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Research paper

Self-defense of *Escherichia coli* against damages caused by nanoalumina

Jing Ma[a,b,1], Meiling Kang[a,1], Yingxia Zhang[a,1], Xuan Guo[c], Zhongjing Tian[a], Chengshi Ding[a,*], Hongmei Wang[a,*]

[a] College of Life Science, Zaozhuang University, Zaozhuang 277160, China
[b] College of Medical Science, Zaozhuang Vocational College, Zaozhuang 277800, China
[c] Research Institute of Chemical Defense, Beijing 102205, China

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ABSTRACT

Although studies showed effects of nanoalumina (nano-Al_2O_3) on *Escherichia coli*, no study completely provides understanding on how bacterial cells respond to damages, especially on how they initiate self-defense. In this study, we showed three types of responses of *E. coli* to damages caused by nano-Al_2O_3. Live, dead, and injured, bacteria showed improved survival rates reaching 104%, 116%, and 104% after exposure to 0.1, 1, and 10 mmol/L of nano-Al_2O_3 respectively. Survival rates improved from 100% to 114%, corresponding to an exposure time of 0–9 h, and from 100% to 127%, corresponding to 0–1000 μg/L Al^{3+}. Improvements were noted in survival rates of *E. coli* K12 MG1655, HB101, DH5α, and K12 MG1655 △*lexA* treated by nano-Al_2O_3 in Luria–Bertani (LB) exposure system or K12 MG1655 in LB, normal saline(NS) and H_2O exposure system. Bacterial cells transformed from long rods to ellipsoidal or nearly spherical as form of self-preservation mechanism; this phenomenon may be related to changes in membrane potential induced by free Al^{3+} released from nano-Al_2O_3 particles. Molecular mechanism of this response involved inhibited gene expression of sythesis and metabolism of carbohydrates, lipids and proteins. Findings presented in this study may improve understanding of potential danger of nanomaterials and control their spread to environmen.

1. Introduction

As one of the most commercialized nanoproducts on the market, nanoalumina (nano-Al_2O_3) are currently used in water treatment technology (Afkhami et al., 2010), ceramics industry (Zielínski et al., 1993), aerospace (Luca et al., 2005; Lewis et al., 2010), catalysts (Martínez Flores et al., 2003), abrasives (Ganguly and Poole, 2003; Sawyer et al., 2003), paints (Khanna, 2008), and flooring material (Landry et al., 2008). According to research, the production of metal oxide nanoparticles (NPs) will reach 1, 663, 168 tons by 2020, with an estimated share of 20% attributed to nano-Al_2O_3 production (Future Markets Inc, 2013; Rittner and Abraham, 1998; Pakrashi et al., 2013). Therefore, growing concentrations of released nano-Al_2O_3 can be observed in environments, especially water resources. For example, Al_2O_3 NPs were used for defluorination or removal of heavy metals from drinking water and for wastewater treatment. After flocculation and precipitation process, 20%–80% of Al_2O_3 NPs remained, and their concentrations ranged to 2 mmol/L in water treated with nano-Al_2O_3 as flocculant (Afkhami et al., 2010; Kumar et al., 2011). Thus, potential ecotoxicity of Al_2O_3 NPs became significant. Many toxicity studies on Al_2O_3 NPs were carried out using different organisms, such as *Ceriodaphnia dubia* (Pakrashi et al., 2013), *Escherichia coli* (Sadiq et al., 2009), *Cupriavidus metallidurans* (Simon-Deckers et al., 2009), *Zea mays*, *Cucumis sativus*, *Glycine max*, *Brassica oleracea*, *Daucus carota* (Yang and Watts, 2005), *Aschelminthes* (Li et al., 2012), *Pheretima* (Heckmann et al., 2011), *Barchydanio rerio* (Zhu et al., 2008) and human cells (Lin et al., 2008).

