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REVIEW

Earthworm's immunity in the nanomaterial world: new room, future challenges

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Abstract

Since the advent of the nanotechnology era, the environmental sink has been continuously receiving engineered nanomaterials as well as their derivatives. Our current understanding of the potential impact of nanomaterials on invertebrate immunity is limited to only a handful of initial studies including those on earthworms. Recently, we reported selective accumulation of silver nanoparticles in the amoebocyte population of *Eisenia fetida* coelomocytes *in vitro*. In this review, we give an overview of available literature on the life-history impacts on earthworms, and what we have learnt of the immune responses to nanoparticles with references to other invertebrate species and vertebrate counterparts. We discuss the significant contribution of amoebocytes as nanoparticle scavengers and suggest a possibility of studying inter-cellular communications in coelomocytes. Implications from the leading researches in vertebrate models tell us that study of the nanoparticle recognition involved in cellular uptake as well as sub- and inter-cellular events may uncover further intriguing insights into earthworm's immunity in the nanomaterial world.

Key Words: nanomaterials, silver nanoparticles, immunogenicity, earthworms, coelomocytes, uptake

Introduction

It was not until the advent of the nanotechnology era that earthworms were on the verge of encountering the "unknown" delivered by the modern human society. The current definition of a nanomaterial adopted by European Commission states "*A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm*". Naturally occurring ultrafine particles of the same size range have existed long before the emergence of nanotechnology. Yet, already in 2005 the extensive review by Oberdörster and colleagues (2005) pointed out possible scenarios of engineered nanomaterials posing threats to ecosystem health. The major concern stemming from rapid development and commercialisation of nanotechnology products is uncertainty of such novel formulations in the modes of action and routes of exposure to living organisms. The environmental sink has received, and is expected to continue receiving, commercially produced nanomaterials as well as their derivatives, environmental transformations and the fate of which have yet to be elucidated in particular in the soil milieus (reviewed in Tourinho *et al.*, 2012).

Immunity is a vital function to maintain organism's well-being, and represents a sensitive physiological indicator that may be affected even at low concentrations of nanomaterials exposure. Only a handful of studies exist so far to aid the current understanding of immune responses to nanomaterials in invertebrates, particularly earthworms. This includes our recent *in vitro* study on *Eisenia fetida* exposed to silver nanoparticles (AgNPs) (Hayashi *et al.*, 2012) supporting molecular responses observed *in vivo* (Hayashi *et al.*, in press). Studies on other earthworm species have been reported by van der Ploeg and co-workers, where *Lumbricus rubellus* was exposed to the carbon-based nanoparticle C_{60} fullerene *in vivo* (2013) and *in vitro* (2012) and likewise exposed to AgNPs (unpublished), as well as partially by the work of Hooper *et al*. (2011) with *E. veneta* exposed to zinc oxide NPs.

In this review we seek to recapitulate what has been learnt from the initial studies on the effects of

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nanoparticles (NPs) on earthworm immunity, with references to other invertebrate species and vertebrate counterparts. The upshot is that different types of immunocytes may respond to NPs in a distinct manner intimately linked to the cell's ability, and that previously uncharacterised aspects of earthworm immunity are emerging in the light of the nanotechnology era.

Sub-lethal impacts of nanomaterials on earthworms

Limited numbers of studies are currently available in the literature on the impact of nanomaterials on earthworms. Carbon-based nanomaterials can affect the life-history traits of *E. veneta* (Scott-Fordsmand *et al.*, 2008), *E. fetida* (Li and Alvarez, 2011) and *L. rubellus* (van der Ploeg *et al.*, 2011). Common to all was reduced reproduction, which could result in a significant decrease in the population growth rate (van der Ploeg *et al.*, 2011). C_{60} fullerenes are also suggested to bioaccumulate in earthworms (*E. fetida*) following soil exposure (Li *et al.*, 2010), whereas carbon nanotubes did not accumulate in *E. fetida* (Petersen *et al.*, 2008a) or *L. variegatus* (Petersen *et al.*, 2008b) when depuration of the gut content was allowed. Ecotoxicological screening of metal-based nanomaterials indicated significant reproductive failure in *E. fetida* exposed to silver or copper NPs (Heckmann *et al.*, 2011). Different types (size and coatings) of AgNPs disturb earthworm's reproductive capacity at 500-1000 mg/kg soil in *E. fetida* (Heckmann *et al.*, 2011; Shoults-Wilson *et al.*, 2011c; 2011b), and in *L. rubellus* significant reproductive toxicity was observed at concentrations as low as 154 mg/kg soil when the AgNPs were well dispersed in soil (van der Ploeg *et al.*, unpublished). Bioaccumulation of AgNPs in earthworms seems relatively low (Coutris *et al.*, 2012; Shoults-Wilson *et al.*, 2011c; 2011b; van der Ploeg *et al.*, unpublished), however, more sensitive endpoints indicate that earthworm's physiological traits are affected already at sub-100 mg/kg soil, for example, avoidance (Shoults-Wilson *et al.*, 2011a), enzyme activities (Hu *et al.*, 2012), oxidative stress responses (Tsyusko *et al.*, 2012) and tissue apoptosis (Lapied *et al.*, 2010).

Other types of NPs (*e.g.* alumina, titania and zinc oxide) have also been tested on earthworms and reviewed elsewhere (Tourinho *et al.*, 2012), and two types of NPs (copper and gold) were observed to biodistribute across the tissues, partly if not all, persisting their nanoparticulate forms (Unrine *et al.*, 2010a; 2010b). Scarcely studied, however, is the potential immunological effect of nanomaterials; which leads us to limit our focus of the review on carbon-based NPs and AgNPs, where insightful observations were revisited in this context.

