decreased and the level of cell
 1748 death again increased. These data reveal the importance of NP-
 1749 induced oxidative stress and the associated induction of
 1750 autophagy as a survival mechanism by which the cell tries to
 1751 combat NP-induced damage.¹⁰⁵

Zhou et al. 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 VO_2 nanocrystals (P- VO_2) or with Y_2O_3 NP autophagy levels were upregulated compared to untreated control cells. Interestingly, in the case of P- VO_2 NPs, this resulted in far less cell death than was the case for the Y_2O_3 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. Expression of HO-1 was found to be dependent on autophagy levels, but interestingly, HO-1 expression was only upregulated by P- VO_2 NPs and not by Y_2O_3 NP for reasons that are yet unclear. This finding does explain the difference between the cytoprotective role of autophagy for P- VO_2 NPs and the pro-death role of autophagy for Y_2O_3 NPs (Figure 17).^{124b}

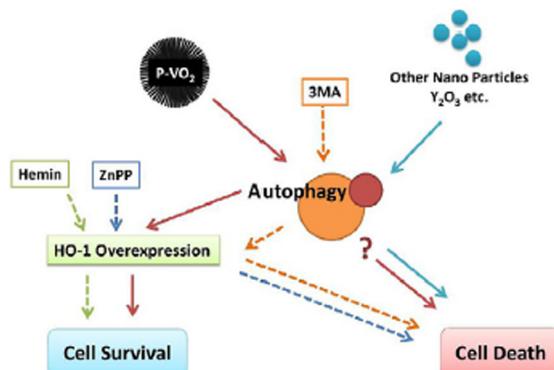


Figure 17. Overview of the cellular effects induced by P- VO_2 and other nanocrystals. P- VO_2 NPs trigger autophagy, which subsequently leads to HO-1 overexpression and cell survival. Accordingly, inhibition of autophagy via 3-MA treatment aborts this overexpression, thus resulting in cell death. A 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 a diverse role in cell death depending on its original trigger. Reprinted with permission from ref 124b. Copyright 2013 IOP Publishing.

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. 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 to penetrate the brain and perturb autophagy are proposed as a potential danger.¹⁶¹ However, their ability to cross the blood–brain barrier also makes them interesting vectors and/or therapeutic agents for combating brain diseases. Since autophagy stimulation has now been linked with multiple

1783 NPs, this suggests they might be able to prevent or help to
 1784 combat the disease depending on the precise conditions of the
 1785 pathology. This hypothesis has been supported by a recent
 1786 study of Lee et al., who evaluated the potential of fullerene-
 1787 mediated autophagy stimulation as a treatment for Alzheimer's
 1788 disease. The authors observed that treatment of neuroblast cells
 1789 with fullerene-based NMs was able to reduce β -amyloid-based
 1790 toxicity, an effect that was partly abrogated by cotreatment with
 1791 3-methyladenine. This indicates the elicited autophagy
 1792 stimulation acts as a cytoprotective process, and accordingly,
 1793 these NPs and other NMs able of stimulating autophagy may
 1794 have therapeutic potential.^{116f} Enhanced clearance of htt
 1795 protein by autophagy was observed upon treatment of PC12
 1796 and Neuro 2a cells with europium hydroxide nanorods,
 1797 indicating that NP-mediated autophagy induction can be
 1798 exploited to accelerate removal of harmful protein aggrega-
 1799 tes.^{119d} It is noteworthy that more NMs are put forward as
 1800 potential therapeutic vectors for treatment of neurodegener-
 1801 ative diseases.¹⁹⁹

