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Original Research Article

Glycoprotein of Coelomic Fluid Triggers Formation of Aggregates/Hetero-Aggregates of ZnO-NPs in Earthworms

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ABSTRACT

Keywords

Nanoparticles, nanomaterials, nanoscavenger, earthworms, aggregation of nanoparticles

With rapid increase in production of engineered nanoparticles, it is crucial to ascertain the fate of nanomaterials in the environment so their availability for environment exposure can be assessed. The present study is focused to assess of fate of nanoparticles to be beneficial or to risk assessments using *Eisenia foetida* earthworm as model organisms. The ZnO-NPs were exposed on earthworms and glycoprotein and aggregates were analyzed in the coelomic fluid of earthworms to understand their possible role as nanoscavenger.

Introduction

With development of various nanomaterials, nanotechnology has gained wide attention because of their novel properties including large surface area and high reaction activity. However, particles of nano-sized range have been present on earth for millions of year and have been used by mankind for thousands of years. But, recently their production and application has been increased due to its wide application in structural engineering, electronics, consumer products, optics, alternative energy, soil and water remediation, diagnostics, drug delivery devices etc. Rapid advancement in this branch of science promises direct benefits to the society and economy. Despite of their bright future outlook there is growing concern about human health, safety and environmental impacts including from exposure to engineered nanomaterials from different environmental sources. With normal use of nanoparticles they may be released from various products and enter into wastewater stream. A major portion of these nanoparticles released into sewage sludge those are disposed of in landfills, incinerated or applied to agriculture lands. Thus, soil system is an alternative sink for large portion of nanoparticles (Gottschalk *et al*., 2009). Turney (2004) stated invariably production, use and disposal lead to release of nanoparticles into the environment. Wastewater treatment streams, landfills and combustion of products containing nanomaterials are means through which they may end up in the environment. However, some nanoparticles are also used in environmental remediation applications. The behavior of nanoparticles in the environment not only depends on their physical and chemical properties but also depends on the characteristics of the receiving environment (Chen *et al.*, 2008; Chen and Elimelech 2008; Saleh *et al.*, 2008).Among the various engineered nanoparticles Zinc oxide nanoparticles (ZnO-NPs) are one of the most abundantly utilized group of nanomaterials and has wide range of application in industrial and household products including toothpaste, mouth-washes, ointments surface coatings, food packaging materials and agriculture. It have been used as dietary supplement in human and livestock to stimulate immune system (Prasad *et al.*, 2008; Rincker *et al.*, 2005).

Many studies demonstrated that ZnO-NPs are toxic to mammalian cells and even more toxic than other nanoscale structures of metallic oxide (Horie *et al.*, 2009; Jeng and Swanson 2006).Li and Haneda (2003) reported that ZnO-NPs in combination of UV exposure generates reactive oxygen species(ROS) like hydroxyl radicals or hydrogen peroxide in aqueous solutions leading to efficient decomposition of organic compounds. Bruner *et al*. (2006) observed that three days exposure of human mesothelioma and rodent fibroblast cells to ZnO-NPs (19 nm) caused DNA and mitochondrial damages. Therefore study is aimed to nullify toxic effects of these nanoparticles with naturally available means. As nature has its ways of resolving imbalances in the environment and organisms is one of the best tools of nature to eliminate toxic pollutants. The biological process of eliminating pollutants (bioremediation) with activities of earthworms may be translated to nullify toxicity of nanoparticles. Our current understanding to remediate/nullify toxicity of nanoparticles by naturally available scavengers is limited. The study is an attempt to testify presence of glycoprotein in coelomic fluid of earthworms to release aggregated or less toxic forms of nanoparticles in soil system.