Immunogenicity of nanomaterials

Recognition, uptake and inflammation: cutting-edge studies in vertebrates

Before remarking rather limited knowledge of the effects of nanomaterials on earthworm's immunity, we begin by capturing potentially relevant prospects that arise from studies of mammalian cells. In general to all eukaryotic cellular machineries size/shape and surface chemistry of nanomaterials are the central parameters in the interaction with immune systems, for example, via biological ligand-receptor signalling (reviewed in Dobrovolskaia and McNeil, 2007). Within the past few years the concepts of dynamic assembly of NPs and biomolecules were established (Shemetov *et al.*, 2012) and this has shed light on how the cells "see" and interact with NPs in the context of receptor-mediated responses (reviewed in Monopoli *et al.*, 2012). Supporting this notion, evidence of NP uptake via conventional endocytic (*e.g.* Lesniak *et al.*, 2013; Wang *et al.*, in press) and phagocytic (*e.g.* Lunov *et al.*, 2011) pathways is emerging. This has a direct implication to innate cellular immunity, which relies mainly on non-self pattern recognition and macromolecule-marking (opsonisation) of particles for phagocytic clearance. On the contrary, relatively less explored is the inflammatory potential of NPs as a result of direct receptor activation (*e.g.* Bastús *et al.*, 2009). This is beautifully vindicated by the activation of integrin receptor signalling in THP-1 cells (a human acute monocytic leukemia cell line) through binding of fibrinogen unfolded upon interactions with NPs (Deng *et al.*, 2011). Inflammatory responses can also be induced indirectly resulting from oxidative stress, a frequently described mode of action of NPs (*e.g.* Hayashi *et al.*, 2012). AgNPs are a good example of nanomaterials that involve oxidative stress (Foldbjerg *et al.*, 2012; Hayashi *et al.*, 2012; Jiang *et al.*, 2013) and modulate pro-inflammatory cytokines including a catalogue of interleukins and TNF-α (reviewed in Klippstein *et al.*, 2010).

Earthworm's immune responses to nanomaterials

The relative simplicity of invertebrate immunity offers a potentially sensitive and accessible means of disentangling the complex interactions of NPs and immune cells. To address this challenge, we have developed an *in vitro* model of *E. fetida* coelomocytes. In our recent report, we observed at the molecular level a cascade of stress responses initiating from oxidative stress genes to immune genes downstream following short-term exposure to AgNPs in coelomocytes and in THP-1 cells (Hayashi *et al.*, 2012). A similar set of genes was affected with a temporal shift when the worms were exposed *in vivo* to the AgNPs in a soil matrix (Hayashi *et al.*, in press). From evolutionary perspectives, a similarity between *E. fetida* coelomocytes and THP-1 cells in the expression patterns of *catalase* (repressed over time), a well-known oxidative stress response gene, and *myeloid differentiation factor 88* (*MyD88*, induced over time), which encodes a central adaptor protein of Toll-like receptors, was an intriguing observation that may comprise a part of immune responses to AgNPs in earthworms but also across the animal kingdom (Hayashi *et al.*, 2012). Signal transduction, primarily through mitogen activated protein kinase (MAPK) pathways, appears to coordinate the cross-talk between oxidative stress and immune responses to AgNPs, as implicated in the expression profiles of *MEK kinase 1* (*MEKK1*) gene both in coelomocytes and THP-1 cells (Hayashi *et al.*, 2012), as well as *in vivo*

