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Differential Autophagic Responses to Nano-Sized Materials

by

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ABSTRACT

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Recent progress in the field of nanotechnology has led to a dramatic increase in the number of engineered nanomaterials currently being produced and introduced in the marketplace, raising concerns regarding the risk of human exposure to these specially designed, highly reactive materials. Engineered nanomaterials exert a diverse range of effects on biological systems, reflecting the heterogeneity and complexity of their properties. The effects of nanomaterials on biological systems, which range from inflammatory response to carcinogenicity and neurodegeneration, originate from the interaction between nanomaterials and cellular components, which also operate at the nanoscale. Cellular clearance mechanisms are typically activated in response to internalization of nano-sized materials. In particular, the cellular response to nanomaterials is mediated by the lysosome-autophagy system, which is the main catabolic pathway in mammalian cells that mediates degradation of a variety of nano-sized materials that are encountered by the cell through the lysosome. The lysosome-autophagy system is thus at the forefront of the cellular response to the uptake of nanomaterials: depending on the nanomaterial's physicochemical properties, this response can vary dramatically and may culminate in enhancement of cellular clearance or
blockage of the autophagic flux and, eventually, cell death. Due to its important role in maintaining cellular homeostasis and survival, defects in the lysosome-autophagy system may have deleterious effects on cells, possibly leading to pathologic conditions.

The objective of this study was to characterize the response of the lysosome-autophagy system to different types of nanomaterials, namely genetically encoded adeno-associated virus (AAV) particles, which are of particular interest due to their potential as gene delivery vectors, and engineered titanium dioxide and zinc oxide nanoparticles, which are used in a variety of consumer products. I investigated a comprehensive set of markers of the lysosome-autophagy system with the ultimate goal of elucidating the specific nature of the autophagic response to nanomaterial uptake and whether it leads to biocompatible or bioadverse effects on cell physiology.

Analyses of the molecular mechanisms underlying the autophagic response to AAV nanoparticles revealed that uptake of AAV induces activation of autophagy, which, in turn, results in a reduction in transgene delivery. Upregulation of autophagy induced by AAV also causes enhanced degradation of potentially toxic autophagic substrates. These results provide important insights for the design of AAV-based gene delivery systems as well as nanotherapeutics for the treatment of diseases characterized by insufficient autophagic clearance.

Titanium dioxide nanoparticles of different primary particle diameters were found to induce transcriptional upregulation of the lysosome-autophagy system.
Prolonged exposure to titanium dioxide nanoparticles, however, induced lysosomal membrane permeabilization, leading to blockage of autophagic flux and accumulation of autophagic substrates. These results point to the complexity of the autophagic response to nanomaterials, which may involve activation of the lysosome-autophagy system, but also impairment of some of its components, leading to accumulation of autophagic vesicles that cannot be cleared by lysosomes.

Exposure to zinc oxide nanoparticles was also found to cause transcriptional upregulation of the lysosome-autophagy system and formation of autophagic vesicles. Cell treatment with bare zinc oxide nanoparticles (~85 nm) or coated with a highly stable silicone derivative (triethoxycaprylylsilane) enhanced the formation and turnover of autophagosomes, while exposure to larger zinc oxide particles (~200-1000 nm) caused blockage of autophagic flux, resulting in accumulation of autophagosomes.

Results from this study provide important insights into the effect of nanomaterials on the lysosome-autophagy system and will inform the design of the next generation of nanomaterials with predictable autophagy-modulating properties for a variety of industrial and medical applications.
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Ephesians 3:20-21

“Now to him who is able to do immeasurably more than all we ask or imagine, according to his power that is at work within us, to him be glory in the church and in Christ Jesus throughout all generations, for ever and ever! Amen.”
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>βCD</td>
<td>2-hydroxypropyl-β-cyclodextrin</td>
</tr>
<tr>
<td>CLEAR</td>
<td>Coordinated Lysosomal Expression and Regulation</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>LAMP</td>
<td>lysosome-associated membrane protein</td>
</tr>
<tr>
<td>LC3</td>
<td>microtubule-associated light chain protein 3</td>
</tr>
<tr>
<td>LINCL</td>
<td>Late Infantile Neuronal Ceroid Lipofuscinosis</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mammalian target of rapamycin protein kinase complex 1</td>
</tr>
<tr>
<td>PSQ</td>
<td>penicillin-streptomycin-glutamine</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SSC</td>
<td>side scattering parameter</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TFEB</td>
<td>Transcription Factor EB</td>
</tr>
<tr>
<td>TiO₂ NP</td>
<td>titanium dioxide nanoparticle</td>
</tr>
<tr>
<td>vgs</td>
<td>viral genomes</td>
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<tr>
<td>ZnOP</td>
<td>zinc oxide particle</td>
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Introduction


Progress in the field of nanotechnology has led to a rapid growth in the number of engineered nanomaterials being produced and introduced into the marketplace [1]. As a result, workers in the nanotechnology industry and consumers are inevitably exposed to these specially designed, highly reactive materials. Due to their nanoscale size, nanomaterials are able to interact with biological components and systems, which also operate at the nanoscale level. The interfaces between nanomaterials and biological systems, including proteins, membranes, and cells, depend on dynamic biophysicochemical interactions [2]. These interactions may lead to cellular uptake, protein conformational changes, immune recognition, or other biocatalytic processes that can have biocompatible or bioadverse outcomes [3]. Surprisingly little, however, is known about the effects that these nano-bio interactions have on cell physiology. Despite growing concern about the potentially
adverse effects of nanomaterials on humans and the environment, most studies focused on elucidating the toxicological profile of nanomaterials have been limited to toxicity and viability measurements [4]. However, even nanomaterials deemed non-toxic according to standard in vitro and in vivo viability assays can still impact fundamental biochemical pathways [5]; and while they may not lead to lethal or pathological outcomes, they can significantly alter cell physiology [5]–[8].

A sophisticated quality control system has evolved in mammalian cells to prevent accumulation of aberrant materials, such as proteinaceous aggregates, through the activation of degradative pathways. When nano-sized materials are internalized into cells, they are likely to be perceived as foreign or toxic and may stimulate activation of cellular clearance mechanisms [9], [10]. Specifically, nanomaterials of different size and composition have been shown to elicit activation of the autophagy system, the main catabolic pathway that mediates degradation of bulk intracellular material [11]. While activation of autophagy can lead to enhanced clearance, it may also be associated with activation of cell death programs [12]. Moreover, nanomaterials that activate autophagy may also lead to a blockage of autophagic flux through impairment of cellular components that mediate degradation.

1.1. The autophagy pathway

Autophagy (“self-eating”) maintains cellular homeostasis by mediating degradation of a variety of intracellular materials ranging from proteins to
organelles. Three forms of autophagy have been described: microautophagy, which proceeds through lysosomal engulfment of cytoplasmic material; chaperone-mediated autophagy, which enables selective degradation of soluble proteins by the lysosome; and macroautophagy, which involves sequestration of cytoplasmic components followed by degradation through the lysosome [11]. Nanomaterials typically induce activation of macroautophagy (hereafter referred to as autophagy), which leads to accumulation of autophagic vesicles that sequester toxic or foreign material [9].

Degradation via autophagy proceeds through (i) cargo recognition by isolation membranes called phagophores and sequestration into double membrane vesicles called autophagosomes; (ii) autophagosome-lysosome fusion, leading to the formation of autolysosomes; and (iii) cargo degradation by lysosomal hydrolytic enzymes [11] (Figure 1). Integrated control of lysosome and autophagosome biogenesis and fusion occurs via regulation of the Coordinated Lysosomal Expression and Regulation (CLEAR) gene network, which is mediated by the transcription factor EB (TFEB) [13]. Activation of TFEB is regulated by the mammalian target of rapamycin protein kinase complex 1 (mTORC1), which is a key component of numerous pathways that regulate cellular growth and proliferation in response to nutrients, energy levels, and growth signals [14], [15]. Under physiologic conditions, mTORC1 localizes onto the lysosomal membrane and phosphorylates TFEB. Phosphorylation of TFEB prevents its translocation into the nucleus [15]. Upon lysosomal stress, mTORC1 is released from the lysosomal
membrane, which results in translocation of TFEB into the nucleus and activation of the CLEAR network [14].

Figure 1. Autophagic degradation of protein aggregates mediated by the transcription factor EB. Autophagy proceeds through a cascade of three main steps: (i) transcriptional activation of autophagy via the transcription factor EB (TFEB) and sequestration of autophagic cargo into autophagosomes, (ii) fusion of autophagosomes with lysosomes, and (iii) degradation of the autophagic cargo in autolysosomes.

Activation of TFEB and the CLEAR network is not only observed upon nutrient deprivation as a means to recycle intracellular components, but also upon aberrant accumulation of intracellular material. A predominantly nuclear localization of TFEB was observed in fibroblasts from mouse models of different lysosomal storage disorders, suggesting that the lysosome-autophagy system is modulated at the transcriptional level in response to lysosomal storage [16]. Modulation of TFEB activation was also observed upon cellular uptake of 2-hydroxypropyl-β-cyclodextrin (βCD) [17], nano ceria [18], and polystyrene
nanoparticles [19], suggesting that transcriptional modulation of autophagy is part of the cellular adaptive response to nanomaterials.

1.2. Nano-sized materials as autophagy activators

1.2.1. Naturally occurring nanomaterials

Autophagy mediates disposal of insoluble nano-sized particles [20]. Inefficient autophagic activity is typically associated with aberrant accumulation of proteinaceous and proteolipidic aggregates, which are linked to the pathogenesis of neurodegenerative diseases such as Parkinson’s, Huntington’s, and lysosomal storage disorders [20]. Impairment of autophagy results in formation of proteinaceous and lipidic inclusions and neurodegeneration, while an increase in autophagic activity results in enhanced clearance of these aberrant nano-sized materials [21].

Autophagy also plays a key role in innate immunity and degradation of intracellular pathogens [22], [23]. The molecular mechanisms underlying activation of autophagy by viral infection are unclear and are likely to be associated with different steps of the viral infectious process [22]. Interestingly, the plasma membrane disturbance induced by human immunodeficiency virus type I [24] and by paromyxovirus particles [25] is sufficient to trigger activation of the autophagic response independent of downstream signal transduction, suggesting that autophagy is activated as a defense mechanism upon entry of this foreign nano-sized material.
In addition to mediating lysosomal degradation of autophagic cargo, activation of TFEB and upregulation of the lysosome-autophagy system may result in enhanced clearance of intracellular material via exocytosis. TFEB overexpression rescues the pathologic phenotypes of \textit{in vitro} and \textit{in vivo} models of lysosomal storage diseases by inducing exocytosis of lysosomes and autolysosomes [26], [27]. Interestingly, autophagy dysfunction induced via pharmacological inhibition or genetic inactivation results in enhanced exocytosis of protein aggregates [28], suggesting that autophagy and exocytosis may be regulated competitively or cooperatively, depending on the nature of the environment.

\subsection*{1.2.2. Engineered nanomaterials}

A number of engineered nanomaterials have been shown to accumulate within autophagosomes and even to enhance formation of autophagosomes. Seleverstov \textit{et al.} first documented the autophagy-inducing effect of quantum dots in 2006 [29]. Different types of nanomaterials were later found to also accumulate within autophagosomes, including silica [30], gold [31], α-alumina [32], rare earth oxides [33], and fullerenes [34]. While the composition and surface chemistry of these nanomaterials vary significantly, the nanoscale size seems to be a common denominator for accumulation into autophagosomes and, possibly, induction of autophagy [10]. Interestingly, the mechanism of autophagy activation varies widely among nanomaterials with different physicochemical properties. Surface modifications affecting the chemical reactivity of nanomaterials increase the complexity of the parameter space that needs to be explored to obtain a
comprehensive understanding of the design rules for engineering nanomaterials that interact with the autophagy pathway in a desired and precisely controlled fashion [5].

1.3. Autophagic response to the cellular uptake of nanomaterials

Despite increasing evidence suggesting a key role of the lysosome-autophagy system in the cell’s adaptive response to nanomaterials, the nature of the autophagic response induced upon nanomaterial uptake remains unclear and seems to consist of a wide and continuous distribution of phenotypes that vary significantly with respect to the nanomaterial’s physicochemical properties. Autophagy plays mainly a cytoprotective role and is commonly activated in response to cellular uptake of foreign materials (foreign body effect) [35]; however, a number of alternative models have been proposed, including nanomaterial-induced toxicity through lysosomal dysfunction [36], oxidative stress [31], [37], mitochondrial damage [38], [39], and direct effects on gene regulation [40] (Figure 2).
Figure 2. Mechanisms of nanomaterial-induced autophagy activation. Nanomaterials may induce autophagy through different mechanisms, including enhanced formation of autophagosomes, in response to the presence of foreign material, induction of oxidative stress, or induction of lysosomal damage. Depending on the nature of the nanomaterial, activation of autophagy may lead to enhanced clearance or blockage of autophagic flux.

Transcriptional activation of the lysosome-autophagy system upon internalization of nano-sized materials may lead to enhanced clearance of endogenous materials or impairment of downstream steps of the autophagy pathway and blockage of autophagic flux. Ceria nanoparticles, for instance, which have been amply investigated due to their growing commercial scale production and risk of human exposure [41], enhance autophagic clearance [42]. Silver nanoparticles, which are also widely used in consumer products and medical
devices due to their excellent anti-microbial properties [43], activate autophagy, but also induce oxidative stress and extensive cytotoxicity [44]. It is unclear whether autophagy is activated as a response due to the foreign body effect or due to reactive oxygen species-induced damage caused by nanosilver [45]. Interestingly, the increased formation of autophagosomes is not followed by enhanced fusion of autophagosomes with lysosomes, suggesting that nanosilver causes a blockage of autophagic flux [44].

1.3.1. Activation of autophagic clearance

TFEB activates the coordinated expression of autophagy and lysosomal genes [13]. Modulation of TFEB activation is thus a promising strategy to enhance clearance of intracellular material, such as proteinaceous and proteolipidic aggregates [13], [46]–[48]. The ability of βCD, an FDA-approved excipient used to enhance drug stability and bioavailability, to induce autophagic clearance was demonstrated in in vitro model systems characterized by aberrant accumulation of distinct biological materials [17], [49]. Internalization of nanoceria stabilized by biocompatible surface coatings (\(N\)-acetyl-glucosamine, polyethylene glycol, or polyvinylpyrrolidone) also results in TFEB-mediated activation of autophagic clearance [42]. The increase in cellular clearance of ceroid lipopigment observed in fibroblasts derived from patients with Late Infantile Neuronal Ceroid Lipofuscinosis (LINCL) [42] and exposed to nanoceria is thus particularly interesting in light of potential therapeutic applications [50].
The autophagy-inducing properties of nanomaterials can also reduce the efficiency of nanomaterial-based delivery systems. The therapeutic effect of the anticancer drug docetaxel delivered into MCF-7 breast cancer cells using poly(lactide-co-glycolide) polymer-based nanoparticles was greatly enhanced upon co-administration of autophagy inhibitors, such as 3-methyladenine or chloroquine. These findings suggest that activation of autophagy observed upon uptake of docetaxel-containing nanoparticles may cause drug degradation and reduced drug delivery [51].

