

CRITICAL REVIEW



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## A systematic review of evidence for silver nanoparticle-induced mitochondrial toxicity

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Silver nanoparticles (AgNPs) are extensively used for their antibacterial properties in a diverse set of applications, ranging from the treatment of municipal wastewater to infection control in hospitals. However, the properties of AgNPs that render them conducive to bactericidal use in commerce may influence their potential toxicity to non-bacterial organisms. Based on the physiological and phylogenetic similarities between bacteria and mitochondria within eukaryotic cells, mitochondria are a likely intracellular target of AgNP toxicity. Mitochondria-specific outcomes of AgNP exposures have been identified in multiple cell types, including (but not limited to) loss of membrane potential, inhibition of enzymes involved in oxidative phosphorylation, and changes in calcium sequestration. However, the biological significance of mitochondrial toxicity due to AgNP exposure is currently incompletely understood. This review examines the existing evidence of mitochondrial toxicity induced by AgNP exposure, with discussions of the role of the physico-chemical properties of the nanoparticles themselves in mitochondrial toxicity. The impacts of potentially differential cell- and tissue-specific significance of AgNP-induced mitochondrial dysfunction are also discussed.

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### Nano impact

Silver nanoparticles (AgNPs) are widely used for their antibacterial properties. Mitochondria (organelles that provide the vast majority of cellular energy) evolved directly from bacteria, and are therefore a target of many antibacterial agents, potentially including AgNPs. Because functionally intact mitochondria are required for maintaining organismal health, it is important to identify environmental nanoparticle exposures that cause mitochondrial damage. This review serves to: 1) critically examine the current understanding of the role of AgNPs in mitochondrial toxicity, with an emphasis on reactive oxygen species production, mitochondrial depolarization, and inhibition of key mitochondrial enzymes; and 2) highlight potential contributions of physicochemical characteristics of AgNPs on mitochondria-specific toxicity.

## Introduction

Silver nanoparticles (AgNPs) are extensively utilized for their antibacterial properties in hospitals [reviewed by ref. 1], in a wide range of consumer and commercial goods,<sup>2</sup> and as a bactericidal agent in the treatment of wastewater.<sup>3</sup> To meet the demand for said applications, between 2.8 and 20 tons of engineered AgNPs are produced annually in the United States alone,<sup>4</sup> with approximately 500 tons produced globally *per annum* (this estimate is an extrapolation).<sup>5</sup> The physico-chemical properties of these engineered AgNPs influence their antibacterial activity, including size,<sup>6</sup> shape,<sup>7</sup> and surface coating.<sup>8</sup> Interestingly, these same properties also influence the toxicity of AgNPs.<sup>9–11</sup> The physical and chemical

behaviors of AgNPs tend to be highly variable depending upon not only their engineered design but also the environmental conditions in which they are found (oxygen availability, tissue-specific microbial populations, route of exposure, aggregation, *etc.*). These unique and variable properties of AgNPs, combined with their high production volume, contribute to the impetus for research on the toxicological impacts of AgNPs.

AgNPs act as antibacterial agents by disrupting the bacterial cell wall and depositing within the membrane,<sup>12</sup> with a potential role for reactive oxygen species entering the membrane after disruption of the bacterium, or the two processes acting synergistically.<sup>13</sup> Smaller AgNPs tend to be more potent antibacterial agents than their larger counterparts,<sup>6</sup> and shape appears to have a significant effect on antibacterial activity, with triangular AgNPs being more effective than spherical or rod-shaped AgNPs.<sup>7</sup>

Mitochondria have long been proposed to have descended directly from proteobacteria, originally inhabiting ancestral

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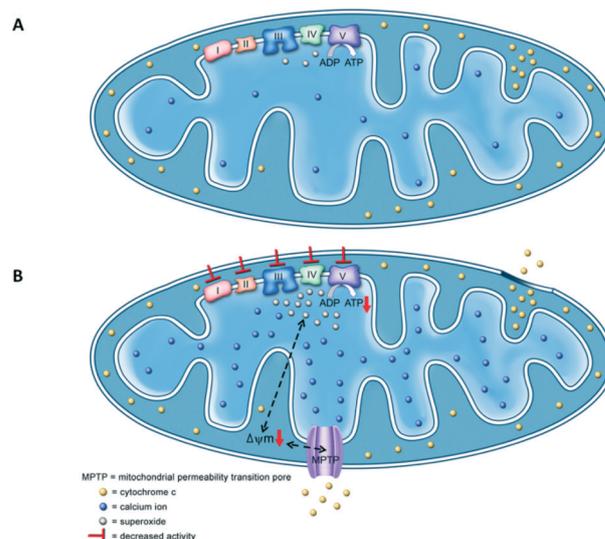
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eukaryotic cells as endosymbionts.<sup>14</sup> Many properties of mitochondria have since been confirmed to be physiologically and/or phylogenetically related to bacteria, including (but not limited to) protein export,<sup>15</sup> membrane biogenesis,<sup>16,17</sup> protein translation,<sup>18</sup> and even coordination of inter-mitochondrial junctions to facilitate information exchange between mitochondria.<sup>19,20</sup> Growing understanding of the mechanisms by which widely-used antibiotics act as mitochondrial toxicants [reviewed by ref. 21–23] demonstrates that there are multiple structures and processes evolutionarily conserved between mitochondria and their bacterial ancestors that may render mitochondria vulnerable to toxicity induced by bactericidal agents. These include, but are not limited to, ribosomes,<sup>24</sup> topoisomerase,<sup>25</sup> DNA mutations,<sup>26</sup> and synthesis of specific enzymes such as cytochrome c oxidase.<sup>27</sup>

