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THE STUDY AND EXPLORATION ABOUT NANOTOXICITY OF OXIDE NANOPARTICLES ON ESCHERICHIA COLI AND EXIGUOBACTERIUM SIBIRICUM

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Date

THE STUDY AND EXPLORATION ABOUT NANOTOXICITY OF OXIDE NANOPARTICLES ON ESCHERICHIA COLI AND EXIGUOBACTERIUM SIBIRICUM

By

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ABSTRACT

THE STUDY AND EXPLORATION ABOUT NANOTOXICITY OF OXIDE NANOPARTICLES ON ESCHERICHIA COLI AND EXIGUOBACTERIUM SIBIRICUM

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Nanoparticles (NPs) have been used in a variety of areas, including cosmetics, pigments, water purification, environmental remediation and drug delivery. However, their toxicological and environmental effects have not been adequately investigated. I found that ZnO and TiO_2 NPs reduced cell viability of *Escherichia coli* K12 (Gram negative) and *Exiguobacterium sibiricum* strain 255-15 (Gram positive) in a dose-dependent manner, while SiO_2 and Fe_3O_4 NPs did not in a 12 h exposure. Among the test NPs, ZnO NPs were more toxic to *E. coli* than to *E. sibiricum*, while TiO_2 NPs were more toxic to *E. sibiricum* than to *E. coli*. The smallest ZnO or TiO_2 NPs, 5-10 nm, were more toxic than the larger particles to both bacteria. Zn^{2+} dissolved from ZnO NPs in both LB and TSB broth, reached saturation at 100-110 ppm. The release of Zn^{2+} ion played a more important role for ZnO NP toxicity to *E. coli* than *E. sibiricum*, due to the greater sensitivity of *E. coli* to Zn^{2+}, while both Zn^{2+} and ZnO NPs showed the toxicity to *E. sibiricum*.

SiO_2 and TiO_2 NPs were internalized in *E. sibiricum* but not in *E. coli* cells as observed by transmission electron microscopy-dispersive spectroscopy, while ZnO NPs were found internal to both of them. No particle larger than 150 nm was found

intracellularly. NPs that can be internalized by microbes may possess the stronger toxicity than those not internalized, which is consistent with my observations.

ZnO and TiO_2 NPs induced production of reactive oxygen species (ROS) in *E. coli* K12 and *E. sibiricum* in a dose-dependent manner. Both material-effect and size-effect were evidenced in ROS induction studies in that the capability for ROS induction increased generally from SiO_2 to TiO_2 to ZnO, and that the smaller TiO_2 NPs induced more ROS than the larger ones. More ROS was induced by SiO_2 NPs in *E. sibiricum* than in *E. coli*, while ZnO NPs induced more ROS in *E. coli* than in *E. sibiricum*, which is consistent with the viability results. Both TiO_2 NPs and ZnO NPs caused significant DNA damage in lymphocytes as measured by the comet assay, and TiO_2 NPs were more genotoxic than ZnO NPs. The latter is probably due to the solubility or smaller size of the ZnO NPs.

Using microarray technology, I found that SiO_2, TiO_2, and ZnO NPs influenced gene expression of *E. coli* and *E. sibiricum* in a variety of function (COG) categories. Genes in the "transport" category were most affected by all treatments. When genes for amino acid and carbohydrate transport were repressed, genes for metal iron transport were induced. Other important categories with altered expression due to NPs were "carbohydrate metabolism", "lipid metabolism", "amino acid metabolism" and "DNA repair". The latter may be caused by ROS damage to the membrane, amino acids in proteins, and DNA. NPs also led to up-regulation of stress-related genes and down-regulation of cellular respiration, also evidence for ROS induction.

To my parents Zhaolan Liang and Xiaoping Yang

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give me their generous love. I hope I can make them proud of me.

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ABBREVIATIONS

Nanoparticles	NPs
Nanomaterials	NMs
Carbon nunotubes	CNTs
Quantum dots	QDs
Reactive oxygen species	ROS
Transmission electron microscopy	TEM
Energy dispersive spectrometry	EDS
Luria-Bertani	LB
Tryptic soy broth	TSB
Tryptic soy agar	TSA
2', 7'-dichlorofuorescin diacetate	DCFH-DA
Polymerase chain reaction	PCR
ATP-binding cassette	ABC
Phosphotransferase system	PTS
Coenzyme A	CoA
Tricarboxylic acid	TCA

