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Engineered nanomaterial-induced lysosomal membrane permeabilization and anti-cathepsin agents

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Abstract

Engineered nanomaterials (ENMs), or small anthropogenic particles approximately < 100 nm in size and of various shapes and compositions, are increasingly incorporated into commercial products and used for industrial and medical purposes. There is an exposure risk to both the population at large and individuals in the workplace with inhalation exposures to ENMs being a primary concern. Further, there is increasing evidence to suggest that certain ENMs may represent a significant health risk, and many of these ENMs exhibit distinct similarities with other particles and fibers that are known to induce adverse health effects, such as asbestos, silica, and particulate matter (PM). Evidence regarding the importance of lysosomal membrane permeabilization (LMP) and release of cathepsins in ENM toxicity has been accumulating. The aim of this review was to describe our current understanding of the mechanisms leading to ENM-associated pathologies, including LMP and the role of cathepsins with a focus on inflammation. In addition, anti-cathepsin agents, some of which have been tested in clinical trials and may prove useful for ameliorating the harmful effects of ENM exposure are examined.

Keywords

Engineered nanoparticles; cathepsins; inflammasome; anti-cathepsin agents; lysosomal membrane permeabilization

Engineered nanomaterials: overview

To understand the potential effects of engineered nanomaterial (ENM) exposures, it is helpful to examine the various applications in which these substances are used. The most commonly cited uses of ENMs include biomedical, industry (e.g. paint), and cosmetics (e.g. sunscreen). While ENMs are increasingly applied to a wide range of industrial pursuits, the biomedical applications are perhaps one of the most promising (Zhao and Castranova, 2011). Metal oxide nanomaterials (both natural and engineered) are being tested for use in drug release systems, medical diagnostics, and sunscreens; metal oxide nanomaterials are highly reactive, with photocatalytic properties in some cases (Zhao and Castranova, 2011; EPA, 2014). ENMs known as quantum dots that are comprised of cadmium selenide, cadmium telluride, and zinc selenide with various possible metal structures are utilized in medical imaging applications, while engineered dendrimers (highly branched polymers),

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composite nanomaterials (synthesized with more than one type), and engineered silver (Ag) nanomaterials (composed of many Ag atoms) are being utilized or proposed for use in medical applications such as drug delivery, cancer detection, and antimicrobial applications (EPA, 2012). ENMs of different types and composition are being applied to focused delivery of chemotherapeutic agents to cancer sites, bypassing healthy tissue that would otherwise be affected. ENMs have also shown promise as targeting delivery agents that regulate lipid metabolism and inflammation to treat atherosclerosis (Zhang et al., 2015a), and the application of ENMs to drug delivery systems for multi-modal chemotherapeutic agents are anticipated to greatly enhance the efficacy of cancer drug protocols and outcomes (Flynn and Wei 2005). Further, because of their unique characteristics ENMs are increasingly being applied towards improved imaging for biomedical diagnostics, leading to earlier detection and improved prognoses of various cancers and other diseases.

While the field of study focusing on ENMs has gained tremendous ground in recent years, there is a distinct imbalance in information between potential applications of ENMs and possible adverse health effects. Further, there has been discussion regarding the most appropriate methods for determining the safety of certain ENMs (Alaraby et al., 2016; Hornos Carneiro and Barbosa, 2016; Hartmann et al., 2015; Kermanizadeh et al., 2016). Therefore, it is important to obtain a better understanding of mechanisms leading to toxicity so that anticipated benefits continue to outweigh risks. To address this paucity of information, intense efforts to understand ENM toxicity have been ongoing in recent years. ENM-activation of inflammatory pathways is one of the most studied areas of concern because of the known role for inflammation in many, if not most, chronic and degenerative health diseases (Stephenson et al. 2016, Maisch et al. 2005, Sethi et al. 2012, Kundu and Surh 2008). In addition to inflammation, concerns regarding oxidative stress, pulmonary toxicity, fibrosis, reproductive system, malignant transformations, granuloma formation, and genetic alterations have been raised (Liu et al., 2012; Shvedova et al. 2008; Farcas et al, 2016; Schramm et al, 2016; Snyder-Talkington et al, 2016; Chakraborty et al, 2017).

Engineered nanomaterials: A human health issue

There is an apparent risk to humans from occupational inhalation exposures to ENMs and exposure through consumer products such as food and cosmetics; however, there has been insufficient time to conduct adequate epidemiology studies. Nonetheless, investigations on workers heavily exposed to titanium dioxide (TiO_2), a nanoparticle (NP) that is commonly used in commercial products, have repeatedly demonstrated its safety (Warheit and Donner 2015). In contrast, *in vivo* and *in vitro* studies consistently suggested that certain types of bioactive nanomaterials represent a significant risk to human health (Bonner et al. 2013, Xia et al. 2013), and many ENMs share similar properties to particles already found in the environment with known adverse human health effects (Peters et al. 2011). For example, carbon nanotubes (CNT) have been characterized as having asbestos-like properties and exposure to the mesothelial lining in mice led to development of mesothelioma—a known outcome of asbestos exposure in some people (Takagi et al., 2012, Sargent et al., 2014, Suzui et al., 2016; Lemen, 2016). Similar to ENMs, differences in toxicity between different types of natural particles and fibers, such as silica and asbestos are thought to arise from various physicochemical properties, including size, morphology, and composition (Nemmar

et al. 2013). It stands to reason, therefore, that many advances toward an increased understanding of the underlying mechanisms associated with environmental particle and fiber exposures, such as asbestos, may also apply to ENMs (Bunderson-Schelvan et al. 2016). In addition, it has been shown in mice that multi-walled carbon nanotubes (MWCNTs), a specific type of ENM, are distributed throughout the body and accumulate over time after an inhalation exposure, including the parietal pleura, respiratory musculature, liver, kidneys, heart, and brain (Mercer et al., 2013); gold nanoparticles have also been shown to extensively redistribute after exposure (Khlebtsov and Dykman, 2011; Hornos Carneiro and Barbosa, 2016).

