Toxicity effects of four typical nanomaterials on the growth of *Escherichia coli*, *Bacillus subtilis* and *Agrobacterium tumefaciens*

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Abstract To offer an insight into the toxicity of nanomaterials (NM) on the growth of bacteria, *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*) and *Agrobacterium tumefaciens* (*A. tumefaciens*) were exposed to nano-Au, nano-Ag, nano-Fe and fullerene (C₆₀) in this study. As an effective bactericide, nano-Ag induced high toxicity on these three bacteria; C₆₀ could inhibit their growth; however, *B. subtilis* and *E. coli* could recover as exposure time extended. Nano-Au and nano-Fe had hardly any effect on three bacteria. *A. tumefaciens* showed the lowest resistance and slowest growth rate during exposure. Images obtained by scanning electron microscope (SEM) revealed that nano-Ag could cause damage to the cell structure of three bacteria at 1 µg/mL. Slight damage on *E. coli* was found when exposed to C₆₀, whereas no obvious physical damage was found after exposure to nano-Au or nano-Fe. It is assumed that surface activities of NM might be responsible for the different toxic effects on these bacteria.

Keywords Nano-Au · Nano-Ag · Nano-Fe · C₆₀ · Bacteria · Growth curve · Physical damage

Introduction

Due to their unique physical and chemical properties, nanomaterials (NM) are nowadays being widely used in daily life. These nanotechnology-based consumer sprays are now leading to increased risk of exposure to humans and other organisms (Nazarenko et al. 2011). The consequent concerns have resulted in considerable researches on their toxicity (Nel et al. 2006; Larese et al. 2009; Song et al. 2009).

Metal oxide nanoparticles with special structure are supposed to have good antibacterial activity. As a potent antimicrobial agent, silver nanoparticles raised great concerns and its toxicity to varieties of microorganisms has been well studied (Rai et al. 2009; Sharma et al. 2009). Nano-Fe has been extensively studied to eliminate pollutants such as chlorinated organic compounds and metal ions (Zhang 2003; Varanasi et al. 2007), and was not considered to be bio-compatible to organisms. It was reported that iron nanoparticles could cause oxidative damage in rats and medaka fish (Zhou et al. 2003; Li et al. 2009). Unique optical properties of gold nanoparticles enable nano-Au to be an effective material in the development of highly sensitive biosensors for DNA analysis, but few studies have reported its potential toxicity (Kim et al. 2007). Fullerene C₆₀ is noted for its multi-functional uses in a variety of biological applications, such as imaging probes, antioxidants and drug carriers (Levi et al. 2006). C₆₀ has been indicated to have genotoxicity, cytotoxicity and potential ability to induce reactive oxygen species in cell lines, rats and fish (Zhu et al. 2006; Jacobsen et al.

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2008; Folkmann et al. 2009). Different reasonable hypotheses were further proposed for the toxicity of those metal NM and fullerene (Fang et al. 2007; Tang et al. 2007; Lyon and Alvarez 2008; Johansen et al. 2009). However, the mechanisms of toxicity have not been clearly discerned.

Microorganisms, including bacteria, fungi and so on, are widely distributed in the environment. They can cause lots of diseases in plants, animals and humans (Lepp et al. 2004; Leslie 2009). However, the cycles of nutrients going through the environment are mainly driven by these microorganisms. Soil microorganisms play an important role in the acquisition and transfer of nutrients from the soil, including decomposition, mineralization, nitrogen cycling, storage and release of nutrients, and carbon cycling. They can also contribute to the removal of pollutants in the environment (Ledin and Pedersen 1996; Richardson 2001; Tiedje and Donohue 2008). Destruction of their community structure can cause disturbance of the substance and energy circulation in the ecosystem. Though certain studies have focused on the effects of single NM on microorganisms, few researches have compared the toxicity between various NM, and especially their effects on the communities of microorganisms.

In this study, the effects and different actions of four typical NM on the bacterial cells were studied. Both Gram-positive (*Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli* and *Agrobacterium tumefaciens*) were chosen because they were all well-studied soil microorganisms, and could be easily cultivated under laboratory conditions. Serial doses of nano-Au, nano-Ag and nano-Fe, as well as fullerene C₆₀ were added to the culture medium of the bacteria. The growth of bacteria during exposure was measured and their growth curves were established. Then, the integrity of cells was examined after exposure, using scanning electron microscopy (SEM). An overview on the toxicity of NM and their potential mechanisms were provided, offering an insight into their influence on various microorganisms.