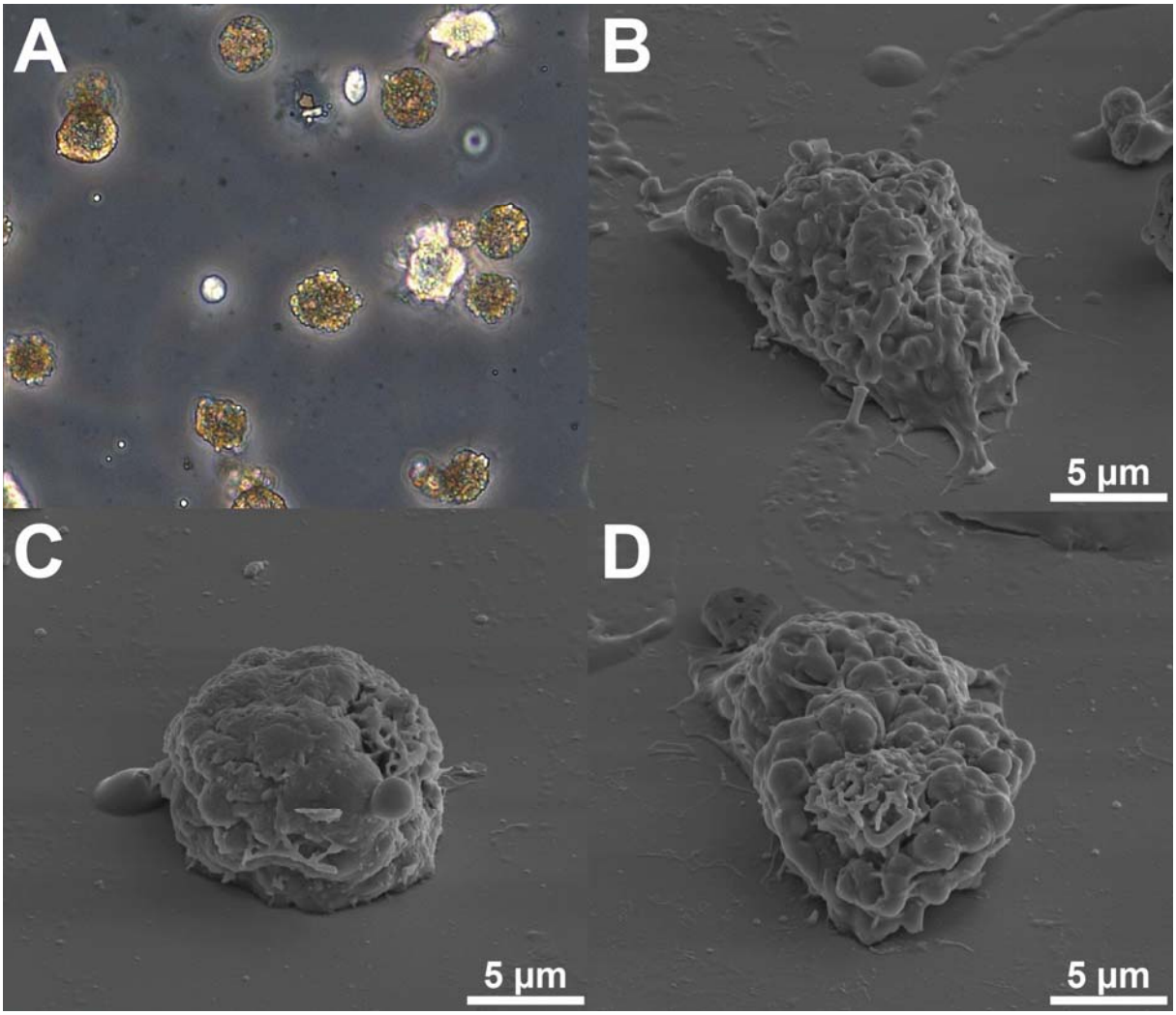

Fig. 1 Light (A) and scanning electron (B, C and D) micrographs of *Eisenia fetida* coelomocytes. The light micrograph was imaged at x200 magnification with a phase-contrast microscopy. The scanning electron micrographs were imaged on the coelomocytes after paraformaldehyde fixation and gold-sputtering (nominal 30 nm in thickness). From morphological observations, panels B and C appear to be amoebocyte populations while panel D is most likely a chloragocyte (characterised by the granule-rich feature of chloragosomes).

in earthworms (Hayashi *et al.*, in press). Phosphorylation states of MAPK families in earthworm coelomocytes were not examined, whereas *in vitro* studies of NPs using haemocytes from *Mytilus* species suggest rapid activation of MAPK cascade members, namely p38 and JNKs, with subsequent nitric oxide production (Canesi *et al.*, 2008; 2010a). The authors further reported effects on other related traits, such as oxidative burst and lysozyme activity. Interestingly, these observations were made with the NP concentrations at which lysosomal membrane stability, a sensitive indicator of cell viability, was not affected. When the molluscs were exposed *in vivo* to NPs in an aquatic system, the digestive gland appeared as the likely target of NPs with signatures of oxidative stress and haemocyte damages (Canesi *et al.*, 2010b). Similarly, haemocytes from freshwater mussels (*Dreissena sp.*) exposed *in vivo* to titania NPs accumulated the NPs intracellularly and showed reduced phagocytic activity as well as activation of ERK1/2 and p38 MAPK families (Couleau *et al.*, 2012).

van der Ploeg and colleagues (2013) exposed *L. rubellus* earthworms *in vivo* to C_{60} fullerenes for two different exposure durations (4-weeks and lifelong), and in both cases observed suppression of *heat shock protein 70* (*HSP70*) gene while enzymes involved in antioxidant mechanisms were unaffected. Of particular interest is significant suppression of *coelomic cytolytic factor 1* (*CCF1*) gene in the lifelong experiment (van der Ploeg *et al.*, 2013); CCF1 is a known pattern recognition receptor in earthworm's immunity (for review see Bilej *et al.*, 2010). Tissue injuries without histological signatures of inflammation were observed both in 4

weeks- and, albeit less severely, lifelong-exposed worms in parallel with decreased *CCF1* expression (van der Ploeg *et al.*, 2013). On this basis, the authors suggested immunosuppressive effects of C_{60}, rather than a result of coelomocyte mortality. This was supported by their *in vitro* work, in which coelomocytes from *L. rubellus* were viable at a wide range of C_{60} concentrations while *CCF1* was down-regulated in concurrence with reduced phagocytic activity (van der Ploeg *et al.*, 2012).

Although these initial studies are only indicative of the extent to which the nanomaterials may interfere with the function of earthworm's immune systems, early warnings are already given; that nanomaterials are a new class of environmental contaminants posing potential threats to earthworms.

Dissecting earthworm's innate immunity

Earthworm immune system consists of cellular (coelomocytes) and humoral components (antimicrobial, cytolytic and pattern recognition molecules) directed towards non-self materials in a natural non-specific manner (reviewed in Cooper *et al.*, 2002). Cytochemical, immunological and functional approaches characterised three major subpopulations (morphological observations by light and electron micrographs are shown in Fig. 1), among which hyaline and granular amoebocytes participate in the cellular effector mechanisms (*e.g.* phagocytosis and encapsulation) while chloragocytes (eleocytes) contribute more to homeostasis and humoral immunity (Engelmann *et al.*, 2004; 2005; Opper *et al.*, 2010). Fig. 2 illustrates the potential room for future challenges in facing the nanotechnology era, supported with evidence in earthworms or other multicellular organisms.

