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Effects of Particle Size and Surface Charge on Mutagenicity and Chicken Embryonic Toxicity of New Silver Nanoclusters

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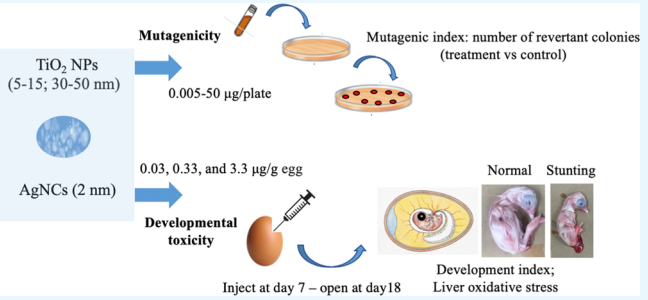
ABSTRACT: Though there are many toxicological studies on metal nanoparticles (NPs), it remains difficult to explain discrepancies observed between studies, largely due to the lack of positive controls and disconnection between physicochemical properties of nanomaterials with their toxicities at feasible exposures in a specified test system. In this study, we investigated effects of particle size and surface charge on in vitro mutagenic response and in vivo embryonic toxicity for newly synthesized silver nanoclusters (AgNCs) at human or environmental relevant exposure and compared the new findings with one of the most common nanoscale particles, titanium dioxide NPs (TiO_2 NPs as a positive control). We hypothesized that the interaction of the test system and physicochemical properties of nanomaterials are critical in determining their toxicities at concentrations relevant with human or environmental exposures. We assessed the mutagenicity of the AgNCs (around 2 nm) and two sizes of TiO_2 NPs (i.e., small: 5−15 nm, big: 30−50 nm) using a *Salmonella* reverse mutation assay (Ames test). The smallest size of AgNCs showed the highest mutagenic activity with the *Salmonella* strain TA100 in the absence and presence of the S9 mixture, because the AgNCs maintained the nano-size scale in the Ames test, compared with two other NPs. For TiO_2 NPs, the size effect was interfered by the agglomeration of TiO_2 NPs in media and the generation of oxidative stress from the NPs. The embryonic toxicity and the liver oxidative stress were evaluated using a chicken embryo model at three doses (0.03, 0.33, and 3.3 μg/g egg), with adverse effects on chicken embryonic development in both sizes of TiO_2 NPs. The non-monotonic response was determined for developmental toxicity for the tested NPs. Our data on AgNCs was different from previous findings on AgNPs. The chicken embryo results showed some size dependency of nanomaterials, but they were more well correlated with lipid peroxidation (malondialdehyde) in chicken fetal livers. A different level of agglomeration of TiO_2 NPs and AgNCs was observed in the assay media of Ames and chicken embryo tests. These results suggest that the test nanotoxicities are greatly impacted by the experimental conditions and the nanoparticle's size and surface charge.



INTRODUCTION

Silver nanomaterials, a group of common materials used in the food industry, are known for their broad-spectrum and high-efficacy antimicrobial activity without inducing drug resistance. Many silver-based compounds are used for physical, biological, and pharmaceutical applications,[1] and they have been incorporated into various polymer matrices and used alone or in combination with other antimicrobial materials to offer robust antimicrobial activity.[2] Silver nanoparticles (AgNPs) are allowed to be used as antimicrobial packaging materials that directly come in contact with food matrices or serve as disinfecting agents during the washing process in production. Similar to AgNPs, silver nanoclusters (AgNCs) are promising agents due to their ultrafine size, high chemical stability, and unexceptionable photostability, which are collections of a small number of metal atoms (typically 2−4 nm) and bridge the gap between isolated atoms and nanoparticles.[3] However, compared to AgNPs, AgNCs have more surface silver atoms available at the same concentration, which leads to a higher antimicrobial activity and low cytotoxicity on human colon cancer cells (HCT 116) in vitro.[4,5] Not much toxicity knowledge is available on AgNCs. Titanium dioxide nanoparticles (TiO_2 NPs) have been widely applied as food additives, integrated into food packaging, and as a whitening agent in confectionary products.[6] The bulk form of TiO_2 is authorized for use as a food additive (E171) with estimated worldwide production of 2.5 million metric tons/year by 2025.[7,8] E171 is a mixture of TiO_2 particles in a dispersed, aggregated, or agglomerated form, with up to 40% E171

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Table 1. Particle Size and Zeta-Potential Measurements of TiO_2 NPs and AgNCs

		DI water			bacterial culture media		
samples	conc. (mg/mL)	diameter (mm)	zeta potential (mV)	PDI	diameter (mm)	zeta potential (mV)	PDI
small TiO_2 NPs	0.125	24.4 ± 0.1	48.4 ± 3.1	0.38	148.9 ± 1.3	−24.7 ± 1.3	0.3−0.8
big TiO_2 NPs	0.125	34.6 ± 0.4	36.5 ± 2.3	0.23−0.49	453.8 ± 39.2	−20.9 ± 3.4	0.34−0.6
AgNCs	0.125	82.2 ± 11.3	−5.0 ± 0.5	0.17−0.60	73.8 ± 3.0	−10.1 ± 1.1	0.16−0.65

containing nano-size particles of dimensions smaller than 100 nm.[8]

The large use of nanomaterials has given rise to safety concerns, with the increased potential of exposure to these nanomaterials from food or migration from food contact materials to the food. Therefore, their adverse effect on human health needs to be carefully assessed. A ban on the sale of food products containing TiO_2 started on January 1, 2020, in France, based on the research findings published by the French Agency for Food reporting on the toxicity of TiO_2 in its nanoscale form.[9] This recent ban on TiO_2 as a food additive stressed the important perspective on nanotoxicities and suggested that TiO_2 NPs could be used as positive controls for comparison with newly synthesized nanomaterials.