7. CONCLUSIONS AND OUTLOOK

1802 This review presents an overview of the most relevant reports
 1803 on NP-mediated autophagy alterations and their impact on
 1804 nanotoxicology and nanomedicine. In general, the field of
 1805 nanotechnology is greatly expanding, increasing the public's
 1806 exposure to NMs at a fast pace. Since many of these (in)organic
 1807 NPs have been proposed to be capable of altering autophagy
 1808 and since autophagy dysfunction itself is associated with
 1809 multiple pathologies (e.g., neurodegenerative diseases), it is of
 1810 vital medical and toxicological importance to determine the
 1811 effect of NPs on autophagy and its consequences. To efficiently
 1812 address this potential danger more in-depth research is
 1813 necessary to determine the role of autophagy in these
 1814 pathologies and at the same time the mechanisms by which
 1815 NPs are able of altering this process. Although most studies
 1816 report on autophagy induction, they often do not prove the
 1817 actual induction and any observed effects may also be explained
 1818 by other processes, such as a reduced turnover of
 1819 autophagosomes. In general, the wide variety of NPs (e.g.,
 1820 differences in size or coating) and used cell types makes it
 1821 difficult to draw any broad conclusions. Several papers do hint
 1822 at possible physicochemical parameters influencing the
 1823 autophagy-modulating effect, but there is still a great need for
 1824 more systematic studies that will aid the design of NPs that do
 1825 not affect autophagy at all or can be tuned to induce autophagy
 1826 to our advantage.

1827 There is growing evidence that NMs are able to activate
 1828 autophagy in a whole variety of cell types, which suggest a
 1829 probable common cellular response toward NP uptake. More
 1830 importantly, NPs have shown intrinsic selectivity in inducing
 1831 autophagy in cancer cells, resulting in their selective destruction
 1832 compared to noncancerous cell types. These exciting findings
 1833 indicate that NMs have huge potential as anticancer
 1834 therapeutics. The many advantages of NPs (e.g., active and
 1835 passive targeting) in combination with this selective toxicity is
 1836 indeed very promising and warrants further investigation.
 1837 However, as the outcome of autophagy induction in cancerous
 1838 tissue may differ depending on multiple factors, the dubious
 1839 role of autophagy in cancer must first be further elucidated in
 1840 order to safely turn these promising findings into practice. In
 1841 conclusion, the findings summarized in this review indicate a
 1842 bright future for NMs in cancer therapy, although more
 1843 research on the autophagic process as well as NP-mediated

autophagy modulation is needed to define the true danger and
 1844 benefit of NPs.
 1845

AUTHOR INFORMATION

Corresponding Authors

*Phone: +32 9 264 8098. Fax: +32 9 264 8189. E-mail: Stefaan. DeSmedt@ugent.be.
 1848
 1849

*Phone: +32 16 330045. Fax: +32 16 330021. E-mail: Stefaan. Soenen@med.kuleuven.be.
 1850
 1851

Notes

The authors declare no competing financial interest.
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Biographies



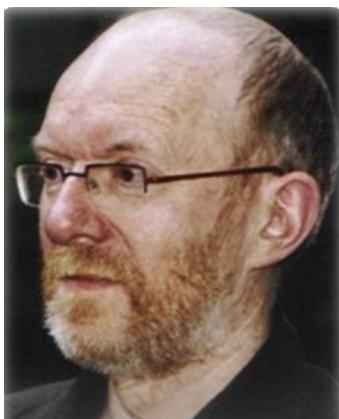
Karen Peynshaert obtained her Bachelor's degree in Pharmaceutical Sciences at Ghent University and continued her study to graduate with a Master's of Science degree in Drug Development. In 2012 she started her Ph.D. studies at the Lab of General Biochemistry and Physical Pharmacy of Ghent University (Belgium) under the supervision of Dr. Stefaan Soenen and Prof. Jo Demeester, in which she studies nanoparticle–cell interaction, mainly focusing on nanoparticle-mediated autophagy modulation.
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Bella B. Manshian completed her Ph.D. degree in Genetic Toxicology studying genotoxic effects of ultraviolet radiation and chemical mixtures. During her Ph.D. studies she worked part time as a research assistant in newborn immunity and allergy studies following onto child developmental medicine and stem cell research in collaboration with Cellgene Cellular Therapeutics, world leaders in stem cell therapy, and the U.K. National Health System. Currently, she is a research officer in the Swansea Nanomedicine department where she investigates the genotoxicity of nanoparticles.
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After obtaining her Bachelor's degree in Pharmaceutical Sciences in
1872 2010, Freya Joris graduated with her Master's of Science degree in
1873 Drug Development in 2012 from the VUB. The same year she started
1874 Ph.D. studies at the Lab of General Biochemistry and Physical
1875 Pharmacy at Ghent University (Belgium) under the supervision of Dr.
1876 Stefaan Soenen and Prof. Stefaan De Smedt, for which she received a
1877 scholarship from the IWT. The focus of her Ph.D. work lies in the
1878 optimization and development of in vitro protocols and models for
1879 studying nanotoxicity.
1880



