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Bioactivity of nanosilver in *Caenorhabditis elegans*: Effects of size, coat, and shape

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ABSTRACT

The in vivo toxicity to eukaryotes of nanosilver (AgNP) spheres and plates in two sizes each was assessed using the simple model organism *Caenorhabditis elegans*. For each shape, smaller AgNP size correlated with higher toxicity, as indicated by reduced larval growth. Smaller size also correlated with significant increases in silver uptake for silver nanospheres. Citrate coated silver spheres of 20 nm diameter induced an innate immune response that increased or held steady over 24 h, while regulation of genes involved in metal metabolism peaked at 4 h and subsequently decreased. For AgNP spheres, coating altered bioactivity, with a toxicity ranking of polyethylene glycol (PEG) > polyvinylpyrrolidone (PVP) ≅ branched polyethyleneimine (BPEI) > citrate, but silver uptake ranking of PEG > PVP > citrate > BPEI. Our findings in *C. elegans* correlate well with findings in rodents for AgNP size vs. uptake and toxicity, as well as for induction of immune effectors, while using methods that are faster and far less expensive, supporting the use of *C. elegans* as an alternative model for early toxicity screening.

1. Introduction

Nanoparticles (NP) are defined as objects that measure between 1 and 100 nm in at least one dimension. Given that the diameter of many cellular macromolecules such as DNA (2 nm) [1], ATP synthase (10 nm) [2], and synaptic vesicles (40 nm) [3] are also in this size range, it is perhaps unsurprising that NP can exhibit bioactivities that are lacking in corresponding bulk materials. NP can be synthesized using a variety of base materials in different sizes and shapes, with each parameter conferring specific physical and bioactive properties [4]. As a result, investigations of novel uses for NP with applications in fields such as electronics, medicine, and food packaging are increasing rapidly [5]. Due to the recent growth in the availability of consumer products that contain NP [6], it has been estimated that gross sales could top one trillion dollars per year by 2015 [7].

Nanosilver (AgNP) has both antibacterial and antiviral activity [8–10], and in in vitro studies has been shown to be as effective as standard antibiotics against several clinically relevant strains of pathogenic bacteria [11,12]. The antimicrobial properties of AgNP have led to a dramatic increase in the availability of commercial products and medical devices that contain AgNP, with estimates of the amount AgNP manufactured per year exceeding 300 tons [13]. While there has also been growth in the number of studies conducted investigating the safety of NP exposure, many lack adequate nanomaterial characterization and/or do not report dosing methods [14]. Additionally, ionic silver

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(Ag^+) can be released from the surface of AgNP in solution [15], yet only a small minority of studies report on the toxicity of supernatants or filtrates of AgNP solutions so that the toxicity of applied AgNP can be evaluated separately from the toxicity of impurities acquired during synthesis, or Ag^+ released during storage [16].

Qualities that can alter the stability and biological response to AgNPs include size, shape, coating, and manufacturing methods. For AgNP spheres, smaller size is associated with increased antibacterial activity [9,17], increased toxicity to zebrafish embryos [18], and higher cytotoxicity and inflammatory marker release in mammalian cell culture models [19]. Silver nanoplates (AgPlates) are high aspect ratio particles that can be used for photoacoustic imaging in medical diagnostics [20]. AgPlates have higher antibacterial activity against *Escherichia coli* than silver nanospheres or rods [21]. Additionally, silver nanoplates were found to be more toxic to zebrafish than silver nanospheres or wires, though this effect was attributed to crystal defects on the surface of the plates having a negative impact when in contact with cellular membranes [22], indicating that different synthesis methods may improve eukaryotic tolerance.

