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Structure Activity Relationships of Engineered Nanomaterials in inducing NLRP3 Inflammasome Activation and Chronic Lung Fibrosis

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Abstract

It has been demonstrated that certain engineered nanomaterials (ENMs) could induce chronic lung inflammation and fibrosis, however, the key structure activity relationships (SARs) that the link the physicochemical properties and the fibrogenic effects have not been thoroughly reviewed. Recently, significant progress has been made in our understanding of the SAR, and it has been demonstrated that ENMs including rare earth oxides (REOs), graphene and graphene oxides (GO), fumed silica, as well as high aspect ratio materials (such as CNTs and CeO_2 nanowires *etc*.) could trigger the NLRP3 inflammasome activation and IL-1β production in macrophages and subsequent series of profibrogenic cytokines, *i.e.* TGF-β1 and PDGF-AA *in vitro* and *in vivo*, resulting in synergistically cell-cell communication among macrophages, epithelial cells, and fibroblasts in a process named epithelial-mesenchymal transition (EMT) and collagen deposition in the lung as the adverse outcomes. Interestingly, different ENMs engage a range of distinct pathways leading to the NLRP3 inflammasome activation and IL-1β production in macrophages, which include frustrated phagocytosis, physical piercing, plasma membrane perturbation or damage to lysosomes due to high aspect ratio, particle structure, surface reactivity, transformation, *etc*. Furthermore, ENM's properties determine the biopersistence in vivo, which also play a major role in chronic lung fibrosis. Based on these progresses, we reviewed recent findings in the literature on the major SARs leading to chronic lung effects.

Graphical Abstract

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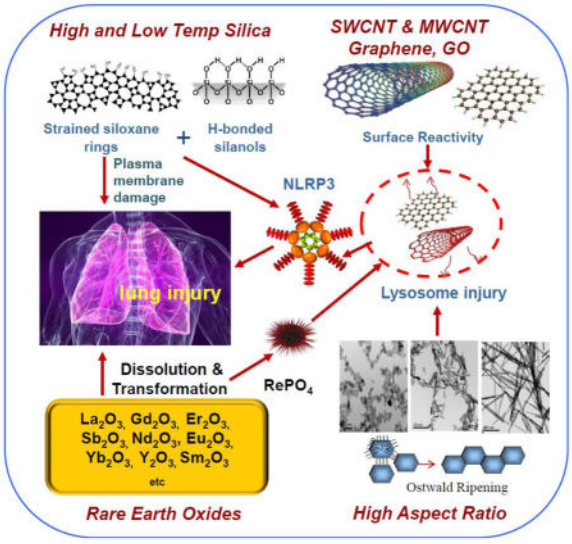

Keywords

Engineered nanomaterial (ENM); NLRP3 inflammasome; lung fibrosis; structure activity relationship (SAR); chronic lung toxicity

Introduction

Engineered nanomaterials (ENMs) with unique physicochemical properties have been used in many commercial products including foods, pharmaceuticals, cosmetics, electronic devices, sunscreens, paints, and other industrial applications.[1–4] However, with the rapid commercialization and increasing production and usage of ENMs, there is an increased exposure potential to human beings and environment, which has generated significant concerns that the ENMs could induce adverse health effects.[5–7] Indeed, many types of ENMs have been shown to be not inherently benign that could induce adverse health effects similar to ambient ultrafine air pollution particles, asbestos, quartz, *etc*. It has been demonstrated that ENMs including metal and metal oxides,[8–13] high aspect ratio materials including single-wall and multiwall carbon nanotubes,[14–17] rare earth metal oxides,[18] fumed silica,[4,19] molybdenum disulfide,[20] graphene and graphene oxide could generate cytotoxicity and pro-inflammatory effects *in vitro* and *in vivo*. Detailed mechanistic studies further revealed that different ENMs could engage a range of distinct pathways leading to the NLRP3 inflammasome activation and IL-1β production,[14] and subsequent series of profibrogenic cytokine production, resulting in synergistically cell-cell communication among macrophages, epithelial cells, and fibroblasts in a process named epithelial-mesenchymal transition (EMT), which lead to chronic collagen deposition in the lung.[14]

In this review, we aim to discuss the chronic lung effects induced by ENMs and we summarized the major ENM physicochemical properties that lead to the NLRP3 inflammasome activation and lung fibrosis. Specifically, we focused on the detailed mechanisms or pathways that lead to the NLRP3 inflammasome activation, which will

provide us strategies for using therapeutic modalities to alleviate the adverse effects. Importantly, it is also possible that through the use of structure-activity relationships (SARs) established and our understanding on the adverse outcome pathways (AOPs), we could use these knowledge for the safer design of engineered nanomaterials (ENMs), which will facilitate the sustainable development of nanotechnology.[21–22]

Chronic lung fibrosis and critical role of the NLRP3 inflammasome activation

Inflammasome is an intracellular multi-protein complex assembled upon various stimuli, which control the activation of caspase-1 and modulate the secretion of cytokines including interleukin-1β (IL-1β) and interleukin-18 (IL-18) in innate immune system.[23–24] The NLRP3 inflammasome is the most studied one in the inflammasome family and it is also the most versatile one that could be induced by various stimuli that are distinct in nature.[25] It has drawn significant attention that NLRP3 inflammasome respond to a wide range of stimuli including dsDNA, RNA, ATP, uric acid, asbestos, α-quartz, and engineered nanomaterials (ENMs),[26–29] and it has been demonstrated that NLRP3 inflammasome activation is associated with many acute and chronic inflammatory diseases,[30] including Alzheimer's disease,[31] gout,[32] type II diabetes,[33] atherosclerosis,[34] and lung fibrosis.[14] Recent studies have suggested that many ENMs could activate the NLRP3 inflammasome, which plays an important role in the generation of chronic granulomatous inflammation and fibrosis in the lung. Mechanistic studies revealed that NLRP3 inflammasome activation induced by ENMs involves frustrated phagocytosis, plasma membrane perturbation and potassium efflux, oxidative stress, lysosomal damage and subsequent cathepsin B release that provides signals for the assembly of the NLRP3 inflammasome.[14,17] IL-1β has been shown to play a major role in triggering the chronic lung effects.[35] (Table 1)

