Differential autophagic responses to nano-sized materials

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Abstract

Autophagy is a complex catabolic pathway that mediates degradation of excess or unwanted cytoplasmic components through the lysosome. Activated by environmental factors, such as nutrient depletion, and intracellular stimuli, such as proteotoxic stress, it provides a highly dynamic quality control mechanism to recycle cellular components, eliminate aberrant materials, and, ultimately, maintain cellular homeostasis. A growing body of evidence suggests that autophagy is also activated upon internalization of engineered nanomaterials, most likely as a protective response to what is perceived as foreign or toxic. This review describes the mechanisms of autophagy activation in response to naturally occurring and engineered nanomaterials. We provide a comprehensive analysis of the impact of nanomaterials on the lysosome-autophagy system, with particular emphasis on cellular markers associated with biocompatible and bioadverse outcomes of autophagy activation, such as clearance of toxic material and autophagy-associated cell death. Potential applications of the next-generation nanomaterials designed to interface with cellular clearance mechanisms with precisely tunable properties are also discussed.

Introduction

Mammalian cells are complex systems that employ highly sophisticated and integrated networks to translate instructions stored in the genome into thousands of molecular components that define their biological properties. As a result, a cell's physiologic state is dictated by the collective behavior of its molecular components, which can vary dramatically depending on endogenous as well as environmental factors. Cellular responses to exogenous stimuli, such as internalization of foreign or toxic materials, are typically activated in an attempt to restore cellular homeostasis through highly plastic quality control mechanisms.

Progress in the field of nanotechnology has led to a rapid growth in the number of engineered nanomaterials currently 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, many of 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 the formation of protein coronas, particle wrapping, intracellular uptake, and biocatalytic processes that can have biocompatible or bioadverse outcomes [2]. Surprisingly little, however, is known about the effect 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 [3]. However, even nanomaterials deemed non-toxic according to standard *in vitro* and *in vivo* viability assays can still impact fundamental biochemical pathways [4]; and while they may not lead to lethal or pathological outcomes, they can significantly alter cell physiology [4].

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 [5]. 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 [6]. While activation of autophagy can lead to enhanced clearance, it may also be associated with activation of cell death programs [7]. Moreover, nanomaterials that activate autophagy may also cause impairment of cellular components that mediate degradation, ultimately leading to blockage of autophagic flux. In this review, we illustrate the mechanisms of autophagy activation in response to naturally occurring and engineered nano-sized materials and discuss cellular markers associated with biocompatible and bioadverse outcomes of nanomaterial-induced autophagy activation.

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 [6]. Nanomaterials typically induce accumulation of autophagic vesicles, which sequester toxic or foreign material, and activation of macroautophagy (hereafter referred to as autophagy) [5].

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 autophagolysosomes; and iii) cargo degradation by lysosomal hydrolytic enzymes [6] (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) [8]. Activation of TFEB is regulated by the mammalian target of rapamycin protein kinase complex 1 (mTORC1), which is a key component of pathways that regulate cellular growth and proliferation in response to nutrients, energy levels, and growth signals [9,10]. Under physiologic conditions, mTORC1 localizes onto the lysosomal membrane and phosphorylates TFEB. Phosphorylation of TFEB prevents its translocation into the nucleus [10]. 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 [9].

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 [11]. Modulation of TFEB activation was also observed upon cellular uptake of 2-hydroxypropyl-beta-cyclodextrin [12], quantum dots [13], and polystyrene nanoparticles [14], suggesting that transcriptional modulation of autophagy is part of the cellular adaptive response to nanomaterials.

Nano-sized materials as autophagy activators

Naturally occurring nanomaterials

Autophagy mediates disposal of insoluble nano-sized particles [15]. 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 [15]. Impairment of autophagy results in formation of proteinaceous and lipidic inclusions and neurodegeneration, while an increase in autophagic activity results in enhanced clearance of toxic material [16].

Formation of autophagosomes involves the recruitment of specific adaptors, including the p62/SQSTM-1/sequestome [17]. Cargo sequestration is mediated by the bifunctional structure of p62, which consists of a ubiquitin association domain that binds

to polyubiquitin chains on misfolded proteins, and a self-aggregation domain that facilitates formation of p62-cargo complexes [18]. Delivery of the cargo to autophagosomes proceeds through the specific interaction of p62 with the mature form of microtubule-associated protein light chain 3 (LC3) on the surface of phagophores. Upon loading of the cargo, phagophores fuse around cargo material to form autophagosomes (Figure 1). In addition to p62, which mediates delivery of proteinaceous material, a number of other autophagic adaptors have been reported to mediate sequestration of other types of cargos, such as intracellular organelles and bacteria [19], through similar mechanisms.