Modulating nanomaterial-induced activation of autophagy to enhance cargo delivery or address safety concerns relies on careful nanomaterial design. Our currently limited understanding of the functional interaction between nanomaterials and the autophagy system precludes the rational design of nanomaterials with predictable autophagy modulating properties.

1.3.2. Blockage of autophagic flux

Extensive autophagic activity is sometimes detected in association with cell death, suggesting that, in addition to a pro-survival role, autophagy may also serve in pro-death function [52]. The observation that several autophagy and apoptosis genes are co-regulated also supports this notion [53]. Evidence pointing to a causal relationship between autophagy and cell death, however, is lacking [12]. While a correlation is sometimes detected [54], the molecular mechanism underlying the toxicity of nano-sized materials and whether it is associated with blockage of autophagic flux or with induction of pro-death autophagy remains unclear.
Autophagic degradation depends on the cooperation of multiple cellular processes, the most critical of which is the fusion of autophagosomes with lysosomes. Thus, whether up-regulation of autophagosome formation is biocompatible (leading to enhanced autophagic clearance) or bioadverse (leading to a blockage of autophagic flux) depends on the context. For instance, an increase in the number of autophagosomes may be due to defective fusion of autophagosomes with lysosomes. On the other hand, defective autophagosome formation may lead to lysosome overload [55]. In both cases, the net result is blockage of autophagic flux, which has been observed in different cell types upon uptake of a variety of nanomaterials [54], [44], [56], [36].

Cellular uptake of carboxylated multiwalled carbon nanotubes results in blockage of autophagic flux, accumulation of autophagosomes, and cytotoxicity. This effect is partly attenuated upon mild stimulation of autophagy using low doses of Bafilomycin A1, which causes extracellular release of nanomaterial from autophagic vesicles [57]. Exocytosis of autophagic vesicles has been proposed as a mechanism of release of nanomaterials. Since most synthetic nanomaterials cannot be degraded through cellular catabolism, activation of autophagy-mediated exocytosis may represent a clearance mechanism for nanomaterials and could potentially be leveraged to provide cellular protection against the toxic effect of engineered, synthetic nanomaterials.

Nanomaterial-induced blockage of autophagic flux has also been investigated for therapeutic applications, particularly for cancer [35], [58]. Targeted delivery of
Autophagy-inducing nanomaterials to cancer cells could enable novel strategies to selectively induce blockage of autophagic flux and cell death [35], [58]. Iron oxide nanoparticles, for instance, were reported to enhance autophagosome formation and cytotoxicity in human cancer cells, but not in normal lung fibroblasts [38], [59]. Iron core-gold shell nanoparticles were also shown to decrease the viability of oral and colorectal cancer cells through activation of autophagy [59].

Perhaps the most common mechanism of nanomaterial-induced blockage of autophagic flux is lysosomal dysfunction. Impairment of lysosomal integrity or inactivation of lysosomal enzymes prevents lysosome-autophagosome fusion, resulting in aberrant accumulation of autophagosomes [9], [60]. Not surprisingly, a number of endocytosed nanomaterials that cause autophagy-associated cell death, including gold nanoparticles [36] and carbon nanotubes [61], are detected in the lysosomes. The mechanism of nanomaterial-induced lysosomal dysfunction is unclear and could be associated with permeabilization or osmotic swelling of lysosomes [62], induction of oxidative stress [44], or alkalinization of the lysosome [38]. A “proton sponge” effect has been proposed as a potential mechanism of lysosomal permeabilization and impairment observed upon cellular internalization of cationic nanoparticles [62]. This model is based on the high proton buffering capacity of the nanomaterial surface that interferes with acidification of the lysosome, thus impairing proton pump activity and inducing lysosomal membrane permeabilization. Lysosomal permeabilization was found to correlate with toxicity in cells treated with cationic polystyrene nanospheres [62]. Interestingly, cationic nanoparticle-induced toxicity was reduced using proton pump inhibitors that
prevent endosomal acidification, suggesting that lysosomal permeabilization is involved in the mechanism of nanomaterial toxicity [62].

Finally, autophagosome transport depends on the integrity and dynamic function of the cytoskeleton [63]. Interaction of nanomaterials with the cytoskeleton through direct impairment of cytoskeleton components [64] or indirectly through induction of oxidative stress [65] typically results in alterations of cell mechanics and blockage autophagic flux. Generally speaking, the interaction of nanomaterials with cellular components may significantly impact the cell’s physiologic state.

The ensemble of autophagic responses to the uptake of nanomaterials is thus likely to represent a phenotypic continuum that is profoundly affected by the combinatorial complexity of the nanomaterial physicochemical properties and that may range from enhancement of cellular clearance to autophagy-associated cell death (Figure 3).
Figure 3. Hypothesized models of the autophagic response to nanomaterials. (A) Endocytosis mediates engulfment of nanomaterial into endosomes. Endosomes fuse with autophagosomes leading to formation of amphisomes. Amphisomes may fuse with lysosomes to form autolysosomes, where degradation occurs. (B) Upon endocytosis, nanomaterials escape endocytic vesicles and are released into the cytoplasm. Nanomaterials may then be sequestered into autophagosomes for autophagy-mediated clearance or exocytosis. Upon release into the cytoplasm, nanomaterials may also cause impairment of cytoskeleton integrity and function, leading to autophagosome dysfunction and blockage of autophagic flux. (C) Nanomaterials released into the cytoplasm, or sequestered into endosomes, may accumulate into lysosomes. Lysosomal storage may result in lysosomal stress, which, in turn, may lead to enhanced autophagic clearance, exocytosis, or lysosomal impairment and blockage of autophagic flux.
Emerging technologies to synthesize and characterize materials at the nanoscale have provided new opportunities to manufacture highly specialized nanomaterials for a variety of applications. As a result, more than 1,600 consumer products containing nanomaterials are currently on the market [66]. The large-yield production of nanomaterials has inevitably increased human exposure, raising awareness of the many unknown and potentially harmful effects of engineered nanomaterials on human health.

The cellular uptake of nano-sized materials has been characterized in detail [67] and the rate at which cells internalize nanomaterials can be controlled with respect to nanomaterial physicochemical properties such as size, charge, and surface chemistry. However, the impact of these physicochemical properties on other cellular processes activated upon cellular internalization of nanomaterials has not been thoroughly investigated. Cellular clearance mechanisms are typically activated in response to internalization of foreign materials and are likely to
represent a common cellular response to materials in the nano-sized range [10]. The goal of this research is to characterize the autophagic response to cellular internalization of nanomaterials with different physicochemical properties. **The underlying hypothesis of this study is that the cellular uptake of nanomaterials impacts the lysosome-autophagy system with modalities that depend on the physicochemical properties of the nanomaterial**, including core material, primary particle size, and surface coating. As a result, the specific nature of the autophagic response, and whether it leads to enhanced clearance or impaired degradation, depends on the nanomaterial’s physicochemical properties. This hypothesis was explored by completing the following specific objectives:

**Specific objective 1: Characterize the effect of adeno-associated virus 2 on the lysosome-autophagy system.** Despite the increasing number of clinical trials based on adeno-associated virus (AAV) as a gene delivery vector [68], questions remain regarding the basic biology of AAV transduction. In particular, intracellular gene delivery requires evasion of cellular degradation and pathogen clearance pathways, including autophagy. The molecular mechanisms underlying activation of autophagy in response to AAV internalization, however, remain uncharacterized. The aim of this study was to elucidate the effect of AAV internalization on the lysosome-autophagy system and to determine whether induction of autophagy mediated by these genetically encoded nanoparticles affects transgene delivery. Results from this study provide a mechanistic understanding of the cellular response to AAV, which will inform the development of strategies to
enhance AAV gene delivery efficiency and may enable engineering of AAV to modulate autophagic clearance.

**Specific objective 2: Characterize the autophagic response to cellular exposure to titanium dioxide nanoparticles.** Despite titanium dioxide being “generally regarded as safe” by the US Food and Drug Administration [69], and an increasing amount of personal care, biomedical, and industrial products containing titanium dioxide nanoparticles (TiO$_2$ NPs) being introduced in the marketplace [70], the effects of TiO$_2$ NPs on cellular systems remain poorly characterized. The aim of this study was to characterize the autophagic response in cells exposed to TiO$_2$ NPs of different sizes. Interestingly, TiO$_2$ NPs and other inorganic nanomaterials seem to affect lysosomal function and integrity [71], which may affect autophagic clearance. I investigated a comprehensive set of markers of the lysosome-autophagy system in cells exposed to TiO$_2$ NPs with anatase crystal structure and primary particle diameters of 15, 50, and 100 nm. Results from this study provide important insights for the design of TiO$_2$ NPs with desired autophagy-modulating properties, and ultimately, for the engineering of safe TiO$_2$ NP-containing products.

**Specific objective 3: Characterize the effect of size and coating on the autophagy-modulating properties of zinc oxide particles.** Although zinc oxide particles (ZnOPs) are currently used in a variety of applications ranging from sunscreens to semiconductors, the health effects of human exposure to ZnOPs are unclear. Cellular exposure to ZnOPs affects cell viability [72], induces DNA damage [73], and produces reactive oxygen species [74]. In this study, I aimed to investigate
the autophagic response to cellular exposure to three ZnOPs with varying
physicochemical properties (bulk, ~200-1000 nm, uncoated; nano, ~85 nm,
uncoated; nano/coated, ~86 nm, coated). Results from this study provide important
insights for the design of ZnOPs with autophagy-modulating properties.
Chapter 3

TFEB-Mediated Activation of the Lysosome-Autophagy System Affects the Transduction Efficiency of Adeno-Associated Virus 2

This work is published in Virology (Popp, L., et al. TFEB-mediated activation of the lysosome-autophagy system affects the transduction efficiency of adeno-associated virus 2. Virology; 2017; 510:1–8.).

3.1. Introduction

Adeno-associated virus serotype 2 (AAV2) is a small (25 nm), non-enveloped, single stranded DNA virus that shows great promise as a gene delivery vector due to its broad tropism for human tissues, efficient cell internalization, and well-characterized safety profile [75], [76]. Despite the increasing number of clinical
trials based on AAV as a gene delivery vector [68], questions remain regarding the basic biology of AAV transduction. In particular, intracellular gene delivery by viral vectors requires evasion of cellular degradation and pathogen clearance pathways, including autophagy [75], [77]. The molecular mechanisms underlying activation of autophagy in response to uptake of AAV, however, remain uncharacterized.

While basal levels of autophagy are essential for the routine turnover of proteins and cytoplasmic components and maintenance of cellular homeostasis, autophagic clearance mechanisms also provide a means for cellular defense, protecting cells against infection by a variety of cellular pathogens, including bacteria and viruses [22], [23], [78], [79]. The interaction of viruses with the lysosome-autophagy system, however, is very complex and may lead to a broad spectrum of outcomes, ranging from degradation of the virus [77], [80], [81] to evasion of autophagy and viral propagation facilitated by autophagic machinery [82]–[84]. Pharmacologic inhibition of other mechanisms of intracellular degradation, including the ubiquitin proteasome system [85], [86], the unfolded protein response [87], and endoplasmic reticulum-associated degradation [88], has been shown to prevent degradation of AAV and, thus, to enhance virus transduction efficiency. However, modulation of autophagy to control AAV intracellular trafficking and cargo delivery or modulation of AAV to evade autophagic degradation remains largely unexplored.

In this study, I sought to elucidate the effect of AAV internalization on the lysosome-autophagy system, and to determine the effect of autophagy activation on
AAV-mediated gene delivery. Specifically, I monitored the effect of AAV2 on a series of markers of autophagy activation, autophagic flux, and autophagic clearance in vitro. The cellular uptake of AAV2 was found to induce activation of autophagy, which, in turn, mediates a reduction in viral transduction efficiency. Results from this study also demonstrate that AAV2-induced activation of autophagy is mediated by TFEB. This study provides a mechanistic understanding of the cellular response to AAV, which will inform the development of strategies to enhance AAV gene delivery efficiency and may enable engineering of AAV to modulate autophagic clearance.

3.2. Results

3.2.1. AAV2 uptake induces TFEB activation and formation of autophagic vesicles

To determine whether cellular uptake of AAV2 induces activation of the lysosome-autophagy system, I first analyzed a transcriptional regulatory network that regulates lysosomal biogenesis and autophagy by monitoring the intracellular localization of TFEB [16]. TFEB localizes predominantly in the cytoplasm of resting cells and translocates to the nucleus upon activation [16]. To investigate the impact of AAV2 uptake on TFEB activation, I tested TFEB intracellular localization in an in vitro model system of TFEB activation, namely HeLa cells stably transfected for the expression of TFEB-3xFLAG (HeLa/TFEB cells) [16]. HeLa/TFEB cells were incubated with AAV2s harboring a GFP transgene (AAV2-GFP) for 24 hrs
(multiplicity of infection (MOI) = 5000 viral genomes (vgs)/cell) and the extent of
TFEB nuclear localization was evaluated by confocal microscopy (Figure 4. A-C).

βCD, a known activator of TFEB [17], was used as a positive control in these
experiments. Colocalization of the TFEB signal (anti-FLAG) with the nuclear stain
was quantified by calculating the Mander’s Correlation Coefficient [89].

Colocalization analyses revealed a 3-fold increase in the average fraction of nuclear
TFEB upon treatment with AAV2-GFP (Figure 4. B) and a 3.4-fold increase in the
fraction of cells presenting TFEB activation (Figure 4. C).

To assess whether the increase in TFEB nuclear translocation observed in
cells incubated with AAV2-GFP results in transcriptional activation of genes
involved in lysosome and autophagosome biogenesis, I measured the expression
levels of representative targets of TFEB. Quantitative RT-PCR analyses revealed
upregulation of TFEB target genes that are part of the lysosome (HEXA and LAMP1)
and autophagy (MAPLC3B and SQSTM1) systems following incubation with AAV2-
GFP (Figure 5) to an extent similar to that observed upon cell exposure to βCD
(Figure A 1).
Figure 4. AAV2 treatment induces TFEB activation. (A-C) Confocal microscopy analyses of TFEB in HeLa/TFEB cells incubated with AAV2-GFP (MOI = 5000 vgs/cell; 24 hrs) or βCD (1 mM; 24 hrs). (A) GFP (green, column 1); colocalization of nuclei (blue, Hoechst stain, column 2) and TFEB (red, anti-FLAG, column 3) is shown in merged images (column 4). The scale bar is 20 μm. UT, untreated. (B) Average fraction of total cellular TFEB that localizes in the nucleus. UT, untreated. Data are presented as mean ± s.e.m. *p<0.05 compared to UT. (C) Percentage of HeLa/TFEB cells with fraction of nuclear TFEB greater than the average fraction of nuclear TFEB in untreated cells (UT, 0.12±0.4). Data are presented as mean ± s.e.m. **p<0.01; ***p<0.001 compared to UT.
Figure 5. AAV2 treatment upregulates autophagy-related genes. Relative mRNA expression levels of MAPLC3B, LAMP1, HEXA, and SQSTM1 in HeLa/TFEB cells treated with AAV2-GFP (MOI = 5000 vgs/cell; 48 hrs). The mRNA expression levels were corrected by the expression levels of house-keeping genes GAPDH and ACTB and normalized to the expression levels in untreated cells (dashed line). Data are presented as mean ± s.d. *p<0.05; **p<0.01 compared to untreated.