While inhalation exposures are the most likely (Oberdörster et al., 2015), environmental exposures from water and food are increasingly of concern (Hendren et al., 2013). Little is known regarding the potential health adverse effects specifically resulting from environmental exposure to ENMs; however, studies demonstrated that silver nanoparticles (Ag-NPs) are bioavailable in estuarine waters (Khan et al. 2012; Gagne et al, 2013) and the same is likely true for other ENMs. ENMs may be released into the environment through both point and non-point sources and remain in suspension or react with other materials (EPA, 2012). ENMs are readily transported over a greater distance than larger particles of the same material; transport in the environment may be affected by surface chemistry, size, as well as biological and abiotic processes (EPA 2014). Therefore, there is little question that environmental exposures to ENMs might occur over time and need to be addressed, and future adverse outcomes associated with ENMs may be linked to the quantity of internalized ENMs. Translocation of ENMs to secondary organs such as liver, kidneys, and heart is known to occur at different rates from inhalation, intratracheal or intranasal instillation, and pharyngeal aspiration exposures (Kermanizadeh et al., 2015; 2016). In general, ENMs are insoluble, persisting in biological fluids for extended periods; however, the toxicity of a few NP is known to be affected by their dissolution in culture media or biological fluids, particularly metal and metal oxide NP (Lai, 2015). Further, it has been recognized that the absorption, distribution, metabolism, and excretion (ADME, which characterizes the disposition of a compound within a biological system), as well as toxicity of silica NP are largely unknown, despite their successful use as effective drug carriers that may, in fact, result in shape-dependent renal damage (Li et al., 2015). Similar data characterizing ADME properties of most ENMs currently in use are also lacking. Ultimately, human health outcomes might reflect a combination of ENM bioactivity and ADME. As such, therapeutic strategies aimed at treating ENM-related diseases may be beneficial for limiting future costs to human health. Here, possible mechanisms underlying pathological outcomes following ENM exposure are examined here as well as potential treatment strategies with a focus on anti-cathepsin agents are presented

Potential risk associated with ENM exposure

Strategies for developing risk assessment protocols for ENMs in commercial products, workplace areas, and the environment are under intense discussion (Cuddy et al. 2015). Currently, there is a significant amount of variation in the types of ENMs being developed (Table 2), as their physical traits are easily manipulated in terms of size, shape, and composition. This has resulted in a significant challenge to toxicologists and policy makers

regarding the safety of these compounds. Consideration of multiple lines of evidence is necessary due to the complex nature and broad range of physicochemical characteristics for existing ENMs, such as specific surface area, surface texture, zeta-potential, particle morphology, aspect ratio, presence of metals, and particle dissolution rate (Simko et al. 2014). Further, ENM-enabled (or containing) products are likely to cause most exposures, and there is a great deal of uncertainty regarding release of ENMs from these products and consequent health impacts along an ENM's lifecycle.

Recently, a significant amount of progress has been made toward identifying the mechanisms associated with ENMs and natural particle toxicity. Inflammation resulting from permeabilization of the lysosomal (alternatively referred to as late endosome) membrane followed by activation of the NLRP3 inflammasome appears to play a critical role (Bunderson-Schelvan et al. 2016). A key event following ENM exposure is release of cathepsins from phago-lysosome vesicles associated with ENM-damaged membranes into the cytoplasm and extracellular milieu. Cathepsins are a family of protein-degrading enzymes that serve a function in a variety of physiological processes. Investigators have increasingly been interested in the role that intracellular cathepsins play in both normal and pathological processes; in fact, drugs targeting cathepsins K and S are in various stages of clinical development.

Particle-induced lysosomal membrane permeability (LMP)

The lysosome is a key component of autophagic and endosomal pathways, which are responsible for sequestering pathogens as well as organelles and proteins including microorganisms, ENMs, amyloid plaques, cholesterol crystals, damaged or dead cells that have become damaged or marked for degradation. This process is triggered by acidic activation of proteolytic digestive enzymes primarily in phagocytic cells such as monocytes and macrophages (Hullin-Matsuda et al. 2014). Autophagosomes possess a double-membrane that fuses with lysosomes to form an autolysosome, which facilitates the breakdown of encapsulated materials that subsequently may be employed for cell survival (Suzuki et al. 2016). Various investigators suggested that nanomaterial-induced autophagy occurs in response to the body perceiving the nanomaterials as foreign, similar to that of bacteria or other pathogens (Peynshaert et al., 2014; Neibert and Maysinger, 2012; Luo et al., 2013). However, autophagy was also found to play both a protective and pro-death role following NP exposures, depending upon the specific particle (Zhou et al., 2013), with autophagic clearance apparently dependent on the charge of the NP (Song et al., 2015). A primary mechanism of ENM-induced inflammation involves the destabilization and pore formation of the lysosomal membrane, termed lysosomal membrane permeability (LMP) (Figure 1). Many diseases associated with LMP provide evidence of autophagy dysfunction, perhaps by preventing the fusion of autophagosomes and lysosomes (Settembre et al. 2008). This process is not completely understood and not unique to ENM (Stern et al. 2012). Since the lysosome organelle is present in all nucleated cells (Alroy et al. 2014), a digestion process related to normal immune system activity is necessary for foreign body removal and antigen presentation. Some particles initiate acute inflammation by destabilizing the lysosomal membrane, which releases the catalytic enzyme contents of the lysosome, triggering inflammatory signaling and potentially cell death (Aits and Jaattela 2014). The