TiO_2 NPs and AgNPs induced developmental toxicity in a few in vivo systems, most in aquatic and soil-related organisms.[10−12] Toxicity of NPs is strongly dependent on the surrounding environment and the properties of the NPs;[11] for example, the smaller size of citrate-coated AgNPs (20 nm) generated higher silver uptake and greater toxicity than the bigger ones (110 nm) in adult zebrafish.[13] Exposure of AgNPs in rat or mouse model induced substantial changes in the levels of inflammatory and serum biochemical markers without changes in the body weight after 29 days of treatment,[14] caused adverse histological changes from 15 days of treatment,[15] or resulted in long retention of silver in the brain and testes after 8 weeks of treatment.[16] The in vivo studies cannot be totally replaced and still serve as the standard because of the complexity of in vivo systems. Therefore, the chicken embryo serves as a promising in vivo model for the evaluation of nanotoxicity, considering the ethical, practical, and technical limits posed on conventional rodent and large animal experiments.[17]

The chicken embryo is a valuable model to evaluate embryonic and developmental toxicity, due to several advantages such as easy manipulation, no blood−brain barrier, and lack of maternal influence.[18] The chicken embryo has also been studied and concluded as an accessible and economical model for many contemporary biomedical research, including studies on mammalian stem cells and cancers.[19] The new applications of chicken embryos provide more valuable insights to determine the correlation between responses of chicken embryos and human diseases, which makes chicken embryos more useful for nanotoxicity studies. It is worth noting that chicken has about 20,000−23,000 genes in its 1 billion DNA base pairs, compared with the human count of 20,000−25,000 genes in 2.8 billion DNA base pairs.[20] The similarity in genome, anatomy, and development of chicken embryos is higher than that in fish embryos compared to the human. Furthermore, the wider presence of nanomaterials in soil, water, air, and food[21] can result in chicken embryo exposure to these nanomaterials, making this in vivo model a relevant testing system for nanotoxicity.[19] Various toxicity endpoints have been reported by different NPs in chicken embryos, including embryotoxicity and teratogenicity, antiangiogenic or pro-angiogenic activity, neurotoxicity, and brain damage.[22]

Nowadays, in vitro bioassays for genotoxicity have become popular due to fewer ethical concerns and faster turnout. Charles et al.[23] systematically reviewed in vitro genotoxicity data for TiO_2 NPs, yet it remained difficult to determine which inherent physicochemical properties (i.e., crystalline form, particle size, and/or surface coating) may explain the reasons for the diverging results observed. The mutagenicity and DNA damage produced by AgNPs has been reported before using different in vitro assays,[24] and the effect of coating and size on the genotoxicity of varied AgNPs and potential mechanisms have been discussed.[25,26] Therefore, it is essential to test new nanomaterials with known nanomaterials as positive controls using the same protocol to assess the impact of physicochemical parameters on the toxic responses.

We hypothesized that the interaction of test conditions and physicochemical properties of nanomaterials are critical in determining their toxicities. To evaluate potential toxicities of one newly synthesized AgNC (around 2 nm), this study aims to assess the mutagenicity using in vitro Ames test and evaluate the developmental toxicity by the in vivo chicken embryonic assay with nanomaterials added at the same ranges of concentrations/doses correlated with human or environmental exposure. We included two sizes of commercial TiO_2 NPs (5−50 nm) as positive controls to compare the toxicity of the new AgNC in response to particle size, surface charge, chemical composition, and test conditions. Different doses or concentrations of NPs were evaluated to determine if there was a nonmonotonic toxicity response (NMDR).

RESULTS

Characterization of TiO_2 NPs and AgNCs. To evaluate the integrity of NP samples in treatment with bacteria or chicken embryos, the three different NP samples were diluted with either DI water or bacterial culture media. The dilution concentrations, hydrodynamic particle sizes, zeta potentials, and polydispersity indexes (PDI) for particle size distributions are summarized in Table 1.

The two TiO_2 NPs (i.e., small: 5−15 nm, big: 30−50 nm) were commercial sample dispersions with original pH values at 1.56 and 1.89, respectively. The original pH value of the stock AgNC solution was 9.37. After dilution with DI water, the hydrodynamic diameters of the two TiO_2 NPs were at around 24.4 and 34.6 nm, with relatively low PDI; the zeta-potential values of TiO_2 NPs were highly positive in DI water (Table 1). Results were consistent with samples descriptions, and the strong acidic solutions ensured the stability and particle size homogeneity of TiO_2 NPs. AgNCs had a hydrodynamic diameter at 82.2 nm with a zeta potential of −5.0 mV after DI water dilution. The physicochemical properties of AgNCs were described in detail in our previous work.[5] The AgNCs showed a size distribution around 2 nm, and the zeta potential of AgNCs was −2.69 mV. Of note, the size of AgNCs was reported with the presence of PMAA and measured by

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Table 2. Mutagenicity of TiO_2 NPs and AgNCs in *S. typhimurium* Test Strains[a]