Materials and Methods

The research design to observe impact of ZnO-NPs in earthworms was developed following published OECD guidelines and Unrine *et al.* (2010). Twenty clitellate adult earthworm *Eisenia foetida* weighing 0.30 ± 0.12 g each in three replicate exposure chambers containing 1 kg dry mass of artificial soil medium were chosen for test experiment. The soil medium consisted of 70% quartz sand, 10% peat moss and 20% kaolin. The pH was adjusted with the addition of a small amount of crushed limestone. Two doses of 50, 35nm ZnO-NPs (5 and 10 mg/kg) were added to dry soil, mixed by homogenizer for 5 minutes and moisture content was maintained for 60%. Soil sub-samples were also taken at the beginning of the exposure from each exposure chamber. After 28 days of exposure, worms collected and washed thoroughly in running tap water followed by rinsing in distilled water. And, placed on wet cotton to ensure complete defecation in order to avoid contamination during harvesting of coelomic fluid. After 2-3 hrs, worms were wiped with cotton wool soaked with 70 % ethyl alcohol to avoid any further contamination. The surface cleaned worms were placed alternately in sterile petridish containing cold extrusion buffer (NaCl 71.2mM; Ethanol 5%; Guaicol-glycerol-ether 50.4mM; EGTA 5mM, pH 7.3) and distilled water at interval of one minute for 8-10 times. Coelomic fluid extruded out through dorsal pores due to external stress condition. After collection of coelomic fluid in cold extrusion buffer, worms were released in soil. The percentage of carbohydrates and protein was estimated using Glycoprotein Carbohydrate estimation kit (Merce). The coelomic fluid was also examined under Transmission Electron Microscopy at The Energy Research Institute, New Delhi.

Results and Discussion

Table 1 depicts the glycoprotein in coelomic fluid of *Eisenia foetida* reared in substrate spiked with different size and doses of ZnO-NPs. Highest concentration of protein (2.12 ± 0.50 mg/ml) and carbohydrate (14.4 ± 0.40 %) was recorded in coelomic fluid of earthworms spiked with 35nm @ 10 mg/kg ZnO-NPs substrate while lowest were in worms cultured in 50nm @ 5 mg/kg spiked substrate (protein 1.20 ± 0.40 mg/ml;carbohydrate 8.9± 1.50 %). Similarly, the highest aggregates of NPs were obtained in coelomic fluid of earthworms reared on 35nm @ 10 mg/kg (Fig 1c) and lowest in worms reared on 50nm @ 5 mg/kg spiked substrates (Fig 1d). Finding shows direct correlation with release of glycoprotein of coelomic fluid with formation of aggregates of nanoparticles. Glycoproteins are proteins which carry covalently-bonded sugar units. The carbohydrate portions of glycoproteins are often called *glycans* (occurs as *oligosaccharide*) and consist 2-30 % of total weight. The oligosaccharides contains hexoses (*Man*, *Gal*, less often *Glc*; *N-accetyl hexose amines*) and often terminates with sialic acid or 1-Fuc residues (*Arbinose*, *1-fucose*, *galactose*, *glucose*, *mannose* and *xylose*).Each glycan of a glycoprotein is linked to a specific aminoacid residue by a single sugar residue. There are two basic types of glycosidic linkages: *O-glycosidic links* involving the hydroxyl groups of *Ser*, *Thr*, *Hylys* or *Hypro* and *N- glycosidic* bonds *via* the amide nitrogen of an *Asn* residue. A single glycoproteins may have both linkages. In general glycoproteins usually find on surface of coelomic cells and secreted actively by endoplasmic reticulum of cells and plays prominent roles in biological systems, being found in cell secretions, having structural roles and occurring as membrane proteins. Biologically active glycoproteins include enzyme and hormones as well many serum proteins as glycoproteins. Their carbohydrate portions of many glycoproteins are carrier of information crucial for functioning of many biological recognition systems. It may be important in controlling the passage of material through the membrane pores. Kornfield and Kornfield (1995) reported that hydrated glycoproteins are major constituents of the extracellular matrix forming effective packing material and efficient lubricant.