release of cathepsin B and possibly cathepsin C (Kono et al. 2012) from the lysosome was reported to initiate NLRP3 inflammasome activation, which subsequently activates Caspase 1 and in the presence of NF-κB co-stimulation, cleaves pro-IL-1β and releases mature IL-1β, inducing an acute inflammatory signal (Figure 1) (Dostert et al. 2008). This acute inflammation might become sustained and result in a chronic inflammatory condition that manifests in various pathologies depending upon the inflammatory area (Bunderson-Schelvan et al. 2016). Recently, Hughes et al (2016) demonstrated increased proteolytic activity from intracellular Caspase 1, extracellular Caspase 1, and cathepsin S in response to silica, alum, and polystyrene particulate exposures suggesting viable markers for lysosomal rupture and acute inflammation. Regarding ENMs, the lungs, skin, and digestive tract are the primary areas of interest, but with the inclusion of ENMs as drug delivery systems, any part of the body is potentially susceptible to LMP-induced inflammation (Donaldson and Seaton 2012, Oberdorster et al. 2007). Further, ENM physicochemical characteristics influence their ability to initiate LMP, and it is not known whether certain ENMs induce LMP (Table 2). In particular, ENM toxicity was noted to be dependent upon cell type, dose, and aspect ratio, with models of longer nanotubes resulting in elevated frequency of cell death, increased changes in morphology, greater tumor necrosis factor alpha release, higher LMP incidence, and enhanced endoplasmic reticulum stress than seen with shorter nanotubes (Wang et al., 2015).

The lysosomal membrane is composed of lipids and proteins (lysosomal membrane associated proteins - LAMP's and kinase/phosphatase enzymes involved in lipid modification) that are involved in a lipid-sorting process where cholesterol is depleted with lower pH and negatively charged bis (monoacylglycero) phosphate (BMP) is subsequently increased (Hullin-Matsuda et al. 2014). BMP is resistant to lysosomal phospholipases and predominantly located on the interior membrane (Hullin-Matsuda et al. 2014), and membrane-stabilizing cholesterol is almost completely absent from lysosomal membranes (Schulze et al. 2009). There is some evidence that cathepsins, specifically B and C, regulate LMP, at least with respect to soluble initiators (Brojatsch et al. 2014), but the exact mechanism underlying ENMs initiating this process is not completely understood (Bunderson-Schelvan et al. 2016). Other potential initiators/mediators of ENM-induced LMP are sphingosine kinases, sphingosine, ceramidase, ceramide, LAMP2, sphingomyelin, and sphingomyelinase (Bunderson-Schelvan et al. 2016). For example, excess sphingosine, in the absence of sphingosine kinase, might alter membrane lipids by neutralizing the negative charge on BMP, subsequently displacing membrane proteins resulting in a permeable lysosomal membrane (Schulze et al. 2009). Unfortunately, there is no experimental evidence that high aspect ratio ENMs initiate this process.

There are several possibilities for how ENMs induce LMP, although none have been proven. One possibility is that ENM physically shears, pokes, or tears the lysosomal membrane. This would be particularly applicable to ENMs that possess a spiny or rigid structure such as some MWCNTs (Palomaki et al. 2011). Another possibility is that the ENMs ionize or solubilize once inside the acidic environment, which would be particularly relevant to metal oxides including zinc (Zn), copper (Cu), and nickel (Ni) oxides and Ag ENMs (Bunderson-Schelvan et al. 2016, Hamilton et al. 2014). This may also explain the bioactivity of some MWCNT contaminated with metals such as Ni or iron (Fe) (Hamilton et al. 2012). There is

some evidence that the amount of Ni contamination on MWCNT is positively correlated with the toxicity/bioactivity of the CNT, indicating that dissolution of metal contaminants of MWCNTs in the acidic environment of the lysosome is at least partially responsible for some of the effects (Hamilton et al. 2012). Still another possibility is that ENMs interact directly with the protein/lipid BMP matrix that composes the internal lysosomal membrane. The protein corona, or innate proteins that have adsorbed to the ENM surface (Kharazian et al., 2016), provides a barrier between ENMs and biological systems, likely affecting ENM recognition and uptake. However, the combination of an acidic environment and degradative enzymes within the phagolysosome was reported to strip off the protein corona (Ma et al., 2015), leaving the raw ENMs to come in physical contact with the interior of the phagolysosome (Bunderson-Schelvan et al. 2016). In addition, formation of a protein corona on ENMs demonstrates that the particles interact and bind proteins outside of the lysosome; therefore, it is possible that this may occur inside the protein/lipid-rich organelle, leading to disruption of the lysosomal membrane (Donaldson et al. 2010; Mahon et al. 2012). It is important to understand that the lysosome was not evolutionally developed to deal with ENM-particle processing, and LMP might simply be a way for the cell to create a distress signal that results in acute inflammation and may have evolved in response to inhaled xenobiotic particles.