To determine whether TFEB activation induced upon cell treatment with AAV2-GFP affects the formation and turnover of autophagosomes, I analyzed the processing of LC3, a protein required for autophagosome formation and maturation [90]. LC3 is a soluble protein that accumulates in the cytoplasm under basal conditions [91]. Upon activation of autophagy, the cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form an LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes [91]. Fusion of autophagosomes with lysosomes results in formation of autolysosomes and degradation of autophagosomal components, including LC3-II, by lysosomal hydrolases; lysosomal turnover of the autophagosomal marker LC3-II thus reflects autophagic activity [92], [93]. While monitoring the levels of LC3-I and LC3-II provides a measurement of autophagosome formation, the autophagic flux is more
accurately monitored by evaluating the LC3-II/LC3-I ratio and the effect of lysosomal protease inhibitors, such as Bafilomycin A1, which inhibit autophagosome-lysosome fusion [93]. Western blot analyses reveal an increase in the ratio of LC3-II/LC3-I in cells treated with AAV2-GFP, suggesting that activation of the lysosome-autophagy system by AAV2 leads to the formation of mature autophagic vesicles (Figure 6; Figure A 2) [93]. Treatment with Bafilomycin A1 further increased the LC3-II/ LC3-I ratio, suggesting that AAV2-GFP uptake induces an increase in autophagic flux [93].

These results indicate that internalization of AAV2 induces activation of TFEB and upregulation of the lysosome-autophagy system.

**Figure 6.** AAV2 treatment induces autophagosome formation and turnover. Quantification of Western blot analyses of LC3 isoforms in HeLa cells treated with AAV2-GFP (MOI = 10000 vgs/cell; 24 hrs) and Bafilomycin A1 (Baf; 1 nM; 1 hr). Data are reported as the ratio of autophagosomal (LC3-II) to cytoplasmic (LC3-I) LC3 and are presented as the mean ± s.d. *p< 0.05; **p< 0.01.
3.2.2. Activation of TFEB affects AAV2 transgene expression

Because TFEB nuclear localization mediates coordinated activation of autophagy and lysosomal biogenesis [13], and enhances autophagic clearance [18], I asked whether the activation of TFEB observed upon AAV2 internalization affects AAV2 transgene expression. To address this question, I compared the transduction efficiency of AAV2-GFP in HeLa/TFEB cells to that of AAV2-GFP in parental HeLa cells by flow cytometry. The population of GFP-positive HeLa cells was found to be about 3.5-fold greater than that of HeLa/TFEB cells (Figure 7. A) and the mean GFP fluorescence intensity of the HeLa cells was found to be almost 4-fold higher than that of the HeLa/TFEB cells (Figure 7. B), corresponding to a 12-fold increase in the transduction index of AAV2 in HeLa cells compared to HeLa/TFEB cells (Figure 7. C).
C). The extent of AAV2 uptake in HeLa cells was found to be very similar to that of HeLa/TFEB cells, as determined by measuring the amount of intracellular viral genomes in cells infected with AAV2-GFP (Figure 8). These results indicate that TFEB activation induced upon internalization of AAV2 affects AAV2 transgene expression.

![Figure 8. Uptake of AAV2 in HeLa and HeLa/TFEB cells.](image)

**Figure 8. Uptake of AAV2 in HeLa and HeLa/TFEB cells.** Number of AAV2-GFP viral genomes (vgs) in HeLa and HeLa/TFEB cells (MOI = 5000 vgs/cell; 2 hrs) measured by quantitative RT-PCR of the virus transgene normalized to the amount of DNA in the cell lysate. Data are presented as mean ± s.d.

To further explore the effect of TFEB activation on AAV2 processing and transgene expression, HeLa/TFEB cells were pre-treated with βCD for 24 hrs to induce activation of autophagy [17] prior to the addition of AAV2-GFP. Pre-activation of TFEB with βCD resulted in a 30% decrease in the transduction index of AAV2 (Figure 9. A-C).
Figure 9. TFEB activation affects AAV2 transduction. (A-C) Analyses of AAV2-GFP transduction (MOI = 5000 vgs/cell; 48 hrs) in HeLa/TFEB cells cultured with and without βCD (1 mM; 24 hrs). (A) Percentage of cells expressing GFP. Data are presented as mean ± s.d. **p<0.01 compared to untreated (UT). (B) Geometric mean fluorescence intensity of cells expressing GFP. Data are presented as mean ± s.d. ***p<0.001 compared to untreated (UT). (C) Relative transduction index (% GFP-positive cells × geometric mean fluorescence intensity). Data are presented as mean ± s.d. ***p<0.001 compared to untreated (UT). (D-F) Analyses of AAV2-GFP transduction (MOI = 5000 vgs/cell; 48 hrs) in HeLa cells pre-treated with TFEB siRNA or a control siRNA for 24 hrs. (D) Percentage of cells expressing GFP. Data are presented as mean ± s.d. (E) Geometric mean fluorescence intensity of cells expressing GFP. Data are presented as mean ± s.d. (F) Relative transduction index. Data are presented as mean ± s.d. *p<0.05 compared to Cntrl siRNA.

To verify that TFEB directly affects the transduction index of AAV2-GFP, TFEB expression was reduced using small interfering RNA (siRNA). Transfection with TFEB siRNA resulted in approximately 80% reduction in TFEB expression.
compared to cells transfected with a control siRNA, as evaluated by quantitative RT-PCR (Figure A 3). Silencing TFEB resulted in nearly a 50% increase in the transduction index of AAV2-GFP in HeLa cells (Figure 9. D-F), providing direct evidence that TFEB expression affects the transduction efficiency of AAV2.

3.2.3. AAV2-induced autophagy results in enhanced autophagic clearance

To investigate whether autophagy induction caused by AAV2 uptake parallels an increase in clearance of autophagic substrates, I used an in vitro model system of protein aggregation consisting of neuroglioma cells stably transfected for the expression of α-synuclein fused to GFP (H4/α-syn-GFP) [95], [96]. H4/α-syn-GFP cells were pre-treated with the proteasome inhibitor MG-132 to enhance formation of α-syn-GFP aggregates [97] and then incubated with AAV2 expressing the iRFP transgene (AAV2-iRFP). Accumulation of α-syn-GFP aggregates was evaluated as previously described [95], [96] by analyzing the colocalization of GFP and ProteoStat® dye, a red fluorescent molecule that specifically interacts with denatured proteins within protein aggregates [97]. Colocalization studies revealed a significant decrease in the amount of aggregated α-syn-GFP in cells transduced with AAV2-iRFP (Figure 10. A-B).

The decrease in α-syn-GFP aggregates upon AAV2 internalization was also confirmed by Western blot [49], which revealed a decrease in the amount of insoluble α-synuclein and an increase in LC3-II/LC3-I levels in cells incubated with AAV2 (Figure 10. C-D; Figure A 4).
These results suggest that AAV2 uptake induces activation of autophagy and results in enhanced degradation of autphagic cargo.

Figure 10. AAV2 induces clearance of α-syn-GFP aggregates. (A-B) Confocal microscopy analyses of α-synuclein aggregation in H4/α-syn-GFP cells pre-treated with MG-132 (10 µM; 16 hrs) and incubated with AAV2-iRFP (MOI = 100000 vgs/cell; 48 hrs). (A) iRFP (cyan, column 1); colocalization of the nuclei (blue, Hoechst stain, column 2), α-syn-GFP (green, column 3), and ProteoStat® dye (red, column 4) is shown in merged images (column 5). The scale bar is 20 µm. “—”, negative control. (B) Average percentage of aggregated α-syn-GFP. Data presented as mean ± s.e.m. *p<0.05 compared to “—”, negative control. (C-D) Quantification of Western blot analyses of (C) insoluble α-synuclein and (D) ratio of LC3-II/LC3-I in H4/α-syn-GFP cells treated with AAV2-GFP (MOI = 10000 vgs/cell; 24 hrs). Data are presented as mean ± s.d. *p<0.05; **p<0.01 compared to “—”, negative control.
3.3. Discussion

AAV has garnered heavy interest as a gene therapy vector over the past two decades [75], [76]; yet, undesirable intracellular processing remains a major hurdle to the optimization of AAV’s transduction efficiency. Autophagy is the cell’s primary degradation mechanism activated in response to accumulation of nano-sized particles [98]; however, the functional interactions between AAV and the lysosome-autophagy system remain largely uncharacterized. In this study, the effect of AAV2 uptake on autophagy was determined by monitoring autophagy activation, autophagic flux, and autophagic clearance in vitro. I determined that cellular uptake of AAV2 induces activation of TFEB and enhances formation of autophagic vesicles. AAV2-induced activation of autophagy was shown to reduce AAV2 transgene expression, and this effect to be specifically mediated by TFEB. These observations are consistent with evidence that modulation of autophagy affects the transduction efficiency of other viruses [80], [81] and are in agreement with the notion that pre-treatment with inhibitors of other cellular degradation pathways (namely the ubiquitin proteasome system [85], [86], unfolded protein response [87], and endoplasmic reticulum-associated degradation [88]) enhances the transduction efficiency of AAV2.

Results from this study demonstrating that TFEB-mediated activation of the lysosome-autophagy system affects the transduction efficiency of AAV point to the critical need to take into account the effects of this degradation pathway in the future design of AAV gene therapy vectors. Modifying the surface chemistry of nano-
sized materials has already been reported as an effective strategy to control the autophagic response to nanomaterial internalization [98]–[100]. Furthermore, some viruses are known to evade autophagy by expressing anti-autophagic maturation factors [83]. Thus, AAV’s transduction efficiency could be potentially enhanced by engineering AAV gene delivery vectors that prevent activation of the autophagy pathway. An alternative route to enhance the efficiency of AAV-based delivery vectors could be co-treatment with chemical inhibitors of autophagy, which, similar to inhibitors of other cellular degradation pathways [88], may result in enhancement of AAV transduction. Finally, the autophagy-inducing properties of AAV could be harnessed to enhance autophagic clearance for the treatment of diseases characterized by deficient autophagy, which results in accumulation of proteinaceous aggregates and dysfunctional organelles [48]. AAV serotype 9 is able to cross the blood-brain barrier [101] and distribute its viral genome throughout neuronal cell bodies, providing the potential for AAV-induced autophagy as a treatment option for neurodegenerative disease [102]. AAV serotype 9 is currently being explored for AAV-based gene therapy of lysosomal storage disorders with neuropathic manifestations [103], [104]. Interestingly, cells of patients affected by a number of different lysosomal storage disorders present partial activation of TFEB, most likely as a physiologic response to lysosomal stress caused by intralysosomal storage of undegraded molecules [16], [105]. TFEB mediated activation of autophagic clearance and the resulting effect on AAV transduction efficiency should thus be taken into account in the design of AAV-mediated gene therapy strategies for delivery of lysosomal proteins. Because pharmacological activation of TFEB is
proven to enhance clearance of lysosomal storage and has emerged as a therapeutic approach for the treatment of lysosomal storage disorders [17], [106], [107], integration of genetic material via AAV-based gene delivery systems will require temporarily decoupling the therapeutic effect of TFEB activation from AAV-mediated replacement of the mutated gene.

While this study provides evidence of TFEB-mediated activation of autophagy induced by AAV2 and points to the effect of AAV2-induced activation of the lysosome-autophagy system on AAV2 transduction efficiency, a number of questions regarding the molecular mechanisms underlying the interaction of AAV with the lysosome-autophagy system remain unanswered. Understanding the features of AAV responsible for activating autophagy and, specifically, whether receptor-mediated endocytosis, endosomal escape, nuclear uptake, or transgene expression are required to activate cellular degradation mechanisms will enable the design of efficient AAV delivery vectors. The effect of autophagy activation on the intracellular fate of AAV also remains to be investigated to determine whether viral particles are sequestered into autophagosomes and whether transgene expression is affected by autophagic degradation of AAV. Considering that LC3 levels and other autophagic markers, which may affect the nature of the cellular response to activation of autophagy [92], vary in a cell-dependent fashion [92], [108], the physiologic impact of AAV-mediated induction of autophagy is also expected to be highly cell type specific. Because of the variable tropism of AAV serotypes [76], the autophagic response to AAV internalization is also likely to be serotype-specific [87]—a property that could be specifically targeted for the development of
therapeutic strategies based on AAV. Finally, it remains to be determined whether
the effect of AAV on the lysosome-autophagy system observed in vitro is also
associated with reduced efficiency of gene delivery in vivo, as expected [109].

In summary, results from this study highlight the important role of the
lysosome-autophagy system in regulating AAV transduction efficiency and will
contribute to defining the design rules for engineering AAV vectors with increased
delivery efficiencies or with desired autophagy-inducing properties.

3.4. Materials and methods

3.4.1. Cell cultures

HeLa cells (ATCC) were cultured in Eagle’s Minimum Essential Medium
supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-
glutamine (PSQ). HeLa/TFEB cells were obtained from Dr. Marco Sardiello (Baylor
College of Medicine) and cultured in Dulbecco’s Modified Eagle Medium (DMEM)
supplemented with 10% FBS and 1% PSQ and selected using G418 (1 mg/mL).
H4/α-syn-GFP cells [95], [96] were cultured in DMEM supplemented with 10% FBS
and 1% PSQ. HEK293T cells (CRL-11268, ATCC) were cultured in DMEM
supplemented with 10% FBS and 1% PSQ. Cells were cultured at 37 °C and 5% CO2
and passaged using TrypLE Express.

3.4.2. Virus production

AAV2 was prepared by Dr. Eric Gomez in Dr. Junghae Suh’s lab [110].
3.4.3. Transduction assays

Viruses were diluted in media and added dropwise onto cells at the desired MOI 24 hours after plating. The virus-containing media was removed and replaced with fresh media after 24 hours. Cells were incubated for another 24 hours, and transgene expression was analyzed by flow cytometry (FACSCanto™ II) using a 488-nm Argon laser. Virus transduction efficiency was assessed by calculating the transduction index (TI = % GFP positive cells × geometric mean fluorescence intensity) [94].