Certainly, high concentrations of NPs and long interaction time between NPs and bacterial cells contribute to toxicity of Al_2O_3 NPs (Wang et al., 2017; Li et al., 2017; Ding et al., 2016; Pakrashi et al., 2013; Qiu et al., 2012). However, over a wide concentration range (0.1–10 mmol/L), Al_2O_3 NPs cause mild growth-inhibitory effects on *E. coli* during 20 h incubation, and dependence of antimicrobial action on concentration is almost negligible (Sadiq et al., 2009). No detrimental effect was observed on *E. coli* after 30 h interaction time with Al_2O_3 slurry between a concentration range of 0.625 and 2.5 mmo/L (Sawai et al., 1995). Survival of resting *Cupriavidus metallidurans* CH34 was stimulated after exposure to 0.1, 1, and 5 mmol/L of Al_2O_3 NPs for 24 h (Simon-Deckers et al., 2009). Aside from bactericidal effects, results indicated low toxicity, nontoxicity, and stimulation of survival rate.

* Corresponding authors.
E-mail addresses: dingchengshi123@126.com (C. Ding), zzxywhm@163.com (H. Wang).
[1] These authors contributed equally to this work.

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However, factors resulting in these contradictory phenomena remain unknown. In damages caused by nano-Al_2O_3, in addition to the passive death, adaptive changes of bacteria remain poorly understood.

After treatment with NPs, bacterial cells either die, become injured, or remain unaffected. However, few available studies discuss ratios of these states and their law of transformation to each other. In the current study, we observed survival rates and three kinds of responses of *E. coli* to damages caused by nano-Al_2O_3. Investigated factors included interaction time between nano-Al_2O_3 and bacterial cells, bacterial strains, and concentrations of dissolved alumina ions and solvent system. Self-defense mechanism of *E. coli* was explored with flow cytometry, transmission electron microscopy (TEM), confocal fluorescent microscopy, genome expression profile microarrays, and determination of intracellular protein.

2. Material and methods

2.1. NP characteristics

A total of 20 wt% nano-Al_2O_3 suspension in water was procured from Sigma-Aldrich (no. product 642991, USA). Dry NP powder was obtained by vacuum freeze-drying (Freeze Dry System, LABCONCO 77530, USA). Morphological features and particle size of nano-Al_2O_3 were characterized using an atomic force microscope (MultiMode 8 AFM, Bruker, USA). Infrared (IR) absorption of characteristic groups were collected with 80 scans per sample, and ranging from 400 cm^{-1} to 4000 cm^{-1} at a resolution of 4 cm^{-1} by KBr disc using an IR spectrophotometer (FTS 6000 Spectrometer, Bio-Rad, USA). After 1 h removal of NPs from suspension by centrifugation at 18,000*g*, inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700, USA) was used to measure concentrations of free Al^{3+} released from 10 mmol/L Al_2O_3 NPs in ultra-pure water, normal saline (NS), or Luria–Bertani (LB) medium.

2.2. Bacterial growth and exposure

E. coli strains K12 MG1655 (ATCC 47076), HB101 (Cat. #9051, not competent; TaKaRa), DH5α (Cat. #D9057A, not competent; TaKaRa), and K12 MG1655 △*lexA* (*lexA* gene deleted) were cultured at 37 °C in an aerobic shaker. A total of 10^9 bacterial cells were exposed to Al_2O_3 NPs in 100 μL interacting system consisting of LB, H_2O, or NS, and stationary culture was carried out in an incubator at 37 °C for 9 h. Then damaged cells were enumerated by pour plate method on BBL™ Trypticase soy agar containing 0.3% yeast extract (TSYA) or Difco™ m Endo Agar (Becton Dickinson and Company, USA) (Ray and Speck, 1973, 1972). Bacteria without exposure to NPs (0 mmol/L NPs) were used as control.

2.3. *E. coli* survival against damages by nano-Al_2O_3

NP suspension or powder was diluted with ultra-pure water to 0.5, 5, 50 mmol/L. These suspensions were treated with ultrasonic vibration for 20 min before usage. In the 100 μL exposure system of LB, final concentrations of Al_2O_3, TiO_2 (< 25 nm, powder, 637254, Sigma-Aldrich), Fe_2O_3 (< 110 nm, 20 wt% in ethanol, 720712, Sigma-Aldrich), and SiO_2 (< 12 nm, powder, 718483, Sigma-Aldrich) NPs were 0.1, 1, and 10 mmol/L respectively. All colony-forming units (cfu) on TSYA plates were considered surviving *E. coli* (Ray and Speck, 1973, 1972).