One logical approach to eliminate ENM-induced LMP may involve manipulation of the cholesterol content of the lysosomal membrane, creating a hyper-stable particle-resistant lysosome as elevated cholesterol content was found to reduce LMP (Appelqvist et al. 2011). This approach has worked with other similar ENMs in the same model. However, the problem with manipulating the lysosome is that it may mimic any of the 51 identified lysosomal storage diseases (LSD) such as Niemann-Pick or Wolman disease and is apparently an unnatural state for the proper operation of the lysosome (Alroy et al. 2014; Alroy and Lyons 2014; Schulz and Sandhoff 2011). Most LSDs result from inadequate or absent enzyme function somewhere along the endosomal/lysosomal pathway. Typically, this occurs from an inherited dysfunction of specific enzymes, but it may also be acquired from certain drugs or plant ingestions (Alroy and Lyons 2014). Patients suffering from adverse health effects attributed to ENM exposure might be responding to conditions of acute stress and inflammation; therefore, a therapeutic intervention for ENM-induced LMP should probably deal with bioactive catalytic enzymes that create or exacerbate inflammation. Of the several cathepsin enzymes released during LMP, cathepsin S is the only one reported to operate at neutral pH (Turk et al. 2012). Cathepsins, in general, are reliable candidates for therapeutic intervention due to their ability to function in a variety of organs and situations and react with a variety of substrates (Reiser et al. 2010). Cathepsin S is currently being studied for its involvement in a number of diseases and inflammatory conditions from cancer to rheumatoid arthritis to chronic pain (Fonovic and Turk 2014). There are numerous cathepsin S inhibitors available and some are already approved for human therapeutic applications (Payne et al. 2014; Petzoldt et al. 2014). Similarly, cathepsin K inhibitors are in various stages of clinical trials (Brömme and Lecaille 2009; Helali et al. 2013).

Cathepsins

Opinions regarding the role of cathepsins within physiological and pathological pathways have dramatically changed since their early description as being primarily responsible for protein turnover. Currently, cathepsins are identified as players in specific biological functions, depending upon type and location. Cathepsins were found to play a role in regulating many key physiological processes, such as generating epitopes for antigen presentation within the immune system (Sadegh-Nasseri and Kim 2015). Cathepsins have garnered a great deal of interest for their apparent role in many disease states, including cardiovascular-related diseases (Platt and Shockey 2015; Zhao et al. 2015), neurodegenerative diseases (Chandra et al. 2015; Bae et al. 2015), cancer (Gomez-Auli et al. 2015; Loser and Pietzsch 2015), and chronic lung disease (Lecaille et al. 2016). Turk et al. (2012) best described cathepsin activity in living organisms as “a delicate balance of expression, targeting, zymogen activation, inhibition by protein inhibitors, and degradation.” Cathepsins B, H, L, C, and X are postulated to be ubiquitously expressed in human tissues as part of normal protein recycling pathways (Turk et al. 2012). Less widely expressed cathepsins, including cathepsin K, W, F, and S are expressed in a more tissue-specific manner—suggesting these proteins serve in a narrower set of cellular functions (Turk et al. 2012).

Cathepsins are small proteins that are primarily monomeric and cleaved into disulfide-linked heavy and light chains during posttranslational modification (Reiser et al. 2010) and that contain unique binding sites. Cathepsins are generally activated in the late endosomes in order to begin proteolytic processing. Once cathepsins become activated, these proteins might be recruited from late endosomes or lysosomes and then secreted into the extracellular space (Reiser et al. 2010). Cathepsins specific to the lysosome or phagolysosome (fused phagosome and lysosome) are D, L, S, C, B, and H (Guha and Padh 2008). Lysosomal cathepsins are activated by low pH (< 5) generated by an ATPase proton-pump mechanism that releases H^+ into the lysosomal organelle (Saftig and Klumperman 2009). Cathepsin S is unique among the lysosomal cathepsins because it is active at neutral and acidic pH and operates outside of the lysosomal structure in the cell cytoplasm or completely independent of the cell (Repnik et al. 2014). Cathepsins are not the only proteolytic enzymes in the lysosome, which also includes glycosidases, lipases, nucleases, phosphatases, and sulfatases. All lysosomal enzymes including cathepsins originate in the rough endoplasmic reticulum and processed through the Golgi apparatus where they receive a terminal mannose-6-phosphate that serves as a recognition marker in the lysosome (Alroy et al. 2014).

Cathepsins are classified according to their active site amino acid as being serine, aspartic, or cysteine cathepsins. The cysteine cathepsin group constitutes the largest family, containing cathepsins B, C, F, H, K, L, O, S, W, V and Z (Reiser et al. 2010). The cysteine cathepsins are synthesized as pre-proenzymes that are directed towards the endoplasmic reticulum where the short N-terminal pre-sequences are cleaved by signal peptidases (Novinec et al. 2014). The proenzyme is then directed to the endolysosomal cell compartments through the mannose-6-phosphate receptor pathway, where the acidic environment cleaves the propeptide region, thereby activating the enzyme. The reducing, acidic environment found in the endolysosomal compartments provides optimal conditions

for maintaining the cysteine cathepsins in their active state. In fact, under conditions of neutral pH, most cathepsins are quickly and irreversibly inactivated (Turk et al. 1995); as previously indicated, one exception is cathepsin S, which is moderately stable even under neutral pH conditions (Wilkinson et al. 2015). Endogenously, inhibitors known as cystatins play a key role in monitoring and controlling cathepsin activity.

Under pathological conditions, the cysteine cathepsins may be secreted extracellularly, where they are known to degrade extracellular proteins in a way that may contribute to tissue injury and disease (Figure 2) (Turk et al. 1995). Degradation of the extracellular matrix following cathepsin activation of matrix-metalloproteinases exerts a destabilizing effect within key signaling pathways and represents a key mechanism by which the cathepsins may contribute to disease development (Christensen and Shastri 2015). The extracellular matrix is a complex network of proteoglycans, collagens, elastin, and other molecules that are highly dynamic and serve as a scaffold to anchor cells, thereby forming tissues and organs. Understandably, the extracellular matrix varies according to its location and is continually being remodeled based upon the physiological needs of the organism or under pathological conditions (Theocharis et al. 2014). The complex relationship between proteases such as cathepsins and functional proteins within the extracellular matrix, such as proteoglycans, has been the focus of a great deal of research (Panwar et al. 2013; Theocharis et al. 2014; Repnik et al. 2015).