3.4.4. Immunofluorescence confocal microscopy

Cells were plated onto glass coverslips and viruses diluted in media were added dropwise onto cells at the desired MOI 24 hours after plating. Cells were washed with PBS, fixed with 4% PFA (30 min), permeabilized with 0.1% Triton X-100 (10 min), and incubated with 8% BSA-PBS blocking solution (60 min). Cells were incubated with primary antibodies against the FLAG tag for one hour (F7425, Sigma), washed, and incubated with the appropriate fluorescent secondary antibody (611-142-002, Rockland) for one hour. Nuclei were stained with Hoechst nuclear stain for 10 minutes. Coverslips were then mounted onto glass slides and imaged using an Olympus IX81 inverted confocal microscope and the FLUOVIEW software. Image analyses were conducted using ImageJ and the JACoP plugin [89] to quantify the degree of colocalization between TFEB (anti-FLAG, red) and the nuclear stain (blue) in each image by calculating the Mander’s Correlation Coefficient.
3.4.5. Quantitative RT-PCR

Quantitative RT-PCR analyses were conducted as previously described [49]. Viruses were diluted in media and added dropwise onto cells at the desired MOI 24 hours after plating. Virus-containing media was removed and replaced with fresh media after 24 hours. Cells were incubated for another 24 hours, and the RNA was extracted using rNAGEM™ reagents. cDNA was synthesized from total RNA using qScript™ cDNA SuperMix and quantified using a NanoDrop 2000. Quantitative RT-PCR reactions were performed using SYBR Green FastMix in a CFX96 Real-Time PCR Detection System with corresponding primers and data analysis was conducted as previously described [17].

3.4.6. Immunoblotting

Viruses were diluted in media and added dropwise onto cells at the desired MOI 24 hours after plating. Samples for LC3 detection were obtained by lysing cells 24 hours post-transduction with Complete lysis-M buffer containing a protease inhibitor cocktail. Lysed cells were centrifuged for 10 minutes at 1500 rpm and 4⁰C and the supernatant was collected for analysis. Protein concentrations were determined using the Bradford assay. Proteins (40 µg) were separated on 15% SDS-PAGE. Membranes were incubated with primary antibodies against LC3 and GAPDH (NB-100-2220, Novus Biologics and sc-25778, Santa Cruz Biotechnology) and the appropriate secondary antibodies (sc-2004, Santa Cruz Biotechnology).

Samples for α-synuclein detection were obtained by lysing cells 24 hours post-transduction with Complete lysis-M buffer containing a protease inhibitor
cocktail and supplemented with 1% Triton X-100. Lysed cells were centrifuged for 60 minutes at 15,000 \( g \) and 4 \(^\circ\)C and the supernatant containing the soluble protein fraction was collected. The pellet was resuspended in Complete Lysis-M buffer supplemented with 2% SDS and 8 M urea and sonicated to collect the insoluble protein fraction. Protein concentrations were determined using the Bradford assay. 4 \( \mu \)g of soluble protein and 40 \( \mu \)g of insoluble protein were loaded onto a 12% SDS-PAGE gel for detection of \( \alpha \)-synuclein and 40 \( \mu \)g of soluble protein for detection of LC3. Membranes were blotted using primary antibodies specific for \( \alpha \)-synuclein, LC3, and GAPDH (S5566, Sigma-Aldrich; NB-100-2220, Novus Biologics; sc-25778, Santa Cruz Biotechnology) and appropriate secondary antibodies (sc-2005, Santa Cruz Biotechnology; sc-2004, Santa Cruz Biotechnology).

The membranes were imaged using an LAS 4000 Imager. Band intensities were quantified with ImageJ analysis software and corrected by GAPDH levels.

### 3.4.7. Viral DNA extraction

Viruses were diluted in media and added dropwise onto cells at the desired MOI 24 hours after plating. Genomic DNA was extracted using an E.Z.N.A.® Tissue DNA Kit. Viral single stranded DNA was quantified via quantitative RT-PCR using primers against the virus transgene. The concentration of viral genomes in each sample was normalized by the total DNA content.
3.4.8. siRNA transfection

siRNA transfections were performed using the HiPerFect® transfection reagent as described in the manufacturer’s manual. Briefly, 75 ng of siRNA (TFEB siRNA, SI00094969, Qiagen; AllStars Negative Control siRNA, 1027280, Qiagen) were diluted in 100 µL of media without serum. 3 µL of the HiPerFect reagent were added to the siRNA and the mixture was vortexed and incubated for 10 minutes at room temperature. The siRNA mixture was then added dropwise onto cells. Media containing the siRNA was replaced with fresh culturing media after 24 hours, and viruses were added dropwise onto cells at the desired MOI. Cells were incubated with the viruses for 24 hours, and transgene expression was analyzed by flow cytometry (FACSCanto™ II) using a 488-nm Argon laser.

3.4.9. Protein aggregation studies

H4/α-syn-GFP cells were seeded onto glass coverslips and treated overnight with 10 µM MG-132 [97]. Following incubation with MG-132, the viruses were diluted in media and added dropwise onto cells at the desired MOI. α-syn-GFP aggregation was evaluated using the ProteoStat® Aggresome Detection Kit according to the manufacturer’s protocol. Confocal analyses were conducted as previously described [96] using a Nikon A-1 confocal microscope and the Nikon NIS Elements C imaging software. Image post-processing was conducted using ImageJ and the JACoP plugin [89] to quantify the degree of colocalization between the ProteoStat® (red) signal and the GFP (green) signal in each image by calculating the Mander’s Correlation Coefficient.
3.4.10. Statistical analyses

All data are presented as the mean ± s.d. with statistical significance calculated using a two-tailed Student’s t-test unless stated otherwise. Differences were considered statistically significant with *p<0.05.
Chapter 4

Autophagic Response to Cellular Exposure to Titanium Dioxide Nanoparticles

This work has been submitted to Acta Biomaterialia (Popp, L. et al. Autphagic Response to Cellular Exposure to Titanium Dioxide Nanomaterials. 2018).

4.1. Introduction

Recent progress in the field of nanotechnology has not only led to the design of multifunctional nanotherapeutics [1] and advanced nano-biosensors [2], but has also resulted in a rapid increase in the number of consumer products containing nanomaterials [3]. The commercialization of nanomaterial-based products has inevitably paralleled an increase in the large-scale production of nanomaterials and a growing concern regarding the potentially adverse effects in exposed employees,
consumers, and patients. Of particular importance is understanding how exposure
to nanomaterials affects human health, which is ultimately a consequence of the
interactions between nanomaterials and cellular components, which also operate at
the nanoscale.

In this study, I investigated the cellular response to titanium dioxide
nanoparticles (TiO_2 NPs), which are used in a number of personal care, biomedical,
and industrial products [70]. Specifically, I monitored a comprehensive set of
markers of the lysosome-autophagy system upon cell exposure to TiO_2 NPs with
three different primary particles sizes (15, 50, and 100 nm). This study reveals that
cellular uptake of TiO_2 NPs induces a response of the lysosome-autophagy system
that is mediated by the transcription factor EB and consequent upregulation of
autophagic flux. Prolonged exposure to TiO_2 NPs, however, was found to induce
lysosomal dysfunction and membrane permeabilization, leading to a blockage in
autophagic flux.

4.2. Results

4.2.1. TiO_2 NPs induce activation of the transcription factor EB

The autophagic response to TiO_2 NPs was investigated using a series of TiO_2
NPs with different primary particle diameters, namely 15, 50, and 100 nm (anatase
crystal structure). The hydrodynamic of diameter of each TiO_2 NP suspended in
water or DMEM was first determined by dynamic light scattering (Figure 11. A).
Media suspensions were prepared by sonicating water suspensions of TiO_2 NPs,
stabilizing NPs through the addition of bovine serum albumin (BSA) [111], and diluting in DMEM. Dynamic light scattering analyses suggest that the TiO₂ NPs form large agglomerates in water and upon dilution into DMEM, with the intermediate size of TiO₂ NPs tested (50 nm) forming the largest agglomerates. Zeta potential measurements of TiO₂ NPs revealed that the 50 nm TiO₂ NPs have a more neutral surface charge in water compared to the 15 and 100 nm TiO₂ NPs (Figure 11. B).

The more neutral surface charge of the 50 nm TiO₂ NPs in water compared to 10 and 100 nm TiO₂ NPs may reduce electrostatic repulsion and favor formation of larger agglomerates. Upon addition of BSA and dilution into DMEM, all three TiO₂ NPs exhibit similar, slightly negative zeta potentials most likely due to the formation of a protein corona [112].

![Figure 11. Dynamic light scattering analyses of TiO₂ NPs.](image)

**Figure 11. Dynamic light scattering analyses of TiO₂ NPs.** Dynamic light scattering analyses of the (A) hydrodynamic diameter and (B) zeta potential of TiO₂ NPs (25 µg/mL) dispersed in water or DMEM. Data are presented as mean ± s.d.

To investigate the molecular mechanisms that mediate the autophagic response to TiO₂ NPs I first monitored the activation of TFEB in cells exposed to
TiO$_2$ NPs of different primary particle sizes [113]. As described in the previous chapters, TFEB mediates integrated control of the lysosome-autophagy system by regulating the expression of the CLEAR network of genes [16], [114]. HeLa/TFEB cells were cultured in media supplemented with TiO$_2$ NPs (0-500 µg/mL; 24 hrs) and TFEB subcellular localization was evaluated by immunofluorescence confocal microscopy using a Hoechst nuclear stain and an anti-FLAG antibody (Figure 12. A). TFEB activation was evaluated by calculating the Mander’s Correlation Coefficient of the TFEB signal (anti-FLAG) and the nuclear stain (Figure 12. B) and by determining the fraction of cells that present more nuclear TFEB than untreated cells (Figure 12. C) [89]. Colocalization analyses reveal that TFEB localizes predominantly in the cytoplasm of untreated HeLa/TFEB cells, as expected (Figure 12. B-C, white bars) [16]. Exposure of HeLa/TFEB cells to TiO$_2$ NPs was found to increase the fraction of TFEB that translocates into the nucleus. Specifically, I observed an increase in the fraction of nuclear TFEB (Figure 12. B) and in the number of cells presenting TFEB nuclear translocation (Figure 12. C) in cells exposed to 15 and 50 nm TiO$_2$ NPs at a media concentration of at least 50 µg/mL and in cells exposed to 100 nm TiO$_2$ NPs at a media concentration of at least 10 µg/mL. The extent of TFEB activation was found not to vary significantly as a function of primary particle size. These results indicate that all three of the TiO$_2$ NPs induce activation of TFEB and that the extent of TFEB activation does not depend on the primary particle size.
Figure 12. TFEB activation in HeLa/TFEB cells exposed to TiO$_2$ NPs. (A) Confocal microscopy analyses of TFEB in HeLa/TFEB cells untreated and treated with 15, 50, and 100 nm TiO$_2$ NPs (50 µg/mL; 24 hrs). Representative images show colocalization of the nucleus (blue, Hoechst stain, column 1) and TFEB (red, anti-FLAG, column 2) in merged images (column 3). Scale bar is 20 µm. (B) Average fraction of total cellular TFEB that localizes in the nucleus. Data are presented as mean ± s.e.m. *p<0.05, **p<0.01, ***p<0.001. (C) Percentage of HeLa/TFEB cells with fraction of nuclear TFEB greater than the average fraction of nuclear TFEB in untreated cells (0.08±0.06). Data are presented as mean ± s.e.m. *p<0.05, **p<0.01, ***p<0.001.

To determine whether activation of TFEB in cells treated with TiO$_2$ NPs depends on the efficiency of cellular internalization, I evaluated the extent of TiO$_2$ NP uptake in HeLa/TFEB cells. Because TiO$_2$ NPs affect intracellular granularity [115], uptake was quantified by measuring the side scattering parameter (SSC) of cells exposed to TiO$_2$ NPs (0-500 µg/mL; 24 hrs) using flow cytometry [116].
Internalization of the TiO$_2$ NPs was found to be concentration-dependent at low concentrations and to plateau at a TiO$_2$ NP media concentration of 200 µg/mL (Figure 13). The primary particle diameter of the TiO$_2$ NPs used in this study was found not to affect TiO$_2$ NP uptake.

![Figure 13. Uptake of TiO$_2$ NPs in HeLa/TFEB cells.](image)

**Figure 13. Uptake of TiO$_2$ NPs in HeLa/TFEB cells.** Side scattering parameter (SSC) of HeLa/TFEB cells exposed to TiO$_2$ NPs for 24 hours measured by flow cytometry. Relative SSC values were calculated by normalizing the SSC of treated cells to that of untreated cells. mean ± s.d. *p<0.05, **p<0.01, ***p<0.001.

To test whether uptake of the TiO$_2$ NPs induces cell toxicity, I tested the viability of HeLa/TFEB cells treated with TiO$_2$ NPs under conditions that resulted in TFEB activation (0-500 µg/mL, 24-72 hrs) using the Trypan Blue dye exclusion technique (Figure 14). I found that cellular exposure to the three TiO$_2$ NPs under the conditions used in this study does not cause a reduction in cell viability.

These results, taken together, indicate that cell exposure to TiO$_2$ NPs results in activation of TFEB, the master regulator of the lysosome-autophagy system, under conditions that do not affect cell viability. Furthermore, the extent of TiO$_2$ NP-
induced TFEB activation does not depend on the primary particle size of the TiO$_2$ NP.

**Figure 14. Viability of HeLa/TFEB cells exposed to TiO$_2$ NPs.** Viability of cells treated with (A) 15, (B) 50, and (C) 100 nm TiO$_2$ NPs for 24, 48, and 72 hours evaluated by assaying Trypan Blue exclusion. Data are presented as mean ± s.d.