Amoebocytes as the scavenger of nanomaterials

Coelomocytes of *L. rubellus*, largely lacking free-circulating chloragocytes (Cholewa *et al.*, 2006), proved capable of internalising polymeric NPs (hydrodynamic diameter of 45 ± 5 nm) apparently involving energy-dependent transport mechanisms (clathrin- and caveolin-mediated endocytosis pathways) (van der Ploeg *et al.*, 2012). Our recent study suggests that the hyaline sub-group of amoebocytes and PMA-differentiated macrophage-like THP-1 cells, but not the monocytic phenotype of THP-1 cells, can accumulate AgNPs of the primary particle sizes around 83 ± 22 nm (Hayashi *et al.*, 2012). Amongst the coelomocyte populations, the hyaline amoebocytes are known to adhere and engulf bacteria (Engelmann *et al.*, 2005), and may thus be considered as the invertebrate counterpart of macrophages. Although NP uptake mechanisms are largely unknown in coelomocytes, the macrophage-like THP-1 cells appear to effect macropinocytosis for the uptake of negatively-charged NPs (Lunov *et al.*, 2011). In mammals, macropinocytosis initiates with cell membrane ruffling via actin rearrangement, suggesting an intriguing possibility of passive uptake of NPs that are membrane-adhered (Fig. 2). Amongst invertebrates, ascidian haemocytes are able to engulf particles via a RGD motif-dependent macropinocytosis (Ballarin and Burighel, 2006), however, such mechanisms are not yet known in earthworms.

Another potential phagocytic pathway is via scavenger receptor class A that is expressed by both human macrophages and macrophage-like THP-1 cells, but not by monocytic THP-1 cells (Lunov *et al.*, 2011). Scavenger receptors are conserved pattern recognition receptors known to bind lipids (lipopolysaccharides and modified low-density lipoproteins) and polyanions for phagocytosis. In particular, a macrophage receptor with collagenous structure (MARCO) is known to recognise and associate with NPs for phagocytic clearance in mammalian cells (Kanno *et al.*, 2007). In invertebrates, haemocytes from insects (Franc *et al.*, 1996) and molluscs (Liu *et al.*, 2011) are known to effect scavenger receptor-mediated uptake of pathogens and apoptotic cells. To date, scavenger receptors are yet to be identified in earthworms; however, their ubiquitous presence suggests an unequivocally conserved role in innate immune recognition that may be involved in NP uptake as in vertebrate counterparts (Fig. 2).

Toxicological implications arising from selective cellular uptake of nanomaterials are profound. Of metal-based nanomaterials that readily dissolve and liberate bioactive metal ions, AgNPs represent the most well-studied type of NPs (*e.g.* Liu *et al.*, 2010). Free Ag^+ ions, the product of oxidative dissolution, itself is highly biologically active and reacts with biomolecules (*e.g.* proteins and DNA) of the cellular components in a similar manner as reactive oxygen species (ROS). AgNPs and Ag^+ ions co-exist extracellularly and/or intracellularly, indicating a multitude of stress pathways not limited to those for the nanoparticulate form but including contribution of liquid-phase silver (*e.g.* Beer *et al.*, 2012; Yang *et al.*, 2011). Intracellular uptake of AgNPs is likely to involve subsequent fusion with lysosomes that may accelerate oxidative dissolution of AgNPs under the acidic milieu (Jiang *et al.*, 2013). This implies that AgNPs may have a targeted impact on amoebocytes as a result of preferential accumulation and subsequent *in situ* molecular damages by liberated Ag^+ ions (Hayashi *et al.*, 2012; see Fig. 2). Time-course profiling of representative gene expressions, in parallel with flow-cytometric analysis of the intracellular ROS level, favour the view that the amoebocyte populations are under oxidative stress that can signal-transduce to immune cascades downstream (Hayashi *et al.*, 2012; and Fig. 2). Recently, the amoebocyte populations, but not chloragocytes, were found to recruit calcium for activation (*e.g.* Homa *et al.*, 2013) and that they may possess a similar biochemistry of calcium signalling as in higher organisms linking stress responses to activation of immune systems (Opper *et al.*, 2010). Studies on how AgNPs affect calcium signalling in amoebocytes may further illuminate the cross-talk between stress and immune responses as known for another highly-conserved signal transduction cascade, MAPK pathways (Fig. 2).