Cathepsin, LMP, and inflammation

Several cathepsins have been associated with inflammatory diseases, while links to LMP were correlated with cathepsins B, C, D, and S (Hughes et al. 2016; Jacobson et al. 2013; Hornung et al. 2008). Cathepsin B has been associated with ischemic cell death resulting from LMP in cerebral ischemia/reperfusion injured rats, an effect that is attenuated by CA074-me, a cathepsin B inhibitor that protects against lysosomal rupture (Xu et al. 2016). Cathepsin B and S activities are also elevated in unstable carotid plaques, which contribute to the inflammatory development of atherosclerosis (Abd-Elrahman et al. 2016), and cathepsin B activity is increased in patients with active arthritis (Däbritz et al. 2016). Cathepsin B was also identified as a key regulator of lysosomal biogenesis (Qi et al. 2016). In a model of oxygen-glucose deprivation/reperfusion-induced apoptosis resulting in LMP, cytosolic levels of cathepsin D were significantly raised, resulting in caspase-dependent apoptosis in astrocytes (Liu et al. 2016). Extracellular cathepsin S and intracellular Caspase 1 were suggested to be regulators of the innate immune response, resulting in release of IL-1β. In particular, their proteolytic activities were associated with LMP following particle exposures (Hughes et al. 2016). Cathepsin S was also correlated with autoimmune responses as well as acute and chronic inflammation. Overexpression of cathepsin S was associated with adverse effects on the immune system (Turk et al. 2012) consistent with some ENM exposures (Kononenko et al. 2015). Excess cathepsin S expression and activity was also correlated with several inflammatory conditions such as rheumatoid arthritis, osteoarthritis, atherosclerosis, acute and chronic lung disease, psoriasis, type II diabetes, and certain forms of heart disease (Fonovic and Turk 2014). The absence of this enzyme was noted in cystic fibrosis patients (Wilkinson et al. 2015). Cathepsin S might be released from phagocytic cells during inflammatory conditions, in the presence of lipopolysaccharides (LPS), and in

wound healing (Guha and Padh 2008). Cathepsin S-deficient mice with defective MHC activity improperly process antigens and was accompanied by unusually enlarged endosomal morphology (Guha and Padh 2008). Because of its role in MHC class antigen presentation, inhibition of cathepsin S is thought to be immunosuppressive (Costantino et al. 2009) and potential side effects have been a barrier against anti-cathepsin S drug development. While epidemiological studies have not directly shown a relationship between ENM exposures and many of these cathepsin-associated diseases, ENMs have been linked to adverse pulmonary effects (Morimoto et al. 2013), a potential for cardiovascular disturbances (Meng et al. 2012), and exacerbation of brain pathologies in diabetic rats (Lafuente et al. 2012). Further, there are few studies examining ENM biopersistence over the long term. However, carbon encapsulated FeNP were shown to be present in the lung and liver one year after intravenous administration in mice (Herrmann et al., 2016); while no adverse effects were observed (i.e. inflammation, fibrosis, necrosis, or carcinogenesis). This study demonstrates the potential for ENMs to persist in biological organisms. Further, potential bioaccumulation of ENM in aquatic organisms and environments (Gagne et al, 2013; Wang et al., 2014) as well as contamination of drinking water under certain conditions (Troester et al., 2016) may result in prolonged exposure conditions, which might produce persistent phagolysosomal membrane damage and promote disease.

Cathepsin inhibitors and anti-cathepsin agents

Cathepsin inhibitors represent a promising area of new drug development (Table 1). Endogenously, cathepsin inhibitors are categorized into three distinct families, including the stefins, cystatins, and kininogens, along with several uncategorized proteins with cystatin-like sequences (Ochieng and Chaudhuri 2010). Cystatins are thought to help sequester unwanted cathepsin activity (Ochieng and Chaudhuri 2010) and aberrant regulation of cystatin expression levels may indirectly contribute to cathepsin-associated diseases. Cystatin levels were found to decline when tumors are approaching end-stage or metastatic categories (Ochieng and Chaudhuri 2010). Further, fetuin A, a cystatin-like protein, was shown to stimulate tumor cell growth both *in vitro* and *in vivo* (Kundranda et al. 2005). Cystatin C is upregulated in patients with dementia, serving a neuroprotective role through pathways that are dependent on inhibition of the cysteine cathepsins (Gauthier et al. 2011). High cathepsin S and low cystatin C levels were correlated with the presence of atherosclerosis in human studies, and it was proposed that cystatin C may be employed as a biomarker for this disease (Lv et al. 2012). Experimentally, cystatin C was observed to be protective against neurodegeneration (Gauthier et al. 2011) and it was proposed as a potential anti-cancer agent (Kos et al. 2014).

In addition to the cystatins, morpholinurea-leucine- homopenylalanine-vinylsulfone phenyl (LHVS) is a cathepsin S inhibitor that was shown to exert neuroprotective effects in a murine model of traumatic brain injury (Xu et al. 2013). LHVS has been utilized to impede antigen presentation in a mouse model as a potential therapy for autoimmune disease (Fujii et al. 2012), specifically multiple sclerosis (Allan and Yates 2015). Cathepsin S inhibitors are currently in development for treatment of numerous pathologies, including neuropathic pain, cancer, rheumatoid arthritis, autoimmune disease, and psoriasis (Reiser et al. 2010; Fonovic and Turk 2014).