### 4.2.2. Cell exposure to TiO$_2$ NPs results in blockage of the autophagic flux

Clearance of autophagic cargo requires the coordinated action of multiple cellular processes that include, in addition to TFEB-mediated upregulation of lysosome and autophagosome biogenesis and function, fusion of autophagosomes with lysosomes to form autolysosomes, where degradation of autophagic cargo occurs [117]. Both lysosomes and autophagosomes must be functionally active for transcriptional activation of the lysosome-autophagy system to translate into enhanced autophagic clearance [118]: indeed, defects in the integrity and function of autophagosomes and lysosomes result in blockage of autophagic flux [119].
Biopersistent NPs, such as TiO$_2$ NPs [120], have been repeatedly reported to induce inactivation of lysosomal enzymes and destabilization of the lysosomal membrane, leading to impairment of autophagic clearance [9], [119], [121], [122]. Interestingly, prolonged cellular exposure to TiO$_2$ NPs has been shown to affect lysosome acidification and lysosomal enzyme function [123], [124]. To test the impact of the TiO$_2$ NPs used in this study on lysosomal integrity, I evaluated the extent of permeabilization of lysosomal membranes under conditions resulting in TFEB activation. Permeabilization of lysosomal membranes was evaluated by monitoring lysosomal galectin puncta [125]. Galectins are soluble, carbohydrate-binding lectins that accumulate in the cytoplasm and rapidly translocate to leaky lysosomes [125]. Galectins bind to sites of lysosomal leakage due to the high concentration of β-galactosides found on the protective glycocalyx lining of lysosomal membranes that become exposed upon membrane permeabilization [126]. Lysosomal membrane permeabilization can thus be monitored by quantifying galectin puncta by immunofluorescence confocal microscopy. HeLa/TFEB cells were exposed to TiO$_2$ NPs under conditions that result in TFEB activation (50, 100, and 500 µg/mL; 24 and 72 hrs) [125]. Cell treatment with L-leucyl-L-leucine methyl ester (2 mM, 2 hrs) was monitored as a positive control in this study [125] and was found to cause lysosomal membrane permeabilization in 49% of the cell population compared to untreated cells that were not found to present punctate galectin signal (data not shown). Cell exposure to the 15, 50, and 100 nm TiO$_2$ NPs for a short time of incubation (50, 100, and 500 µg/mL; 24 hrs) did not result in the formation of significant punctate galectin signal (**Figure 15**, white bars). Prolonged cell exposure
to the three TiO₂ NPs (72 hrs), however, was found to cause lysosomal membrane permeabilization (Figure 15, grey bars; Figure A 5). Specifically, punctate galectin structures were observed in about 20-40% of cells treated with each of the three types of TiO₂ NPs. These data suggest that prolonged exposure to TiO₂ NPs at concentrations that activate TFEB induces lysosomal damage.

Figure 15. Lysosomal membrane permeabilization in HeLa/TFEB cells exposed to TiO₂ NPs. Permeabilization of lysosomal membranes in HeLa/TFEB cells incubated with TiO₂ NPs (50, 100, or 500 µg/mL; 24 or 72 hrs) as measured by confocal microscopy analyses of galectin-1. Data are presented as mean ± s.e.m. *p<0.05.

Because autophagic clearance depends on fusion of lysosomes with autophagosomes, the effect of cell exposure to TiO₂ NPs on autophagosomal turnover was tested under conditions that impair lysosomal integrity. The effect of short (24 hrs) and prolonged (72 hrs) exposure of HeLa cells to TiO₂ NPs on autophagosome accumulation was evaluated by testing the levels of LC3-I and LC3-II by immunoblotting [93]. A concentration-dependent increase in the LC3-II/LC3-I ratio was observed after 24 hours of treatment with 15, 50, and 100 nm TiO₂ NPs,
suggesting formation of autophagosomes (**Figure 16; Figure A 6**). Addition of Bafilomycin A1 (10 nM; 1 hr) was found to increase the LC3-II/LC3-I ratio in cells exposed to low concentrations of TiO$_2$ NPs (10 µg/mL; 24 hrs), but not in cells exposed to high concentrations of TiO$_2$ NPs (100 µg/mL; 24 hrs) (**Figure 16. A-C; Figure A 6. A-C**). These results suggest that short exposure to low concentrations of TiO$_2$ NPs results in enhancement of autophagic activity without accumulation of autophagosomes, while increasing the concentration of TiO$_2$ NPs in the culturing media causes accumulation of autophagosomes, which is typically associated with blockage of autophagic flux. Interestingly, accumulation of autophagosomes was also observed upon prolonged cell exposure to even low media TiO$_2$ NP concentrations (10 µg/mL) (**Figure 16. D-F; Figure A 6. D-F**), indicative of a blockage of autophagic flux.

In summary, analyses of the ratio of LC3-II/LC3-I indicate that cell exposure to TiO$_2$ NPs induces the formation of autophagosomes in a concentration-dependent manner, with prolonged exposure to TiO$_2$ NPs inducing accumulation of autophagosomes. The observed increase in accumulation of autophagosomes parallels the increase in permeabilization of lysosomal membranes, suggesting that autophagosomal accumulation is likely to be associated with lysosomal impairment and blockage of autophagic flux.
Figure 16. LC3 protein expression levels and processing in HeLa cells exposed to TiO2 NPs. LC3 levels in HeLa cells incubated with TiO2 NPs (10 or 100 µg/mL) for (A-C) 24 hours or (D-F) 72 hours and in the presence of Bafilomycin A1 (Baf; 10 nM; 1 hr) determined by Western blot and reported as ratio of autophagosomal (LC3-II) to cytoplasmic (LC3-I) isoforms. Data are presented as mean ± s.d. *p<0.05, **p<0.01, ***p<0.001.

Autophagic flux can be further monitored by correlating the intracellular levels of LC3 (autophagosome formation) with the colocalization of LC3 and
lysosomal membrane protein LAMP-2 (lysosome-associated membrane protein) (autolysosome formation) [127], [128]. Low levels of LC3 and LC3-LAMP colocalization are typically indicative of basal autophagic activity. An increase in LC3 levels that is not paralleled by an increase in LC3-LAMP colocalization indicates a blockage in autophagic flux, while an increase in LC3 levels paralleled by an increase in LC3-LAMP colocalization is characteristic of enhanced autophagic flux [128].

HeLa/TFEB cells were treated with TiO$_2$ NPs (50, 100, and 500 µg/mL; 24 or 72 hrs) and immunofluorescence confocal microscopy images were analyzed to quantify the number of LC3 puncta per cell and the extent of colocalization of LC3 and LAMP-2 (Figure 17; Figure A 7). Cells treated with TiO$_2$ NPs for a short time (24 hrs) were predominantly characterized by an increase in both the average number of LC3 puncta per cell and the extent of LC3-LAMP colocalization, a pattern indicative of an increase in autophagic vesicles and enhanced autophagic flux (Figure A 7. A). Prolonged exposure to TiO$_2$ NPs (72 hrs) resulted in an increase in the average number of LC3 puncta per cell that does not parallel an increase in LC3-LAMP colocalization (Figure A 7. B). The high levels of autophagosomal markers associated with a basal level of formation of autolysosomes upon prolonged exposure to TiO$_2$ NPs is indicative of blockage of autophagic flux, as it is likely due to accumulation of autophagosomes that do not fuse with lysosomes to form autolysosomes.

These results, taken together, suggest that prolonged exposure to the TiO$_2$ NPs used in this study leads to lysosomal membrane permeabilization and
autophagosome accumulation, resulting in a blockage of autophagic flux, and that this TiO₂ NP-mediated effect on the lysosome-autophagy system is independent of the primary particle size of the TiO₂ NPs.

Figure 17. Autolysosome formation in HeLa/TFEB cells exposed to TiO₂ NPs. Quantification of LC3 puncta and colocalization of LC3 and LAMP in HeLa/TFEB cells incubated with (A) 15, (B) 50, and (C) 100 nm TiO₂ NPs (50, 100, and 500 µg/mL; 24 or 72 hrs) as determined by confocal microscopy. Data points represent images of 10-20 cells. The average basal level of LC3-LAMP colocalization (0.26 ± 0.03) and LC3 puncta/cell (1.5 ± 0.49) of untreated cells are reported as solid black lines.

4.2.3. Prolonged exposure to TiO₂ NPs impairs clearance of autophagic substrates

To test whether TiO₂ NP-induced activation of autophagy results in enhanced clearance of autophagic cargo, I monitored the intracellular accumulation of aggregated α-synuclein, which is a model substrate of autophagic clearance [49]. Specifically, I tested the accumulation of aggregated α-synuclein in H4/α-syn-GFP cells [95], [96] exposed to TiO₂ NPs. H4/α-syn-GFP cells were first transfected to
express TFEB-3XFLAG to evaluate the extent of TFEB activation induced by exposure to TiO$_2$ NPs. Transfected cells were incubated with 15, 50, and 100 nm TiO$_2$ NPs (100 µg/mL; 24 hrs). Confocal microscopy analyses show that TFEB is predominately found in the cytoplasm of untreated cells and in the nucleus of cells treated with the TiO$_2$ NPs, indicating that exposure of H4/α-syn-GFP cells to TiO$_2$ NPs leads to transcriptional activation of autophagy (Figure 18).

**Figure 18.** TFEB activation in H4/α-syn-GFP cells exposed to TiO$_2$ NPs. Confocal microscopy analyses of TFEB subcellular localization in H4/α-syn-GFP cells transfected with TFEB-3XFLAG and incubated with TiO$_2$ NPs (100 µg/mL, 24 hrs). Representative images of colocalization of nuclei (blue, Hoechst stain, column 1) and TFEB (red, anti-TFEB, column 3) is shown in merged images (column 4). UT, untreated. Scale bar is 20 µm.
Exposure of H4/α-syn-GFP cells to TiO₂ NPs (100 µg/mL, 24 or 72 hrs) was also found to increase LC3-II/LC3-I levels (Figure 19. A; Figure A 8), confirming activation of autophagy and formation of autophagic vesicles. The effect of TiO₂ NPs on autophagic clearance was evaluated by quantifying the amount of soluble and insoluble α-synuclein in H4/α-syn-GFP cells treated with TiO₂ NPs (100 µg/mL; 24 or 72 hrs) by Western blot (Figure 19. B; Figure A 8) [49]. Short exposure (24 hrs) to the TiO₂ NPs resulted in a reduction in the ratio of insoluble to soluble α-synuclein (Figure 19. B; Figure A 8. A-B), suggesting that TiO₂ NP-induced activation of autophagy enhances the clearance of α-synuclein aggregates. The ratio of insoluble to soluble levels of α-synuclein in cells exposed to TiO₂ NPs for prolonged times (72 hrs) were found to be comparable to, or higher than, that of untreated cells (Figure 19. B; Figure A 8. C-D), suggesting that activation of the autophagic response does not parallel enhanced clearance upon prolonged exposure to TiO₂ NPs, and may instead result in blockage in autophagic flux and accumulation of autophagic cargo.
Figure 19. Accumulation of α-syn in H4/α-syn-GFP cells exposed to TiO2 NPs. (A) Quantification of LC3-II/LC3-I levels in the soluble protein fraction of H4/α-syn-GFP cells treated with TiO2 NPs (100 µg/mL; 24 or 72 hrs) measured by Western blot. UT, untreated. Data are presented as mean ± s.d. *p<0.05, **p<0.01, ***p<0.001 compared to UT. (B) Quantification of α-synuclein levels in soluble and insoluble protein fractions of H4/α-syn-GFP cells treated with TiO2 NPs (100 µg/mL; 24 or 72 hrs) determined by Western blot. UT, untreated. Data are presented as mean ± s.d. *p<0.05, **p<0.01.

4.3. Discussion

This study provides a comprehensive analysis of the autophagy-inducing properties of three TiO2 NPs of anatase crystal structure with varying primary particle sizes (15, 50, and 100 nm) with the goal of understanding if primary particle diameter has an effect on the autophagy-modulating properties of TiO2 NPs. Specifically, the effects of TiO2 NP uptake on the autophagy pathway were assessed by monitoring regulation of the lysosome-autophagy system at the transcriptional level, formation of autophagic vesicles and fusion of autophagic vesicles with lysosomes, and, ultimately, clearance of endogenous autophagic substrates. I found that the cellular uptake of TiO2 NPs results in activation of the lysosome-autophagy
system via TFEB. Prolonged exposure to TiO$_2$ NPs resulted in lysosomal dysfunction and lysosomal membrane permeabilization, which leads to a blockage in autophagic flux. I observed a minimal effect of the primary particle size on the autophagic response under the experimental conditions used in this study. It remains to be determined, however, whether TiO$_2$ NP agglomeration [129] affects the interaction of this nanomaterial with the lysosome-autophagy system and whether the size TiO$_2$ NP agglomerates plays a role in shaping the response of this important homeostatic pathway.

4.4. Materials and methods

4.4.1. Nanomaterial characterization

TiO$_2$ NPs were purchased from NanoAmor (5430MR, 15 nm) and mkNano (MK-TiO2-A050, 50 nm and MKN-TiO2-A100, 100 nm). Stock solutions (10 mg/mL) were prepared by suspending TiO$_2$ NPs in ultrapure DI water (18.2 MΩ·cm). The stock suspensions were sonicated immediately prior to use using a probe sonicator for 5 minutes at an 80% pulsation regime, stabilized with BSA through addition of an 80 mg/mL BSA-H$_2$O solution at a NP to protein mass ratio of 1:1, and further diluted in DMEM immediately prior to use in biological assays [111]. Particle hydrodynamic diameter and zeta potential measurements (25 µg/mL) were obtained using a Beckman Coulter DelsaMax Pro system.
4.4.2. Cell cultures

HeLa cells were cultured in DMEM supplemented with 10% FBS and 1% PSQ. HeLa/TFEB cells were cultured in DMEM supplemented with 10% FBS and 1% PSQ and selected using G418 (1 mg/mL). H4/α-syn-GFP cells [95], [96] were cultured in high glucose DMEM supplemented with 10% FBS and 1% PSQ and selected using blasticidin (5 µg/mL). Cells were cultured at 37 °C in 5% CO₂ and passaged using TrypLE Express.

4.4.3. Immunofluorescence microscopy

Immunofluorescence studies were conducted as previously described [119]. Briefly, cells were seeded on glass coverslips. HeLa/TFEB cells were cultured in the presence of TiO₂ NPs for 24 or 72 hours. H4/α-syn-GFP cells were first transfected for the expression of TFEB-3XFLAG using the TFEB-3XFLAG plasmid from Dr. Marco Sardiello (Baylor College of Medicine), then cultured in the presence of TiO₂ NPs for 24 hours. Cells were washed three times with PBS, fixed with 4% PFA (15 min), permeabilized with 0.1% Triton X-100 (10 min), and incubated with 8% BSA-PBS (30 min).

TFEB subcellular localization studies were conducted as previously described [130]. Cells were incubated with an anti-FLAG or anti-TFEB antibody (F7425, Sigma or ab70729, Abcam) for one hour, washed, incubated with a fluorescent secondary antibody (611-142-002, Rockland or 610-143-002, Rockland) for one hour, washed, and then incubated with a Hoechst nuclear stain for
10 minutes. Images were obtained using a Nikon A-1 confocal microscope and the Nikon NIS Elements C imaging software. Image post-processing and colocalization analyses were conducted using the JACoP plugin [89] in ImageJ. The fraction of nuclear TFEB was quantified by calculating the Mander’s Correlation Coefficient for each image. Average values were calculated from analyzes of ~50 cells per sample collected from at least three independent experiments. The percentage of cells presenting TFEB nuclear localization was determined by calculating the fraction of cells presenting the nuclear localization of TFEB as determined by evaluating the Mander’s Correlation Coefficient greater than the average fraction of TFEB nuclear localization in untreated cells plus one standard deviation.