treatments (μg/plate)		number of revertants/plate in *S. typhimurium* strains (M ± SD) and mutagenic index (MI)					
		TA98 (±)		TA100 (±)		TA102 (±)	
negative control (DI water)		7 ± 1	19 ± 4	79 ± 4	77 ± 4	106 ± 1	82 ± 8
positive control		131 ± 4[b]**	388 ± 14[e]**	756 ± 47[c]**	754 ± 47[e]**	759 ± 38[d]**	734 ± 78[e]**
		Small TiO_2 NPs (5−15 nm)					
0.005	6 ± 1 (0.8)	11 ± 4 (0.6)	93 ± 1 (1.2)	81 ± 2 (1.0)		90 ± 1 (0.8)	72 ± 13 (0.9)
0.05	7 ± 1 (1.0)	16 ± 5 (0.8)	88 ± 4 (1.1)	82 ± 4 (1.1)		86 ± 4 (0.8)	69 ± 11 (0.8
0.5	4 ± 1 (0.6)	7 ± 2 (0.4)	80 ± 3 (1.0)	82 ± 6 (1.1)		85 ± 12 (0.8)	67 ± 11 (0.8)
5	11 ± 1 (1.5)	10 ± 1 (0.5)	65 ± 4 (0.8)	77 ± 1 (1.0)		81 ± 8 (0.8)	69 ± 12 (0.8)
50	10 ± 1 (1.4)	8 ± 2 (0.4)	65 ± 4 (0.8)	70 ± 1 (0.9)		81 ± 11 (0.8)	70 ± 1 (0.8)
		Big TiO_2 NPs (30−50 nm)					
0.005	11 ± 1 (1.5)	11 ± 4 (0.6)	77 ± 5 (1.0)	86 ± 3 (1.1)		110 ± 4[#] (1.0)	74 ± 14 (0.9)
0.05	12 ± 1 (1.7)	17 ± 7 (0.9)	70 ± 4 (0.9)	94 ± 2 (1.2)		89 ± 6 (0.8)	70 ± 13 (0.8)
0.5	8 ± 1 (1.1)	17 ± 3[#] (0.9)	89 ± 8 (1.1)	98 ± 4[*,#] (1.3)		93 ± 8 (0.9)	76 ± 7 (0.9)
5	7 ± 1 (1.0)	16 ± 1[#] (0.9)	74 ± 6 (0.9)	100 ± 3[*,##] (1.3)		87 ± 7 (0.8)	79 ± 11 (1.0)
50	9 ± 1 (1.2)	10 ± 3 (0.5)	81 ± 5[#] (1.0)	83 ± 1[##] (1.1)		91 ± 13 (0.9)	61 ± 12 (0.7)
		AgNCs (around 2 nm)					
0.005	10 ± 2 (1.4)	21 ± 3 (1.1)	110 ± 3* (1.4)	96 ± 4 (1.2)		110 ± 3 (1.0)	68 ± 5 (0.8)
0.05	12 ± 2 (1.6)	17 ± 3 (0.9)	98 ± 4 (1.2)	104 ± 3* (1.4)		101 ± 5 (0.9)	69 ± 4 (0.8)
0.5	9 ± 1 (1.2)	15 ± 3 (0.8)	26 ± 8 (0.3)	86 ± 4 (1.1)		101 ± 6 (1.0)	54 ± 6 (0.7)

[a]Differences were evaluated using one-way ANOVA followed by Tukey's test, and statistical significance was indicated by $p < 0.05$ and $p < 0.01$ (* means there are significant differences between the treated group and negative control. # means there is significant differences between two sizes of TiO_2 NPs at the same dose). Data was shown as mean ± s.d. revertants/plate from two independent trials. [b]Positive controls: 2-NF (1 μg/plate). [c]NaN_3 (1 μg/plate). [d]Mitomycin C (1 μg/plate). [e]2-AA (5 μg/plate).

Table 3. Mortality Rate and Malformation Rate of Chicken Embryos Treated with Two Sizes of TiO_2 NPs and AgNCs at Three Doses (0.03, 0.33, and 3.3 μg/g)[a]

treatment	non-treated	placebo group (PBS)	Small TiO_2 NPs (μg/g)			Big TiO_2 NPs (μg/g)			AgNCs (μg/g)		
			0.03	0.33	3.3	0.03	0.33	3.3	0.03	0.33	3.3
∑fertilized eggs	12	12	8	8	8	8	8	8	8	8	8
mortality rate	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)	12.5% (1)	0.0% (0)	12.5% (1)	0.0% (0)	0.0% (0)	0.0% (0)	12.5% (1)
malformation rate	0.0% (0)	0.0% (0)	0.0% (0)	25.0% (2)	0.0% (0)	0.0% (0)	12.5% (1)	0.0% (0)	0.0% (0)	0.0% (0)	0.0% (0)

[a]The data was summarized from the data in two trials. The number in parentheses represents the number of dead chicken embryo or malformation chicken embryo. Sizes of TiO_2 nanoparticles were small: 5−15 nm and big: 30−50 nm (anatase) and that of AgNCs was around 2 nm.

scanning electron microscopy (SEM) in the previous study. Because of its low surface charge, AgNCs were not stable with DI water dilution; therefore, they exhibited a larger particle size and PDI measured by DLS.

All three nanomaterials aggregated aggressively when mixed with bacterial culture media at high concentrations (>1.25 mg/mL, data not shown), which indicated that the phosphate buffered solution (PBS) or other buffering compounds in bacterial culture media could disrupt the stability of NP dispersion. Mixed with media at a final concentration of 0.125 mg/mL, the particle size of small and big TiO_2 NPs increased to 148.9 and 453.8 nm, with high PDI; the zeta potentials of the two TiO_2 NPs changed from highly positive values to negative values. Interestingly, the particle size of AgNCs stayed at the similar level of particles mixed with DI water, while the zeta-potential values of AgNCs slightly increased. The different behavior of particle aggregation could be explained by the different original pH values of the NPs. Apparently, the media will cause a dramatic change in the size distribution of NPs; we will use the measured size in certain media as the actual size of NPs for further discussion.

Ames Test on TiO_2 NPs and AgNCs. For the Ames test, three *Salmonella* tester strains (i.e., TA98, TA100, and TA102) were used with a pre-incubation method. These strains of *Salmonella typhimurium* were used to detect point mutations, including the substitution, addition, or deletion of one or a few DNA base pair(s). Shown in Table 2, the positive control of each bacteria strain, with or without S9, produced a statistically significant increase in the number of revertant colonies and in the historical ranges of our laboratory, which confirmed the sensitivity and accuracy of the test system. Exposure to AgNCs at 0.005 and 0.05 μg/plate (TA100) increased revertant numbers and had a mutagenic index (MI) of 1.4 in the absence and presence of S9 mixture. This finding suggested that AgNCs showed the sign of mutagenic activity (0.005 and 0.05 μg/plate), even though it did not exceed the critical value of 2.0. For AgNCs > 0.5 μg/plate; no colony was found, indicating strong antimicrobial efficacies of AgNCs.