While anti-cathepsin agent development focused on the discovery of selective substrates and small-molecule inhibitors, this field has benefited from discovery of important regulatory molecules that served as models for subsequent drug design. In addition to the cystatins discussed above, glycosaminoglycans facilitate autocatalysis of the cathepsin proenzyme, and in some cases, modulate that activity. As such, they are known to play a crucial role in the binding between cysteine cathepsins and their protein substrates (Aguda et al. 2014). In particular, negatively charged glycosaminoglycans are known to modulate the activity of cathepsin S (Sage et al. 2013), which was associated with autoimmune diseases (Stoeckle et al. 2012; Baugh et al. 2011), cancer (Zhang et al. 2015b), and atherosclerosis (Figueiredo et al. 2015). As a primary component of the extracellular matrix, the glycosaminoglycans are covalently-linked negatively-charged polysaccharides that are highly variable and able to interact with many of the other components within the matrix as well as growth factors, cytokines, and chemokines (Theocharis et al. 2010). Drugs aimed at modulating the interaction between glycosaminoglycans and their respective proteases, such as the cathepsins, display potential for treatment of many diseases associated with aberrant cathepsin activity, including potential pathologies related to ENM exposures (Figure 2).

Concluding Remarks

In summary, the use of ENMs is rapidly increasing and ENMs have many useful purposes. However, our ability to screen these materials for human health risk and develop regulatory mechanisms for protecting the public has lagged behind their release into the market. These products were found not only in specific commercial products and applications, but increasingly as contaminants in the environment. As such, exposure risks are becoming a significant concern. In addition, studies describing serious pathological outcomes in animal models following exposure to certain types of ENMs suggest that over time there may be human exposure cases resulting in disease. Therefore, preemptive discussions regarding possible treatment options are timely. The most likely mechanism of ENM-induced pathologies may be attributed to phagolysosomal membrane permeabilization. This results in the aberrant release of cathepsins that may contribute to disease development, as well as activation of the NLRP3 inflammasome and an increase in release of inflammatory mediators. There is a significant body of evidence regarding the utilization of anti-cathepsin agents, both in clinical and lab settings. Therefore, there is great potential to capitalize on this information to preemptively prepare for an expected rise in ENM-associated illnesses. Further, there is a great deal of interest in re-purposing drugs that are currently approved by the FDA—potentially saving time and money that would otherwise be required for development of new pharmaceuticals. As such, future research aimed at inhibiting the pathological effects of LMP-associated cathepsin release may provide prevention or treatment strategies that minimize the harmful effects of ENM exposure.

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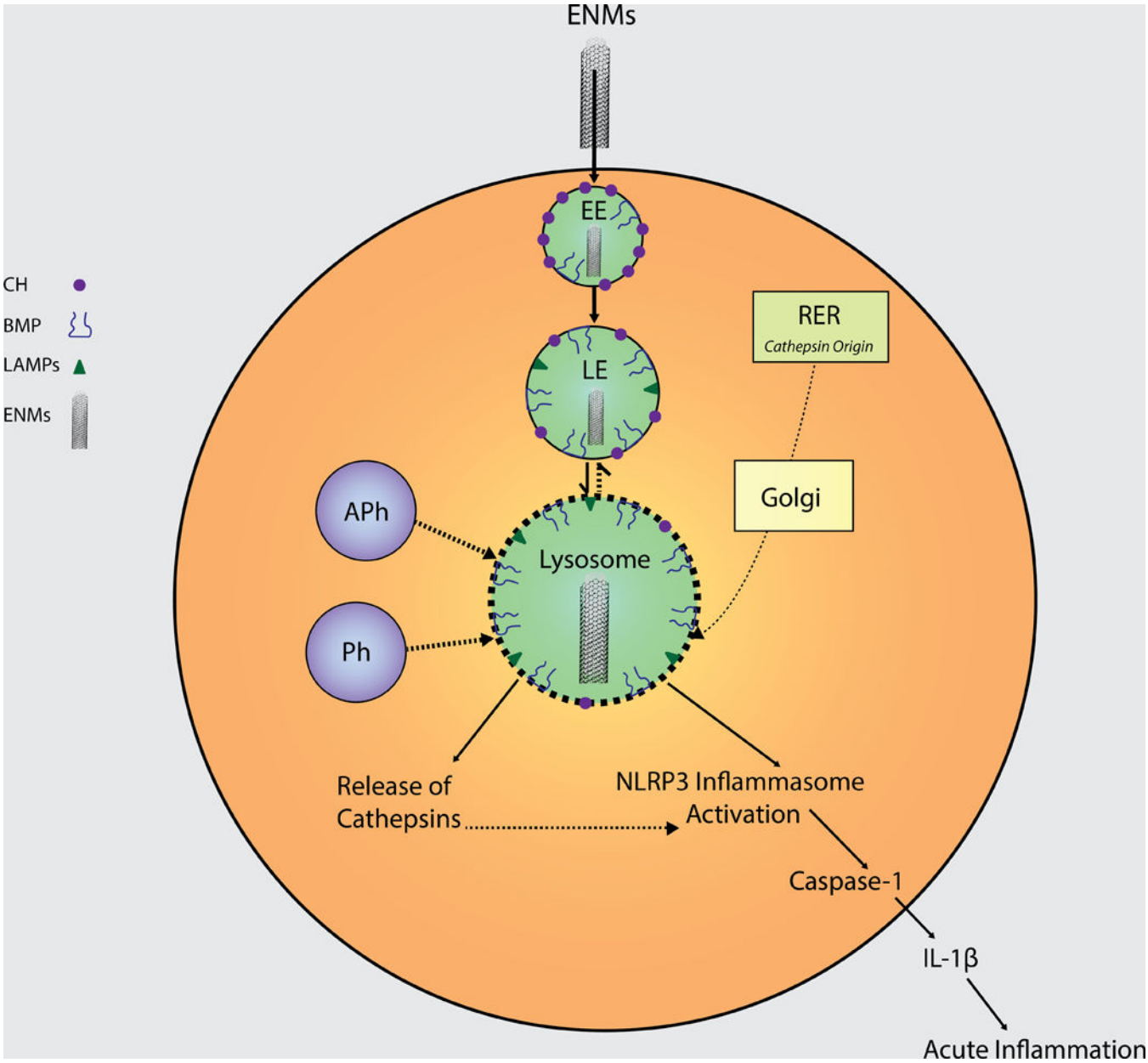

Figure 1.