By analogy, because AgNPs possess bactericidal properties, mitochondria may well also be important intracellular targets of AgNP-induced toxicity. This may be true even when AgNP toxicity results from dissolution, since ionic silver is also bactericidal.<sup>28,29</sup> Mitochondria provide the majority of the energy required for proper cellular function, and damage to mitochondria resulting in decreased or inefficient energy production has the potential to hinder ATP-dependent cellular mechanisms. The ability of a cell to correct or adapt to low levels of ATP varies, and depends on inherent local demand for ATP (which can itself be highly dynamic). Thus, cells residing within tissues that are highly metabolically active are potentially more sensitive to mitochondrial toxicants, as has been observed in drug-induced mitochondrial poisoning [reviewed by ref. 30] and is also observed for inherited mitochondrial diseases [reviewed by ref. 31 and 32]. Because mitochondrial biology is also highly variable with developmental stage, if AgNPs are mitochondrial toxicants, there may be developmental windows of susceptibility [reviewed by ref. 33].

In support of the possibility that AgNPs cause mitochondrial toxicity, a number of publications have reported mitochondrial alterations after AgNP exposures (summarized schematically in Fig. 1). On the other hand, there are few reports of AgNPs being translocated to mitochondria, raising the question of whether mitochondrial effects are direct or indirect.

Furthermore, while mitochondrial toxicity induced by AgNP exposure has begun to be investigated, the relative contribution of mitochondrial dysfunction to cellular and organismal AgNP-induced toxicity remains to be fully elucidated. To guide future research in this area, this review serves as a systematic analysis of the existing research on AgNP induced mitochondrial toxicity, addressed in terms of the cell-specific significance of the observed mitochondrial toxicity (search methods described in Table 1). We categorize the results into four categories of mitochondrial modes of toxicity for which significant evidence exists in the literature: reactive oxygen species production, loss of mitochondrial inner membrane potential, inhibition of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction, and inhibition



**Fig. 1** Schematic illustrating published effects of AgNP exposure on mitochondria. We review literature reporting data consistent with AgNP-induced mitotoxicity, organized into 4 major categories discussed in the text (panel A represents a healthy mitochondrion, panel B represents a mitochondrion after AgNP exposure). 1) Mitochondrial oxidative stress: one study reported increased mitochondrial superoxide production;<sup>43</sup> 2) mitochondrial depolarization: in 20 studies, decreased mitochondrial membrane potential was identified;<sup>9,32,39,43,67,68,70–82,107</sup> 3) metabolic activity by MTT assay (largely reflecting succinate dehydrogenase activity in the mitochondria): 6 studies reported decreased MTT reduction, which includes both mitochondrial and cytosolic reductive capacity;<sup>9,72,81,88,89,108</sup> 4) inhibition of mitochondrial functions: 2 studies measured decreased ATP content,<sup>79,101</sup> and one study was conducted each on ETC activity,<sup>93</sup> calcium-induced MPTP,<sup>86</sup> increased mitochondrial calcium sequestration,<sup>86</sup> release of cytochrome c.<sup>68</sup> These results are reflective of specific findings within a limited number of *in vivo* studies and across both cancerous and non-cancerous cell types *in vitro*; thus, this figure represents a generalized view of AgNP mitotoxic effects. Specific effects are likely to differ by cell type and other variables. It also should be noted that the mitotoxicity shown in this figure is not exhaustive of all essential mitochondrial signaling pathways and functions which may be ultimately affected by AgNPs. Dashed arrows denote mechanisms that likely influence one another. Note that our literature review and schematic reflect specific mitochondrial measurements, although most of these measurements (e.g., MTT reduction, ATP levels, and oxidative stress) are frequently carried out in a way that simultaneously measures the parameters outside of the mitochondrion.

of the electron transport chain. Throughout, to the extent possible based on the data, we discuss mitochondrial toxicity in terms of the physicochemical properties of AgNPs themselves.