No increase in the number of revertants to three *Salmonella* tester strains was determined after exposure to the small sizes of TiO_2 NPs tested at 0.005 to 50 μg/plate compared with the negative control, while the significantly increased numbers (MI 1.3) were observed for the big TiO_2 NPs in TA100 strains with S9 activation. Of note, the MI levels were high (1.5 or 1.7) for big TiO_2 NPs in TA 98 without S9 activation. But one should be careful to explain it as an indication of high mutagenic activity, because the differences between all treatments in strain TA 98 and negative control were not significant.

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Table 4. Ratio of Embryo to Egg Weight, LSI (%), and Weight of Embryo and Organs of Chicken Embryo at Day 18, after Injection of Two TiO_2 NPs and AgNCs[a]

groups	dose (ng/g)	REEW	LSI (%)	weight		
				embryo	liver	heart
non-treated	N/A	0.39 ± 0.021	2.26 ± 0.06	24.34 ± 2.23	0.59 ± 0.01	0.22 ± 0.01
placebo	PBS	0.36 ± 0.021	2.20 ± 0.10	24.03 ± 1.34	0.51 ± 0.02	0.22 ± 0.02
small TiO_2 NPs	0.03	0.40 ± 0.014	2.17 ± 0.11	22.34 ± 3.23	0.52 ± 0.01	0.19 ± 0.03
	0.33	0.39 ± 0.023	1.87 ± 0.44	22.03 ± 2.12	0.46 ± 0.15	0.20 ± 0.07
	3.3	0.36 ± 0.020	2.10 ± 0.09	23.02 ± 2.45	0.48 ± 0.04	0.20 ± 0.01
big TiO_2 NPs	0.03	0.40 ± 0.014	1.99 ± 0.23	24.01 ± 1.89	0.48 ± 0.08	0.20 ± 0.03
	0.33	0.37 ± 0.012	2.06 ± 0.06	22.32 ± 4.01	0.46 ± 0.04	0.20 ± 0.01
	3.3	0.40 ± 0.006	2.27 ± 0.01	23.02 ± 3.27	0.58 ± 0.01	0.21 ± 0.00
AgNCs	0.03	0.40 ± 0.007	2.00 ± 0.11	21.82 ± 3.44	0.48 ± 0.00	0.21 ± 0.03
	0.33	0.40 ± 0.020	2.18 ± 0.42	22.23 ± 1.28	0.54 ± 0.08	0.21 ± 0.02
	3.3	0.38 ± 0.009	2.03 ± 0.00	23.21 ± 2.03	0.50 ± 0.01	0.23 ± 0.02

[a]Differences were evaluated by one-way ANOVA and followed by Tukey's test, and statistical significance was indicated by $p < 0.05$ (*$p < 0.05$). REEW: ratio of embryo to egg weight, LSI: liver somatic index. All values are expressed as mean ± s.d. from two independent trials. Sizes of TiO_2 nanoparticles were small: 5−15 nm and big: 30−50 nm (anatase) and that of AgNCs was around 2 nm.

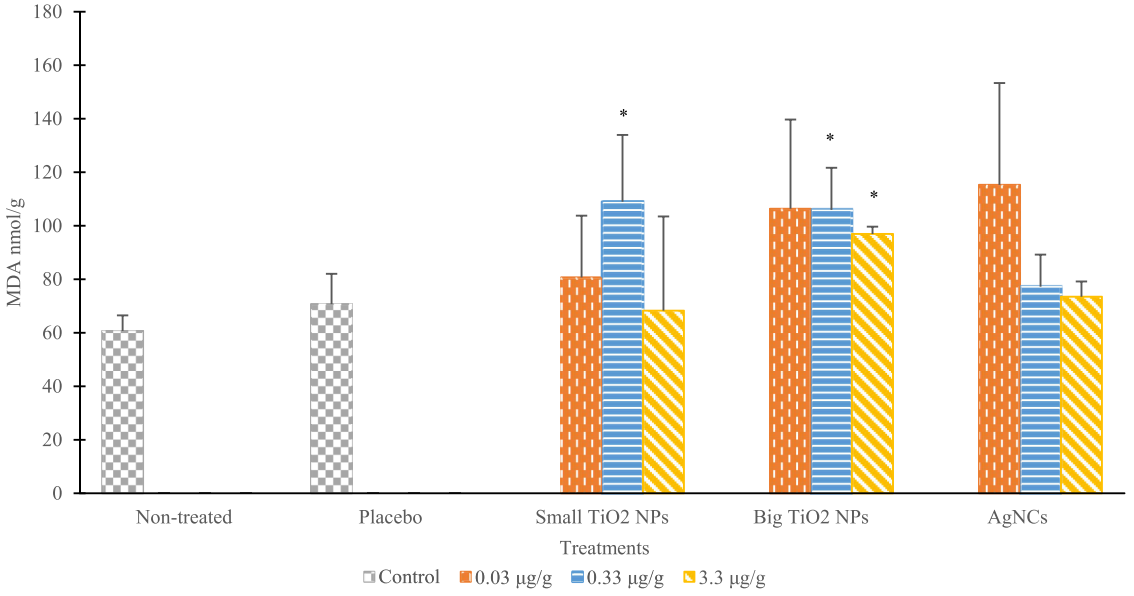

Figure 1. Impacts of two TiO_2 NPs and AgNCs on MDA of livers in chicken embryos. Values are expressed as mean ± s.d. from two independent trials. Sizes of TiO_2 nanoparticles were small: 5−15 nm and big: 30−50 nm (anatase) and that of AgNCs was around 2 nm. Differences were evaluated using one-way ANOVA followed by Tukey's test between each sample group and non-treated group, and statistical significance was indicated by $p < 0.05$ (*, $p < 0.05$).