Chronic lung fibrosis or fibrotic reactions in the airways or the lung interstitium constitute a common pathologic outcome following exposure to toxic substances such as inhaled particles, fibers, and metals.[27,36] There is cumulative understanding of the importance of cooperation among epithelial cells, macrophages, and fibroblasts in the pathogenesis of lung fibrosis.[14–15,36] Epithelial injury can lead to EMT, which represents a gradual cellular transformation process in which the epithelial cells acquire reversible mesenchymal features that could culminate in their differentiation to myofibroblasts or fibroblasts.[14–15,36] The release of chemokines by injured epithelial cells attracts macrophages contributing to EMT through the production of IL-1β, TGF-β1, PDGF-AA, and proteases.[14–15,36] Triggering of IL-1β production in macrophages by xenobiotics or ENMs involves the activation of various signaling pathways leading to the assembly of the NLRP3 inflammasome, which is responsible for converting pro-IL-1β to IL-1β. IL-1β acts in synergy with epithelial produced TGF-β1 and PDGF-AA to promote EMT, which culminates in the formation of fibroblast-like cells that deposit collagen in the lung (Figure 1).[14–15,36] In addition, biopersistence of ENMs also plays an important role in determining their chronic profibrogenic potential. Studies have demonstrated that surface properties, *e.g.*, charge, hydrophobicity, affect the retention of ENMs in the lung. Prolonged retention of ENMs in

the lungs can have a significant toxicological effect due to increased production of reactive oxygen species (ROS) and, in turn, increased profibrogenic potential.[37]

Surface reactivity of carbon nanotubes is responsible for lysosome damage and NLRP3 inflammasome activation

One type of engineered nanomaterial that has been widely studied and demonstrated that could induce chronic lung inflammation and lung fibrosis is carbonaceous nanomaterials. Engineered carbonaceous nanomaterials (ECNs) include single-wall carbon nanotubes (SWCNTs), multiwall carbon nanotubes (MWCNTs), graphene, and graphene oxides (GO). ECNs are drawing greater attention because of their widely potential applications in electronics, optics, and drug delivery due to their unique physicochemical properties including high conductivity, tensile strength, surface area, flexibility as well as hydrophilicity and dispersibility in aqueous solutions when surfaces are properly functionalized.[21,38] However, the extensive use of ECNs also raises safety concerns, and literature shows that ECNs are capable of inducing both acute and chronic lung injury. For instance, SWCNTs and MWCNTs generate acute lung injury as well as subchronic granulomatous inflammation and fibrosis in the rodent lung, whether through intratracheal instillation, oropharyngeal aspiration, or aerosolized inhalation.[39–42] Similarly, graphene and GO have also been shown to be capable of inducing pulmonary inflammation and fibrosis.[41,43] One type of CNT is needle-like long aspect ratio tubes that are tube aggregates resembling asbestos fibers, such as the famous Mitsui 7 carbon nanotubes. It's been demonstrated that needle-like CNTs could activate NLRP3 inflammasome in macrophage due to "frustrated phagocytosis" that induce oxidative stress, lysosomal damage, cathepsin B release that activates NLRP3 inflammasome and induce the secretion of IL-1β.[14] However, strictly speaking, these materials should be defined as carbon fibers instead of carbon nanotubes and they comprise only a small part of carbon nanotube family. For typical CNTs, the tubes are thinner and bendable unlike the needle-like nanofibers (Figure 2), moreover they tend to agglomerate or bundle yielding large entangled "bird nest"-like structures in a non-modified bulk material after aerosolization,[44–45] suggesting that the mechanism of "frustrated phagocytosis" would not apply to typical CNTs. In this case, studies have found that tube length, charges, suspension state, and surface catalytic properties are major contributors of CNT-induced NLRP3 inflammasome activation.

To systematically dissect the proportional contribution of each property, Wang and Li *et al.* established a MWCNT library containing as-prepared (AP), purified (PD), and tubes with different functionalization and charge including carboxylated (COOH), polyethylene glycol (PEG), amine (NH_2), sidewall amine (sw-NH_2), and polyetherimide (PEI)-modified MWCNTs. AP- and PD-MWCNTs are hydrophobic with the only major difference in purity that PD have very low transition metal content because of the purification processes involving acid wash.[15,46] COOH-, PEG- are hydrophilic with negative charge, NH_2-MWCNTs and sidewall amine are neutral or weakly cationic due to incomplete neutralization of carboxylate groups in COOH-tubes, while PEI-MWCNTs are highly cationic due to the high amine density of PEI polymer. In general, the NLRP3 inflammasome activation and IL-1β production induced by these nanotubes are in the order

of PEI- ≫ AP > PD > NH_2 or sw-NH_2 ≫ COOH- or PEG-, reflecting their differences in surface charge or coating, purity, and hydrophobicity. Cellular screening in lung epithelial cell (BEAS-2B) and macrophages (THP-1) showed that, compared to AP-MWCNTs, anionic functionalization (COOH- and PEG-) decreased the production of pro-fibrogenic cytokines and growth factors (including IL-1β, TGF-β1, and PDGF-AA), while neutral and weak cationic functionalization (NH_2 and sw-NH_2) showed intermediary effects. In contrast, the strongly cationic PEI-functionalized tubes induced the most robust biological effects. These differences could be attributed to differences in cellular uptake and NLRP3 inflammasome activation induced by MWCNT with different charges. Because particles with strong positive charges are prone to be taken up by macrophages and therefore tend to induce lengthened retention time *in vivo*, they could lead to significant toxicological effects due to enhanced membrane binding and damage, increased cellular uptake, proton sponge effects that induced lysosomal damage, which leads to NLRP3 inflammasome activation, IL-1β production, and inflammation (Figure 2).[31,37,46] Compared to pristine MWCNTs, strong cationic PEI-MWCNTs induced significant IL-1β, TGF-β1 and PDGF-AA production as well as lung fibrosis, while carboxylation significantly decreased the extent of pulmonary fibrosis. These results demonstrate that surface charge plays an important role in the structure-activity relationships that determine the pro-fibrogenic potential of functionalized CNTs in the lung (Figure 2).