## Oxidative stress as a mechanism of cytotoxicity – relation to mitochondrial toxicity

The generation of intracellular reactive oxygen species (ROS) is postulated to be an important mitochondrial mechanism of AgNP toxicity, and has been documented in cell

**Table 1** Database results for relevant search terms. PubMed search terms used to retrieve manuscripts reporting mitochondrial effects of silver nanoparticle exposure. Detailed experimental specifics of primary research articles for which toxicological effects were observed are included in Table 2

Database searched	Search terms used	Number of primary research articles retrieved	Number of review articles retrieved	Date of retrieval
PubMed	“Silver nanoparticle, mitochondria”	44	4	08.25.2015
	“Silver nanoparticles, mitochondrial”	86	6	08.25.2015
	“Silver nanoparticles, mitochondrial toxicity”	51	5	08.25.2015
	“Silver nanoparticles, mitochondria toxicity”	27	3	08.25.2015

culture<sup>9,34–36</sup> and across tissues in laboratory animals.<sup>37–39</sup> Multiple mechanisms exist for AgNP-mediated reactive oxygen species (ROS) generation, including nanoparticle surface chemistry, depletion of antioxidant molecules *via* binding by dissolved ions with thiol groups, altered enzymatic production of reactive oxygen species, and inhibition of the electron transport chain.<sup>40,41</sup> In most cell types and under most circumstances, mitochondria are the major source of ROS production, and mitochondrial perturbations such as inhibition of the electron transport chain can significantly increase mitochondrial ROS production [reviewed by ref. 42]. Mitochondria are also a major target for oxidative damage [reviewed by ref. 43, 44].

Most reports of potential mitochondrially-related ROS effects resulting from AgNP exposure are correlative, consisting of nonspecific cellular increases in ROS associated with significant metrics of mitochondrial dysfunction. However, recent work has reported AgNP-mediated, mitochondria-specific ROS effects that were size-dependent. In human blood monocytes, both 5 nm and 28 nm PVP-coated AgNPs induce elevated production of mitochondria-specific ROS as measured by the mitochondria-specific dye MitoSOX. However, the 5 nm AgNPs induced lysosomal swelling, whereas mitochondrial swelling and direct uptake of 28 nm particles was observed.<sup>45</sup> In the same study, 100 nm PVP-coated AgNPs were taken up by monocytes, but did not induce mitochondrial or lysosomal changes.<sup>45</sup> These data suggest that mitochondria-specific uptake and subsequent or correlative changes in function is dependent upon AgNP size. However, it is important to note that monocytes are macrophage precursor cells and are primarily phagocytic in nature [reviewed by ref. 46]. It is likely that uptake of AgNPs differs between phagocytic and non-phagocytic cells. Cellular AgNP uptake observed in monocytes may not reflect rates of physiological uptake of AgNPs in non-phagocytic cell types, and intracellular destinations may vary. Because cellular uptake mechanisms of the particles likely influence the amount and subcellular localization of AgNPs, the mechanisms by which the AgNPs gain entry to the cell are also likely related to resultant organelle-specific toxicity. Cells such as macrophages that are capable of generating an NADPH oxidase-mediated oxidative burst are also unusual in their capacity to generate ROS in response to nanoparticle exposure [reviewed by ref. 47].

Ionization of AgNPs within the intracellular space has the potential to cause mitochondrial dysfunction *via* mechanisms including oxidative stress, but has yet to be investigated as a

primary factor in the development of mitochondrial toxicity. Because dissolution of AgNPs to silver ions occurs *in vivo*<sup>11,48</sup> and may be accelerated by localization to acidic compartments such as lysosomes,<sup>49,50</sup> silver ions derived from AgNPs have the potential to exert cell-specific and organelle-specific toxicity. *In vitro*, silver ions are taken up by cells and move from the soluble to the insoluble cellular fraction over time, bind to metallothionein, and inhibit ETC enzymes.<sup>51</sup> Additionally, a considerable amount of data shows that silver ions are bactericidal.<sup>28,29,52–56</sup> Silver ions exert antimicrobial effects through various mechanisms in bacteria, including (but not limited to) suppression of succinyl co-A synthetase expression,<sup>28</sup> an enzyme located in the mitochondrial matrix and required for the citric acid cycle; binding to bacterial DNA;<sup>29</sup> inhibition of the oxidation of glucose, glycerol, fumarate, succinate, and lactate;<sup>55</sup> and competition with copper ions.<sup>56</sup> This evidence lends credence to the possibility that dissolution of AgNPs results in mitochondrial toxicity *via* mechanisms that are conserved between bacteria and mitochondria. For example, a high degree of homology exists between the superoxide dismutase enzymes found in mitochondria and *E. coli*.<sup>57</sup> Superoxide dismutase *E. coli* deletion mutants are highly sensitive to AgNP toxicity associated with (but not singularly caused by) silver ionization.<sup>58</sup> This provides for the possibility that AgNP exposure may elicit a parallel ROS-mediated toxicity in mitochondria. Additionally, work by Schreurs and Rosenberg showed that silver ions cause phosphate and metabolite efflux from *E. coli*, acting both as an uncoupler and an inhibitor of the respiratory chain.<sup>59</sup> ROS-induced toxicity of AgNPs due to dissolution has also been seen *in vitro*.<sup>60</sup> Thus, dissolution of AgNPs is likely to cause mitochondrial toxicity due to silver ion-induced bactericidal mechanisms that are phylogenetically and physiologically conserved between bacteria and mitochondria.