Additionally, we observed that at the same dose, the exposure to larger size TiO_2 NP (30−50 nm) resulted in more revertant colonies than to those in smaller size treatment (5−15 nm). As indicated in Table 2, compared to the small TiO_2 NPs at the same dose, the significantly increased ($p < 0.05$) number of revertants in the big NPs group was found in all three strains. This finding was more apparent at the higher dosage ranging from 0.5 to 50 μg/plate.

Embryonic Toxicity and Liver Oxidative Stress for TiO_2 NPs and AgNCs. In this study, we assessed the developmental toxicity of two sizes of TiO_2 nanoparticles (i.e., small: 5−15 nm, big: 30−50 nm, anatase) and AgNCs (2.2−2.4 nm) at three doses (0.03, 0.33, and 3.3 μg/g) using a chicken embryo model. Two negative control groups were included: an untreated group and a placebo group (injected PBS solution). No death or deformed embryos were found in these control groups (Table 3). However, 12.5% death rate of chicken embryos was observed after exposure to small TiO_2 NPs and AgNCs at 3.3 μg/g while to a lower dosage for big TiO_2 NPs (0.33 μg/g). The result indicated that big TiO_2 NPs may have the embryo toxicity with NMDR. Moreover, growth retardation (stunting and edema) was observed for both TiO_2 NPs at 3.3 μg/g, but not for AgNC treatment at all dosages.

As shown in Table 4 the ratio of embryo weight to egg weight (REEW) slightly increased after exposure to two sizes of TiO_2 NPs and AgNCs at all doses compared to the placebo group; however, the difference was not significant ($p > 0.05$). The increase in this index might be due to a systemic edema, one of a gross pathological change.[27] Additionally, liver somatic index (LSI) is a general indicator of health and response to the environmental contaminant exposure. The LSI value of each group was around 2.1%, with no significant difference among each group. The heart weight between each group had only small fluctuations around 0.20 g (Table 4).

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The non-treatment group had the highest liver weight of 0.59 g. Among all the treatment groups, the exposure of big TiO_2 NPs at 0.33 μg/g resulted in the lowest liver weight of 0.46 g, which decreased by 22% when compared with those in the non-treatment group (p = 0.054). The potential of NMDR is present if the dose response is considered for the 3 test doses of big TiO_2 NPs, which is similar to its pattern of embryonic death.

Moreover, the malondialdehyde (MDA) levels for each treatment group were determined in order to evaluate the oxidative stress level of chicken fetal liver (Figure 1). As one important toxicity mechanism caused by the generation of excess reactive oxygen species (ROS) exceeding the antioxidant capacity in cells, oxidative stress can lead to cell death and the production of toxic and reactive aldehyde metabolites, known as free radicals.[28] As the primary final product of lipid peroxidation, the MDA level is commonly used to evaluate oxidative damage.[29] Among the total 11 experimental groups, the non-treated group had the lowest MDA level at 60.67 nmol/g (Figure 1). Even though there were no increase of MDA levels up to 2-fold, significant differences were observed for the 3 treatment groups when compared to the non-treated group ($p < 0.05$). Compared to the non-treated group, the big TiO_2 NPs at 0.33 and 3.3 μg/g doses and the small TiO_2 NPs at 0.33 μg/g significantly increased the MDA value ($p < 0.05$). Big TiO_2 NPs seemed to show more developmental toxicity and warranted further investigations. The potential adverse effect in the chicken embryo from big TiO_2 NPs might be due to the generation of high oxidative stress or the anti-angiogenesis effect[30] but remains to be confirmed.

DISCUSSION

Chicken Embryonic Assay Is a Cost-Effective In Vivo Method for the Evaluation of Nanotoxicity. Thus far, only a few in vivo studies reported have the embryonic toxicity of two major food-related NPs (TiO_2 NPs and AgNPs) and none for AgNCs using chicken embryo or zebrafish embryo model.[31,32] Using the chicken embryo model, the developmental deformity (omphalocele and flexed limbs) and interference in canonical Wnt signaling were found for TiO_2 NPs (88.6 nm) at low doses only (10 and 25 μg/mL), while the inhibition on blood vessel formation and the lymph follicles in the bursa of Fabricius were reported after AgNPs exposure.[33,34] The chicken embryo model can provide fast and precise assessment in developmental effects, tissue morphology, and gene expression level related to lipid metabolism and hormone homeostasis.[35,36] Additionally, compared with other traditional in vivo tests, the chicken embryo model using early-stage animals has been proved to be cost-effective and efficient.[37] Our findings also suggested that chicken embryo is a valuable in vivo model for evaluating the toxicity of nanomaterials. It is important to use the relevant dosage of the two test nanoparticles in the chicken embryonic assay, and the design of the experiments should include the correlation of human exposure levels with the treatment dosage to better reflect the impacts of the nanomaterials on the human health. In our study, we chose the test dosage using the available human consumption levels or environmental exposure levels of the two nanoparticles as well as the human equivalent dose factor (18.5 for conversion from chicken, Agency for Toxic Substances and Disease Registry) and human safety factor.