Lysosomal membrane permeabilization was evaluated by detecting lysosomal galectin-1 puncta as previously described [125]. Briefly, HeLa/TFEB cells were seeded on glass coverslips and incubated with TiO₂ NPs for 24 or 72 hours. Cells were washed three times with PBS and fixed with 4% PFA (15 min). Background fluorescence was reduced by incubating the cells with a 50 mM ammonium dichloride solution (15 min). Cells were permeabilized with 0.1% Triton X-100 (10 min) and incubated with 8% BSA-PBS (30 min). Cells were incubated with anti-galectin-1 (ab25138, Abcam) and anti-LAMP-2 (354301, BioLegend) antibodies for one hour, washed, and incubated with the appropriate fluorescent secondary antibodies (611-142-002, Rockland; 072-03-15-06, KPL) for one hour. Images were obtained using a Nikon A-1 confocal microscope and the Nikon NIS Elements C imaging software. Image post-processing was conducted using ImageJ to determine the number of LAMP-2-positive galectin-1 puncta on a
cell-by-cell basis. Puncta were counted manually and average values were calculated by analyzing ~30 cells per sample from at least three independent experiments.

LC3-LAMP colocalization was evaluated as previously described [119]. Cells were incubated with anti-LC3 (NB100-2220, Novus Biologics) and anti-LAMP-2 (354301, BioLegend) antibodies for one hour, washed, and incubated with appropriate fluorescent secondary antibodies (611-143-002, Rockland or 610-142-002, Rockland) for one hour. Images were obtained using a Nikon A-1 confocal microscope and the Nikon NIS Elements C imaging software. Image post-processing and colocalization analyses were conducted using the JACoP plugin [89] in ImageJ. The level of LC3-LAMP colocalization was quantified by calculating the Mander’s Correlation Coefficient for each image. The number of LC3 puncta per cell was determined by thresholding images of LC3 and using the ‘analyze particles’ function in ImageJ.

4.4.4. Nanoparticle uptake

Cellular uptake of TiO₂ NPs was evaluated as previously described [116], [131]. Briefly, HeLa/TFEB cells were exposed to TiO₂ NPs for 24 hours, washed three times with PBS, and collected in cell culture media. Uptake was quantified by measuring the SSC of cells exposed to TiO₂ NPs using a BD FACSCanto™ II Flow Cytometer with the 488 nm Argon laser and a 488 nm emission filter.
4.4.5. Cell viability

HeLa/TFEB cells were cultured in media supplemented with TiO₂ NPs for 24, 48, or 72 hours. Cells were collected in PBS and mixed with a 0.4% Trypan Blue solution at a 1:1 volume ratio. At least 100 cells per biological replicate were counted using a hemocytometer, and the percentage of viable cells was determined using the following formula: \( \% \text{ viable} = 1 - \frac{\text{number of stained cells}}{\text{total number of cells}} \times 100\% \).

4.4.6. Western blot

LC3 levels in HeLa cells were measured by incubating cells with TiO₂ NPs for 24 or 72 hours. Cells were collected and lysed with Complete Lysis-M buffer (Roche) containing a protease inhibitor cocktail. Total protein concentrations were determined using the Bradford assay and 40 µg of protein from each sample were separated by 15% SDS-PAGE. Membranes were incubated with primary antibodies (LC3, NB-100-2220, Novus Biologics; GAPDH, sc-25778, Santa Cruz Biotechnology) and the appropriate secondary antibody (sc-2004, Santa Cruz Biotechnology). Imaging was performed on an LAS 4000 Imager and relative band densities were determined using ImageJ.

\( \alpha \)-synuclein levels in H4/\( \alpha \)-syn-GFP cells were measured by incubating cells with TiO₂ NPs for 24 or 72 hours. Cells were lysed using Complete Lysis-M buffer (Roche) containing a protease inhibitor cocktail and supplemented with 1% Triton X-100 for 30 minutes on ice. Lysed cells were centrifuged for 60
mins at 15,000 \( g \) and 4 °C, and the supernatant containing the soluble protein fraction collected for analysis. The pellet was resuspended in Complete Lysis-M buffer supplemented with 2% SDS and 8 M urea and sonicated to collect the insoluble protein fraction. Protein concentrations were determined using the Bradford assay. 4 µg of soluble protein and 40 µg of insoluble protein were separated by 12% SDS-PAGE for detection of α-synuclein and 40 µg of soluble protein for detection of LC3. Membranes were blotted using primary antibodies (α-synuclein, S5566, Sigma-Aldrich; LC3, NB-100-2220, Novus Biologics; GAPDH, sc-25778, Santa Cruz Biotechnology) and appropriate secondary antibodies (sc-2005, Santa Cruz Biotechnology; sc-2004, Santa Cruz Biotechnology). Imaging was performed on an LAS 4000 Imager and relative band densities were determined using ImageJ.

4.4.7. Statistical analyses

All data are presented as mean ± s.d. with the statistical significance calculated using a two-tailed Student’s \( t \)-test unless stated otherwise. Differences were considered statistically significant with *\( p<0.05 \).
Chapter 5

Effects of Size and Coating on the Autophagy-Modulating Properties of Zinc Oxide

5.1. Introduction

The global production of zinc oxide nanoparticles is expected to exceed 40,000 tons per year by the year 2020, with about 80% of this production being used in the cosmetic industry and, particularly, for the production of sunscreens [132]. The widespread use of zinc oxide nanoparticles is mainly due to their unique antibacterial, UV-filtering, and photocatalytic properties [132]. More recently, zinc oxide nanoparticles have also been used in biomedical applications including imaging, drug delivery, and gene delivery [133]. The increase in production and use of zinc oxide nanoparticles has paralleled rising concern over the health effects associated with accidental exposure to these particles. Cell exposure to zinc oxide
nanoparticles has been shown to affect cell viability [72], induce DNA damage [73], and produce reactive oxygen species [74], and studies indicate that even sub-toxic doses of nanomaterials may induce bio-adverse effects on cellular systems [134]–[136].

This study provides a comparison of zinc oxide particles (ZnOPs) of different sizes and coating, namely bulk ZnOPs (~200-1000 nm, uncoated), nano ZnOPs (~85 nm, uncoated), and nano/coated ZnOPs (~86 nm, coated), with the ultimate goal of understanding the effects of particle size and coating on the autophagy-modulating properties of ZnOPs.

5.2. Results

5.2.1. Differential cytotoxicity and release of Zn$^{2+}$ ions from ZnOPs

The cellular response to exposure to ZnOPs was investigated using commercially available ZnOPs in the bulk (200-1000 nm), nano (~85 nm), and nano/coated formats (~86 nm). The selection of these three particles was motivated by a specific interest in understanding how the size and coating of commercially relevant ZnOPs affect interactions of the particles with the autophagy system. All three ZnOPs consist of a zinc oxide core. The nano/coated ZnOPs are coated with a silicone derivative (triethoxycaprylylsilane) that is expected to be highly stable even at low pH [137]. To determine the behavior of these particles in solution, I measured the hydrodynamic diameter of each particle type suspended in DMEM using dynamic light scattering (Figure 20). Light scattering analyses indicate
that the bulk ZnOPs form a polydisperse suspension with an average hydrodynamic
diameter of 361.5 ± 95.8 nm (Figure 20. A) and a polydispersity index of 0.5 ± 
0.1 (Figure 20. B) with the majority of particles (>80%) ranging in size from 100 to 
1000 nm in diameter (data not shown). Analyses of the suspensions of nano ZnOPs 
and nano/coated ZnOPs revealed these particles to have average hydrodynamic 
diameters of 157.0 ± 8.1 nm and 143.7 ± 5.9 nm (Figure 20. A), respectively, and a 
low polydispersity index (0.3 ± 0.0) (Figure 20. B).

**Figure 20. Characterization of ZnOPs.** Dynamic light scattering analyses of the (A) 
average hydrodynamic diameter and (B) average polydispersity index of ZnOPs 
dispersed in DMEM. Data are presented as mean ± s.d.

ZnOPs of different shapes and sizes and presenting different surface 
functionalities were reported to cause cytotoxicity in a variety of cells types [72], 
[121], [138], [139]. It is unclear, however, whether the size and coating of the ZnOPs 
plays a role in the cytotoxic effects observed upon cell exposure to ZnOPs [140]– 
[142]. To assess the cytotoxic effect of the ZnOPs used in this study and to 
determine experimental conditions that are not associated with induction of
excessive cytotoxicity, which would preclude accurate characterization of the effect of ZnOPs on autophagy, I analyzed markers of apoptosis in HeLa cells exposed to ZnOPs (0-25 µg/mL; 24 hrs). Specifically, I monitored membrane rearrangement, which is characteristic of early apoptosis, by quantifying binding of Annexin V (Figure 21. A), and membrane fragmentation, which is characteristic of late apoptosis and cell death, by quantifying binding of propidium iodide (PI) (Figure 21. B). Significant induction of early apoptosis and cell death was observed upon exposure to all three ZnOPs at a media concentration of 25 µg/mL, similar to previously published studies [143], [144]. The viability of HeLa cells was shown to be affected similarly by the bulk and nano ZnOPs (comparable to results shown by Kim et al. and Song et al. [143], [144]), while the nano/coated ZnOPs were found to also induce early apoptosis at a lower concentration of 20 µg/mL.

Figure 21. Viability of Hela cells exposed to ZnOPs. (A-B) Flow cytometry analyses of (A) Annexin V and (B) PI binding in HeLa cells incubated with ZnOPs (24 hrs). Data are presented as mean ± s.d. *p<0.05, **p<0.01, ***p<0.001.
Previous studies suggest that the toxicity of ZnOPs is due to intracellular dissolution of Zn$^{2+}$ ions from the ZnOPs [121], [144], [145]. To test whether cellular exposure to the three ZnOPs results in different intracellular levels of Zn$^{2+}$, and to investigate the correlation between intracellular Zn$^{2+}$ levels and cell viability, I monitored the levels of intracellular Zn$^{2+}$ in HeLa cells exposed to ZnOPs (0-25 µg/mL; 24 hrs) by measuring FluoZin-3 fluorescence using flow cytometry (Figure 22). The intracellular levels of Zn$^{2+}$ were found to depend on the ZnOP media concentration for all three ZnOPs, as expected [144]. FluoZin-3 fluorescence measurements also suggest that intracellular dissolution of the bulk ZnOPs is comparable to the nano ZnOPs for all media concentrations tested (similar to what was previously reported by Song et al. [144]), while cell treatment with nano/coated ZnOPs at media concentrations of 20 and 25 µg/mL resulted in significantly higher levels of Zn$^{2+}$. The differences in intracellular Zn$^{2+}$ levels observed in cells treated with nano/coated ZnOPs compared to cells treated with bulk or nano ZnOPs may explain the differences in cytotoxic effect of the ZnOPs observed in apoptosis assays (Figure 21.A), with cell exposure to nano/coated ZnOPs resulting in higher intracellular Zn$^{2+}$ levels and induction of apoptosis at lower media concentrations.

To determine whether ZnOP format affects the extent of cellular uptake, ZnOP internalization was quantified by measuring the SSC of HeLa cells exposed to ZnOPs using flow cytometry (Figure 23). Similar uptake levels were observed for the bulk, nano, and nano/coated ZnOPs at all concentrations tested (0-25 µg/mL; 24 hrs), indicating that the effect of ZnOPs on apoptosis and the extent of Zn$^{2+}$
dissolution are not due to differences in the extent of cellular uptake, but are likely associated with the different physicochemical properties of the ZnOPs tested.

**Figure 22. Intracellular Zn$^{2+}$ release by ZnOPs.** Flow cytometry analyses of FluoZin-3 fluorescence in HeLa cells incubated with ZnOPs (24 hrs). Relative fluorescence values were calculated by normalizing the fluorescence of ZnOP-treated cells to that of untreated cells. Data are presented as mean ± s.d. *p<0.05, **p<0.01.

**Figure 23. Cellular uptake of ZnOPs.** Flow cytometry analyses of the side scattering parameter (SSC) of HeLa/TFEB cells exposed ZnOPs (24 hrs). Relative SSC values were calculated by normalizing the SSC of ZnOP-treated cells to that of untreated cells. Data are presented as mean ± s.d. *p<0.05, **p<0.01.
5.2.2. Cell exposure to ZnOPs results in activation of the transcription factor EB

Activation of the lysosome-autophagy system has been reported in association with cell exposure to ZnOPs [74], [145]–[147]. To investigate the molecular mechanisms involved in activation of the autophagic response observed upon cell exposure to ZnOPs, I monitored the activation of TFEB (Figure 24). TFEB nuclear localization in HeLa/TFEB cells cultured in the presence of ZnOPs (0-25 µg/mL; 24 hrs) was quantified by immunofluorescence confocal microscopy using a Hoechst nuclear stain and an anti-FLAG antibody (Figure 24. A). TFEB activation was quantified by calculating the Mander’s Correlation Coefficient of the TFEB signal (anti-FLAG) and the nuclear stain (Figure 24. B) and by determining the fraction of cells that present activation of TFEB higher than untreated cells (Figure 24. C). Colocalization analyses revealed a concentration-dependent increase in the fraction of nuclear TFEB and in the number of cells presenting nuclear translocation of TFEB greater than untreated cells in HeLa/TFEB cells exposed to at least 10 µg/mL of ZnOPs. Interestingly, cell exposure to the bulk, nano, and nano/coated ZnOPs resulted in similar extents of TFEB activation.
Figure 24. TFEB activation in HeLa/TFEB cells exposed to ZnOPs. (A) Confocal microscopy analyses of TFEB in HeLa/TFEB cells untreated and treated with ZnOPs (20 µg/mL; 24 hrs). Representative images show colocalization of the nucleus (blue, Hoechst stain, column 1) and TFEB (red, anti-FLAG, column 2) in merged images (column 3). Untreated, UT. Scale bar is 20 µm. (B) Average fraction of total cellular TFEB that localizes in the nucleus. Data are presented as mean ± s.e.m. *p<0.05, **p<0.01, ***p<0.001. (C) Percentage of HeLa/TFEB cells with fraction of nuclear TFEB greater than the average fraction of nuclear TFEB in untreated cells (0.17±0.05). Data are presented as mean ± s.e.m. *p<0.05, **p<0.01, ***p<0.001.

5.2.3. ZnOPs induce blockage of autophagic flux in a size-dependent fashion

To determine whether transcriptional activation of the lysosome-autophagy system mediated by TFEB in cells exposed to ZnOPs parallels an increase in the
formation and turnover of autphagic vesicles, I monitored the intracellular levels of LC3-I and LC3-II. LC3 levels in cells exposed to ZnOPs were first monitored by Western blot (Figure 25. A; Figure A 9). HeLa cells were treated with ZnOPs (20 µg/mL; 24 hrs) and Bafilomycin A1 (10 nM; 1 hr). Cell exposure to all three ZnOPs was found to induce an increase in the ratio of LC3-II/LC3-I, indicating an increase in the formation of autophagic vesicles. A further increase in the LC3-II/LC3-I ratio was observed in cells exposed to nano or nano/coated ZnOPs and Bafilomycin A1, but not in cells exposed to bulk ZnOPs and Bafilomycin A1. These results suggest that, while all three ZnOPs cause an increase in formation of autophagosomes, exposure to the bulk ZnOP also results in blockage of autophagic flux.