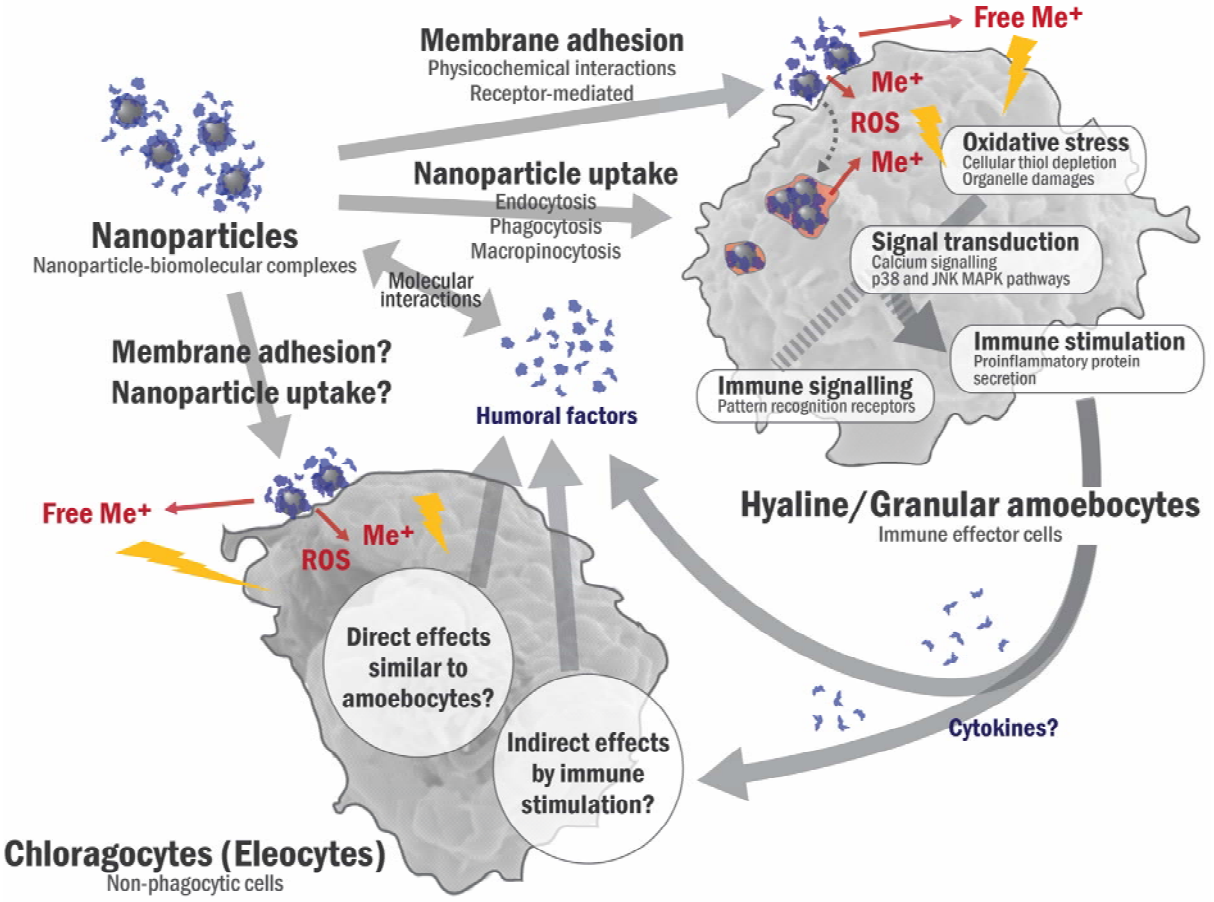

Fig. 2. Schematic illustrating the room and future challenges to progress further in our understanding of the effects of nanomaterials on earthworm's immunity. See the main text for references to each heading. Headings with a question mark show subjects that are relatively less understood in coelomocytes and in vertebrates. Drawings are not to scale. Me^{+}; metal ions, ROS; Reactive oxygen species.

Inter-cellular communications: an indirect effect?

Phagocytes secrete cytokines as a biological means of cellular communication to initiate *e.g.* inflammation but also other immunological functions, such as acute phase responses by liver cells. As described earlier in this review, cytokine secretion/modulation is a documented effect of NPs while much less is known for secondary impacts related to altered cytokine profiles (Fig. 2).

Direct evidence, however, has not been discovered for conserved and novel cytokines in earthworms. In contrast, other invertebrate organisms (especially insects) have already provided sufficient data for cytokine-mediated immune functions (*e.g.* haematopoiesis) (Malagoli, 2010; Malagoli *et al.*, 2012; Söderhäll *et al.*, 2005). The conservation and existence of proinflammatory cytokines in earthworms are not a fairy-tale; earlier we observed positive reactions of coelomocytes to monoclonal antibodies targeted for mammalian TNF-α (Engelmann *et al.*, 2002), and recently the work of Fuller-Espie and colleagues (2008) supports enhanced phagocytic activity of hyaline amoebocytes treated with mammalian cytokines (notably IL-1β, IL-2 and TNF-α).

The coelomic fluid of earthworms is sometimes assumed in the immunological context equivalent to blood plasma in mammals, both representing a protein-rich immune-competent circulatory system. Distinct from the mammalian counterpart is the existence of (migratory and sessile) chloragocytes involved in the regulation of essential minerals, haemoglobins and metallothioneins in response to natural stressors (Molnár *et al.*, 2012). This is probably by functional analogy with the hepatic/renal systems of vertebrates, and chloragocytes may contribute to regulation of the total protein balance in the coelomic fluid. For example in echinoderm, immunostimulants and invertebrate cytokines were able to regulate secretion of acute phase proteins from coelomocytes (Beck *et al.*, 2002). Its relation to immunostimulation remains unclear, but in *Eisenia* earthworms the expression and secretion of the cytolytic/antimicrobial molecule lysenin was increased in migratory chloragocytes upon Gram-

positive bacterial challenge of total coelomocytes (Opper *et al.*, 2013). Given that these characteristics partly resemble those of vertebrate hepatic cells, acute phase response in coelomocytes may await further investigations using nanomaterials as a tool to selectively target amoebocytes and modulate cytokine profiles. This also suggests an interesting feature of using coelomocytes as a mixed-population model integrating the cell-to-cell signalling events (especially of immune effector cells to hepatic-like cells) that is otherwise difficult to study in vertebrate *in vitro* models of monocultured cell lines. We also note that secretion of humoral factors and deposition of chloragosomes (specialised lysosome-like structures of chloragocytes) may lead to dynamic interactions of NPs and these biomolecules (Fig. 2).