CNT dispersion state also plays a major role in lung fibrogenic effects. Pristine MWCNTs are typically hydrophobic because of bare carbon structures, which render them to form large agglomerates in aqueous solutions, impacting their bioavailability, surface reactivity and potential toxic effects.[47] In order for biomedical applications, MWCNTs are often dispersed using dispersants in solution, including bovine serum albumin (BSA), dipalmitoylphosphatidylcholine (DPPC), Pluronic copolymer, *etc*. We demonstrate that the dispersal of as-prepared (AP), purified (PD), and carboxylated (COOH) MWCNTs by BSA and DPPC influences TGF-β1, PDGF-AA, and IL-1β production *in vitro* and *in vivo* (Figure 2).[14,47] The effect of dispersal was most noticeable in AP- and PD-MWCNTs, which are more hydrophobic and unstable in aqueous buffers than hydrophilic COOH-MWCNTs. Well-dispersed AP- and PD-MWCNTs were readily taken up by BEAS-2B, THP-1 cells, and *ex vivo* alveolar macrophages (AM) and induced more prominent TGF-β1 and IL-1β production *in vitro* and IL-1β, TGF-β1, and PDGF-AA production *in vivo* than non-dispersed tubes. Moreover, there was good agreement between the profibrogenic responses *in vitro* and *in vivo* as well as the ability of dispersed tubes to generate granulomatous inflammation and fibrosis in airways of the lungs.[14,47] Taken together, these results indicate that the dispersal state of MWCNTs affects profibrogenic cellular responses that correlate with the extent of pulmonary fibrosis and are of potential use to predict pulmonary toxicity. Using similar approach, it is also possible to rule out the CNT properties that independently contribute to chronic effects. Recently, it was found that the electronic properties and chirality of single-walled carbon nanotubes (SWCNTs), semiconducting (S-SWCNT) or metallic (M-SWCNT), do not show differences in impacting NLRP3 inflammasome activation. They trigger similar amounts of IL-1β and TGF-β1 production in THP-1 and BEAS-2B cells. Oropharyngeal aspiration confirmed that both SWCNT variants induce

comparable fibrotic effects in the lung. These results are significant to the safety assessment of carbon nanotubes by industry.[48]

Although we know CNTs could induce lysosomal damage after cellular uptake and frustrated phagocytosis is unlikely the major pathway, the detailed mechanism on the physicochemical property that is responsible for the lysosomal membrane damage is not entirely clear at this moment. There are several possibilities, however. First, the pristine bare carbon surface of CNT materials (also graphene and GO) is typically hydrophobic, which has been suggested that they are able to extract large amounts of phospholipids from the cell membranes because of the strong dispersion interactions between carbon structure and lipid molecules.[49] In addition, surface defects and functional groups on CNTs and graphene oxides could induce abiotic ROS generation. These all could contribute to the membrane damage induced by these carbonaceous materials. The surface reactivity of CNT and graphene being the reason behind their ability to induce lysosomal damage is supported by the finding that the profibrogenic effect of CNTs could be inhibited by a tri-block copolymer (Pluronic F108 (PF108)) coating on CNTs.[14,41,50] PF108 is composed by two hydrophilic poly(ethylene oxide) (PEO) chains and an interspersed hydrophobic poly(propylene oxide) (PPO) domain. Pluronic coating not only confers excellent MWCNT dispersion but also reduces the pro-fibrogenic effects of these tubes *in vitro* and in intact animal lungs *in vivo*. The mechanism of this effect appears to be prevention of lysosomal damage in macrophages as well as possibly other cell types. PF108 could passivate the tube surface by forming a protective “brush-like” layer that provides steric hindrance and interferes in tube aggregation as well as damaging interactions with the lysosomal membrane. In contrast, tube dispersal by BSA plus DPPC does not protect the lysosome, likely because of the instability of this coating in the acidified lysosomal environment that may come off the CNT surface and be degraded in lysosomes, which allows the interaction between reactive CNTs surface and lysosomal membrane. While we do not know the exact molecular mechanism by which the tube surface could induce lysosomal membrane damage, it was possible to demonstrate that BSA-coated tubes lead to cathepsin B release, NLRP3 inflammasome activation and IL-1β production in phagocytic cells. In contrast, the PF108 coating appears to be more resistant to removal from the surface under acidic conditions.[14,41] Further studies are needed to probe the interactions between carbon surface structure and membranes that lead to membrane damage. Nonetheless, these results suggest that PF108 coating could be used as a safe design approach for not only MWCNTs, but also other ECNs when considering their possible use for biomedical applications, such as drug delivery and imaging.

Similar to MWCNTs, other ECNs including SWCNTs and graphene have also been shown to follow the same SAR. Wang *et al.* established an ECN library comprising three different types of SWCNTs (8 tubes by three synthesis methods), graphene, and graphene oxide (two sizes) for comparative analysis according to the NLRP3 inflammasome pathway that plays a role in the pathogenesis of pulmonary fibrosis.[41] SWCNTs synthesized by Hipco, arc discharge and Co-Mo catalyst (CoMoCAT) methods were obtained in their as-prepared (AP) state, following which they were further purified (PD) or coated with Pluronic F108 (PF108) or bovine serum albumin (BSA) to improve dispersal and colloidal stability. GO was prepared as two lateral sizes, GO-small (S) and GO-large (L), while the pristine graphene (not GO) samples were coated with BSA and PF108 to enable dispersion in aqueous

solution.[41] *In vitro* screening showed that AP- and PD-SWCNTs, irrespective of the method of synthesis, as well as graphene (BSA) and GO (S and L) could trigger IL-1β and TGF-β1 production in macrophage (THP-1) and epithelial (BEAS-2B) cell lines, respectively. These data suggest that the common carbon structure of SWCNTs is the determining factor in triggering the inflammasome pathways, not the composition and content of metal impurities. Oropharyngeal aspiration in mice confirmed that AP-Hipco tubes, graphene (BSA-dispersed), GO-S and GO-L could induce IL-1β and TGF-β1 production in the lung in parallel with lung fibrosis. Notably, GO-L was the most pro-fibrogenic material among these ECNs. In contrast, PF108-dispersed SWCNTs and pristine graphene failed to exert fibrogenic effects.[41]

The differences between small and large GO are likely due to their differences in membrane adsorption and cellular uptake. Ma *et al.* showed that GO-L had stronger adsorption onto the plasma membrane with less phagocytosis, likely due to the unfavorable uptake kinetics for GO with large lateral size.[51] One outcome of plasma membrane adsorption is that it allows more robust GO interaction with toll-like receptors (e.g., TLR4), which activates NF-κB pathways, and possibly more NLRP3 and pro-IL-1β protein expression and together with GO induced lysosomal damage after cellular uptake result in stronger NLRP3 inflammasome activation. Consequently, larger GO enhanced production of inflammatory cytokines and recruitment of immune cells. In contrast, GOS sheets were more likely taken up by cells, which lacks TLR4 activation and limit its capability to induce NLRP3 inflammasome activation. It was also demonstrated that the reactive surface, rather than metal impurities or other factors of the SWCNTs, graphene and GO, is the primary physicochemical characteristic in this mechanistic paradigm. The space constraints in the lysosome allow close contact of the ECNs with the organelle membrane, allowing the reactive surface and oxygen radical generation induced by NADPH oxidase to deliver an injurious impact to the lysosome membrane.[16] This notion is further supported by the finding that PF108 coating prevents the damaging effects of SWCNT and graphene surfaces in the lysosome, leading to reduced IL-1β production, which is also true in the scenario of MWCNTs exposure as described above.[14,41,43,50] Collectively, these data indicate that lateral size of GO and the dispersal state and surface reactivity of ECNs play key roles in triggering the NLRP3 inflammasome pathway, which could prove helpful for hazard ranking and a proposed tiered testing approach for large ECN categories (Figure 2).