Materials and methods

Chemicals

Nano-Ag (aqueous, 99.99%) was purchased from Shanghai Huzheng Nano Technology Company. Nano-Au (aqueous, 99.99%) and nano-Fe (powder, dissolved in double-distilled water later) were obtained from Toda Kogyo Corporation. Fullerene C₆₀ (powder 99.5%) was purchased from Alpha Aesar Company. C₆₀ solution was prepared according to the reference Yamakoshi et al. (1994). C₆₀ (0.8 mg) dissolved in toluene (1.0 mL) was added to

2.0 mL CHCl₃ containing 100 mg of polyvinylpyrrolidone (PVP K-30, 95%). The solution was well mixed and then the solvent was thoroughly evaporated at 50° under vacuum. The residue was dissolved in 2.0 mL water and the solution was mixed by ultrasonic for 10 min to obtain a clear brown solution. The size and shape of nanoparticles were visualized and determined by transmission electron microscope (JEM-100CXII).

Bacterial culture

Gram-negative *E. coli*, *A. tumefaciens* and Gram-positive *B. subtilis* were obtained from State Key Laboratory of Agricultural Microbiology, Huazhong Agriculture University. The bacteria were maintained and incubated in shaking incubators, using LB medium (Tryptone:yeast extract:NaCl = 2:1:2), at a temperature of 37°C for *E. coli* and *B. subtilis*, 28°C for *A. tumefaciens*.

Growth experiment

Eugonic colonies of the three bacteria were inoculated into 90 µL fresh LB medium and then transferred into 96-well plates. The suspensions were then thoroughly mixed with 10 µL diluted solutions of NM, giving exposure doses of NM at 1 and 5 µg/mL for nano-Ag, 1 and 5 µg/mL for nano-Au, 50 and 100 µg/mL for nano-Fe, 20 and 40 µg/mL for C₆₀, as well as 0.125 and 0.25% PVP to exclude the effect of PVP in C₆₀ solution. Controls were set up in the absence of NM, and each treatment contained four replicates. The plates were then incubated in a shaking incubator for 10 h, at 37°C for *E. coli* and *B. subtilis*, 28°C for *A. tumefaciens*. The growth rates and bacterial concentrations were determined by measuring optical density (OD) at 600 nm every hour, using LB medium as the spectrophotometer reference.

Scanning electron microscope

Scanning electron microscope (SEM) was used later to examine the surface morphology of these bacteria. The following exposure concentrations of the NM were selected to carry out this experiment: 10 µg/mL for nano-Au, 1 µg/mL for nano-Ag, 100 µg/mL for nano-Fe and 20 µg/mL for C₆₀, and 0.25% PVP as a control to C₆₀. A shaking incubator followed the same procedure as the growth experiment for 10 h. The bacteria were harvested by centrifugation (3,000 rpm for 10 min) and washed twice in sterilized double-distilled water. Furthermore, bacteria exposed to C₆₀ and PVP were partly collected at 7 h. Then the samples were fixed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer at pH 7.2) overnight at 4°C, followed by three rinses in double-distilled water. Again, the samples

were washed by sterilized double-distilled water for two times, then serially dehydrated in increasing concentrations of ethanol: 30, 50, 70, 85, 95, 100 and 100%, and finally dried in a vacuum freeze dryer. After coating with gold by sputter coater (E-1010, HITACHI), the cellular structure of these bacteria was observed under scanning electron microscope (S-3000 N, HITACHI and JSM-6390).

Results

As shown in Fig. 1, the size and shape of those nanoparticles can be observed clearly. The diameter of single nanoparticle ranged from 10 to 60 nm. Nano-Au, nano-Ag as well as C_{60} were well dispersed, while small clusters of nano-Fe existed in the solution.

As shown in Fig. 2, 5 $\mu\text{g/mL}$ nano-Ag led to 100% mortality of the three bacteria, though a recovery was observed in *B. subtilis* at the end of exposure. *A. tumefaciens* seemed to be more sensitive to nano-Ag, as 1 $\mu\text{g/mL}$

nano-Ag could cause obvious growth delay (Fig. 2c). SEM images showed serious physical damage of *E. coli* cells; ruptures on cell wall and shrunken cells could be easily observed as depicted in Fig. 3. The physical damage of *E. coli* was as the same as the other two bacteria (images not shown).

Nano-Fe and nano-Au seemed to be less adverse to the three bacteria, as no obvious toxic effect was observed within the studied concentration range (Figs. 4, 5). Growth delay in *B. subtilis* occurred after exposure to 100 $\mu\text{g/mL}$ nano-Fe for 4 h, but this effect was not significant (Fig. 4). Correspondingly, no obvious physical damage to the bacterial cell integrity was observed, regardless of the concentrations of nano-Fe and nano-Au (SEM images not shown).