An important logistical consideration related to silver ion toxicity is the effect of AgNP sample storage conditions. Recent evidence indicates that when determining ion-specific and AgNP-specific toxicity, storage conditions of the AgNPs themselves may be a significant factor. The length of storage of AgNPs exhibits a positive correlation with silver ion release in solution and subsequent cellular toxicity.<sup>61</sup> Other considerations for AgNP storage include inert gas conditions<sup>60,62</sup> or completely anaerobic conditions,<sup>63</sup> as significant oxidative dissolution of AgNPs to silver ions occurs when AgNPs are stored under atmospheric conditions. Incomplete

investigation or reporting of dissolution-related factors complicates interpretation of experiments reporting “particle-specific” effects.

Finally, despite evidence of ROS-dependent mechanisms of toxicity, AgNP exposure can result in cytotoxic changes in which no increase in ROS is observed. For example, Gliga *et al.*<sup>64</sup> found that AgNP-induced cell death instead depended on particle size, with only the smallest tested particles (10 nm) resulting in measurable cytotoxicity in a study utilizing immortalized human bronchial epithelial cells; the observed changes were linked to intracellular silver ion release. These data suggest that regardless of induction of ROS-dependent mechanisms, cytotoxicity is dependent upon AgNP size, with smaller AgNPs eliciting a more toxic response than relatively larger ones. Additionally, intracellular ionization of AgNPs appears to be important for cytotoxicity even when increased ROS were not detected. We have also found that in the whole organism model *Caenorhabditis elegans*, most of the AgNPs tested caused their toxicity largely *via* dissolution, with a smaller role for ROS production detectable for a subset of AgNPs.<sup>11</sup>

## Mitochondrial depolarization

Depolarization of the mitochondrial membrane potential ( $\Delta\psi_m$ ) can occur as a response to a variety of insults, both endogenous and exogenous. Sufficient mitochondrial membrane polarization is required for essential mitochondrial functions, including canonical protein import mechanisms,<sup>65,66</sup> ATP production,<sup>67</sup> and proper responses to cellular calcium concentration flux.<sup>68</sup> Loss of  $\Delta\psi_m$  is the most frequently studied mitochondrial outcome as a result of AgNP exposure.<sup>9,34,41,69–80</sup> Loss of  $\Delta\psi_m$  has been observed across a diverse range of tissues and cell types (both cancerous and non-cancerous) in mammals and in whole organisms: liver (fibroblasts,<sup>34</sup> hepatocytes,<sup>73</sup> hepatoblastoma cells<sup>77</sup>); brain (neuroblastoma cells,<sup>71</sup> glioblastoma cells<sup>69</sup>); lung (alveolar macrophages,<sup>9</sup> alveolar basal epithelial carcinoma cells<sup>78,74</sup>); the gastrointestinal tract (colorectal adenocarcinoma cells,<sup>72,80</sup> colon carcinoma cells,<sup>76,77</sup> oral squamous carcinoma cells<sup>81</sup>); the immune system (dendritic cells,<sup>82</sup> macrophages,<sup>75,83</sup> acute monocytic leukemia cells<sup>84</sup>); the skin (keratinocytes)<sup>75,79</sup> in *Candida albicans*<sup>70</sup> and in *Caenorhabditis elegans*.<sup>41</sup>

Interestingly, AgNP coating/conjugation did not correlate with likelihood of observing loss of  $\Delta\psi_m$  (bare AgNPs = 11/19 studies; coated/conjugated AgNPs = 10/19 studies; these numbers add up to more than 100% because some studies reported that both bare and coated/conjugated AgNPs cause loss of  $\Delta\psi_m$ ) (Table 2). Further, the average size of the nanoparticle did not differ significantly between bare AgNPs and those that contained a coating (Fig. 2), suggesting that particle size did not confound the relationship between coating and observance of loss of  $\Delta\psi_m$ . An important point with regard to the interpretation of these results is that a significant portion of these studies utilize cancerous cell lines. There are fundamental functional differences between mitochondria in

cancerous cells and mitochondria in non-cancerous cells [reviewed by ref. 85]. Because nanoparticle-facilitated delivery of chemotherapeutic drugs is emerging as a promising cancer treatment technology [reviewed by ref. 86], the interpretation of AgNP-induced mitochondrial depolarization specifically within cancer cells may be one of therapeutic significance rather than toxicity. However, the particular physiological impact of mitochondrially-targeted depolarization as a result of AgNP exposure in cancerous cells may be different from non-cancerous cells, as tumor cells produce energy largely *via* aerobic glycolysis yet still require mitochondrial function for the production of macromolecules [reviewed by ref. 87].