Autophagy also plays a key role in innate immunity and degradation of intracellular pathogens [19,20]. 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 [20]. Interestingly, the plasma membrane disturbance induced by human immunodeficiency virus type I (HIV-I) [21] and by paromyxovirus particles [22] 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 foreign material.

In addition to mediating lysosomal degradation of autophagic cargo, activation of TFEB and upregulation of the lysosome-autophagy system may result in clearance of intracellular material via exocytosis. TFEB overexpression rescues the pathologic phenotypes of *in vitro* and *in vivo* models of lysosomal storage diseases by inducing exocytosis of lysosomes and autophagolysosomes [23,24]. Interestingly, autophagy dysfunction induced via pharmacological inhibition or genetic inactivation results in enhanced exocytosis of protein aggregates [25], suggesting that autophagy and exocytosis may be regulated competitively or cooperatively, depending on the nature of environment.

Engineered nanomaterials

A number of engineered nanoparticles were found to accumulate within autophagosomes and even to enhance formation of autophagosomes. Seleverstov *et al.* first documented the autophagy-inducing effect of quantum dots [26]. Different types of nanomaterials were later found to accumulate within autophagosomes, including silica [27], gold [28], α -alumina [29], rare earth oxides [30], and fullerenes [31]. While the composition and surface chemistry of these nanoparticles vary significantly, the nanoscale size seems to be a common denominator for accumulation into autophagosomes and, possibly, induction of autophagy. Interestingly, the mechanism of autophagy activation varies widely among nanoparticles with different physicochemical properties. Surface modifications affecting

the chemical reactivity of nanoparticles 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 [4].

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 reaction 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: the autophagic response to nanomaterials may thus represent a cellular reaction to what is perceived as foreign or toxic [32]. A number of alternative models, however, have been proposed and include nanomaterial-induced toxicity through lysosomal dysfunction [33], oxidative stress [28,34], mitochondrial damage [35] and selective degradation of mitochondria by autophagy (or mitophagy) [36], as well as direct effects on gene regulation [37] (Figure 2).

Transcriptional activation of the lysosome-autophagy system upon internalization of nanoparticles may lead to enhanced clearance of endogenous materials or impairment of downstream steps of the 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 [38], enhance autophagic clearance [39]. Silver nanoparticles, which are also widely used in consumer products and medical devices due to their excellent anti-microbial properties [40], activate autophagy, but also induce oxidative stress and extensive cytotoxicity [41]. It is unclear whether autophagy is activated as a response to foreign material or to ROS-induced damage caused by nanosilver [42]. Interestingly, the increased formation of autophagosomes is not followed by enhanced fusion of autophagosomes with lysosomes, suggesting that nanosilver may cause blockage of autophagic flux [41].

Activation of autophagic clearance

TFEB activates the coordinated expression of autophagy and lysosomal genes [8]: modulation of TFEB activation is thus a promising strategy to enhance clearance of intracellular material, such as proteinaceous and proteolipidic aggregates [8,43]. The ability of 2-hydroxypropyl-beta-cyclodextrin, 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 [12,44]. Interestingly, 2-hydroxypropyl-beta-cyclodextrin-

mediated activation of TFEB was found not to depend on its ability to alter cellular levels of cholesterol [44]. Internalization of nanoceria stabilized by biocompatible surface coatings (*N*-acetyl-glucosamine, polyethylene glycol, or polyvinylpyrrolidone) also results in TFEB-mediated activation of autophagic clearance [39]. Nanoceria functions as a radical scavenger and protects cells from radiation-induced damage and oxidative stress [45]. The increase in cellular clearance of ceroid lipopigment observed in fibroblasts derived from patients with Late Infantile Neuronal Ceroid Lipofuscinosis [39] is thus particularly interesting in light of potential therapeutic applications [46].

The autophagy-inducing properties of nanomaterials can also reduce the efficiency of nanoparticle-based delivery systems. The therapeutic effect of the model 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 [47].

Modulating nanomaterial-induced activation of autophagy to enhance cargo delivery or address safety concerns of nanoparticles relies on careful nanomaterial design. Our currently limited understanding of the functional interaction between nanoparticles and the autophagy system precludes the rational design of nanomaterials with predictable autophagy modulating properties. High-affinity surface coating peptides were found to abrogate autophagic activity of lanthanide nanocrystals. Interestingly, significant activation of autophagy was observed using a bifunctional peptide combining a high binding affinity moiety with the RGD cell adhesion domain [48]. A battery of 81 multiwalled carbon nanotubes presenting high surface ligand structural diversity was screened to select ligands that enhance LC3 processing [49]. Interestingly, autophagy induction was observed in cells treated with carbon nanotubes functionalized with surface ligands, but not in cells treated only with the ligands, suggesting that autophagy is activated in response to uptake of the nanomaterials and that the specific nature of the autophagic response depends on the mode of interaction between the nanomaterial and the lysosome-autophagy system [49].