CHAPTER 1

INTRODUCTION: TOXICITY OF NANOMATERIALS TO MICROORGANISMS

Abstract

The unique and advanced functions of nanomaterials (NMs) make them broadly useful and powerful for research, industrial development and many products. Their use, however, also brings uncertainties regarding their bio-safety and environmental impacts. The goal of this study is to introduce the main types of NMs and review their environmental adverse effect, with a particular emphasis on microorganisms. I also collate published papers regarding the antibacterial activity of different NMs, and describe several possible mechanisms of NM toxicity. Production of reactive oxygen species (ROS) is the most favored explanation for NP toxicity. ROS can attack DNA, amino acids in proteins and lipids in membranes, causing extensive damage to cells. Direct contact between NMs and microorganisms is also necessary for NP toxicity.

Introduction

Nanotechnology, a broad interdisciplinary field revolutionizing research development and new product and process, is one of the most rapidly growing fields. In nanotechnology and nanoscience, NMs, nanotools and nanodevices are the important segments, and the global nanotech market is estimated to grow to $25.2 billion by 2011 (75). The Royal Society stated that "Nanotechnologies are the design, characterization, production and application of structures, devices and systems by controlling shape and size at nanometer scale", when there have also been a few definitions offered by other organizations (25). Most of these definitions refer only to the size range or new functionalities, but neglect risks and uncertainties which also need to be considered and resolved. The development of nanotechnology and nanoscience requires multidisciplinary integration of physics, chemistry, biology, and engineering, and mainly consists of nano-medicine, nano-fabrication, nano-metrology and nano-materials (59).

Manufactured NMs are defined by the U.S. National Nanotechnology Initiative as materials that have at least one dimension in the 1 to 100 nm range (88). Recently more and more NMs have been synthesized and applied in various fields, such as environmental remediation, drug delivery, cosmetics, electronics, energetics and photonics (3). For instance, 2954 kg of fullerenes were synthesized world-wide in 2003 (10). In 2005, more than ten billion dollars was spent internationally on nanotechnology development, and it is estimated that by 2015 the global market for nanotechnology and

NMs will be around $1 trillion (35). Because of wide and abundant applications, NMs may enter environment during their life cycle and end up as environmental contaminants of water, air and soil (93). The concerns about the toxicological and environmental impacts of NMs have been increasing rapidly, and the research conducted about safety of NMs remains focused on the issues related to occupational health and human safety. The concerns about environmental safety of NMs need attention; these materials are too small to detect and track in environment. The entrance of NMs to environment can be divided into intentional releases and unintentional releases, such as atmospheric emission and liquid waste streams.

Nanoparticles (NPs), one of the most useful subsets of NMs, are widely applied in water purification, catalysis, pigments, food, cosmetics, and lubrication (3, 31, 38, 86, 116). NPs have natively existed in nature, while the exposure to the NPs may have some adverse effect on the environmental microorganisms(81). The mechanisms of NP toxicity are not yet clear, and the studies associated on their potential influence on public health and environments are inadequate. In this chapter, I focus on the toxicity of NPs to microorganisms. Understanding NP toxicity to microorganism is important to evaluate the risk of NPs to environment, since microorganisms play a fundamental role of the food chain as the lowest level and are responsive for much of biogeochemical cycles. Moreover, they grow rapidly and are sensitive to some toxicants, which make them as a good test model for NP toxicity. These studies will supply helpful information for

regulatory policy concerning the manufacture, application and disposition of NPs.

Classes of NMs and their toxicity

NMs have always existed in the environment, from both natural and anthropogenic sources. The natural NMs include clays and particulate organic matter in soil, volcanic ash and forest-fire smoke, and, colloid and ocean spray in aquatic systems. The engineered NMs possess many unique characteristics, which are attributable to their small size, large surface area, chemical composition, surface structure, solubility, shape, and aggregation. The main types of manufactured NMs include:

Fullerene

The manufacture of carbon-base fullerenes, spherical molecules comprising 60 carbon atoms, opened the gate of the nanotechnology era (58). Its configuration is like a football with 20 hexagons and 12 pentagons and its diameter is about 1 nm (Figure 1.1(a)). Underivatized fullerenes are extremely insoluble in water, and subject to precipitation and aggregation (4, 54). Fullerene has been applied in polymer and structural composites, electromagnetic shielding, electron field emitters, super capacitors and batteries, due to their high conductivity, high surface area and unique electronic properties (3, 74).