As shown in Fig. 6, growth of those three bacteria was inhibited by 10 and 20 $\mu\text{g/mL}$ of C_{60} . *E. coli* and *B. subtilis* recovered from their growth delay as the exposure time prolonged (Fig. 6a, b). However, the result shown in Fig. 6c differs from this. While similar restraint on

Fig. 1 Images of nanoparticles taken by transmission electronic microscope: **a** Au nanoparticles, **b** C_{60} nanoparticles, **c** Fe nanoparticles, **d** Ag nanoparticles. Magnifications and plotting scales are marked out in each picture

Fig. 2 Growth curves of **a** *E. coli*, **b** *B. subtilis* and **c** *A. tumefaciens* in absence of nano-Ag (filled diamonds control, filled squares 1 $\mu\text{g/mL}$ nano-Ag, filled triangles 5 $\mu\text{g/mL}$ nano-Ag). Data represents the mean $\pm$ standard error of four replicate experiments

Fig. 3 Images of *E. coli* taken by SEM after Exposure to nano-Ag. **a** Control, **b** 1 $\mu\text{g/mL}$ nano-Ag. Magnifications and plotting scales are marked out in each picture

Fig. 4 Growth curves of **a** *B. subtilis*, **b** *E. coli* and **c** *A. tumefaciens* in absence of nano-Fe (filled diamonds control, filled squares 50 $\mu\text{g/mL}$ nano-Fe, filled triangles 100 $\mu\text{g/mL}$ nano-Fe). Data represents the mean $\pm$ standard error of four replicate experiments

Fig. 5 Growth curves of **a** *B. subtilis*, **b** *E. coli* and **c** *A. tumefaciens* in absence of nano-Au (filled diamonds control, filled squares 1 $\mu\text{g/mL}$ nano-Au, filled triangles 5 $\mu\text{g/mL}$ nano-Au). Data represents the mean $\pm$ standard error of four replicate experiments

A. tumefaciens occurred after about 7 h, the bacteria recovered after the growth inhibition. Only slight damage on a few cells of *E. coli* was observed (Fig. 7b) but not to *B. subtilis* or *A. tumefaciens* (data not shown), when exposed to 20 $\mu\text{g/mL}$ C_{60} for 7 h. As shown in Fig. 7d, the damage reduced as exposure time prolonged. PVP solvent showed low toxicity to all three bacteria (Fig. 6), and the bacterial cells were not affected by this solvent (Fig. 7c), which means the joint toxicity effects of PVP can be excluded.

Discussion

It is known that silver has the highest electrical and thermal conductivity of all metals but the lowest contact resistance (Seiler et al. 1988). Because of these properties, this metal has been used in a wide variety of applications (Drake and Hazelwood 2005). Silver ions and salts have been used for decades as antimicrobial agents in various fields for their growth-inhibitory capacity against microorganisms. Similarly, nano-Ag is considered to be toxic to varieties of

Fig. 6 Growth curves of **a** *B. subtilis*, **b** *E. coli* and **c** *A. tumefaciens* in presence of C_{60} (filled diamonds control, filled squares 10 $\mu\text{g/mL}$ C_{60} , filled triangles 20 $\mu\text{g/mL}$ C_{60} , times 0.125% PVP, asterisks

0.25% PVP). Data represents the mean $\pm$ standard error of four replicate experiments

Fig. 7 Images of *E. coli* taken by SEM after exposure to C_{60} . **a** control, **b** 20 $\mu\text{g/mL}$ C_{60} for 7 h, **c** 0.25% PVP for 10 h and **d** 20 $\mu\text{g/mL}$ C_{60} for 10 h. Magnifications and plotting scales are marked out in each picture

organisms (Lee et al. 2007; Larese et al. 2009). It was reported that nano-Ag had a concentration-dependent interaction with *E. coli* (Dror-Ehre et al. 2009). In this study, nano-Ag exhibited its toxicity to bacteria at a low concentration and led to 100% mortality of bacterial cells at concentration higher than 5 $\mu\text{g/mL}$. Liu et al. (2010) exposed human cell lines to nano-Ag with different sizes. By using ICP-MS they showed that smaller nanoparticles enter cells more easily than larger ones, which may be the reason of higher toxic effects. One study indicated that the toxicity of nano-Ag was linked with its interaction with sulfhydryl compounds, which were found in the respiratory enzymes of bacterial cells (Rai et al. 2009). Others assumed that it was related to the formation of free radicals and subsequent free radical-induced membrane damage. Hsin et al. (2008) confirmed that apoptosis induced by

nano-Ag was associated with the generation of reactive oxygen species (ROS) and JNK activation (Kim et al. 2007; Hsin et al. 2008). Images obtained from SEM here revealed the damage of nano-Ag to cellular structure in all these three bacteria. Due to its high reactive properties, it is most likely that Ag nanoparticles could adhere to the surface of bacteria, followed by reaction with peptidoglycan, then lead to the destruction of cell structure integrity and eventually to cell death (Pal et al. 2007; Panacek et al. 2006).