The estimated daily human consumption of food-grade E171 TiO_2 was 0.2−2 mg/kg body weight (bw), while much higher average daily consumption is found (i.e., 10.4 mg/kg bw) for children, with up to 32.4 mg/kg bw per day (EFSA 2016) due to higher contents of E171 TiO_2 in candies and chewing gums.[38] A previous study reported a level of 40% of TiO_2 NPs in the food-grade E171, which makes the estimated daily human consumption of TiO_2 NPs at around 0.08−0.8 mg/kg bw. Considering human equivalent dose factor of 18.5 for conversion from chicken and human safety factor (10) in animal study,[39,40] the equivalent human exposure level for the median dose (0.33 mg/kg) in our chicken embryonic assay was $0.33 \times 18.5/10 = 0.61$ mg/kg; this was comparable with the estimated daily human consumption exposure but lower than the daily exposure level in children. In a report[41] provided by the World Health Organization, it was suggested that where Ag salts are used for drinking-water disinfection, a concentration of 0.1 mg/L could be tolerated without risk to health. The allowable concentration is slightly higher than the low dose used in this study.

Chicken embryos are more sensitive to different variety of chemicals and physical agents, which is an important factor for first-line screening model. Carnegie stage is a standardized system of 23 stages to compare different embryo development among species.[42,43] This shows that the chicken embryo model is efficient and the selection of embryos at different developmental stage for exposure of test chemicals is easy to perform. The animal development stage should be considered in toxicity studies because the treatments might cause more negative health effects on the sensitive developmental period. In a recent study,[44] exposure to E171 TiO_2 (contain 17−36% of nanoparticles) in fruit flies (*Drosophila melanogaster*) at an estimated daily human consumption concentration (0.014 mg/mL) for 20 generations resulted in a change in the normal developmental and reproductive dynamics, increased genotoxicity, and several other negative health impacts. The larval stages were at a higher risk of sustaining damages from E171 as they had a slower elimination rate of TiO_2 compared to the adults, and the genotoxic effect of E171 was statistically higher in each subsequent generation compared to the previous one. Additionally, one study using the zebrafish embryos demonstrated that TiO_2 NPs would accumulate in the brain of zebrafish larvae and result in ROS generation and loss of dopaminergic neurons.[45] Generally, the embryonic toxicity of NPs is affected by the size, concentration, different ion types, agglomeration formation, and surface charge along with different test embryonic systems. For example, the toxicity of TiO_2 NPs could be related to the reactivity of the NPs due to different environmental complex. Although only 17−35% of primary particles in food-grade TiO_2 were below 100 nm, they were more active (bound with more cationic dyes) than P25 TiO_2.[46]

In our study, the NMDR is determined for developmental toxicity for the tested NPs. The NMDR has been reported in endocrine-disrupting chemical response, and the nanomaterials have been proved as one type of endocrine disruptors.[47,48] Unlike the traditional dose−response curve, the response curve slope in NMDR will change, which means that the lower dose might show a higher effect than that of the higher dose. Therefore, it is not uncommon to observe that the highest response was found in the middle dose.

Particle Size and Chemical Compositions of Nanoparticles Are Important for Understanding the Mutagenicity of Nanomaterials. AgNCs are a new kind of silver nanomaterial, and two of our co-authors[5] have previously

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reported strong antimicrobial activities from the same AgNCs used in our study. The potential mutagenicity risk is determined in our study for AgNCs (around 2 nm) at 0.005 and 0.05 μg/plate using the Ames test. Our data on AgNCs was different from that of previous findings on AgNPs,[49,50] and the discrepancy might be related to the different particle sizes, coating, and chemical compositions of silver nanomaterials. Considering the characterization of AgNCs, the particle size did not change much after adding in the bacterial culture medium, and the size distribution range was broad, which means that the smaller size of particles around 3−4 nm may still exist. We also used much lower dose levels ranging from 0.005 and 0.05 μg/plate, which did not have strong antimicrobial effects. A previous study reported no mutagenic activity with or without S9 for 40−59 nm of AgNPs at 100 to 500 μg/plate using four *S. typhimurium* strains (TA98, TA100, TA1535, and TA1537) in the Ames tests.[49] Noteworthy, the same AgNPs showed genotoxicity (measured as one type of DNA breakage) in mammalian cell lines with a dose-dependent response range from 0.01 to 10 μg/mL by Comet and MN assays.[49] Based on their research findings,[49] it was recommended to develop a suitable battery of assays for the assessment of nanoparticle genotoxicity with considerations of particle size and coating. In another study,[50] the tested AgNPs (4−12 nm) had a major increase in cytotoxicity above concentrations of 20 g/mL, so the toxicity limited the doses for Ames test to be assayed at 2.4−38.4 μg/plate, and no increases in mutant frequency over the vehicle control were found for the assayed concentrations. However, in the same study, the micronucleus frequency was increased (3.17-fold) by AgNPs (at 30 μg/mL) in the human lymphoblastoid TK6 cells, showing a weak positive genotoxicity response.[50] In summary, both previous studies reported different genotoxicity responses when using different assays and concentrations. Our study added new findings on mutagenicity of the different silver nanomaterials at much lower concentrations. Because the potential genotoxicity is closely related to carcinogenesis after exposure to humans, more study is needed to confirm the genotoxicity potential of the AgNCs.