Fullerenes are redox active and can generate ROS, which may cause damage to DNA,

membranes and amino acids (20, 100). Fullerenes have been observed to alter the membrane lipid composition, phase transition temperature and membrane fluidity in *Pseudomonas putida* and *Bacillus subtilis* (100). The antibacterial activity of fullerene on *Escherichia coli* and *B. subtilis* has been reported in literature, as a decreased aerobic respiration rate (28, 63). Surface-modified fullerene can generate singlet oxygen, causing lipid peroxidation and exhibiting antibacterial activities (48, 64, 67). Carboxyfullerene caused the inhibition on Gram positive species by damage to cell membranes, i. e. *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus faecalis*, but not for Gram negative *E. coli*, *Pseudomonas aeroginosa*, *Salmonella typhmuriumi* and *Klebsiella pneumoniae* (108, 109). However, toxicity of a carboxy-fullerene derivative to *E. coli* was detected in a dose-dependent manner (107).

Some other fullerene derivatives were also studied for their antibacterial activities. For instance, one positively charged fullerene derivative inhibited the growth of *Mycobacterium tuberculosis*, and alkylated fullerene derivatives could inhibit the growth of *E. faecalis*, *S. aureus*, *S. epidermidis*, and *Enterococcus hirae* (11, 67). Their toxicity to *E. coli* was explained as respiratory chain inhibition and hydrogen peroxide production (68, 69). Moreover, fullerene derivatives had a mutagenic effect on *S. typhimurium* (6). It was also reported that fullerene can alter the microbial community in soil (106).

Nanotubes and nanowires

Carbon nanotubes (CNTs), first observed by Sumio Iijima in 1991, are a particular form of fullerenes, including single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) (Figure 1.1(b)) (44). Both SWCNBs and MWCNTs show antibacterial activity: direct contact between SWCNBs and bacteria can cause membrane damage and lead to cell death (49, 85). Dr Elimelech's group published a serial of papers about CNT toxicity. They provided the direct evidence that both SWCNTs and SWCNTs exhibited strong antimicrobial activity on *E. coli*, and SWCNBs were more toxic to bacteria than MWCNTs (50, 51). They also reported that these CNBs could regulate gene expression of *E. coli* and induce stress-related genes, and that the size of CNT could influence the antibacterial effects (49). Meanwhile, the surface coating is also important for CNT antimicrobial activity (85). Nanowires are ultrafine wires or linear arrays of dots formed by self assembly, and used as interconnectors in nanoelectronic devices (25). There are not many studies of nanowire toxicity, and perhaps they are less likely to be toxic due to their long dimension.

Nanoparticles

One important subset of NMs are NPs, which are particles with a diameter less than 100 nm. The materials of NPs include metal, metal oxides and silica. The metal oxide NPs can be consisted of individual oxides, such as ZnO, TiO_2, CeO_2 and CrO_2, and binary oxides, such as $BaTiO_3$ and $LiCoO_2$.

NPs of Silicon oxide (SiO_2), Titanium oxide (TiO_2) and Zinc oxide (ZnO) are the popular ones among the oxide NPs. SiO_2 is used as an additive in food, drugs, cosmetics and printer toners (Figure 1.1(a)). It is also a good semiconductor and an electrical insulator. TiO_2 is used as a photocatalyst for disinfection of bacteria, and a number of the papers explored this subject (9, 14, 66, 72, 94, 96, 104). It is also employed as a pigment in paints and a sunscreen product in cosmetics. ZnO is widely used in pigments, semiconductors, and cosmetics. It was estimated that globally 1000 tons of NPs were used in sunscreen products in 2004 (10).

There have been a number of reports on the toxicity of SiO_2, TiO_2 and ZnO NPs. SiO_2 NPs are the least toxic among these three NPs. Their antibacterial activity on *E. coli* and *B. subtilis* was reported while some studies indicated that they were not toxic (1). Due to the wide application of TiO_2 NPs, their toxicity on microorganisms has been well studied. The TiO_2 NP photocatalytic inactivation of *E. coli*, *S. aureus* and *E. faecalis* was reported, and scientists also demonstrated antibacterial activity to *Streptococcus* spp., *Enterococcus* spp., *Vibrio fischeri*, *Serratia marcescens*, *Bacillus megaterium* and some fungi (29, 36, 76, 94, 95, 118). N-doped TiO_2 NPs and Ag-coated TiO_2 NPs also showed inhibition of *E. coli* and *Micrococcus lylae*, respectively (60, 61, 123). Matsunaga et al. reported that TiO_2 can oxidize CoA and cause cell death (72, 73). Moreover, the cell membrane may be oxidized prior to the oxidation of CoA, and a partial decomposition of outer membrane is followed by disordering of the cytoplasmic membrane, leading to cell