Understanding immune responses to nanomaterials: the challenges

In the light of our current understanding in nanomaterials and their immunogenicity, we have in this review given an account for a summary of available literature on the life-history impacts of nanomaterials on earthworms, and what we have learnt of the immune responses to nanomaterials. The phagocytic population of the coelomocytes, namely hyaline amoebocytes, seems a susceptible target of nanomaterials, as a result of which indirect responses by chloragocytes are conceivable.

Environmental toxicants (such as pesticides and heavy metals) compromise the host's immune system. These pollutants inhibit the cellular immune functions (*e.g.* phagocytosis) while humoral components (*e.g.* lysozyme production) are elevated. At the dawn of knowledge about the impact of nanomaterials on invertebrate immunity, it is still obscured whether they influence cellular and/or humoral arms of immunity in a fashion that has not been previously documented. We have discussed a significant contribution of amoebocytes as NP scavengers and proposed a possibility of studying inter-cellular communications in coelomocytes. Implications from the leading researches in vertebrate models tell us that study of the NP recognition involved in cellular uptake as well as sub- and inter-cellular events may uncover further intriguing insights into earthworm's immunity in the nanomaterial world.

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References

Ballarin L, Burighel P. RGD-containing molecules induce macropinocytosis in ascidian hyaline amoebocytes. J. Invertebr. Pathol. 91: 124-130, 2006.

Bastús NG, Sánchez-Tilló E, Pujals S, Farrera C, López C, Giralt E*, et al.* Homogeneous conjugation of peptides onto gold nanoparticles enhances macrophage response. ACS Nano. 3: 1335-1344, 2009.

Beck G, Ellis TW, Habicht GS, Schluter SF, Marchalonis JJ. Evolution of the acute phase response: Iron release by echinoderm (*Asterias forbesi*) coelomocytes, and cloning of an echinoderm ferritin molecule. Dev. Comp. Immunol. 26: 11-26, 2002.

Beer C, Foldbjerg R, Hayashi Y, Sutherland DS, Autrup H. Toxicity of silver nanoparticles - nanoparticle or silver ion? Toxicol. Lett. 208: 286-292, 2012.

Bilej M, Procházková P, Šilerová M, Josková R. Earthworm immunity. In: K. Söderhäll, (Ed.), Invertebrate immunity. Springer US, pp 66-79, 2010.

Canesi L, Ciacci C, Betti M, Fabbri R, Canonico B, Fantinati A*, et al.* Immunotoxicity of carbon black nanoparticles to blue mussel hemocytes. Environ. Int. 34: 1114-1119, 2008.

Canesi L, Ciacci C, Vallotto D, Gallo G, Marcomini A, Pojana G. *In vitro* effects of suspensions of selected nanoparticles (C60 fullerene, TiO_2, SiO_2) on *Mytilus* hemocytes. Aquat. Toxicol. 96: 151-158, 2010a.

Canesi L, Fabbri R, Gallo G, Vallotto D, Marcomini A, Pojana G. Biomarkers in *Mytilus galloprovincialis* exposed to suspensions of selected nanoparticles (nano carbon black, C_{60} fullerene, nano-TiO_2, nano-SiO_2). Aquat. Toxicol. 100: 168-177, 2010b.

Cholewa J, Feeney GP, O'Reilly M, Stürzenbaum SR, Morgan AJ, Płytycz B. Autofluorescence in eleocytes of some earthworm species. Folia histochemica et cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society. 44: 65-71, 2006.

Cooper EL, Kauschke E, Cossarizza A. Digging for innate immunity since Darwin and Metchnikoff. BioEssays. 24: 319-333, 2002.

Couleau N, Techer D, Pagnout C, Jomini S, Foucaud L, Laval-Gilly P*, et al.* Hemocyte responses of *Dreissena polymorpha* following a short-term *in vivo* exposure to titanium dioxide nanoparticles: Preliminary investigations. Sci. Total Environ. 438: 490-497, 2012.

Coutris C, Hertel-Aas T, Lapied E, Joner EJ, Oughton DH. Bioavailability of cobalt and silver nanoparticles to the earthworm *Eisenia fetida*. Nanotoxicology. 6: 186-195, 2012.

Deng ZJ, Liang M, Monteiro M, Toth I, Minchin RF. Nanoparticle-induced unfolding of fibrinogen promotes Mac-1 receptor activation and inflammation. Nat Nano. 6: 39-44, 2011.

Dobrovolskaia MA, McNeil SE. Immunological properties of engineered nanomaterials. Nat Nano. 2: 469-478, 2007.

Engelmann P, Pál J, Berki T, Cooper EL, Németh P. Earthworm leukocytes react with different mammalian antigen-specific monoclonal antibodies. Zoology. 105: 257-265, 2002.

Engelmann P, Molnár L, Pálinkás L, Cooper EL, Németh P. Earthworm leukocyte populations specifically harbor lysosomal enzymes that may respond to bacterial challenge. Cell Tissue Res. 316: 391-401, 2004.

Engelmann P, Pálinkás L, Cooper EL, Németh P. Monoclonal antibodies identify four distinct annelid leukocyte markers. Dev. Comp. Immunol. 29: 599-614, 2005.

Foldbjerg R, Irving ES, Hayashi Y, Sutherland DS, Thorsen K, Autrup H*, et al.* Global gene expression profiling of human lung epithelial cells after exposure to nanosilver. Toxicol. Sci. 130: 145-157, 2012.