However, an important caveat to these studies is that they were not specifically designed to test whether mitochondrial impacts were a primary mode of toxicity, or occurred as a secondary response resulting from the failure of other cellular homeostatic processes. Loss of  $\Delta\psi_m$  has not yet been directly correlated with mitochondrial uptake of AgNPs, which would provide compelling evidence of mitochondrial depolarization as a primary mechanism of toxicity. Mitochondrial depolarization associated with AgNP exposure could instead be part of one or more signaling cascades involving crosstalk between the mitochondrion and other cellular components, since exposure to AgNPs results in calcium-induced opening of the mitochondrial permeability transition pore<sup>88</sup> and induction of mitochondrially-mediated apoptotic mechanisms.<sup>70,75,89</sup>

## Measurement of AgNP toxicity using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

AgNP exposure results in reduced mitochondrial activity or cell viability as measured by the MTT assay.<sup>9,74,83,90,91</sup> AgNPs exposures *in vitro* have caused MTT reduction in conjunction with mitochondrial depolarization.<sup>9,74,83</sup> In macrophages, exposures in the range of 5  $\mu\text{g mL}^{-1}$ –30  $\mu\text{g mL}^{-1}$  AgNPs resulted in mitochondrial depolarization, with a reduction in cellular viability at concentrations up to 50–100  $\mu\text{g mL}^{-1}$ .<sup>9,83</sup> Loss of mitochondrial membrane potential was observed at a range of 100–200  $\mu\text{g mL}^{-1}$  AgNPs, whereas a decrease in cellular viability in epithelial carcinoma cells was observed at lower concentrations.<sup>74</sup> This suggests that mitochondrial effects, such as loss of membrane potential, may be a primary effect in AgNP exposure only in certain cell types, or that mitochondrial depolarization is a secondary effect and arises concurrently with cell death in other cell types. This conclusion, however, is dependent upon the measured parameters within the few studies listed (differing sizes, types, and concentrations of AgNPs used, differing *in vitro* conditions, *etc.*) and thus may not be extrapolated to categorize primary or secondary mitochondrial effects due to AgNP exposure, nor to different cell types. The MTT assay, primarily utilized in toxicological studies as a measure of overall cellular health, is

**Table 2** Physicochemical properties of AgNPs and corresponding tissue-specific and cell-specific mitochondrial toxicity. Results of primary research articles investigating AgNP-induced mitochondrial toxicity (details of search terms and retrieval in Table 1). Inclusion criteria: primary research article, written in English, and quantified mitochondrial outcomes of AgNP exposure