Exposure to engineered nanomaterials (ENMs, represented here as multi-walled carbon nanotubes) results in their uptake by early endosomes (EE) and subsequent transport to the lysosome via the late endosome (LE) in a potentially iterative process that results in an increase in permeability of the lysosomal membrane. Lysosomes are more susceptible to permeabilization than the LE and EE, attributable to lower membrane cholesterol (CH, purple dot) content and higher bis (monoglycero) phosphate (BMP, lipid symbol) in the lysosome than those of the LE and EE, which have progressively higher amounts of CH in their membranes, respectively. Intra-lysosomal cathepsins originate in the rough endoplasmic reticulum (RER) and are processed by the Golgi apparatus for activation and recognition by the lysosome. The increased permeability of the lysosome causes an aberrant

release of these cathepsins, a process that is modulated by autophagosomes (APh) and phagosomes (Ph). This, in turn, affects many aspects of normal cell physiology, including activation of the NLRP3 inflammasome and the subsequent cascade of acute inflammation that has been associated with various inflammatory pathological conditions. EE, early endosomes; LE, late endosomes; IL-1β, interleukin-1 beta; ENMs, engineered nanomaterials; CH, cholesterol; BMP, bis (monoglycero) phosphate; LAMPS, lysosomal membrane proteins

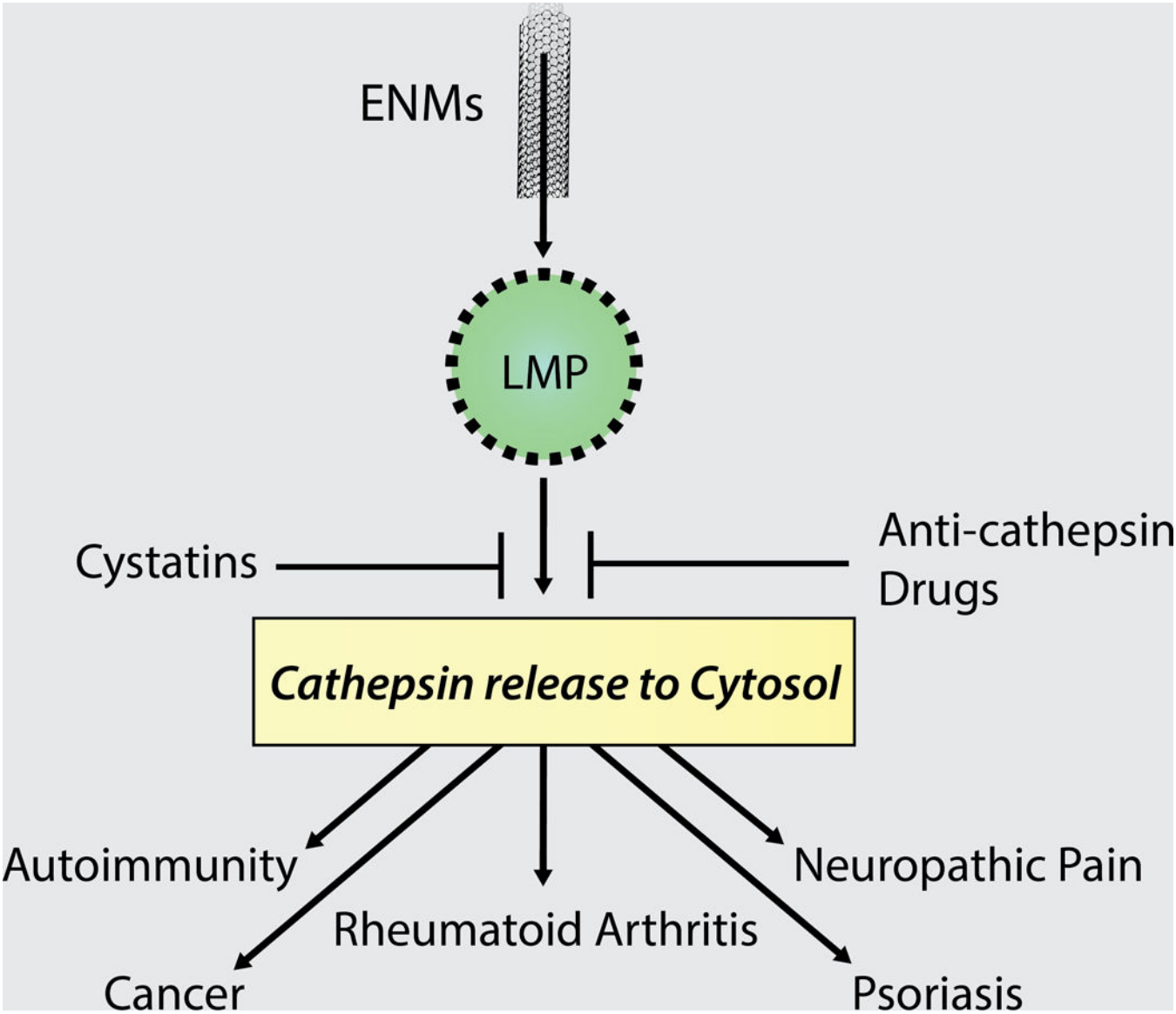

Figure 2.