On entry of nanoparticles in earthworms glycoproteins of coelomic fluid facilitate nanoparticles to dissolution; speciation (association with other ionic or molecular dissolved chemical substances); biological and chemical transformation to other chemicals and may be complete mineralization (to carbon dioxide and water); agglomeration (may be disagglomeration) and settling of the particles. And, may be expected to behave according to well understood phenomenon of Van der Waal forces to become dominate over repulsive electrostatic forces and steric hinderance. As consequences particles adhere to each other and then excreted out either through coelomic fluid or cast. The dominant factor in this stability of aggregates under natural conditions may be pH, ionic strength and presence of natural organic matter (Lead and Wilkinson, 2006). However the surface properties of the nanomaterials are also very important for their aggregation behavior. The humic acid and fulvic acids with varying pH may have combined effects on the fate nanoparticles resulting into higher level of aggregation. Surface modification of nanoparticles may influence fate and behavior of nanoparticles.

As carbon nanotubes are considered to be highly hydrophobic and have a tendency to aggregate, they are expected to settle in natural environment. Kannedy *et al*., (2008) reported that surface modifications (including functional groups and coating) with natural organic matters play important role in stability of aggregation of nanoparticles. The nanoparticles preferentially bind with the natural organic matter (NOM) in gut of worms and thus become less bio-available.

Table.1 Estimation of glycoprotein in coelomic fluid of earthworms reared in ZnO-NPs spiked substrate

Sl No.	Sample	Protein Concentration (mg/ml)	Total Carbohydrate Content (%)
01.	Control (without nanoparticle spiked substrate)	0.25 ± 0.12	3.2 ± 0.80
02.	35nm @ 5 mg/kg spiked substrate	1.84 ± 0.40	10.2 ± 0.40
03.	35nm @ 10 mg/kg spiked substrate	2.12 ± 0.50	14.4 ± 0.40
04.	50nm @ 5 mg/kg spiked substrate	1.20 ± 0.40	8.9± 1.50
05.	50nm @ 10 mg/kg spiked substrate	1.95 ± 0.50	12.3± 0.50

Fig.1 Transmission electron microphotograph of coelomic fluid of Eisenia foetida reared on different substrate; a. control; b. 35nm @ 5 mg/kg; c, 35nm @ 10 mg/kg ;d. 50nm @ 5 mg/kg; e. 50nm @ 10 mg/kg