Coats or capping agents are used to stabilize NP in solution by altering surface charge or introducing steric repulsion. One of the most commonly used AgNP stabilizing agents is citrate, which gives the resultant particles a negative surface charge [23,24]. Polyvinylpyrrolidone (PVP) is a polar, hygroscopic compound used as a wetting or binding agent for cosmetics and pharmaceuticals. PVP also imparts a slight negative charge to AgNP, though less than citrate [15]. In zebrafish embryos, PVP coated AgNP is more toxic than citrate coated AgNP of the same size and shape [18]. Branched polyethyleneimine (BPEI) is a relatively new coating material for NP which provides a positive surface charge due to amino and amide groups [25]. Positively charged amine groups have been shown to increase NP protein absorption and cell membrane interactions in vitro [26], but information on how positively charged NPs behave in vivo is scarce. Polyethylene glycol (PEG) is manufactured in a variety of molecular weights and chemical variants for use in processed foods and pharmaceuticals. PEGylation of NP inhibits agglomeration and protein absorption [27]. While it is known that functional coatings can alter the bioactivity of NP [28], there are only a few published studies on the relative toxicity of different coats on NPs of a specific size and shape. Base material and synthesis methods can also alter NP toxicological profiles [29,30], making generalized safety assessments difficult. Therefore, it is important that each NP species be tested individually, in a model that is appropriate for assessed exposure route [31].

In vitro toxicity testing using cellular models is simple, fast, and inexpensive when compared to traditional in vivo toxicity testing using mammals, yet results from in vitro tests using cellular models frequently do not correlate with in vivo data [32]. Another drawback of many types of in vitro models for predictive toxicology is that they cannot mimic the effects of biological fluids or enzymatic metabolism that occurs in vivo [33]. Therefore, a small whole animal model such as the microscopic soil dwelling nematode *Caenorhabditis elegans* is likely to prove more predictive for oral toxicity screening than cell based assays. *C. elegans* have a short generation time and are easily maintained under laboratory conditions in axenic media. Many types of assays in *C. elegans* can be completed in a week or less. Previously, we and others have demonstrated that *C. elegans* assays can predict mammalian toxicity ranking [34–36], and that 10 nm AgNP spheres, while toxic to *C. elegans* at high concentration, are less toxic than Ag^+, which also correlates with data from in vivo rodent and in vitro mammalian cell culture studies [30]. Here we assess the effects of AgNP size, shape, and coat on bioactivity in *C. elegans* using larval growth assays, organismal silver uptake measurements, and gene expression.

2. Materials and methods

2.1. Reagents and test materials

ReagentPlus grade silver acetate was purchased from Sigma–Aldrich, and fresh solutions of 1 mg/mL silver ion (Ag^+) equaling 1.55 mg/mL silver acetate were prepared weekly. For evaluations of NP coat toxicity, mPEG-OH, MW 5000 was supplied by Laysan Bio; citrate, PVP, and BPEI were supplied in solution by nanoComposix. BioPure NPs were purchased from nanoComposix (San Diego, CA). The manufacturer characterizes each batch with transmission electron microscopy (TEM) to determine size and shape distributions, UV-visible spectroscopy to measure the optical properties, particle hydrodynamic diameter with dynamic light scattering, and particle surface charge with a zeta potential measurement. BioPure NPs are extensively washed with the suspending solvent to remove residual reactants from the manufacturing process. Mass concentration is determined with inductively coupled plasma mass spectroscopy (ICP-MS). The particles are sterile filtered and tested for endotoxin contamination before delivery. Throughout the study, nanomaterials were stored at 4 °C and tested regularly by dilution in diH_2O followed by UV-visible spectroscopy (UV-vis) using a UV-1800 (Shimadzu) to verify the stability of each NP suspension. NPs with altered UV–vis spectra were replaced. For assessment of agglomeration or degradation effects in nutrient media, NPs were also mixed to 100 μg/mL in CeHM as above, then diluted 1:20 in diH_2O, and tested by UV–vis. Supernatants of all test articles were obtained by high-speed centrifugation of silver suspensions according to published guidelines [37]. Briefly, suspensions of 10nmAgCit were subjected to centrifugation at 40,000 × *g* for 120 min, and all other AgNP suspensions at 25,000 × *g* for 90 min, with a WX Ultra Series centrifuge in a F50L-24 × 1.5 rotor (ThermoScientific). Supernatants were carefully separated from pellets and assessed for toxicity and silver concentration.

2.2. Experimental design

C. elegans feed by pharyngeal pumping, which pulls liquid phase components from their surroundings into their digestive system, making them a good model for oral toxicity as test articles can be applied to the media in which they are maintained. Some of the endpoints and methods