Chronic lung fibrosis is a complex pathological condition that may involve other pathways in addition to the NLRP3 inflammasome pathway. Previous studies demonstrated that MWCNTs could directly stimulate fibroblast proliferation and collagen deposition.[47,52] It is also found that CNTs could trigger TGF-β/Smad pathway in a NLRP3 inflammasome independent manner.[53–54] This suggests that additional pathways may also be involved in the fibrogenic process by engineered nanomaterials, thus further studies are needed to understand these processes mechanistically.

High aspect ratio determines the nanowire induced NLRP3 inflammasome activation

High aspect ratio ENMs, such as nanorods and nanowires, have attracted great attention due to their unique chemical, mechanical, electric, and optical properties and their applications in nanodevices. It is known that high aspect ratio materials including asbestos, metabolic products such as fibrillar peptide amyloid-β and monosodium urate (MSU) crystals could activate NLRP3 inflammasome.[27] Similarly, recent studies have suggested that high aspect ratio ENMs could also activate the NLRP3 inflammasome, which plays an important role in the generation of chronic granulomatous inflammation and fibrosis in murine lung.[25,27,55] Although various high aspect ratio materials (asbestos, needle-like carbon fibers) have been shown to induce NLRP3 inflammasome activation, it is not clear what critical lengths and aspect ratios are needed and how these materials induce this effect.[27] To answer these questions, Ji *et al.* synthesized a series of cerium oxide (CeO_2) nanorods and nanowires with precisely controlled lengths and aspect ratios.[56–57] This study is environmentally relevant because the commercial cerium oxides contain not only spherical nanoparticles, but also nanorods, thus results of numerous studies on cerium oxide toxicity in the literature require cautious interpretation because aspect ratio obviously play an important role in toxicity. From a material point of view, it is reasonable to visualize particles with different aspect ratios in commercial products because phosphate and chloride ions that are often present in the raw materials used for ceria synthesis also play a major role in the synthesis of high aspect ratio nanostructures. In a study by Ji *et al*, high-resolution TEM analysis shows that single-crystalline CeO_2 nanorods/nanowires were formed along the direction by an “oriented attachment” mechanism, followed by Ostwald ripening. By controlling the phosphate and chloride content, Ji *et al.* successfully created a comprehensive CeO_2 nanorod/nanowire combinatorial library, which allows, for the first time, the systematic study of the effect of aspect ratio on lysosomal damage, cytotoxicity, and IL-1β production by the macrophages (THP-1). This *in vitro* toxicity study demonstrated that, at lengths ≥200 nm and aspect ratios ≥22, the CeO_2 nanorods could progressively induce more lysosomal damage, cathepsin B release, NLRP3 inflammasome activation and IL-1β production than spheres in THP-1 cells (Figure 2). SEM analysis showed that at the critical length of 200 nm, CeO_2 nanorods formed micrometer-sized stacking bundles, which could pierce the cell membrane and result in the failure of phagocytosis in the macrophages, a feature known as “frustrated phagocytosis”. The stacking bundles were also capable of piercing lysosomes (1.1–2.9 μm in diameter) that leads to lysosome damage and following cathepsin B release, which induce NLRP3 inflammasome activation and IL-1β production. The high aspect ratio CeO_2 is also relevant to chronic lung toxicity *in vivo* in comparative studies. Although oropharyngeal aspiration could induce acute lung inflammation for CeO_2 nanospheres and nanorods, only the nanorods with the highest AR induced significant IL-1β and TGF-β1 production in the bronchoalveolar lavage fluid at 21 days but did not induce pulmonary fibrosis. However, after a longer duration (44 days) exposure to 4 mg/kg of the nanorods, more collagen production was seen with CeO_2 nanorods *vs.* nanospheres after correcting for Ce lung burden. In addition to CeO_2 studies, a separate study by Hamilton *et al.* also demonstrated that the high aspect ratio titanium dioxide nanobelts (60–300 nm in diameter and 15–30 μm in length) induced inflammasome activation and the release of inflammatory cytokines

through lysosomal damage and cathepsin B release, while the spherical (60–200 nm in diameter) and short nanobelts (60–300 nm in diameter and 0.8–4 μm in length) did not. These results suggest that both length and diameter components of aspect ratio should be considered when addressing the chronic pulmonary effects of high aspect ratio materials.

The role of biotransformation in rare earth oxide induced NLRP3 inflammasome activation