In our study, AgNCs showed the highest mutagenic activity in *Salmonella* strain TA100 among three groups but had less chicken embryonic toxicity than TiO_2 NPs. The highest mutagenic activity of AgNCs can be explained by the particle size effect. Although all three NPs agglomerated when mixed with bacterial culture media, the particle size of AgNCs maintained at nano-size scale. It is generally assumed that solubility may increase as particle size decreases; therefore, the higher mutagenicity may be explained by the smaller size of Ag agglomerates, which can release more Ag^+ ions to directly interact with DNA. The release of Ag^+ ions has not been measured in the present study; however, our collaborator tested the Ag^+ ion releasing profile of AgNC embedded zein films submerged in water and determined that the small AgNCs released much more Ag^+ ions than other AgNPs with larger sizes (Figure S4, ref 13)

In a review about the underlying mechanisms of toxic effects of Ag Nanoparticles,[51] the authors summarized substantial evidence, suggesting that the effects induced by AgNPs are mediated by Ag ions that are released from the surface of the particles. For example, the use of electron spin resonance showed that the active surface of AgNPs can directly induce the generation of free radicals. Furthermore, the dissolution of AgNPs into Ag ions triggers the production of hydroxyl radicals in acidic endo/lysosomes.[52] The cytotoxicity and genotoxicity of bovine serum albumin (BSA)-coated AgNPs on Chinese hamster ovary cells were investigated and revealed that both BSA-AgNPs and Ag ions generated ROS that oxidized DNA to oxidative adducts and induced the formation of micronuclei.[53]

Compared with the genotoxicity findings on silver nanomaterials, more studies with conflicting results are reported on TiO_2 NPs. In a previous report for TiO_2 NPs in the Ames test, it was suggested that no mutagenicity sign was detected for TiO_2 NPs at 10 nm; however, they conducted only the Ames test without S9 activation.[54] In another study, positive genotoxicity responses were detected for the TiO_2 NPs (100 nm) on plants and human lymphocytes using the Comet assay and DNA laddering test, and the highest increase in the extent of DNA damage was observed at 4 mM.[55] In our study, we determined that the TiO_2 NPs became bigger when mixed with culture media, which may explain the insensitivity of some Ames tests and discrepancies with other studies. In addition, various intrinsic physicochemical properties could contribute to the inconsistent results on mutagenicity of NPs. In some studies, smaller nanoscale particles of TiO_2 would be more genotoxic than their micro-sized counterparts,[56,57] while others showed no significant effects of size.[58,59] Furthermore, the crystalline form seems to have an impact on the genotoxic effect of TiO_2 NPs. Anatase is generally expected to be more cytotoxic than rutile, because of its stronger photocatalytic properties.[60] However, some studies have shown that more severe toxic effects may be induced with the rutile form or mixture of rutile and anatase.[58,61,62] All these clearly indicate that it is essential to report both test system compositions and physicochemical properties of nanomaterials with their toxicities.

Interaction between Different Surface Charged Nanoparticles and the Test System Compositions Impact Nanotoxicities. Our results revealed that the big TiO_2 NPs, which had a larger particle size in both DI water and bacterial culture media, showed more adverse chicken embryonic effect and a higher mutagenic activity than the small TiO_2 NPs. The result may be explained by the higher MDA level observed in the big TiO_2 NPs, rather than revealing the inverse size effect of TiO_2 NPs. It has been reported that the MDA, as a vital biomarker of lipid peroxidation, serves as an important contributor to DNA damage and mutation in bacterial and mammalian cells.[63] The oxidative stress has been observed as a contributor of the mutagenicity in the Ames test after TiO_2 NPs exposure.[64] The mechanism of mutagenicity induced by NPs could be the result of two factors: one is the size-dependent internalization of the particles and the other is the generation of oxidative stress by the catalytic potential of the particles.[23] In the case of TiO_2 NPs, because their agglomerated forms exceeded the nano-scale size, the generation of oxidative stress from the NPs contributed mainly to the observed toxicity.

In addition, we did not neglect the impacts of test conditions on the toxicity results of in vitro and in vivo tests. The nominal particle sizes of AgNCs, small TiO_2, and big TiO_2 NPs were 2, 5−15, and 30−50 nm, respectively. However, the NPs were not stable and underwent different levels of agglomeration depending on multiple media properties (ionic strength, composition, pH, viscosity, and hydrophobicity). The media properties can affect the toxic response of NPs by modifying particle aggregation, cellular uptake, and bioactivity.[23] In this

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study, the original TiO_2 NP (small TiO_2: 1.56, big TiO_2: 1.89) dispersions were highly positively charged under acidic pH and the pH was dramatically different from the pH of bacterial culture media (usually near neutral); therefore, the agglomeration of TiO_2 NPs can be primarily explained by the pH or buffering ability of the media.[65] Slurries and flocs were observed due to the significant agglomeration when the solution pH was near the isoelectric point (IEP) of NPs; in the case of TiO_2 NPs, the IEP was around 6.2. The particle size of AgNCs was also affected by environmental solution but most likely due to the dilution of its inherent stabilizer. The original pH of the AgNCs used in this study was 9.37 and mixing with bacterial culture media increased their surface charge, indicating that the pH of AgNCs was away from its IEP. Therefore, AgNCs were able to stay at the nano-sized scale in Ames tests, explaining their strong antimicrobial activity and mutagenicity.

Furthermore, egg albumen contain proteins as the major portion that could cause the well-known protein corona formation,[66,67] which may alter nanoparticles dissolution in vivo when using the chicken embryonic assay. Albumen proteins such as ovalbumin contain four free thiol groups, and the role of organic thiols in the dissolution of AgNPs is complex, depending on the concentration. If the thiol concentration is too low, a thiolate ligand shell forms around the NP and protects it from further dissolution; at higher concentrations, AgNPs can release Ag(+1) species by forming a soluble silver thiolate complex.[21] Solubility is also dependent on the pH, which affects the protonation state. In the chicken embryonic assay, the pH of the egg liquid during embryogenesis was near neutral and became alkaline with the embryo growth.[68] Due to the possible interaction between AgNPs and the test system compositions, further studies on the NP dissolution and distribution in chicken embryos are needed for understanding the chicken embryonic toxicity of AgNPs.