death (19, 76, 104). Later, Saito et al. found rapid leakage of K^+ ion and slow release of proteins and RNA. They explained that the cell death was due to a significant disorder in cell permeability (7, 96). The toxicity of TiO_2 NPs can be influenced by NP size and light intensity (2, 39). ZnO NPs are probably the most toxic among these three NPs. They inhibited the growth of *E. coli*, *S. aureus*, *V. fishceri*, *B. subtilis*, *S. typhimurium* and *Streptococcus agalactiae* (43, 83, 92, 122). The release of Zn^{2+} ion from ZnO NPs, direct contact between ZnO NPs and membranes, and ROS induction all contributed to their antibacterial activity. A NP size-effect was also demonstrated in ZnO toxicity studies, and internalization of ZnO was found in *E. coli*, *S. agalactiae*, *Exiguobacterium sibiricum* and *S. aureus* (Figure1.1 (b)) (12, 43, 120).

Recently there have been a few reports about the antimicrobial activity of some other NPs, and on the interaction between NPs and microorganisms (12, 15, 29, 89, 90, 103). CuO NPs inhibited the growth of *V. fischeri*, and the growth of *E. coli* was found to be inhibited by N-doped ZrO_2, CeO_2, Ag/Al_2O_3 and Fe_2O_3 NPs (5, 15, 80, 105). Both CaO and MgO NPs demonstrated antibacterial activity to *B. subtilis*, *E. coli*, *S. aureus*, *S. typhimurium* and *B. megaterium* (41, 42, 65, 97, 98).

Silver NPs are one of the most promising metal NPs for future applications. They are antibacterial, antimicrobial and antifungal (24). Silver NPs can also interact with DNA, and interrupt RNA replication and microbial reproduction (79, 110).The toxicity of Ag NPs to *E. coli* was reported by Sondi and Salopek-Sondi, and Ag NPs also inhibited the

growth of *Salmonella typhi*, *P. aeruginosa*, and *Vibrio cholerae* (79, 90, 102). The toxicity of gold NPs was demonstrated to depend on surface coating. Cationic gold NPs are moderately toxic and anionic gold NPs are quite nontoxic (30, 117). Organic compound NPs also possess antibacterial activity, such as the growth inhibition of *K. pneumonia*, *S. aureus*, and *Streptococcus mutans* by polyethylenimine NPs and Ag-loaded polystyrene NPs (8, 89).

Quantum dots (QDs).

Quantum dots, which are small assemblies of metal, metal oxide or semiconductor materials in size of 2-10 nm, were first created in the early 1980s (25). Quite a few QDs contain some noble or transition metals, such as CdSe, InAs and ZnS. The components for QD shell or core can be quite different (18, 52). The application of QDs covers biomedical imaging, drug delivery and electronics industry (34). They can be coated by some organic molecules, which can influence their characteristics and toxicity (40). QDs can induce ROS and nick supercoiled DNA to cause genotoxicity (32, 46).

Other NMs

There are some other types of NMs, such as dendrimers and biopolymers. Few toxicity studies have been conducted on these NMs, perhaps due to their limiting applications.

Mechanisms of NP toxicity

There have been a few hypothesized mechanisms of NP toxicity, such as disruption of cell membranes, induction of ROS, dissolution of hazardous constituents and interruption of energy production (54, 84). NPs may cause toxicity to bacteria via one or more of these mechanisms.

ROS induction

Induction of ROS by NPs is currently the most favored explanation for the toxicity of NPs. In 1997, Rahman et al. found the increasing induction of ROS by nano-sized TiO_2 in human and rat alveolar macrophages (91). That TiO_2 NPs were toxic to some cells and caused oxidative stress in different cell types, including bacteria and mammal cells (2, 33, 62, 101, 113-115).

The chemical reactions by which TiO_2 NPs induce ROS are showed below (16, 17, 123). In the first equation, a hole (e^-)/electron (h^+) in the valence band and conduction band is induced by light (λ<390 nm) and TiO_2 NPs. The e^- is absorbed onto the surface of a TiO_2 NP and available for electron transfer, which can reduce O_2 to O_2^- (reaction 2), and produce H_2O_2 by further reducing O_2^- (reaction 3). The OH radicals can be produced by the reaction of superoxide with H_2O_2 (reaction 4) and reduction of H_2O_2 by e^- (reaction 5). The OH radicals can also be formed by the reactions of H_2O or OH^- with h^+ and electron abstraction from absorbed oxidizable species by h^+ (reaction 6 and 7). H_2O_2

can be produced by either a reductive pathway (reaction 3) or an oxidative pathway (reaction 8), while it can be decomposed into OH radicals by light (reaction 9).