Rare earth oxides (REOs) are increasingly used in products such as catalysis, electronics, fuel additives (*e.g.*, CeO_2) as well as biosensors, luminescence probes, *etc*. Growing global application of rare earth oxides (REOs) for commercial and biological use has increased the possibility of human exposure and adverse health effects. Human exposure to rare earth has shown to induce not only pneumoconiosis (lung fibrosis) in polishers and RE mining workers, but also systemic fibrosis in patients with renal impairment, a.k.a., nephrogenic systemic fibrosis (NSF) after administration of Gd-based MRI agents. Understanding the mechanisms for inducing these adverse pro-fibrogenic effects is important for the safety assessment of REO particles as well as opportunities for safer design. In a study by Li *et al*, a well-prepared library of 10 commercial REOs was prepared and a mechanistic understanding was obtained on how REOs induce cellular and pulmonary damage. A distinct mechanism of toxicity emerged, which involves compartmentalized intracellular biotransformation process in lysosomes that results in NLRP3 inflammasome activation, pro-fibrogenic growth factor production and lung fibrosis. It was found that REOs are unstable in acidic physiological environment and macrophage uptake and lysosomal processing of REO nanoparticles lead to enhanced, pH-dependent particle dissolution. The released RE ions (III) are rapidly bound by bystander phosphates resulting in the crystallization of $REPO_4$ deposits on the particle surfaces. This results in nanoparticle biotransformation into urchin shaped structures for light RE and mesh-like structures for heavy RE. Once the free phosphates in lysosomal fluid are depleted, the released RE ions are capable of stripping phosphate groups from the surrounding lipid bilayer on lysosomal membrane, leading to organelle damage, cathepsin B release, NLRP3 inflammasome activation and IL-1β release from the macrophages. IL-1β then participates in a progressive march of events that include the production of TGF-β1 and PDGF-AA by epithelial cells, ultimately culminating in lung fibrosis (Figure 3). These findings could explain the development of RE-induced pneumoconiosis in occupational settings and may also be involved in the pathogenesis of NSF by Gd-containing MRI contrast agents. Taking this SAR into account, namely the high binding affinity between RE and phosphates, Li *et al*. demonstrated that prior phosphate coating at a neutral pH constitutes a simple, yet effective way to decrease the hazard potential of REOs. In addition, a more effective coating by ethylenediamine tetra(methylenephosphonic acid) (EDTMP) surface coating provides the most stable REOs and rare earth based upconversion nanoparticles (UCNPs) for imaging purposes, which not only maintain their imaging intensity, but also do not induce pro-inflammatory/fibrogenic effects *in vitro* and *in vivo*. The mechanism of EDTMP protection of RE involves organophosphate ligand coordination to the lanthanide atoms in a hexadentate fashion, involving two nitrogen and four oxygen atoms. The tetrahedral phosphonic groups in this complex limit the free space around the central RE atoms, which

are shielded from interacting with ligands such as water molecules, thus preventing dissolution of RE materials. Interestingly, EDTMP can also effectively chelate divalent metal ions, including Co(II), Fe(II), Zn(II), and Cu(II). It is possible, therefore, this phosphonate moiety could also be used effectively to coat the surfaces of transition metal nanoparticles, including highly soluble materials such as ZnO and CuO. In summary, the EDTMP coating can be used as a safer design principle for producing metal/metal oxide nanoparticles for biological use.

Surface silanol density determines fumed silica induced plasma membrane perturbation and NLRP3 inflammasome activation

Fumed silica production ranks the third among largest industrial aerosol commodity by value and the fourth largest by volume.[58] Fumed silica has been generally recognized as safe (GRAS) for use in food products by the Food and Drug Administration (FDA). However, emerging evidence from experimental studies suggests that fumed silica exhibit hazardous potentials due to its siloxane ring structure, high silanol density, and “string-of-pearl-like” aggregate structure, which could combine to cause membrane disruption, generation of reactive oxygen species and pro-inflammatory effects in macrophage and epithelial cells[19,59–60] as well as acute inflammatory responses in animal lungs after inhalation[4] and incidence of liver fibrosis after long term exposure by oral intake.[61]

Mechanistic study by Zhang, *et al.* demonstrated that the membrane lytic potential and cytotoxicity of fumed silica could be attributed to specific surface properties as a result of the reconstruction of strained three-membered rings (3MRs) and surface display of silanols groups.[19] The density of surface silanol groups (≡Si-OH), which are partially deprotonated at physiological pH to form ≡Si-O$^-$, determines the magnitude of electrostatic interaction between the fumed silica surface and plasma membrane phospholipids.[19,62–63] Silanol groups on fumed silica surface serve as hydrogen donors with quaternary and phosphate ester groups of phospholipid membrane components or to lesser extent secondary amide groups of proteins. Moreover, anionic deprotonated silanols interact electrostatically with positively charged tetra-alkylammonium-containing phospholipids. This could lead to disruption of plasma membrane integrity, resulting in hemolysis of red blood cells.[19,64–65] In addition, it is suggested that ring strain (as opposed to or in addition to mechanical grinding) results in preferential hemolytic cleavage of three-membered rings to form surface radicals that can further react with water, oxygen, or hydrogen peroxide to generate hydroxyl radicals that could further lead to activation of a cascade of downstream signaling pathways,[19] which includes the NLRP3 inflammasome.[19,66–67]

Compared to other ENMs (rare earth oxides,[18] graphene[41] and high aspect ratio materials such as carbon nanotubes,[25] CeO_2,[37] AlOOH[20] and Ag nanowires[16]), fumed silica exhibits a distinct pathway leading to NLRP3 inflammasome activation. Rather than originating from the cellular interior (lysosomes), fumed silica induced inflammasome activation is plasma membrane perturbation-mediated (detailed above). Fumed silica interacts strongly with the plasma membrane, without significant cellular uptake due to its large chain-like structure.[4] As a results, fumed silica nanoparticles were mostly found to be associated with the external

cell surface membrane, where the particles stimulated filopodia formation and membrane ruffling, and surface silanol groups (≡Si–OH) selectively promote interactions with cell membranes.[19] The strength of the binding interaction of the fumed silica surface with the cell membrane leads to plasma membrane perturbation and functional changes, including K^+ efflux, which is mechanistically linked to the NLRP3 inflammasome activation (Figure 4).[25,68–69] In support of this structure-activity relationship (SAR), it has been demonstrated that by reducing the surface silanol density through calcination and metal doping, fumed silica nanoparticles exhibit reduced membrane perturbation, potassium efflux, NLRP3 inflammasome activation and cytotoxicity in macrophages and bone marrow-derived macrophages (BMDMs). The decrease in inflammatory potential is also confirmed in reduced acute lung inflammation induced by Al-/Ti-doped fumed silica.[4] These SAR results demonstrate the possibility of a safer design approach for future commercial use of fumed silica (Figure 4).[4]

Recently, Sun *et al.* also demonstrated that fumed silica exhibits sub-chronic injury potential in the lung during repetitive dosing.[70] Single bolus dose instillation of 21 mg/kg fumed silica did not induce sustained IL-1β production or sub-chronic pulmonary effects, due to the comparatively high rate of dissolution and clearance of fumed silica from the lung. In contrast, repetitive dosing could lead to biopersistence with a change in lung toxicity, and the NLRP3 inflammasome pathway was continuously activated by repetitive dose administration of 3 × 7 mg/kg fumed silica, one week apart. Repetitive dosing can trigger increased collagen production, even at 3 × 3 mg/kg. The unique physicochemical properties of fumed silica, *e.g.*, its chain-like morphology, high density display of reactive silanols, and ability to generate ROS, appear to be equally important for the sub-chronic as for the acute hazard potential in the lung. This is confirmed by the reduction in collagen deposition in the lung if titanium-doped fumed silica particles are used, which both reduce the silanol density and the potential to generate ROS.[4]