Figure 25. Autophagosome formation and turnover in HeLa cells exposed to ZnOPs. (A) Western blot analyses of LC3 levels in HeLa cells incubated with ZnOPs (20 µg/mL; 24 hrs) and Bafilomycin A1 (Baf; 10 nM; 1 hr). The ratio of autophagosomal (LC3-II) to cytoplasmic (LC3-I) isoforms is reported. Data are presented as mean ± s.d. *p<0.05, **p<0.01, ***p<0.001. (B) Flow cytometry analyses of HeLa/GFP-LC3 cells incubated with ZnOPs (20 µg/mL; 24 hrs) and Bafilomycin A1 (Baf; 10 nM; 1 hr) and treated with saponin. Relative GFP fluorescence values were calculated by normalizing the GFP signal of cells treated with ZnOPs to that of untreated cells (UT). Data are presented as mean ± s.d. *p<0.05, **p<0.01.
The accumulation of autophagic vesicles was also investigated using HeLa cells stably transfected to express LC3 fused to GFP (HeLa/GFP-LC3) by measuring the fluorescence of cells exposed to ZnOPs by flow cytometry (Figure 25. B).

HeLa/GFP-LC3 cells were incubated with ZnOPs (20 µg/mL; 24 hrs) and Bafilomycin A1 (10 nM; 1 hr) and the cells were treated with saponin prior to flow cytometry analyses. Saponin treatment allows extraction of the soluble cytoplasmic form of LC3 (LC3-I) and enables measurement of the autophagosome-associated LC3-II form [148]. Cell treatment with ZnOPs and Bafilomycin A1 was first confirmed not to affect the expression of GFP-LC3 by measuring the fluorescence of HeLa/GFP-LC3 cells not treated with saponin (Figure A 10). Cell exposure to all three ZnOPs was found to induce an increase in GFP fluorescence compared with untreated cells, indicating an increase in the amount of LC3-II accumulation upon ZnOP treatment. Exposure to the nano or nano/coated ZnOPs and Bafilomycin A1 caused a further increase in GFP fluorescence, indicating that treatment with the nano or nano/coated ZnOPs enhances autophagosomal turnover, which is blocked upon addition of Bafilomycin A1. Exposure to the bulk ZnOPs and Bafilomycin A1 did not induce an increase in GFP fluorescence, indicating that treatment with the bulk ZnOP alone induces accumulation of autophagosomes. These results confirm the results obtained by Western blot (Figure 25. A) and suggest that exposure to the bulk ZnOP results in a blockage of autophagic flux.

The impaired turnover of LC3-II observed in the results of Western blot and flow cytometry analyses suggest reduced fusion of autophagosomes with lysosomes. To directly evaluate the effect of cellular exposure to ZnOPs on autophagosome-
lysosome fusion and formation of autolysosomes, I monitored the colocalization of LC3 and LAMP-2 in cells exposed to ZnOPs (Figure 26). Specifically, HeLa cells were treated with ZnOPs (20 µg/mL; 24 hrs) and the colocalization of LC3 and LAMP-2 was measured by immunofluorescence confocal microscopy using anti-LC3 and anti-LAMP-2 antibodies (Figure 26. A). All three ZnOPs were found to increase the levels of LC3-LAMP-2 colocalization, as determined by calculating the Mander’s Correlation Coefficient for LC3 and LAMP-2 (Figure 26. B), with the bulk ZnOP inducing the lowest levels of colocalization. These results suggest that the increase in transcriptional activation of autophagy and formation of autophagosomes observed upon exposure to the bulk ZnOP may not parallel efficient autophagosome-lysosome fusion, resulting in suboptimal turnover of autophagosomes compared to what is observed in cells treated with the nano or nano/coated ZnOPs.
Figure 26. LC3-LAMP colocalization in HeLa cells exposed to ZnOPs. (A) Confocal microscopy analyses of LC3 and LAMP-2 in HeLa cells exposed to ZnOPs (20 µg/mL; 24 hrs). Representative images show colocalization of the nucleus (Hoechst, blue, column 1), autophagosomal membrane marker LC3 (green, column 2), and lysosomal membrane marker LAMP-2 (red, column 3) in merged images (column 4). Untreated, UT. Scale bar is 20 µm. (B) Quantitative analyses of confocal microscopy images. Untreated, UT. Data are presented as mean ± s.e.m. *p<0.05, **p<0.01, ***p<0.001.
These results, taken together, suggest that exposure to the nano or nano/coated ZnOPs results in the enhanced formation of autophagosomes, fusion of autophagosomes with lysosomes, and, finally, autophagosome turnover. Cell exposure to the nano or nano/coated ZnOPs did not result in significant differences in autophagosome formation and turnover under the conditions tested, suggesting that the triethoxycaprylylsilane coating on ZnOPs does not play a significant role in shaping the autophagic response to uptake of ZnOPs. Cell exposure to the bulk ZnOP, on the other hand, resulted in enhanced formation of autophagosomes, but also in blockage of autophagic flux, ultimately leading to accumulation of autophagosomes. These results suggest that hydrodynamic diameter of ZnOPs plays a key role in determining the response of the lysosome-autophagy system to internalization of ZnOPs.

5.2.4. ZnOP-induced upregulation of autophagic clearance depends on ZnOP size

To test whether ZnOP-induced TFEB activation leads to enhanced autophagic clearance, I monitored the degradation of autophagic cargo using fibroblast cells derived from patients with Late Infantile Neuronal Ceroid Lipofuscinosis (LINCL), which are characterized by the accumulation of ceroid lipopigment, an autofluorescent lipofuscin-like material that is normally degraded through autophagy [149]. The effect of ZnOPs on autophagic clearance was evaluated by quantifying the total amount of ceroid lipopigment fluorescence in LINCL cells treated with ZnOPs (20 µg/mL; 24 hrs) by confocal microscopy (Figure 27).
Microscopy analyses showed that accumulation of ceroid lipopigment was reduced by approximately 25% in cells treated with the nano or nano/coated ZnOPs (Figure 27. B). Cellular treatment with the bulk ZnOP under the same conditions, however, did not lead to a reduction of ceroid lipopigment autofluorescence, suggesting that TFEB activation by the bulk ZnOP does not enhance autophagic clearance.

Figure 27. Accumulation or clearance of autophagic substrates in LINCL cells exposed to ZnOPs. Confocal microscopy analyses of accumulation of ceroid lipopigment in LINCL cells incubated with ZnOPs (20 µg/mL; 24 hrs). (A) Representative images of ceroid lipopigment (green) and nuclei (blue). Untreated, UT. Scale bar is 50 µm. (B) Total ceroid lipopigment fluorescence signal detected in LINCL cells. Untreated, UT. Data are reported as mean ± s.e.m. *p<0.05, **p<0.01.
5.3. Discussion

There is growing concern over the effect of increasing human exposure to nano-sized zinc oxide through the production and use of ZnOPs in commercial products. In this study, I characterized the autophagic response to three ZnOPs (bulk, nano, nano/coated) by analyzing markers of the lysosome-autophagy system, from transcriptional activation to clearance of a model autophagic substrate. I discovered that the autophagic response to ZnOPs is mediated by TFEB and may result in either a biocompatible or a bioadverse outcome depending on the specific physicochemical properties of the ZnOP. Particularly, cellular treatment with the bulk ZnOP was found to induce accumulation of autophagosomes, resulting in a blockage of autophagic flux in HeLa and LINCL cells, while the nano and nano/coated ZnOPs were found to enhance autophagic degradation. These results indicate that bulk ZnOPs cause blockage of autophagic flux. While disruption of lysosomal stability and trafficking are typically the cause of defects in autophagic clearance, it remains to be determined whether other mechanisms, such as disruption of the cytoskeleton or formation of reactive oxygen species [9] may be involved.

In summary, results from this study indicate that the size of ZnOPs plays a critical role in determining the nature of the autophagic response to zinc oxide that manifests in response to cellular uptake of ZnOPs. These results also indicate that the effect of ZnOP size should be taken into account in the design of ZnOPs that may come into contact with biological systems.
5.4. Materials and methods

5.4.1. Nanomaterial characterization

ZnOPs (bulk, nano, and nano/coated) were purchased from Making Cosmetics®. The bulk (200-1000 nm) and nano (~85 nm) ZnOPs consist of super purity zinc oxide, and the nano/coated ZnOPs consist of ~98% super purity zinc oxide and 2% hydrophobic coating material (triethoxycaprylylsilane) (~86 nm). Stock solutions (20 mg/mL) were prepared by suspending ZnOPs in filtered dimethyl sulfoxide (bulk and nano) or methanol (nano/coated). Stock suspensions were sonicated immediately prior to use in a bath sonicator for 15 minutes and then diluted in DMEM. Particle hydrodynamic diameter measurements were obtained using a Malvern Instruments Zen 3600 Zetasizer system.

5.4.2. Cell cultures

HeLa cells were cultured in DMEM supplemented with 10% FBS and 1% PSQ. HeLa/TFEB cells were cultured in DMEM supplemented with 10% FBS and 1% PSQ and selected using G418 (1 mg/mL). HeLa cells stably expressing GFP-LC3 (HeLa/GFP-LC3) were created by transfecting HeLa cells with pBABEpuro GFP-LC3 (Addgene) using jetPRIME® (Polyplus Transfection) according to the manufacturer’s instructions. The transfection media was replaced with fresh media 24 hours post-transfection. HeLa/GFP-LC3 cells were cultured in DMEM supplemented with 10% FBS and 1% PSQ and selected using puromycin (0.5 µg/mL). LINCL cells derived from patients were from Coriell Cell Repositories (GM16486) and were grown in
DMEM supplemented with 20% FBS, 1%PSQ, and non-essential amino acids. Cells were cultured at 37 °C in 5% CO$_2$ and passaged using TrypLE Express.

5.4.3. **Cell viability**

Analyses of cell viability were conducted as previously described [42]. Briefly, HeLa cells were cultured in media supplemented with ZnOPs for 24 hours. Cell toxicity was tested using the Dead Cell Apoptosis Kit with Annexin V and propidium iodide (Life Technologies) according to the manufacturer’s instructions and analyzed by flow cytometry using a BD FACSCanto™ II Flow Cytometer with the 488 nm Argon laser.

5.4.4. **ZnOP uptake**

Cellular uptake of ZnOPs was evaluated as previously described [116], [131]. Briefly, HeLa/TFEB cells were exposed to ZnOPs for 24 hours, washed three times with PBS, and collected in PBS. Uptake was quantified by measuring the SSC of cells exposed to ZnOPs using a BD FACSCanto™ II Flow Cytometer with the 488 nm Argon laser.

5.4.5. **Intracellular zinc ion measurements**

Analyses of intracellular levels of Zn$^{2+}$ ions were conducted using the cell permeant form of FluoZin™-3 AM (Life Technologies) according to the manufacturer’s instructions. HeLa cells were cultured in media supplemented with ZnOPs for 24 hours. The ZnOP-containing media was replaced with serum-free
media containing 500 nM FluoZin™-3 AM and cells were incubated for 30 minutes at room temperature. Cells were washed once with serum-free media to remove any dye associated with the cell membrane, and then incubated in serum-free media for another 30 minutes to allow complete de-esterification of the dye molecules. Cells were then collected in PBS and analyzed by flow cytometry using a BD FACSCanto™ II Flow Cytometer with the 488 nm Argon laser.

5.4.6. Immunofluorescence microscopy

Immunofluorescence studies were conducted as previously described [119]. Briefly, cells were seeded on glass coverslips. HeLa or HeLa/TFEB cells were cultured in the presence of ZnOPs for 24 hours. Cells were washed three times with PBS, fixed with 4% PFA (15 min), permeabilized with 0.1% Triton X-100 (10 min), and blocked in 8% BSA-PBS (30 min).

TFEB subcellular localization studies were conducted as previously described [130]. HeLa/TFEB cells were incubated with an anti-FLAG antibody (F7425, Sigma) for one hour, washed, incubated with a fluorescent secondary antibody (611-142-002, Rockland) for one hour, washed, and then incubated with a Hoechst nuclear stain for 10 minutes. Images were obtained using a Nikon A-1 confocal microscope and the Nikon NIS Elements C imaging software. Image post-processing and colocalization analyses were conducted using the JACoP plugin in ImageJ [89]. The fraction of nuclear TFEB was quantified by calculating the Mander’s Correlation Coefficient for each image. Average values were calculated over ~50 cells per sample collected from at least three independent experiments.
The percentage of cells presenting TFEB nuclear localization was determined by calculating the fraction of cells presenting nuclear localization of TFEB (as determined by evaluating the Mander's Correlation Coefficient) greater than the average fraction of TFEB nuclear localization in untreated cells plus one standard deviation.

LC3-LAMP colocalization studies were conducted as previously described [119]. Cells were incubated with an anti-LAMP-2 antibody (354301, BioLegend) and an anti-LC3 antibody (NB100-2220, Novus Biologics) for one hour, washed, and incubated with the appropriate fluorescent secondary antibodies (611-143-002, Rockland and 610-142-002, Rockland) for one hour. Images were obtained using a Nikon A-1 confocal microscope and the Nikon NIS Elements C imaging software. Image post-processing and colocalization analyses were conducted using the JACoP plugin in ImageJ [89]. The level of LC3-LAMP colocalization was quantified by calculating the Mander’s Correlation Coefficient for each image. Average values were calculated over ~10 images per sample collected from at least three independent experiments.

5.4.7. Western blot

LC3 levels in HeLa cells were measured after incubating cells with ZnOPs for 24 hours. Cells were collected and lysed with Complete Lysis-M buffer (Roche) containing a protease inhibitor cocktail. Total protein concentrations were determined using the Bradford assay and 40 µg of protein from each sample were separated by 15% SDS-PAGE. Membranes were incubated with primary antibodies...
(LC3, NB-100-2220, Novus Biologicals; GAPDH, NB300320, Novus Biologicals) and the appropriate secondary antibody (sc-2004, Santa Cruz Biotechnology; sc-2020, Santa Cruz Biotechnology). Imaging was performed on an LAS 4000 Imager and relative band densities were determined using ImageJ.

5.4.8. GFP-LC3 measurements

Cells were collected in PBS, pelleted, and washed with either PBS or saponin (0.05% in PBS) [148]. Cells were pelleted and resuspended in PBS for analysis by flow cytometry using a BD FACSCanto™ II Flow Cytometer with the 488 nm Argon laser.