Due to its highly effective ability to catalyze redox processes, nano-Fe has been widely applied in the degradation of halogenated organic compounds and other persistent toxic substances (Li et al. 2009). Oxidative damage and the low cytotoxicity caused by nano-Fe have been reported (Zhou et al. 2003; Li et al. 2009; Song et al. 2009). Regardless of the synthesis method or particle morphology,

exposure to air or oxygen at room temperature leads to a thin oxide shell cladding on nano-Fe which is typically 2–3 nm thick (Wang et al. 2009; Song et al. 2009). In this study, exposure to ambient air before use may be responsible for the low activity of the nano-Fe surface, leading to the low toxicity to the three bacteria.

Among the various metals, Au has some rather peculiar properties: high resistance to corrosion and oxidation, highest ductility among metals, as well as very high electron negativity (Choudhary and Goodman 2002). Therefore, nanoparticles of this metal are now widely used in colorimetric DNA targeting biosensors, as well as nanoparticles assembly techniques. However, so far its toxicity has been studied only in a few papers. One study showed that nano-Au of 30 nm had no crucial antimicrobial effects compared to nano-Ag (Kim et al. 2007). In this study it is concluded that nano-Au does not exhibit adverse effects to the studied bacteria, and the negative surface property may be responsible for its nontoxicity.

Several studies have shown the low toxicity of fullerenes (Fiorito et al. 2006; Oberdörster et al. 2006). However, some studies came to the opposite conclusion (Sayes et al. 2004; Fang et al. 2007; Tang et al. 2007; Lyon and Alvarez 2008). C₆₀ is supposed to have high electron affinity and the ability to generate superoxide anions, and thus it is hypothesized that the toxicity of C₆₀ is related to its ability to induce oxidative stress (Sayes et al. 2005). It was indicated that water-soluble C₆₀ inhibited the growth of *E. coli*, but the bacteria could recover from damage at low concentrations (Tang et al. 2007). Similarly, in this study, *E. coli* and *B. subtilis* recovered from their growth delay during exposure of C₆₀. Furthermore, images obtained by SEM showed the integrity of cell structure at the end of exposure. An explanation for the toxicity of fullerene is that C₆₀ exerts ROS-independent oxidative stress in bacteria, which leads to protein oxidation, changes in cell membrane potential, and interruption of cellular respiration (Lyon and Alvarez 2008). It is assumed that C₆₀ could enter into the cell and then generate reactive oxygen species (ROS), which damages DNA, leading to the growth delay of bacteria and damage to the integrity of cellular structure. Meanwhile, the cell itself could later clear the redundant ROS by involving its ROS scavenging system and recovers from the damage.

The three bacteria used in this paper, Gram-positive (*B. subtilis*) and Gram-negative (*E. coli* and *A. tumefaciens*), are representative of the ubiquitous microbes in the environment. They are long believed to be harmful because their relation with diseases in plants, animals and humans. However, their positive roles in the environment have also been well known (Conrad 1996; Ledin and Pedersen 1996; Richardson 2001). Communities of microorganisms, including these three bacteria, are part in the C cycle and N

cycle (Conrad 1996; Zak et al. 2000). Soil microorganisms play an important role in acquisition and transfer of soil nutrients (Richardson 2001). Some microorganisms have even been used to decompose or remove pollutants in the environment (Joshi et al. 1985; Ledin and Pedersen 1996). Following the wide use of NM, some of these materials or their derivatives can be released into the ambient environment during manufacture, application, disposal and recycling (Kashiwada 2006; Nel et al. 2006; Bystrzejewska-Piotrowska et al. 2009; Larese et al. 2009). Long-term exposure of some of these materials could cause potential risks to microorganisms, and cause disturbance to the substance and energy distribution in ecosystems. Therefore, the assessments of the toxicity of NM are necessary before extending their applications.

Conclusion

An overview of the effects of NM on the growth of bacteria is presented in this paper. It is concluded that nano-Ag is the most toxic among the studied NM, as it could cause mortality of the bacteria within the exposure concentration range. SEM observation of the bacteria indicated physical damage of nano-Ag on the structure of all the three bacteria. Exposure of C₆₀ led to slight growth delay of *B. subtilis* and *E. coli*, although they recovered from the adverse effect with time. However, *A. tumefaciens* proved to be more sensitive to C₆₀, with apparent inhibition after 7 h of exposure. Results from SEM showed slight damage of *E. coli* when exposed to C₆₀ for 7 h, but the cells soon recovered from the inhibition; while no damage could be observed in the other two bacteria. However, nano-Au, and nano-Fe showed little or no effect on the growth of bacteria, and no obvious damage could be observed after exposure to the two NM. Comparing the different results above, it is suggested that their various surface properties may be responsible for the toxic or nontoxic effects of NM. On the other hand, further researches are needed to evaluate their potential risks on the biodiversity within the microbial populations and communities in the environment.

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