CONCLUSIONS

Among the three different sizes of NPs, the small AgNCs had the highest mutagenicity, because the AgNC particles maintained the smallest nano-size scale in the Ames test. For TiO_2 NPs, the size effect was interfered by the agglomeration of TiO_2 NPs in media and the generation of oxidative stress from the NPs. More adverse effects on chicken embryo development were observed in both TiO_2 NPs than in AgNCs, which were well correlated with the MDA levels in the liver of chicken embryos. The NMDR is determined for developmental toxicity for the tested NPs. The test system properties had a major impact on the particle size of NPs both in vitro and in vivo. It is recommended to take protocol conditions into consideration and carefully select stable and homogenous solutions of NPs to help reveal the real effects of physicochemical parameters on the toxicity of NPs.

EXPERIMENTAL SECTION

Materials. Two sizes of TiO_2 NPs (i.e., small: 5−15 nm, big: 30−50 nm, anatase) were purchased from US research nanomaterials (TX, USA), and the AgNCs was kindly provided by Dr. Qin Wang's group from the University of Maryland. *S. typhimurium* tester strains (TA 98, TA 100, and TA 102), Oxoid nutrient broth no. 2, metabolic activation mixture (S9), minimal glucose agar, and top agar were purchased from Molecular Toxicology Inc. (Boone, NC, USA). The PBS was purchased from Fisher Scientific.

Characterization of Nanoparticles. The AgNCs were synthesized by reducing $AgNO_3$ by 60 min UVA exposure with polymethacrylic acid (PMAA) as a stabilizer and have a size distribution around 2 nm confirmed by SEM.[5] Two sizes of TiO_2 NPs (i.e., small: 5−15 nm, big: 30−50 nm, anatase) were aqueous solutions with a stock concentration of 150 mg/mL. Particle size distribution and zeta potential of the three samples were measured by a Wyatt mobius DLS Zeta Potential (Wyatt Technology, CA, USA). The samples were diluted with either DI water or bacterial culture media (without S9 mixture). Particle size and zeta potential were calculated by a software with the Smoluchowski model. All measurements were performed at 25 °C in triplicate.

Ames Test. Ames test was conducted using the *S. typhimurium* tester strains TA98, TA100, and TA102 pre-incubated as described by earlier[69] and improved by Woodruff et al.[54] The strains were incubated overnight in Oxoid nutrient broth no. 2 at 37 °C and 100 rpm to reach cell densities of 1−2 × 10^9 CFU/mL. The metabolic activation mixture (S9) from livers of Sprague−Dawley rats was freshly prepared before each test. Then, 0.05 mL of test compounds (diluted by autoclaved DI water) was added to 0.5 mL of S9 mixture (or 0.5 mL PBS in treatments without S9 mixture) and 0.1 mL of bacterial culture, and the mixture was incubated at 37 °C for 4 h. The test concentrations for two sizes of TiO_2 NPs were 0.005, 0.05, 0.5, 5, and 50 μg/plate, whereas the doses for AgNCs included only 0.005, 0.05, and 0.5 μg/plate due to its high antimicrobial activity. Then, 2 mL of top agar was added, and the mixture was poured on to a plate containing minimal agar. After 48 h incubation, the His+ revertant colonies on plates were counted manually. Each test was repeated in two independent trials and duplicated for each trial.

Chicken Embryonic Assay. Fertilized eggs (60 ± 2.4 g) were obtained from the University of Delaware research farm. The eggs were weighed and randomly divided into 11 groups and placed in the incubator at 38 °C and 60% humidity for 7 days. These groups included one untreated control group, one placebo group that received PBS, and nine experimental groups that were injected with three kinds of nanoparticles at concentrations of 10, 100, and 1000 μg/mL. The TiO_2 NPs and AgNCs were all diluted by autoclaved DI water.

At day 7 of incubation, the eggs were candled to locate the air cell, and a suitable location was selected for injection and marked. A hole was drilled at the marked location, and 0.2 mL of each chemical solution or vehicle control was injected slowly into the eggs, yielding 0.03, 0.33, and 3.3 μg/g egg. The hole was then sealed with Duco Cement and eggs were placed back in the incubator. At day 18, all eggs were placed in the refrigerator overnight to euthanize the chicken embryos, and then, the eggs were opened at day 19.

The number of dead embryos was recorded every 2 days by candling. The embryos were weighted and then dissected. Liver and heart weight were taken. LSI was calculated for all individuals (LSI = liver mass/body mass × 100%). The liver samples from each group were collected for lipid peroxidation measurement. MDA, as an index of lipid peroxidation, was measured in chicken embryonic liver samples following the protocol of TBARS (TCA Method) Assay Kit (no. 700870) (Cayman Chemical, MI, USA). MDA reacts with thiobarbituric acid (TBA) as a thiobarbituric acid reactive substance to produce a red complex that has peak absorbance at 532 nm

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using a microplate spectrophotometer (BioTek Synergy^2). Results were expressed as nmol/g tissue. In addition, the pH value of liquid in chicken embryo egg was measured at day 13 and day 17 using a pH meter (Fisherbrand, MA, USA).

Data Analysis. The results were analyzed with the statistical software package JMP (JMP PRO 13). In the chicken embryonic assay, the significant difference in morphological, developmental endpoints, and lipid oxidation levels were determined using a one-way ANOVA followed by Tukey's test for multiple comparisons between treatment group and the non-treated control group or placebo group (PBS treatment). Changes were considered statistically significant if $p < 0.05$. In the Ames test, the data (revertants/plate) was assessed by means of the one-way ANOVA followed by Tukey's test. The MI was also calculated for each concentration (MI = #revertants per plate with the test compound/#revertants per plate with the negative (solvent) control). A tested compound was considered mutagenic when a 2-fold increase in the number of mutants (MI mutagenic index ≥ 2) was observed in at least one concentration or the sample was considered to present signs of mutagenicity.[70] If there was only $p < 0.05$ when compared with the solvent control group through one-way ANOVA followed by Tukey's test but the increase was < 2-fold, we reported it as sign of mutagenicity.

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Notes

The authors declare no competing financial interest.

The authors confirm that the data supporting the findings of this study are available within the article.

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