ENMs could affect autophagy that regulate activated NLRP3 inflammasome complexes

While all of the above mentioned nanomaterials are capable of inducing the assembly of the NLRP3 inflammasome complex, there are material-specific differences in precisely how the lysosomal membrane is damaged by the composition, shape/aspect ratio, redox potential and surface reactivity of the materials. Although the activation of the NLRP3 inflammasome by selected ENMs is of considerable importance in terms of material toxicity, it is also important to consider that ENMs could exert an effect on the abundance and turnover of the activated inflammasome complexes through the autophagy pathway. It is known that a variety of ENMs (such as REOs, CNTs, and quantum dots) are inducers of autophagy, which plays a role in mopping up and delivery of ubiquitinated protein complexes and organelles (e.g., mitochondria) to the lysosome for clearance. This could be one of the major connections of ENMs with the autophagy pathway in that autophagy speeds up homeostatic removal of activated inflammasome complexes, with the implication that interference in autophagic flux could lead to exaggerated IL-1β production (Figure 5). Not only could this information be important from the perspective of the toxicological effects of ENMs, but it is

well known that autophagy deregulation is involved in a variety of human diseases such as cancer, Parkinson's disease, diabetes, *etc*.

In order to clarify whether there are differences in the effect of different ENMs on the autophagy pathway and the implications of those differences in terms of their pro-inflammatory effects, Li *et al*. prepared two important classes of ENMs, MWCNTs and REOs, both of which could act as NLRP3 inflammasome inducers and therefore establish a background against which the similarities or differences of the homeostatic regulation of IL-1β production could be assessed. Although exhibiting quite different sizes, shapes and surface functionalities, MWCNTs and REO nanoparticles are potent inducers of NLRP3 inflammasome assembly, which is homeostatically regulated by autophagic flux. However, while MWCNTs induced NLRP3 assembly and IL-1β production as a result of tube-specific injury mechanisms (surface reactivity) to the lysosome, homeostatic regulation by autophagy remains intact and is capable of swiftly removing activated NLRP3 complexes. Thus, not only did MWCNTs fail to induce the accumulation of LC3-labeled autophagosomes, but fusion with the lysosome could be enhanced by the autophagy inducer, Rapamycin. In contrast, the REO-specific mechanism of lysosomal injury (biotransformation and phosphate stripping from lipid bilayers), damages this organelle to the extent that it disrupts autophagosome fusion and removal of the activated NLRP3 complexes; this leads to exaggerated IL-β production. Under these conditions, Rapamycin had a comparatively minor effect because it could not speed up removal by the damaged lysosomes. Quartz also induced IL-1β production that was subject to Rapamycin down regulation, while other transition metal oxides, COOH-MWCNTs and mesoporous silica failed to affect inflammasome activation. In addition, Li *et al*. demonstrate another unique mechanism of lysosome damage by rare earth oxides such as La_2O_3, which results in interference in lysosome acidification and disruption of phosphoproteins by stripping phosphate groups from lysosomal enzymes. These data show that the autophagy pathway is important as a homeostatic mechanism for removal of NLRP3 complexes induced by ENMs, with the potential to impact the severity of lung inflammation.

Conclusions

In this review, we summarized major SARs for ENMs including carbon nanotubes, CeO_2 nanowires, graphene and graphene oxide, rare earth oxides, and fumed silica. The properties involved include aspect ratio, surface reactivity, biotransformation, and ability to catalyze ROS generation, *etc*. The interactions at the nano-bio interface determine the plasma membrane perturbation or lysosomal damage and a series of subsequent signaling transductions that lead to NLRP3 inflammasome activation and IL-1β production, which is tightly controlled by autophagic flux, a main homeostatic intracellular mechanism that regulate the pro-inflammatory responses. These SARs provide opportunities to mitigate the toxicological responses induced by ENMs by signaling pathway inhibition or safer design by modifying the major toxic properties of ENMs. We need to mention that there are other ENM-induced pathways that could also lead to chronic lung effects, so more research is needed to elucidate additional SARs for ENMs, which will facilitate the sustainable development and application of nanotechnology that will benefit mankind.

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Highlights

It has been demonstrated that certain engineered nanomaterials (ENMs) could induce chronic lung inflammation and fibrosis, however, the key structure activity relationships (SARs) that the link the physicochemical properties and the fibrogenic effects have not been thoroughly reviewed. Recently, significant progress has been made in our understanding of the SAR, and it has been demonstrated that ENMs including rare earth oxides (REOs), graphene and graphene oxides (GO), fumed silica, as well as high aspect ratio materials (such as CNTs and CeO_2 nanowires etc.) could trigger the NLRP3 inflammasome activation and IL-1β production in macrophages and subsequent series of profibrogenic cytokines, i.e. TGF-β1 and PDGF-AA in vitro and in vivo, resulting in synergistically cell-cell communication among macrophages, epithelial cells, and fibroblasts in a process named epithelial-mesenchymal transition (EMT) and collagen deposition in the lung as the adverse outcomes. Interestingly, different ENMs engage a range of distinct pathways leading to the NLRP3 inflammasome activation and IL-1β production in macrophages, which include frustrated phagocytosis, physical piercing, plasma membrane perturbation or damage to lysosomes due to high aspect ratio, particle structure, surface reactivity, transformation, etc. Furthermore, ENM's properties determine the biopersistence in vivo, which also play a major role in chronic lung fibrosis. Based on these progresses, we reviewed recent findings in the literature on the major SARs leading to chronic lung effects.

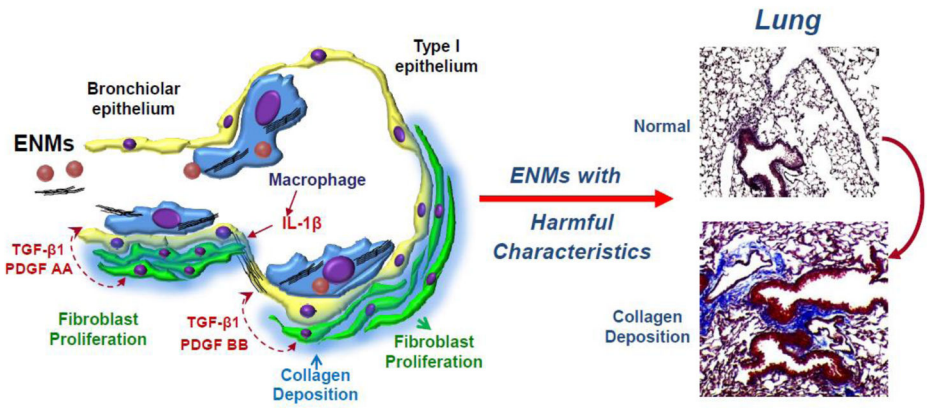

Figure 1. Schematic to explain the fibrogenic effects induced by engineered nanomaterials

Chronic lung fibrotic reactions in the airways or the lung interstitium constitute a common pathologic pathway following exposure to ENMs including high aspect ratio and rare earth nanomaterials. Nanomaterials could induce lysosomal damage and cathepsin B release after cellular uptake, which triggers NLRP3 inflammasome activation and IL-1β production by macrophages. IL-1β acts in synergy with TGF-β1 and PDGF that are produced by lung epithelial cells to promote epithelial–mesenchymal transition (EMT), which results in deposition of collagen in the lung.