Jo Demeester (born 1951) received his M.S. (1974) and Ph.D. degrees
1881 in Pharmaceutical Sciences in Ghent University in 1980. He became
1882 Professor at the same university in 1989 at the Laboratory of General
1883 Biochemistry and Physical Pharmacy (LGBPP), which has directed
1884 since 1997. Since 1994 he has been Director of the International
1885 Centre for Standards of the International Pharmaceutical Federation
1886 (F.I.P.), and since 1998 he has been an expert in the Group on
1887 Biological Products of the European Pharmacopeia. Since 2003 he has
1888 been President of the Enzyme Commission of the International
1889 Pharmaceutical Federation, and since 2006 he has been President of
1890 the Belgian Society of Pharmaceutical Sciences.
1891



Stefaan De Smedt (born 1967) studied pharmacy at Ghent University
1892 (Belgium) and graduated in 1995. He joined the pharmaceutical
1893 development group of Janssen Research Foundation. In 1999 he
1894 became Professor at Ghent University, where he is chairing the Ghent
1895 Research Group on Nanomedicines. Since 2004 he has served as
1896 European Associate Editor of the *Journal of Controlled Release*. His
1897 research is at the interface between drug delivery, biophysics, material
1898 sciences, and advanced optical imaging. He received the 2006 CRS
1899 Young Investigator Award and the 2010 APV Research Award for
1900 Outstanding Achievements in Pharmaceutical Sciences. He is scientific
1901 founder of spin-off Memobead Technologies.
1902



Kevin Braeckmans obtained his M.S. degree in Physics at Ghent
1903 University (Belgium) in 1999 and received his Ph.D. degree from
1904 Ghent University (Belgium) on advanced optical microscopy methods
1905 for which he received first prize (FBBF) in 2005. He stayed at the
1906 group of Prof. Braüchle at the Ludwig-Maximilians Universität
1907 München developing algorithms for single-particle tracking. Later, he
1908 returned to Ghent University as a FWO postdoctoral researcher. In
1909 2008 he became a professor at the Faculty of Pharmaceutical Sciences
1910 of Ghent University, where he is leading the Biophotonic Imaging
1911 Group, focusing on microscopy techniques for measuring molecular
1912 dynamics in gene therapy.
1913



1914 Stefaan J. Soenen (born 1983) graduated with his M.S. degree in
1915 Industrial Sciences (Biochemistry) in 2005 and a second M.S. degree
1916 in Molecular Medical Biotechnology at Ghent University (Belgium) in
1917 2006. He then pursued Ph.D. studies until 2010 at the lab of Prof.
1918 Marcel De Cuyper (KULeuven, Belgium) focusing on cell–nano-
1919 particle interactions. His work has been awarded the best biochemistry
1920 thesis in 2005 and a 2010 frontispiece issue in *Small*. Presently, he is a
1921 post-doctoral fellow at the Biophotonics Imaging group (Ghent
1922 University) under the guidance of Prof. Kevin Braeckmans and Prof.
1923 Stefaan De Smedt, focusing on biomaterials use in live cells.

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