AgNP size (nm; NS = not specified)	AgNP coating	Concentration @ which mitochondrial effects observed	Mitochondrial effects	Target organ or cell type used	Reference
15, 100	None	25 and 50 $\mu\text{g mL}^{-1}$	Concentration-dependent decrease in membrane potential (Rhod123)	Immortalized rat liver fibroblasts	34
15	None	100 $\mu\text{g mL}^{-1}$	Reduced mitochondrial activity (MTT assay)	Immortalized rat adrenal pheochromocytoma cells (PC-12)	90
25	Hydrocarbon, polysaccharide	25 $\mu\text{g mL}^{-1}$	Reduced mitochondrial membrane potential (both coatings exhibited lower mmp than control; polysaccharide to a greater extent than hydrocarbon)	Immortalized mouse neuroblastoma cells (N2A)	71
15, 30, 55	None	15 nm@5 $\mu\text{g mL}^{-1}$ 30 nm@10 $\mu\text{g mL}^{-1}$ 55 nm@50 $\mu\text{g mL}^{-1}$	Reduced mitochondrial activity (MTT assay)	Rat alveolar macrophages	9
6–20	Starch-capped	100, 200, 400 $\mu\text{g mL}^{-1}$ in U251 & IMR-90	Reduced mitochondrial membrane potential (both 15 nm and 30 nm @ 5 $\mu\text{g mL}^{-1}$ ; not observed @ 55 nm) Reduced ATP content	Immortalized human lung fibroblasts (IMR-90) and immortalized human glioblastoma cells (U251)	103
5–45	None	10, 25, 50 ppm	Decreased respiratory chain complex activity: Brain (complex I & III @ all three concentrations; complex II & IV @ 25 and 50 ppm) Liver (complex I, II, III, and IV @ all three concentrations) Muscle (complex I @ 50 ppm; complex II @ 25 and 50 ppm; complex III and IV @ all three concentrations) Heart (complex I, II, III, and IV @ 25 and 50 ppm)	Rat brain, liver, muscle, heart	95
30–50	0.2% PVP	10, 12.5, 15, 20 $\mu\text{g mL}^{-1}$	Decreased mitochondrial activity (MTT assay), <i>although, to a lesser extent than AgNO<sub>3</sub></i>	Immortalized human alveolar basal epithelial carcinoma cells (A549)	91
40, 80	None	2 $\mu\text{g}$ , 5 $\mu\text{g}$ for both 40 nm & 80 nm	Decreased state 3 and state 4 respiration; increased susceptibility to calcium-induced mitochondrial permeability transition	Liver mitochondria isolated from Wistar rats	96
4–9	Chitosan nanocarrier	24, 48 $\mu\text{g mL}^{-1}$	Decreased mitochondrial membrane potential	Immortalized human colorectal adenocarcinoma cells (HT29)	72
100	None	3 $\mu\text{g mL}^{-1}$	Increased mitochondrial calcium sequestration	Immortalized Chinese hamster lung fibroblasts (V79-4)	88
3	None	2 ng $\text{mL}^{-1}$	Loss of mitochondrial membrane potential; release of cytochrome c	<i>Candida albicans</i>	70
40	mPEG-SH	6.25, 12.5, 25, 50, 100 $\mu\text{g mL}^{-1}$	Significantly decreased mitochondrial membrane potential	Immortalized human hepatocytes (HL-7702)	73
14	PVP	5 $\mu\text{g mL}^{-1}$	Induction of mitochondrial apoptosis pathways	Immortalized rat adrenal pheochromocytoma cells (PC-12)	89
2–3	PVP	0.5, 1 $\mu\text{g mL}^{-1}$	Decrease in mitochondrial membrane potential (trend; not statistically significant)	Immortalized mouse dendritic cell line (DC2.4)	82
5, 28, 100	PVP	0.9 & 1.15 $\mu\text{g mL}^{-1}$ for 5, 28 nm superoxide 0.9 $\mu\text{g mL}^{-1}$ for decreased membrane integrity	Increased mitochondrial superoxide production; decrease in membrane integrity (trend; not statistically significant)	Primary human monocytes	45

Table 2 (continued)

AgNP size (nm; NS = not specified)	AgNP coating	Concentration @ which mitochondrial effects observed	Mitochondrial effects	Target organ or cell type used	Reference
43.9	Citrate	30 $\mu\text{g mL}^{-1}$ for decreased mitochondrial membrane potential 100 $\mu\text{g mL}^{-1}$ for decreased viability (MTT)	Decreased membrane potential (microscopy; not quantified) Decreased viability (MTT)	Immortalized mouse macrophages (RAW 264.7)	83
182.7	None	50, 100, 200 $\mu\text{g mL}^{-1}$ for decreased MTT	Decreased viability (MTT)	Immortalized human alveolar basal epithelial carcinoma cells (A549)	74
13, 33, 46 10–65	Tannic-acid modified (13, 33, 46; 10–65 unmodified)	100, 200 $\mu\text{g mL}^{-1}$ for decreased mitochondrial membrane potential $\geq 2.5 \mu\text{g mL}^{-1}$ for 33, 46 nm; $\geq 2.5 \mu\text{g mL}^{-1}$ for 13, 33, 46 and 10–65 for decreased mmp in keratinocytes $\geq 1 \mu\text{g mL}^{-1}$ for 13, 33, 46 nm for caspase-9 release in monocytes; $\geq 1 \mu\text{g mL}^{-1}$ for 33 & 46 nm for caspase-9 release in keratinocytes; for 10–65, $\geq 5 \mu\text{g mL}^{-1}$ for keratinocytes and 10 $\mu\text{g mL}^{-1}$ for monocytes 1, 5, 10 $\mu\text{g mL}^{-1}$ for decrease in mitotracker staining intensity (simultaneously with loss of calcein staining)	Decreased mitochondrial membrane potential Decreased mitochondrial membrane potential (JC-1) Caspase-9 release	Immortalized mouse keratinocytes (291.03C) and monocytes (RAW 264.7)	75
20	None	139 $\mu\text{g mL}^{-1}$	Decreased intensity of mitotracker red staining	Primary rat cortical cell cultures	109
346.8 $\pm$ 65.2	Alginate-chitosan-blended nanocarrier	139 $\mu\text{g mL}^{-1}$	Decrease in membrane potential (JC-1)	Immortalized human glioblastoma cells (U87MG)	69
3, 11, 30	None	4, 8 $\mu\text{g mL}^{-1}$	Qualitative decrease in membrane potential (JC-1)	Immortalized human acute myocytic leukemia cells (SHI-1)	84
<100	None	40, 125 $\mu\text{g mL}^{-1}$	Decrease in membrane potential (TMRE)	Human colon carcinoma cells (Caco-2)	76
27.3 hydrodynamic; 20.4 TEM	Citrate	1, 10, 20 $\mu\text{g mL}^{-1}$ HepG2 10, 20 $\mu\text{g mL}^{-1}$ Caco-2	Decrease in membrane potential (R123)	Human hepatoblastoma cells (HepG2)	77
20 nm hydrodynamic; 20–100 nm DLS	None	25 $\mu\text{g mL}^{-1}$	Decrease in MTT	Human colon carcinoma cells (Caco-2)	77
Bare = 35.1 nm PVP8 = 47.7 nm PVP38 = 108 nm 20 nm	PVP; bare	Bare = 0.041 $\text{mg L}^{-1}$ PVP8-AgNPs = 0.607 $\text{mg L}^{-1}$ PVP38-AgNPs = 3.262 $\text{mg L}^{-1}$ Bio-AgNPs = 25 $\mu\text{g mL}^{-1}$ Chem-AgNPs = 70 $\mu\text{g mL}^{-1}$ Nuclear-targeted = 0.4 nM	Decrease in mitochondrial membrane potential (TMRE) with bare AgNPs, PVP8	Human neuroblastoma cells (SH-SY5Y)	110
42 $\pm$ 12	PEGylated	4, 8, 16, 33 ppm	Qualitative decrease in membrane potential (JC-1) Decrease in ATP	Human astrocytoma cells (D384) <i>C elegans</i>	41
16 $\pm$ 2.0	None	4, 8, 16, 33 ppm	Decrease in mitochondrial membrane potential (Rhod123)	Human astrocytoma cells (D384)	110
30	None	4 $\mu\text{g mL}^{-1}$	Qualitative decrease in mitochondrial membrane potential (Rhod123)	Human astrocytoma cells (D384)	41
			Qualitative decrease in membrane potential (JC-1) Decrease in MMP (MitoProbe DiOC <sub>2</sub> ) Decrease in ATP	Immortalized human alveolar basal epithelial carcinoma cells (A549)	78
			Decrease in mitochondrial membrane potential (Rhod123)	Human oral squamous carcinoma cells (HSC-3)	81
			Qualitative decrease in mitochondrial membrane potential (Rhod123)	Human keratinocytes (HaCat)	79
			Qualitative decrease in mitochondrial membrane potential (Rhod123)	Human colon adenocarcinoma cells (COLO 205)	80