5.4.9. Ceroid lipopigment accumulation

The accumulation of ceroid lipopigment in LINCL cells was measured as previously described [17]. Briefly, cells were incubated with ZnOPs for 24 hours, washed three times with PBS, and fixed with 10% formaldehyde (10 min). Cell nuclei were stained using a Hoechst nuclear stain (10 min). Coverslips were then mounted onto glass slides, imaged using a Nikon A-1 confocal microscope, and analyzed using the Nikon NIS Elements C imaging software. Image post-processing was conducted using ImageJ. Average ceroid lipopigment signal was determined by calculating the corrected total cell fluorescence [CTCF = Integrated Density – (Area of selected cell x Mean fluorescence of background reading)] for individual cells in each image.
5.4.10. Statistical analyses

All data are presented as mean ± s.d. with the statistical significance calculated using a two-tailed Student’s $t$-test unless stated otherwise. Differences were considered statistically significant with *p<0.05.
Chapter 6

Summary and Future Directions

6.1. Summary

Emerging technologies to synthesize and characterize novel nano-sized materials have provided opportunities to engineer highly specialized materials for a variety of applications. This increasing use and commercialization of new nanomaterials has led to concerns over the many unknown and potentially harmful effects associated with human exposure to these nanomaterials. Most engineered nanomaterials are processed by uptake and routing pathways evolved to recycle and degrade intracellular nano-sized materials; these pathways converge to the lysosome-autophagy system. Impairment of this fundamental homeostatic system is associated with the development of a variety of ailments, including cancer, immune diseases, and degenerative diseases, paralleling the known effects of engineered nanomaterials on human health. Characterizing the impact of nanomaterials on the
lysosome-autophagy system, which mediates the main cellular response to nanomaterial uptake, is important to understand the impact of nanomaterials on cell physiology and human health. A better understanding of the functional interactions of nanomaterials with the lysosome-autophagy system will inform the design of safe nanomaterials and nanotherapeutics with desired autophagy-modulating properties. The objective of this research was to characterize the response of the lysosome-autophagy system to cellular uptake of genetically encoded nanoparticles based on AAV protein capsids and engineered TiO₂ and ZnO nanomaterials, and to correlate the autophagic response induced by these nanomaterials with biocompatible or bioadverse cellular responses associated with autophagy activation.

Analysis of the effect of AAV on the lysosome-autophagy system reported in Chapter 3 revealed that cellular uptake of AAV induces TFEB-mediated upregulation of the lysosome-autophagy system and formation of autophagosomes. I found that AAV-mediated induction of autophagy decreases AAV transduction efficiency and that this effect is mediated directly by TFEB. Results from this study provide a mechanistic understanding of the response of the lysosome-autophagy system to AAV internalization and will inform the design of AAV-based nanotherapeutics.

In Chapter 4, I reported a detailed analysis of the response of the lysosome-autophagy system to cell exposure to TiO₂ NPs with varying primary particle size. I found cellular uptake of TiO₂ NPs to induce activation of TFEB. I also found the time of exposure to TiO₂ NPs to have a dramatic effect on the outcome of the autophagic
response. I observed an increase in the formation of autolysosomes and enhanced clearance of autophagic cargo after short exposure times (24 hrs). Prolonged exposure (72 hrs) to TiO$_2$ NPs, however, induced lysosomal membrane permeabilization, which, in turn, causes blockage of the autophagic flux and accumulation of autophagic substrates. Results from this study revealed that the primary particle size does not affect the autophagic response to TiO$_2$ NPs; it remains to be determined, however, whether TiO$_2$ NP agglomeration plays a role.

The analysis of the autophagy-modulating properties of ZnOPs reported in Chapter 5 revealed that cellular uptake of ZnOPs induces activation of TFEB and autophagy in a particle size-dependent fashion. I found cellular exposure to nano and nano/coated ZnOPs to enhance autophagic clearance and cellular exposure to bulk ZnOPs to cause blockage of autophagic flux and accumulation of autophagic substrates. Results from this study indicate that the size of ZnOPs plays a critical role in determining the nature of the response of the lysosome-autophagy system to ZnOPs.

6.2. Future directions

Further work will be aimed at addressing some of the shortcomings of the current study and extending the approaches developed here to explore the autophagic response to nanoparticles in other in vitro systems of biomedical relevance. It is of particular interest to conduct these studies using cell lines that are representative of cell types at the forefront of human exposure to nanomaterials,
such as human alveolar epithelial cells (A549), human skin cells (HaCaT), human colon cells (Caco-2), and human macrophage cells (THP-1). Characterization of the autophagic response to nanomaterials in immortal monocultures should ideally be paralleled by studies conducted using primary cell lines and co-culture models [150].

Estimates of relevant doses in vitro are often based on worst-case assumptions or minimal treatment concentrations associated with induction of a measurable cellular response [150]. In vitro studies, however, should ideally be conducted using nanomaterial concentrations that recapitulate the effect of human exposure. The maximum permissible occupational exposure limits for inhalation of nanoscale (ultrafine) TiO$_2$ and ZnO provided by the United States Occupational Safety and Health Administration are 0.3 mg/m$^3$ (10 hr time-weighted average) [151] and 5 mg/m$^3$ [152] (8 hr time-weighted average), respectively, as calculated based on the airborne mass fraction of total inhalable TiO$_2$ or ZnO particles. These recommendations are based on laboratory observations of the effects of inhalation of nano-sized metal oxide powders in animal models [120], [153], [154]. While it is difficult to establish relevant nanomaterial concentrations for in vitro studies that provide insights into the effect of human exposure to nanomaterials, dose values may be estimated using different methods including the In vitro Sedimentation, Diffusion and Dosimetry (ISDD) model [155] and the Multiple-Path Particle Dosimetry (MPPD) model [156]. These models predict the actual cellular dose based on the nanomaterial’s physicochemical properties and concentration in suspension.
and the exposed cellular surface area. It should be noted that nanomaterial concentrations used for *in vitro* studies, such as those presented in this thesis, might be multiple orders of magnitude larger than those used for *in vivo* studies. Nevertheless, these studies typically generate knowledge of the molecular mechanisms underlying the response of the lysosome-autophagy system to nanomaterials that could not be obtained from *in vivo* studies and that are necessary to proceed to clinical evaluations.

A number of questions remain regarding the functional interaction of nanomaterials with the lysosome-autophagy system, especially concerning the molecular mechanisms underlying activation of the autophagic response and the route of accumulation of nanomaterials within autophagosomes. It is widely documented that nanoparticles interact with proteins in cellular media and intracellular milieu, leading to formation of protein corona [157], [158]. It remains to be determined whether nanomaterial sequestration into autophagic vesicles is mediated by mechanisms involved in canonical autophagy or selective autophagy. Selective autophagy is activated upon interaction between specific receptors and cargo molecules, which, in turn link the cargo to the autophagic machinery through interaction with a scaffold protein [159]. Published evidence suggests that nanomaterials may be ubiquitininated as a means of targeting what is perceived as foreign material to autophagosomes [9], [160]. It would be important to investigate whether nanomaterials are recognized by the autophagy system through a ubiquitin-dependent mechanism and to what extent the resulting effect on the
lysosome-autophagy system resembles the mechanisms of autophagy activation induced by naturally occurring nano-sized proteinaceous particles.

The cellular outcome of the blockage of autophagic flux induced upon exposure to nanomaterials also remains to be determined. Blockage of autophagic flux induced by biopersistent nanomaterials has primarily been observed in association with accumulation of the nanomaterial in subcellular vesicles, including autophagosomes, lysosomes, and multivesicular bodies, and cell death [38], [57], [119], [161]. Because nano-sized materials have been shown to exit cells via different mechanisms of secretion [162], it would be interesting to investigate whether inducing exocytosis of nanomaterials could ameliorate the phenotypes associated with blockage of autophagic flux. Characterizing the cellular outcome of nanomaterial-induced blockage of autophagic flux and, specifically, determining whether it is associated with exocytosis of nanomaterials and whether it leads to autophagy-associated cell death would provide a more comprehensive understanding of the impact of nanomaterials with autophagy-modulating properties on cell fate.

Another mechanism that may be activated in response to nanoparticle internalization is LC3-associated phagocytosis. LC3-associated phagocytosis was recently discovered upon detection of autophagy-associated proteins on the surface of phagosomes containing bacterial particles [163]. LC3-associated phagocytosis seems to be dedicated to sequestration and degradation of waste from the extracellular environment such as pathogens and cell debris [164]. While
phagocytosis primarily occurs in professional phagocytic cells, such as macrophages, phagocytosis may also occur in other cell types including fibroblasts and epithelial cells [165], which are typically affected by accidental exposure to engineered nanomaterials through inhalation or dermal contact [166]. Interestingly, phagocytosis of latex beads in murine bone-derived macrophages [167] and of polystyrene microspheres in rat alveolar macrophages [168] has already been reported. Further investigations are required to understand the mechanism of phagocytosis of nanomaterials, and whether this endocytic pathway results in LC3-associated phagocytosis.

Characterization of the autophagic response to nano-sized materials provides new opportunities to engineer highly specialized nanomaterials for biomedical applications ranging from drug and gene delivery systems [1], [169] to nanotherapeutics for cancer treatment [170]. Upregulation of the lysosome-autophagy system and enhancement of autophagic clearance in response to nanomaterial uptake may be desirable for the treatment of diseases characterized by inefficient autophagic clearance [48], but may also have an adverse effect on the delivery of therapeutic agents, as autophagy activation may enhance degradation of cargo delivered using nanomaterials. For instance, integration of genetic material using AAV-based gene delivery systems for the treatment of lysosomal storage disorders [103], [104] would require temporarily decoupling the therapeutic effect of autophagy activation by AAV from AAV-mediated delivery of the therapeutic gene [171]. The autophagy-inducing properties of nanomaterials may also adversely
affect the efficiency of synthetic nanomaterial-based drug and gene delivery systems. The therapeutic effect of the anticancer drug docetaxel delivered using polymeric nanoparticles, for example, was affected by co-administration of autophagy modulating agents [51], suggesting that effective delivery of materials using nanoparticles is likely to require inhibition of autophagy to counteract the effect of the nanomaterial delivery system of cellular clearance. Blockage of autophagic flux caused by nanomaterials, on the other hand, could be exploited to induce selective blockage of autophagic flux and cell death [35], [38], [58], [59] or chemosensitization [172] through targeted delivery to cancer cells [35], [58].
References


[69] FDA, “Title 21: Food and Drugs, Part 73 - Listing of Color Additives Exempt From Certification.”


Appendix A

Figure A 1. AAV2 and βCD treatment induce upregulation of autophagy-related genes. Relative mRNA expression levels of representative genes encoding lysosome (HEXA) and autophagy (SQSTM1) proteins in HeLa/TFEB cells treated with AAV2-GFP (MOI = 5000 vgs/cell; 48 hrs) or βCD (1 mM; 24 hrs). The mRNA expression levels were corrected by the expression levels of house-keeping genes GAPDH and ACTB and normalized to the expression levels in untreated cells (1.0). Data are presented as mean ± s.d. *p<0.05 compared to untreated.

Figure A 2. AAV2 and βCD treatment induce formation of autophagosomes. Western blot analyses of LC3 isoforms (cytoplasmic LC3-I and autophagosome-associated LC3-II) in HeLa cells incubated with AAV2-GFP (MOI = 5000 vgs/cell; 24 hrs) and Bafilomycin A1 (Baf; 1 nM; 1 hr). GAPDH served as a loading control.
Figure A 3. TFEB silencing in HeLa cells. TFEB gene expression in HeLa cells treated with QIAGEN All Stars Negative Control siRNA and TFEB siRNA. Data are presented as mean ± s.d. **p<0.01 compared to Cntrl siRNA.

Figure A 4. AAV2 induces clearance of α-syn-GFP aggregates. Western blot analyses of α-synuclein in the insoluble protein fraction and LC3 in the soluble protein fraction from H4/α-syn-GFP cells treated with AAV2-GFP (MOI = 10000 vgs/cell; 24 hrs). GAPDH served as a loading control.
Figure A 5. Lysosomal membrane permeabilization in HeLa/TFEB cells exposed to TiO$_2$ NPs. Confocal microscopy analyses of lysosomal membrane permeabilization in (A) untreated HeLa/TFEB cells (UT) and HeLa/TFEB cells incubated with (B) 15, (C) 50, and (D) 100 nm TiO$_2$ NPs (50, 100, or 500 µg/mL; 72 hrs). Representative images show the colocalization of LGALS1 (red, anti-LGALS1, column 1) and LAMP-2 (green, anti-LAMP-2, column 2) in merged images (column 3). Yellow arrows indicate puncta of colocalized LGALS1 and LAMP-2 (zoomed image in column 4). Scale bar is 50 µm.
Figure A 6. LC3 protein expression levels and processing in HeLa cells exposed to TiO_{2} NPs. Western blot analyses of LC3 isoforms (cytoplasmic LC3-I and autophagosome-associated LC3-II) in HeLa cells incubated with TiO_{2} NPs (10 or 100 µg/mL) for (A-C) 24 or (D-F) 72 hours and Bafilomycin A1 (Baf; 10 nM; 1 hr). GAPDH is used as a loading control.
Figure A 7. LC3-LAMP colocalization in HeLa/TFEB cells exposed to TiO$_2$ NPs. Confocal microscopy analyses of LC3-LAMP colocalization in untreated HeLa/TFEB cells (UT) or HeLa/TFEB cells incubated with TiO$_2$ NPs (100µg/mL) for (A) 24 or (B) 72 hours. Representative images show nuclei (blue, Hoechst stain, column 1) and the colocalization of LAMP (red, anti-LAMP-2, column 2) and LC3 (green, anti-LC3, column 3) in merged images (column 4). Scale bar is 20 µm.
Figure A 8. Accumulation of autophagic substrates in H4/α-syn-GFP cells exposed to TiO₂ NPs. Western blot analyses of α-synuclein and LC3 in the insoluble and soluble protein fractions of H4/α-syn-GFP cells treated with TiO₂ NPs (100 µg/mL) for (A-B) 24 or (C-D) 72 hours. GAPDH is used as a loading control. Untreated, UT.
Figure A 9. LC3 protein expression and processing in HeLa cells exposed to ZnOPs. Western blot analyses of LC3 isoforms (cytoplasmic LC3-I and autophagosome-associated LC3-II) in HeLa cells incubated with ZnOPs (20 µg/mL; 24 hrs) and Bafilomycin A1 (Baf; 10 nM; 1 hr). GAPDH is used as a loading control.

Figure A 10. GFP fluorescence of HeLa/GFP-LC3 cells exposed to ZnOPs. Flow cytometry analyses of HeLa/GFP-LC3 cells incubated with ZnOPs (20 µg/mL; 24 hrs) and Bafilomycin A1 (Baf; 10 nM; 1 hr). Relative GFP fluorescence values were calculated by normalizing the GFP signal of cells treated with ZnOPs to that of untreated cells (UT). Data are presented as mean ± s.d.