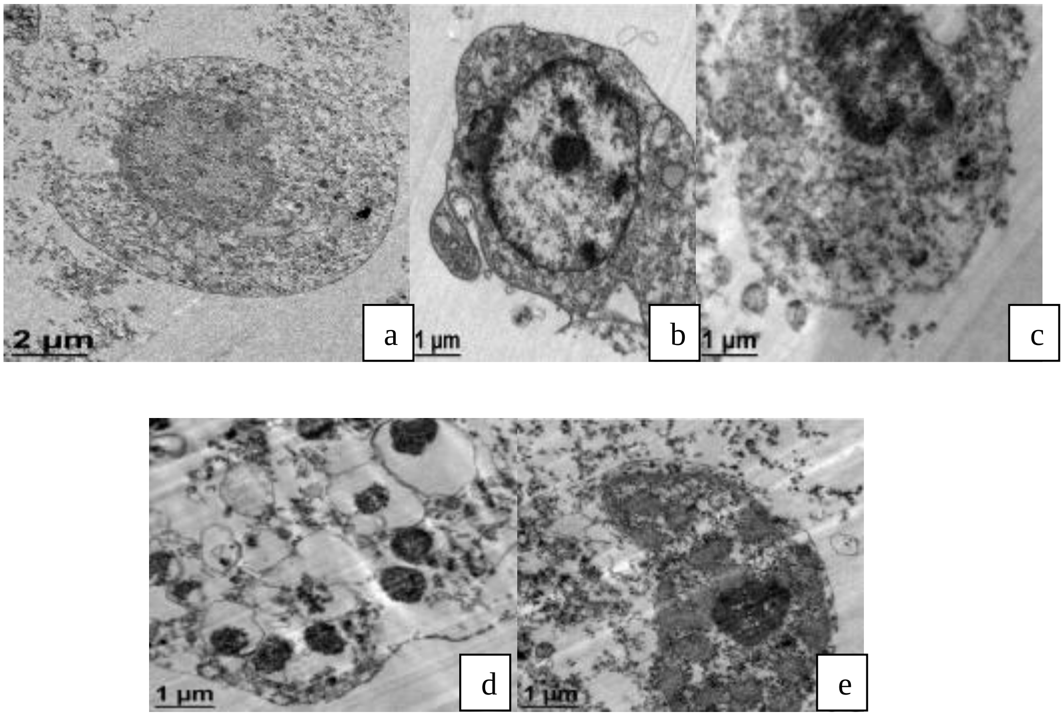

Fig.2 Calciferous glands

This association with organic matter may reduce or even eliminate antibacterial activity (Li *et al.*, 2008) in gut microbiome. Natural organic matter consists of mainly fulvic and humic substances. Fulvic compounds typically make up 40 to 80% of NOM compounds and, due to their small size they can coat the surfaces of particles and may alter the surface charge and have been shown to stabilize with an effect similar to surfactants (Domingos *et al*., 2009). Natural organic matter attaches to the surface of particles in a variety of ways *viz*., irreversible adsorption onto the surfaces of iron oxide *via* ligand exchange between carboxyl/hydroxyl functional groups of humic acid and iron oxide surfaces (*Gu et al*., 1994) or hydrophobic interactions with carbon based nanomaterials (Hyung *et al*., 2007). Stabilization usually results from NOM forming a charged stabilizing layer on the outside of the particle. Destabilization, on the other hand, results from particles being bridged by larger NOM molecules, such as rigid biopolymers (Ernest *et al*., 2010).

Large-molecular-weight biomolecules and bio-macromolecules, including proteins, polypeptides, and amino acids (Mc Keon and Love, 2008; Moreau *et al*., 2007; Vamunu *et al*., 2008), also affect aggregation of NPs in earthworms.

The stabilization of aggregates may also depends microorganism which proliferated in the ingested materials in the gut or develop after the mixture has been excreted and nanoparticles in the worm's intestine may be cemented by calcium humate '*formed from ingested decomposing organic matter and calcite excreted by the calciferous glands'* as suggested by Satchell (1967). The stabilization in these aggregates might be due to secretions of earthworms obtain from intestinal and body wall mucus secretions and to measures the extent to which they interact with soil components and nanoparticles to produce water stable aggregates.

Hayes (1983) suggested the gum like nitrogenous mucus materials, probably mucoproteins left behind when earthworm pass over the soil surface, readily adhere to the surfaces with which they come into contact. Such body wall secretions stabilize the wall of the burrows (Edwards and Lofty, 1971) and may be available nanoparticles readily formed into aggregates. In case where aggregate-forming secretions are resistant to microbial attack, the micro-aggregates and aggregates formed could be expected to be stable (Hayes, 1983). Further, the soil particles in passing through the earthworm gizzard; aggregates wouldn't survive this process, although domains could, and some very small micro-aggregates might. Clearly a re-association of aggregate forming components must take place as the materials pass through the earthworm intestine. Hayes (1983) suggested that calciferous glands (figure 2) provides appropriate amount of calcium to allow divalent cations bridging to take place between the humic substances and the negatively charged inorganic colloids. This bridging is actually resulted into formation of aggregates, which takes place in the intestine in presence of excess water in the intestine.

Drying of the casts after ejection brings the particles into close proximity and allow to polymers secreted in the intestine, as well as the indigenous humic substances and polysaccharides to form the necessary bridges(aggregates). The humus substance provides appropriate polymers for binding of these particles and stabilizing aggregates. These polymers may have to be of appropriate size and in abundance, to bridge the distances between the components of aggregates/hetero-aggregates. The larger the aggregates, the longer and the more numerous of these bridges must be to confer stability of nanoparticles.

In conclusion, the study concluded the presence of glycoproteins and naturally organic materials plays significant role in formation of aggregates the nanoparticles in body cavity of earthworms and finally discharges the aggregates or hetero-aggregates of ingested nanoparticles either through the cast or coelomic fluid. However, present investigation is needs to obtain the complete understanding of formation of aggregates in the body cavity of earthworms and to apply this principle to develop technology to reduce the impact of nanoparticles in soil system.

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