### Interaction of AgNP size and Coating/Conjugation on Mitochondrial Depolarization

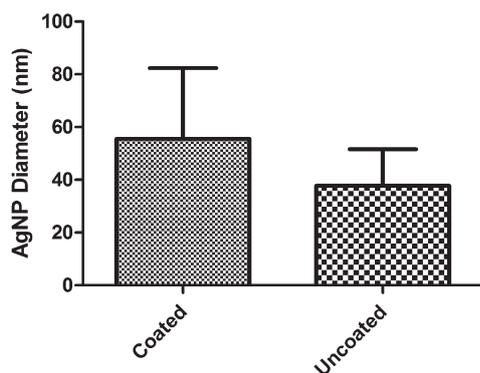


Fig. 2 Effect of particle size on likelihood of observing mitochondrial depolarization between coated and uncoated AgNPs. Average reported diameter of AgNPs for coated/conjugated AgNPs ( $n = 12$ ) and bare AgNPs ( $n = 13$ ) (Table 2). Error bars represent standard error of mean AgNP diameter. Studies reporting a range of values for the average diameter were not included in this analysis.

sometimes specifically referred to as a cellular proliferation assay or a mitochondrial activity assay in the literature. The assay utilizes reduction of the MTT reagent with the substrates succinate, NADH, and NADPH,<sup>92</sup> and is not mechanistically confined to mitochondria as only 25–45% of the MTT-formazan product co-localizes with mitochondria,<sup>93</sup> but rather includes reduction at cytoplasmic, endoplasmic reticulum, and lysosomal membranes.<sup>94</sup> Thus, results from MTT assays do not permit definitive classification of mitochondrial response, and caution should be exercised when interpreting the results in terms of organellar specificity. Other cellular metabolic activity assays used to measure general cytotoxicity include Alamar Blue, XTT, and WST-1. Based on the same general biochemical principles as the MTT assay, these assays must be used judiciously when referring to mitochondrial activity, as the readout does not distinguish between reduction of the assay reagent by mitochondrial or cytoplasmic enzymes. It is advised that these assays be employed in addition to more direct mitochondrial assays to provide a complete picture of cytotoxicity after AgNP exposure.