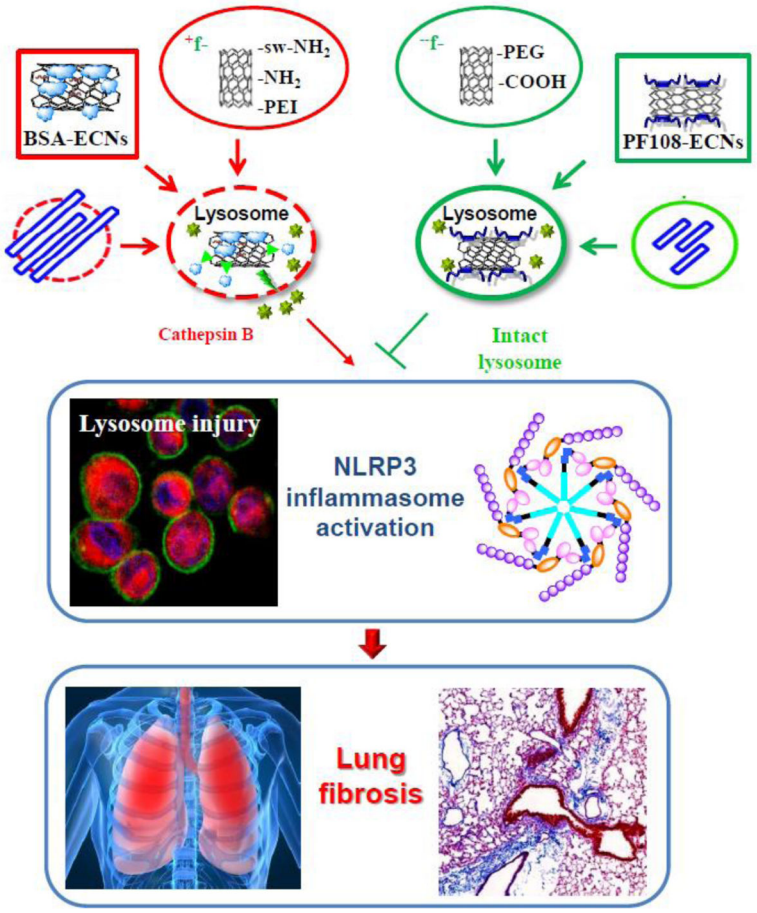

Figure 2. Major mechanisms of NLRP3 inflammasome activation by carbonaceous nanomaterials (ECNs) and long aspect ratio (LAR) nanoparticles

ECNs and LAR nanoparticles are capable of inducing chronic lung injury via NLRP3 inflammasome activation. For typical ECNs, including SWCNT, MWCNT, graphene and graphene oxide *etc.*, the surface charges, suspension state, and surface catalytic properties or surface reactivity are major contributors of CNT-induced lysosomal damage and NLRP3 inflammasome activation in macrophages. Pluronic F108 (PF108), coating not only confers excellent dispersion but also reduces the pro-fibrogenic effects of these tubes *in vitro* and in intact animal lungs via prevention of lysosomal damage in macrophages.

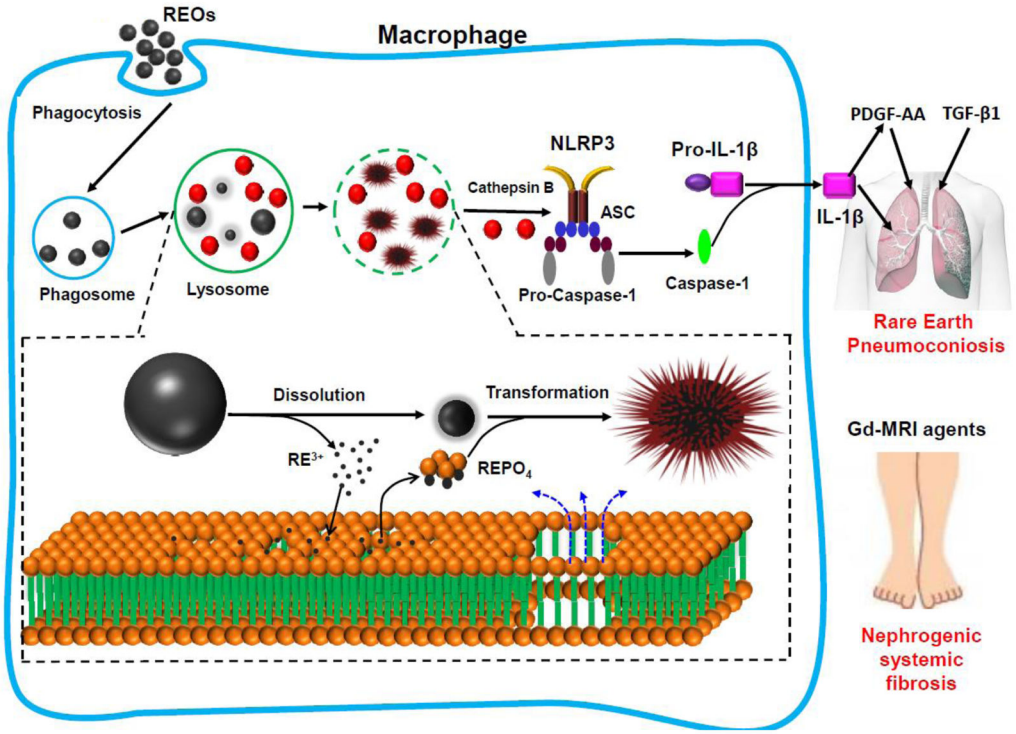

Figure 3. Schematic to explain the effects of REOs on autophagy and NLRP3 inflammasome regulation

After internalization into lysosomes, REOs dissolve in the acidic environment and the dissolved ions bind with phosphate to transform from spheres to sea urchin structure. This biotransformation leads to stripping of phosphate from lipid bilayers on lysosomal membrane, resulting in lysosomal damage and cathepsin B release, which lead to the assembly of the NLRP3 inflammasome and IL-1β production. IL-1β acts in synergy with TGF-β1 and PDGA to induce lung fibrosis. Similar mechanism is involved in the nephrogenic systemic fibrosis induced by Gd containing MRI reagent.