Franc NC, Dimarcq J-L, Lagueux M, Hoffmann J, Ezekowitz RAB. Croquemort, a novel *Drosophila* hemocyte/macrophage receptor that recognizes apoptotic cells. Immunity. 4: 431-443, 1996.

Fuller-Espie SL, Goodfield L, Hill K, Grant K, DeRogatis N. Conservation of cytokine-mediated responses in innate immunity: A flow cytometric study investigating the effects of human proinflammatory cytokines on phagocytosis in the earthworm *Eisenia hortensis*. Invert. Surv. J. 5: 124-134, 2008.

Hayashi Y, Engelmann P, Foldbjerg R, Szabó M, Somogyi I, Pollák E*, et al.* Earthworms and humans *in vitro*: Characterizing evolutionarily conserved stress and immune responses to silver nanoparticles. Environ. Sci. Technol. 46: 4166-4173, 2012.

Hayashi Y, Heckmann LH, Simonsen V, Scott-Fordsmand JJ. Time-course profiling of molecular stress responses to silver nanoparticles in the earthworm *Eisenia fetida*. Ecotoxicol. Environ. Saf. doi: 10.1016/j.ecoenv.2013.08.017, *in press*.

Heckmann L-H, Hovgaard M, Sutherland D, Autrup H, Besenbacher F, Scott-Fordsmand J. Limit-test toxicity screening of selected inorganic nanoparticles to the earthworm *Eisenia fetida*. Ecotoxicology. 20: 226-233, 2011.

Homa J, Zorska A, Wesolowski D, Chadzinska M. Dermal exposure to immunostimulants induces changes in activity and proliferation of coelomocytes of *Eisenia andrei*. J Comp Physiol B. 183: 313-322, 2013.

Hooper HL, Jurkschat K, Morgan AJ, Bailey J, Lawlor AJ, Spurgeon DJ*, et al.* Comparative chronic toxicity of nanoparticulate and ionic zinc to the earthworm *Eisenia veneta* in a soil matrix. Environ. Int. 37: 1111-1117, 2011.

Hu C, Li M, Wang W, Cui Y, Chen J, Yang L. Ecotoxicity of silver nanoparticles on earthworm *Eisenia fetida*: Responses of the antioxidant system, acid phosphatase and ATPase. Toxicol. Environ. Chem. 94: 732-741, 2012.

Jiang X, Foldbjerg R, Miclaus T, Wang L, Singh R, Hayashi Y*, et al.* Multi-platform genotoxicity analysis of silver nanoparticles in the model cell line CHO-K1. Toxicol. Lett. 222: 55-63, 2013.

Kanno S, Furuyama A, Hirano S. A murine scavenger receptor MARCO recognizes polystyrene nanoparticles. Toxicol. Sci. 97: 398-406, 2007.

Klippstein R, Fernandez-Montesinos R, Castillo PM, Zaderenko AP, Pozo D. Silver nanoparticles interaction with the immune system: Implications for health and disease. In: P. P. D, (Ed.), Silver nanoparticles. Intech Open Access Publishers, pp 309-324, 2010.

Lapied E, Moudilou E, Exbrayat J-M, Oughton DH, Joner EJ. Silver nanoparticle exposure causes apoptotic response in the earthworm *Lumbricus terrestris* (oligochaeta). Nanomedicine. 5: 975-984, 2010.

Lesniak A, Salvati A, Santos-Martinez MJ, Radomski MW, Dawson KA, Åberg C. Nanoparticle adhesion to the cell membrane and its effect on nanoparticle uptake efficiency. J. Am. Chem. Soc. 135: 1438-1444, 2013.

Li D, Fortner JD, Johnson DR, Chen C, Li Q, Alvarez PJJ. Bioaccumulation of $^{14}C_{60}$ by the earthworm *Eisenia fetida*. Environ. Sci. Technol. 44: 9170-9175, 2010.

Li D, Alvarez PJJ. Avoidance, weight loss, and cocoon production assessment for *Eisenia fetida* exposed to C_{60} in soil. Environ. Toxicol. Chem. 30: 2542-2545, 2011.

Liu J, Sonshine DA, Shervani S, Hurt RH. Controlled release of biologically active silver from nanosilver surfaces. ACS Nano. 4: 6903-6913, 2010.

Liu L, Yang J, Qiu L, Wang L, Zhang H, Wang M*, et al.* A novel scavenger receptor-cysteine-rich (SRCR) domain containing scavenger receptor identified from mollusk mediated PAMP recognition and binding. Dev. Comp. Immunol. 35: 227-239, 2011.

Lunov O, Syrovets T, Loos C, Beil J, Delacher M, Tron K*, et al.* Differential uptake of functionalized polystyrene nanoparticles by human macrophages and a monocytic cell line. ACS Nano. 5: 1657-1669, 2011.

Malagoli D. Cytokine network in invertebrates: The very next phase of comparative immunology. Invert. Surv. J. 7: 146-148, 2010.

Malagoli D, Accorsi A, Sacchi S, Basile V, Mandrioli M, Pinti M*, et al. Drosophila* helical factor is an inducible protein acting as an immune-regulated cytokine in S2 cells. Cytokine. 58: 280-286, 2012.

Molnár L, Engelmann P, Somogyi I, Mácsik LL, Pollák E. Cold-stress induced formation of calcium and phosphorous rich chloragocyte granules (chloragosomes) in the earthworm *Eisenia fetida*. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 163: 199-209, 2012.

Monopoli MP, Aberg C, Salvati A, Dawson KA. Biomolecular coronas provide the biological identity of nanosized materials. Nat Nano. 7: 779-786, 2012.