2.4. Three different responses of *E. coli* to nano-Al_2O_3 as determined by pour plate method

In LB exposure system, final concentrations of Al_2O_3 NPs were respectively 1, 10, 20, 40, and 100 mmol/L. Cfus on TSYA plates indicated *E. coli* survival, whereas that on selective Endo Agar represented live *E. coli* cells with strong viability. Injured bacterial cells growing on TSYA plates but did not grow on Endo Agar plates (LeChevallier et al., 1983; McFeters et al., 1986). Survival was indicated by the total number of live and injured bacterial cells.

2.5. Effect of exposure factors on bacterial survival

Exposure factors included exposure time, bacterial strains, free Al^{3+}, and exposure system. Survival rates and live rates were measured. Exposure times measured 0, 3, 6, 9, 12, and 24 h. Bacterial strains including K12 MG1655, HB101, DH5α, and K12 MG1655 △*lexA* were used. The release of Al^{3+} from Al_2O_3 NPs in ultrapure water were detected by ICP-MS. Concentrations of free Al^{3+} were 0, 1, 10, 100, and 1000 μg/L. LB, NS, and H_2O exposure systems were selected.

2.6. Flow cytometry and confocal microscopy

To further confirm responses of *E. coli* to damages caused by nano-Al_2O_3 and their transformation laws, two fluorescent dyes for nucleic staining, SYTO9 and propidium iodide (PI) from LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, Basel, Switzerland), were used to analyze live, dead, or injured states of *E. coli* cells. Exposure system of LB consisted of 10^9 *E. coli* K12 MG1655 cells and 1 or 40 mmol/L Al_2O_3 NPs; this system was washed thrice with phosphate-buffered saline (PBS, pH 7.4, 0.2 mol/L) and was diluted to 10^6 cells/mL. A total of 1 mL of each sample was stained with a mixture of 5 μmol/L SYTO9 and 30 μmol/L PI and incubated at room temperature in the dark for 25 min before analysis. Flow cytometric measurements were performed on FACSCalibur (BD Biosciences, NJ, USA) with 488 nm excitation from a blue laser. Optical filters were used to measure green fluorescence (fluorescence lane 1 (FL1), 535 nm) and red fluorescence (FL2, 585 nm). Cells without exposure to NPs were used as negative control, and cells boiled for 10 min served as positive control (dead and injured cells). A total of 10^4 cells were collected by FL1 and FL2, and specific gates for bacterial detection in dot plots were determined (Fig. 3) according to manufacture's instructions on fluorescence dyes.

Bacterial membrane potential was detected using BacLight™ Bacterial Membrane Potential Kit (B34950, Molecular Probes, Switzerland) according to manufacture's instructions. The carbocyanine dye 3, 3′-diethyloxacarbocyaninc iodide ($DiOC_2$) was used. Cells treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were used as positive control.

Morphology and dead/live states of above cells stained by SYTO9 and PI were monitored using a ZEISS LSM 710 laser scanning confocal microscope (Carl Zeiss, Germany). Cells were coated on a 35 mm petri dish (Corning, NY, USA), and their images were obtained with emission filter for red and green lights.

2.7. TEM analysis

The exposure system of LB consist of 10^9 *E. coli* K12 MG1655 cells and 1 mmol/L Al_2O_3 NPs stood at 37 °C in incubator for 9 h. Treated cells were fixed in a solution of 2.5% glutaraldehyde in 0.1 mol/L PBS (pH 7.4) at 4 °C for 2 h. Then cells were washed twice with 0.2 mol/L citric acid solution, and fixed with 2% OsO_4 in anhydrous acetone containing 8% dimethoxypropane at 4 °C for 2 days. Next, the dehydration was performed by enthanol gradient method, and cells were embedded in Epon812 resin. The samples were cut to 50 nm-width slices with an ultramicrotome (Leica ultracut-R, German). Afterwards, staining with 2% aqueous uranyl acetate for 15 min and Reynold's lead citrate for 5 min were carried out. Finally, samples were observed and photographed with a philips CM120 TEM (Philips, Holland).

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