### Tissue-specific inhibition of oxidative phosphorylation as a result of AgNP exposure

Direct inhibition of electron transport chain (ETC) enzymes occurs across multiple tissues as a result of AgNP exposure, with the quantity and identity of ETC enzyme inhibition varying in a tissue-specific, concentration dependent manner.<sup>95</sup> Of the tissues studied, liver mitochondria were the most sensitive, with Complexes I, II, III, and IV inhibited at all

concentrations of AgNPs tested.<sup>95</sup> Additionally, Teodoro, *et al.*, found that AgNP exposure resulted in decreased state 3 and state 4 respiration in isolated liver mitochondria.<sup>96</sup> Costa *et al.* found that brain, heart, and muscle mitochondria displayed differing patterns of ETC enzyme inhibition, despite being tissues of commonly high metabolic demand: complex I and III were more sensitive to inhibition than II and IV in brain; complex I, II, III, and IV in heart were in general less sensitive than in brain and muscle, with complex I the least sensitive; and in muscle, complex III and IV were the most sensitive to inhibition, complex II exhibited intermediary sensitivity, and complex I was most resistant to inhibition by AgNPs.<sup>95</sup> While the contribution of silver ions themselves were not assessed in ETC enzyme inhibition, the dissolution of AgNPs likely plays a role, as silver ions bind readily to thiol groups.<sup>97</sup>

### Conclusions

Numerous beneficial applications of AgNPs exist due to their antibacterial properties. However, the very physicochemical properties of AgNPs and mechanisms associated with these unique properties that render them useful as bactericidal agents may cause parallel cytotoxic mechanisms. Mitochondrial endpoints in AgNP exposure studies are of particular interest, not only because mitochondria perform an essential suite of cellular functions, but also due to the phylogenetic and physiological overlap between bacteria and mitochondria within eukaryotic cells. Further, subsets of the population may be susceptible to mitochondrial toxicity caused by AgNPs, as is the case for antibiotics that poison mitochondria [reviewed by ref. 22]. Antibiotic toxicity usually occurs in patients with underlying mitochondrial vulnerabilities or aberrations, including specific mtDNA mutations.<sup>26,98,99</sup> This indicates that gene–environment interactions are causative factors in antibiotic-induced mitochondrial toxicity, and suggests that some individuals would be similarly sensitive to AgNP exposure if AgNP toxicity is significantly mediated by direct mitochondrial toxicity.

However, mitochondrial dysfunction as a primary mode of cytotoxicity during AgNP exposure can be difficult to distinguish from secondary mechanisms in which mitochondria actively sense and respond to other organelles' signaling mechanisms. This is because a number of mitochondrial responses to toxicity are integrated into and mediated by signaling cascades of nonmitochondrial origin, including apoptosis [reviewed by ref. 100] and calcium sequestration.<sup>101,102</sup> To date, a small number of studies have investigated direct mitochondrial uptake of AgNPs,<sup>45,103,104</sup> and a subset of these studies have found evidence of AgNP entry into the mitochondrion.<sup>45,103</sup> The magnitude of AgNP exposure resulting in mitochondria-specific dysfunction (such as decreased ATP production, observed in fibroblasts and glioblastoma<sup>103</sup> and oral squamous carcinoma cells<sup>81</sup>) in the cell as a whole varies and is dependent upon factors such as the rate of cellular turnover and age of the organism. Thus, the biological

significance of cytotoxicity introduced by mitochondrial injury (whether off-target or primary in nature) is likely influenced by cell type and inherent demand for mitochondrially-derived energy sources. This is directly related to the likelihood that AgNPs are distributed to, and exert mitochondrial toxicity within, tissues with relatively high metabolic demand. Silver nanoparticles are distributed throughout the body upon oral administration<sup>105–107</sup> and inhalational exposure,<sup>108</sup> with liver being the predominant target tissue in oral exposure<sup>105</sup> and both liver and lung in inhalational exposure.<sup>108</sup> However, long-term clearance kinetics of silver after oral AgNP exposure indicates that elimination of silver from AgNPs is slowest in tissues with biological barriers (brain and testis).<sup>106</sup> Widespread tissue distribution of AgNPs indicates that there are likely differential tissue-specific functional outcomes if mitochondria are indeed a target of AgNP toxicity. This is likely to be mediated by cellular composition of the tissues (*i.e.* postmitotic neurons in brain), localized or heterogeneous demands for mitochondrially-derived energy, and the ability of the cell or tissue to cope with disturbances to mitochondrial function, among others. Elucidation of the definitive role of the mitochondrion in AgNP-induced toxicity (beyond that summarized in Fig. 1), related to the physicochemical characteristics of the AgNPs themselves and in terms of tissue specificity, requires further, more targeted studies. Studies may be targeted toward more specific mitochondrial endpoints that have yet to be systematically investigated and include (but are not limited to) morphology, biogenesis, respiration, mitochondrial DNA damage, transcription of mitochondrial proteins, and protein import into the mitochondrion, among others.

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