Oberdörster G, Oberdörster E, Oberdörster J. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. Environ. Health Perspect. 113: 823-839, 2005.

Opper B, Németh P, Engelmann P. Calcium is required for coelomocyte activation in earthworms. Mol. Immunol. 47: 2047-2056, 2010.

Opper B, Bognár A, Heidt D, Németh P, Engelmann P. Revising lysenin expression of earthworm coelomocytes. Dev. Comp. Immunol. 39: 214-218, 2013.

Petersen EJ, Huang Q, Weber JWJ. Bioaccumulation of radio-labeled carbon nanotubes by *Eisenia foetida*. Environ. Sci. Technol. 42: 3090-3095, 2008a.

Petersen EJ, Huang QG, Weber WJ. Ecological uptake and depuration of carbon nanotubes by *Lumbriculus variegatus*. Environ. Health Perspect. 116: 496-500, 2008b.

Scott-Fordsmand JJ, Krogh PH, Schaefer M, Johansen A. The toxicity testing of double-walled nanotubes-contaminated food to *Eisenia veneta* earthworms. Ecotoxicol. Environ. Saf. 71: 616-619, 2008.

Shemetov AA, Nabiev I, Sukhanova A. Molecular interaction of proteins and peptides with nanoparticles. ACS Nano. 6: 4585-4602, 2012.

Shoults-Wilson W, Zhurbich O, McNear D, Tsyusko O, Bertsch P, Unrine J. Evidence for avoidance of ag nanoparticles by earthworms (*Eisenia fetida*). Ecotoxicology. 20: 385-396, 2011a.

Shoults-Wilson WA, Reinsch BC, Tsyusko OV, Bertsch PM, Lowry GV, Unrine JM. Role of particle size and soil type in toxicity of silver nanoparticles to earthworms. Soil Sci. Soc. Am. J. 75: 365-377, 2011b.

Shoults-Wilson WA, Reinsch BC, Tsyusko OV, Bertsch PM, Lowry GV, Unrine JM. Effect of silver nanoparticle surface coating on bioaccumulation and reproductive toxicity in earthworms (*Eisenia fetida*). Nanotoxicology. 5: 432-444, 2011c.

Söderhäll I, Kim Y-A, Jiravanichpaisal P, Lee S-Y, Söderhäll K. An ancient role for a prokineticin domain in invertebrate hematopoiesis. The Journal of Immunology. 174: 6153-6160, 2005.

Tourinho PS, van Gestel CAM, Lofts S, Svendsen C, Soares AMVM, Loureiro S. Metal-based nanoparticles in soil: Fate, behavior, and effects on soil invertebrates. Environ. Toxicol. Chem. 31: 1679-1692, 2012.

Tsyusko OV, Hardas SS, Shoults-Wilson WA, Starnes CP, Joice G, Butterfield DA*, et al.* Short-term molecular-level effects of silver nanoparticle exposure on the earthworm, *Eisenia fetida*. Environ. Pollut. 171: 249-255, 2012.

Unrine JM, Hunyadi SE, Tsyusko OV, Rao W, Shoults-Wilson WA, Bertsch PM. Evidence for bioavailability of au nanoparticles from soil and biodistribution within earthworms (*Eisenia fetida*). Environ. Sci. Technol. 44: 8308-8313, 2010a.

Unrine JM, Tsyusko OV, Hunyadi SE, Judy JD, Bertsch PM. Effects of particle size on chemical speciation and bioavailability of copper to earthworms (*Eisenia fetida*) exposed to copper nanoparticles. J. Environ. Qual. 39: 1942-1953, 2010b.

van der Ploeg MJ, van den Berg JH, Bhattacharjee S, de Haan LH, Ershov DS, Fokkink RG*, et al. In vitro* nanoparticle toxicity to rat alveolar cells and coelomocytes from the earthworm *Lumbricus rubellus*. Nanotoxicology. doi:10.3109/17435390.2012.744857, 2012.

van der Ploeg MJC, Baveco JM, van der Hout A, Bakker R, Rietjens IMCM, van den Brink NW. Effects of C_{60} nanoparticle exposure on earthworms (*Lumbricus rubellus*) and implications for population dynamics. Environ. Pollut. 159: 198-203, 2011.

van der Ploeg MJC, Handy RD, Heckmann L-H, van der Hout A, van den Brink NW. C_{60} exposure induced tissue damage and gene expression alterations in the earthworm *Lumbricus rubellus*. Nanotoxicology. 7: 432-440, 2013.

van der Ploeg MJC, Handy RD, Waalewijn-Kool PL, van den Berg JHJ, Herrera Rivera ZE, Bovenschen J*, et al.* Effects of silver nanoparticles (NM-300K) on *Lumbricus rubellus* earthworms and particle characterisation in relevant test matrices, including soil. Environ. Toxicol. Chem., Submitted.

Wang F, Yu L, Monopoli MP, Sandin P, Mahon E, Salvati A*, et al.* The biomolecular corona is retained during nanoparticle uptake and protects the cells from the damage induced by cationic nanoparticles until degraded in the lysosomes. Nanomedicine: Nanotechnology, Biology and Medicine. doi: 10.1016/j.nano.2013.04.010, in press.

Yang X, Gondikas AP, Marinakos SM, Auffan M, Liu J, Hsu-Kim H*, et al.* Mechanism of silver nanoparticle toxicity is dependent on dissolved silver and surface coating in *Caenorhabditis elegans*. Environ. Sci. Technol. 46: 1119-1127, 2011.