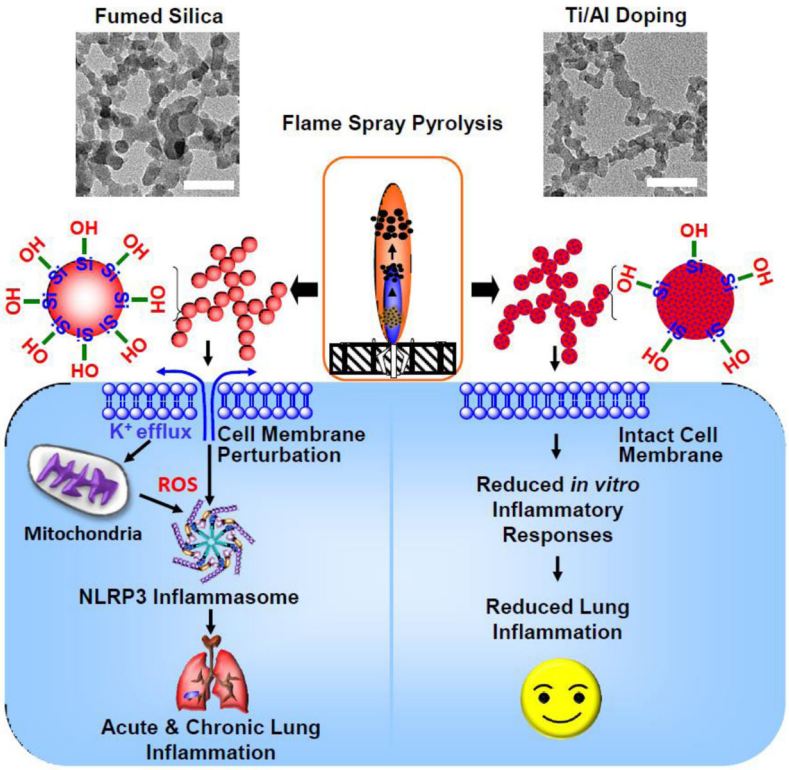

Figure 4. Reduction of acute inflammatory effects of fumed silica nanoparticles in the lung by adjusting silanol display through metal doping

Different from CNTs, GO, and REOs, the presence of silanols on fumed silica and its chain-like structures could enhance plasma membrane perturbation without cellular uptake and the generation of potassium efflux that leads to the assembly of the NLRP3 inflammasome. Metal doping including Ti and Al could reduce surface silanol density, leading to dose-dependent reduction in hydroxyl radical generation, membrane perturbation, potassium efflux, NLRP3 inflammasome activation and cytotoxicity *in vitro*. Similarly, Ti- and Al-doping, ameliorated acute and chronic pulmonary inflammation *in vivo* induced by fumed silica.

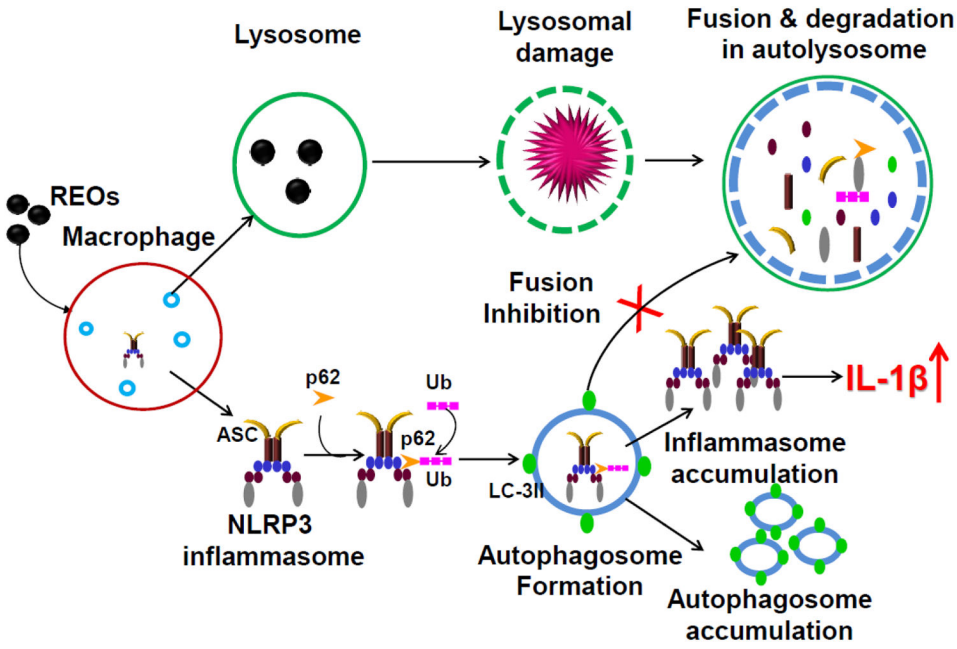

Figure 5. Mechanism of REOs on autophagy and NLRP3 inflammasome regulation

Phagocytosed REOs transform to RE-phosphate with sea urchin structure, which leads to lysosomal dysfunction and inhibition of autophagosome–lysosome fusion, resulting in accumulation of autophagosomes and the failure to degrade the activated NLRP3 inflammasome complexes. Loss of NLRP3 inflammasome homeostasis leads to exaggerated IL-1β production that could induce adverse health effects including lung fibrosis.

Table 1

ENM physicochemical properties and NLRP3 inflammasome activation pathways in the lung

ENMs	Physicochemical Properties	NLRP3 inflammasome activation pathways
CNTs	Dispersion, charge, surface functionalization	Lysosomal damage, cathepsin B release[21,56–57,60,62–63]
GO	Size, surface functional groups	TLR4 activation, Lysosomal damage, cathepsin B release, ROS generation[51,58,71]
Fumed silica	Surface silanol, Three-membered ring	Plasma membrane perturbation, potassium efflux, ROS generation[19,48]
REOs	Dissolution, phosphate binding, transformation	Lysosomal damage, cathepsin B release[18]