Many of the intra-lysosomal cathepsins have been directly associated with a variety of inflammation-related diseases (Table 1), and an emerging group of therapeutics/inhibitors have been developed for the study or treatment of cathepsin-related disorders. It is possible, therefore, that anti-cathepsin agents can be designed for the treatment of diseases associated with ENM exposure, since the mechanism of lysosomal membrane permeabilization is similar to other endogenous inflammatory agents (cholesterol crystals, amyloid plaques, uric acid crystals, etc.).

Table 1.

Inhibitors against LMP-related cathepsins and associated diseases

LMP Cathepsins	Active Site Amino Acid	Inhibitor		Associated Diseases/References/Study Details	
		Experimental Reagent	Drug Candidate		
Cathepsin B	Cysteine	Ca-074 (CA-074-Me) E64d	PADK	Cancer Lung Disease, RA, OA Neurodegenerative Diseases	Appelqvist 2013, Review; Gondi 2004, mice injected via tail vein and evaluated for 72 h; Reiser 2010, Review; Sevenich 2010, knockout mice; Tummalapalli 2007, *in vitro*; Vasiljeva 2006, *in vitro*
Cathepsin C (Dipeptidyl Peptidase I)	Cysteine	Semi-carbazides Non-peptidic Cyanamides Dipeptide-derived Nitriles	Semi-carbazides Non-peptidic Cyanamides Dipeptide-derived Nitriles	Immune Disorders Lung Diseases	Laine 2010, Review; Reiser 2010, Review
Cathepsin D	Aspartic Acid	Pepstatin A Hydroxyethyl-amine isosteres	Pepstatin A derivatives Hydroxyethyl-amine isosteres	Cancer Alzheimer's Disease	McConnell 2006, *in vitro*; Yan 1999, *in vitro*
Cathepsin H	Cysteine	Cystatins α2-macroglobulin		Cancer, Lung Diseases	Gocheva 2006, knockout mice and 2010, *in vitro* and *in vivo*; Reiser 2010, Review
Cathepsin K	Cysteine		Odanacatib MIV-711 Balicatib Relicatib ONO-5334 SAR114137	Atherosclerosis, Cancer,Metabolic Syndrome Lung Disease, RA, OA, Osteoporosis	Buhling 2004, mice and humans; Dejica 2008, humans; Duong 2016, Review; Fonovic 2014, Review; Gocheva 2006, knockout mice and 2010, *in vitro* and *in vivo*; Lutgens 2006, knockout mice; Schurigt 2008, transgenic mice; Svelander 2009, daily dose (25 mg/kg) in mice for 14 days; Yang 2008, humans
Cathepsin L	Cysteine	Z-FY-CHO PADK		Atherosclerosis Cancer Metabolic Syndrome Lung Disease Immune Disorders RA, osteoarthritis	Gocheva 2006, knockout mice and 2010 *in vitro* and *in vivo*; Honey 2002, *in vitro and in vivo*; Hsieh 2002, *in vitro*; Huang 2003, diabetic mice; Kitamoto 2007, knockout mice; Maehr 2005, diabetic mice; Nakagawa 1999, cathepsin null mice; Reiser 2010, Review

LMP Cathepsins	Active Site Amino Acid	Inhibitor		Associated Diseases/References/Study Details	
		Experimental Reagent	Drug Candidate		
Cathepsin S	Cysteine	Cystatin C Paecilopeptin LHVS	RWJ-445380 VBY-036 VBY-891 CRA-028129 SAR114137	Atherosclerosis Cancer Metabolic Syndrome, lung disease, OA Immune Disorders Rheumatoid Arthritis Neuropathic Pain Psoriasis Abdominal Aortic Aneurism	Fonovic 2014, Review; Gocheva 2006, knockout mice and 2010, *in vitro* and *in vivo*; Hsieh 2002, *in vitro*; Nakagawa 1999, cathepsin null mice; Payne 2014, humans; Sukhova 2003, LDL deficient mice; Reiser 2010, Review

RA, rheumatoid arthritis; OA, osteoarthritis; LHVS morpholinurea-leucine-homophenylalanine-vinylsulfone-phenyl; PADK, Z-phe-ala-diazomethylketone

Table 2.

Top 11 nanomaterial classes (Committee to Develop a Research Strategy for Environmental, Health, and Safety Aspects of Engineered Nanomaterials, 2012) and effects.

Engineered Nanomaterial	Induces LMP?	Possible Associated Pathologies	Study Model	References
Ceramic Nanoparticles	Possibly, shown to be bioactive	Wear and tear of joint prostheses	Raw264.7 cells Human MΘ	Zhang, 2011 Lucarelli 2004
Carbon Nanotubes	Yes	Apoptosis	Hepatocytes	Zhu 2016
	Yes	Lung Disease	Raw267.7 cells Murine MΘ	Tahara 2012 Jessop 2017 Yang 2014
Nanoporous Materials	Unlikely			Tanaka 2010 Korhonen 2016
Graphene	Possible, lysosomes are increased when used as a drug carrier	Unknown	HepG2 cells	Yang 2016b
Metal Nanoparticles	Possible, when exposed to UV light (WO3/Pt)	Cardiovascular Disease	THP-1 cells	Clark 2016 Xu 2015
	Yes (AgNPs)	Unknown	4T1 breast cancer cells	Jimeno-Romero 2017
Nanoscale Encapsulation	Unlikely			
Fullerenes	Possible, shown to cause mitochondrial damage	Unknown	Isolated mitochondria	Yang 2016a
Dendrimers	Possible, may cause endosomal rupture	Unknown	Numerical simulation	Mukherjee 2013
Nanostructured Metals	Unknown			
Nanowires	Likely	Cell death	Human MΘ	Müller 2010
Quantum Dots	Likely	Reproductive toxicity Pulmonary inflammation	Invertebrate Human lung fibroblasts	Yan 2016 Stan 2015

MΘ, macrophage; LMP, lysosomal membrane permeabilization