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 a pro-death function [51]. Activation of cell death mechanisms in cells presenting enhanced formation of autophagosomes is observed upon treatment with a variety of nanomaterials, including polymeric nanoparticles [52] and iron oxide nanoparticles [35].

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 [7]. While a correlation is sometimes detected [54], the molecular mechanism underlying nanoparticle toxicity 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 beneficial or detrimental 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 autophagy flux, which has been observed in different cell types upon uptake of a variety of nanomaterials, including fullerenol [54], silver [41], rare earth oxide [56], and gold [33] nanoparticles.

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 nanoparticles 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 cytoprotection against the toxic effect of engineered nanomaterials.

Nanomaterial-induced blockage of autophagic flux has also been investigated for therapeutic applications. Targeted delivery of autophagy-inducing nanoparticles to cancer cells could enable novel strategies to selectively induce blockage of autophagic flux and cell death [58]. Iron oxide nanoparticles, for instance, were reported to enhance autophagosome formation and cytotoxicity in human cancer cells, but not in normal lung fibroblasts [35,59]. 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 induction of lysosomal dysfunction. Impairment of lysosomal integrity or inactivation of lysosomal enzymes prevents lysosome-autophagosome fusion, resulting in aberrant accumulation of autophagosomes [5,60]. Not surprisingly, a number of endocytosed nanomaterials that cause autophagy-associated cell death, including gold

nanoparticles [33] and carbon nanotubes [61], are detected in the lysosomes. The mechanism of nanoparticle-induced lysosomal dysfunction is unclear and could be associated with permeabilization or osmotic swelling of lysosomes [62], induction of oxidative stress [41], or alkalinization of the lysosome [35]. A “proton sponge” effect has been proposed as a potential mechanism of lysosomal permeabilization and impairment observed upon cellular internalization of cationic nanoparticles. 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 [33,62]. 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 nanoparticle toxicity [33,62].

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).

Conclusions and outlook

Emerging technologies to synthesize and characterize nanomaterials have provided new opportunities to manufacture highly specialized nanomaterials for a variety of applications. As a result, more than 1,600 consumer products containing nanoparticles are currently on the market [1]. The large-yield production of nanoparticles has inevitably increased human exposure, raising awareness of the many unknown and potentially harmful effects of nanoparticles on human health.

A wealth of information is available about the cellular uptake of nanoparticles, and the rate at which cells internalize nanoparticles can be controlled with respect to nanoparticle size, charge, and surface properties. However, the impact of these physicochemical properties on other cellular processes has not been investigated in a systematic fashion. 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 nanosize range. Understanding the functional interaction of autophagy with nanoparticles is expected to profoundly impact the design of nanomaterials, as it will open the way to engineer the next generation of nanotherapeutics with tunable properties at the interface with cellular clearance pathways and to develop safe nanomaterials for a variety of consumer products.

Acknowledgements

This work was funded by the National Science Foundation (CBET-1336053 and CBET 1254318) and the Welch Foundation (C-1824).

Figure Legends

Figure 1. Selective autophagic degradation of protein aggregates mediated by p62. The cargo-loading adaptor p62 binds to polyubiquitin chains on misfolded proteins and self-aggregates, inducing formation of p62-protein complexes. The specific interaction of p62 with LC3-II results in cargo delivery to autophagic membranes (phagophores). Upon loading of the cargo, fusion of the phagophore surrounds the cargo material, resulting in formation of autophagosomes. Autophagosomes deliver cargo material to lysosomes through the formation of autophagolysosomes, where degradation occurs.

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, and induction of lysosomal damage. Depending on the nature of the nanomaterial, activation of autophagy may lead to enhanced clearance or blockage of autophagic flux.

Figure 3. Hypothesized models of 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 autophagolysosomes, 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.

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Annotated References and Recommended Readings

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

Lee Y-H, Cheng F-Y, Chiu H-W, Tsai J-C, Fang C-Y, Chen C-W, Wang Y-J:

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- Uptake of silver nanoparticles results in induction of oxidative stress and growth arrest or apoptosis. The authors detect enhanced expression of LC3-II and accumulation of autophagosomes; however, lack of autophagosome-lysosome fusion is indicative of blockage in autophagic flux.

Li R, Ji Z, Qin H, Kang X, Sun B, Wang M, Chang CH, Wang X, Zhang H, Zou H, et al.: **Interference in Autophagosome Fusion by Rare Earth Nanoparticles Disrupts Autophagic Flux and Regulation of an Interleukin-1 β Producing.** *ACS Nano* 2014, **8**:10280–10292.

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Figure 1

Figure 2

Figure 3