$TiO_2 + h\nu \rightarrow TiO_2 (e^- + h^+)$ (1)

$e^- + O_2 \rightarrow O_2^-$ (2)

$O_2^- + 2H^+ + e^- \rightarrow H_2O_2$ (3)

$H_2O_2 + O_2^- \rightarrow {}^\bullet OH + OH^- + O_2$ (4)

$e^- + H_2O_2 \rightarrow {}^\bullet OH + OH^-$ (5)

$h^+ + OH^- \rightarrow {}^\bullet OH$ (6)

$h^+ + H_2O \rightarrow {}^\bullet OH + H^+$ (7)

$2h^+ + 2H_2O \rightarrow 2H^+ + H_2O_2$ (8) $h\nu$

$+ H_2O_2 \rightarrow 2{}^\bullet OH$ (9)

An interfacial charge transfer has been reported to occur between TiO_2 NPs and *E. coli* membrane (82). Reaction 10 and 11 exhibit the surface charge trapping on TiO_2 NPs. During the process of interfacial charge transfer, h^+ reacts with peptidoglycan (PGN) and lipo-polysaccharides (LPS) on microbial cell wall membranes, such as *E. coli* (reac*tion* 12). Meanwhile, h^+ can be scavenged by PE, LPS or *E. coli* during surface oxidation (reaction 13).

$e^- + Ti(IV)O\text{-}H \rightarrow Ti(III)O\text{-}H^-$ (10)

$h^+ + Ti(IV)O\text{-}H \rightarrow Ti(IV)O^\bullet\text{-}H^+$ (11)

$h^+ + RH \rightarrow R^\bullet + H^+$ (12)

$Ti(IV)O^\bullet\text{-}H^+ + RH \rightarrow OH + R^\bullet + H^+$ (13)

ROS can be transferred through membranes, and oxidize fatty acids of phospholipids and cause lipid peroxidation. This process can change membrane permeability and fluidity, and make bacteria more vulnerable to osmotic stress and interrupt nutrient uptake (13). ROS can also oxidize and damage DNA to cause genotoxicity. For instance, there have been a few reports about the DNA lesions caused by ROS induction by TiO_2 NPs (22, 33, 37, 57, 70, 111-113). Moreover, ROS can oxidize amino acids in proteins, and especially attack sulfur-iron assembly, which can release Fe ions, induce Fenton chemistry and lead to more production of ROS (45). The induction of ROS by ZnO NPs was also hypothesized as an explanation for their toxicity, although the mechanisms for ZnO NP toxicity are not yet well understood (99). ZnO NP toxicity may be caused by the binding of the NPs to the bacterial surface due to electronic forces (103, 122). The internalization of NPs is not required for ROS induction. ROS can be transferred into bacteria via direct contact between NPs and their membranes, although the lifetime for ROS is very short (10^{-9} second).

Release of toxic components from NPs

Hazardous components can be released by some NMs. Silver NPs are a good example, as release of silver ions has been hypothesized as one of the mechanisms for toxicity of silver NPs (79). That the interaction between silver ions and thiol groups in proteins can inactivate some enzymes (71). Moreover, silver ions can also attack membranes and prevent DNA replication (27). ZnO NPs can release Zn^{2+} ion, which is

antibacterial (121). Kirchner et al. reported that QDs (CdSe/ZnS) released toxic Cd^{2+}, which increased their toxicity (53). Meanwhile, both free Cd and Se from QDs was found inside *S. aureus* before QDs was internalized, which confirmed the release of components from QDs (56).

Damage to membrane integrity

Membrane integrity is important for microorganisms to maintain cell activity, such as transport and energy transduction, and to protect cells from potential damage. Some NMs can attack bacterial membranes directly. ZnO NPs were internalized into *E. coli* cells (12). *E. coli* and *E. sibiricum* cells with broken membrane and leaking cytoplasm were found when they were exposed to ZnO NPs in our studies. QDs were also found to enter bacterial cells (55). However, there is no clear explanation for how NP internalization occurs, since NPs are still too big for channels or transporters to transport through membranes. Moreover, Ag NPs can also interact with sulphur-containing amino acids in membranes, prohibiting transport through membranes and causing cell death (102, 119). The embedding of silicon NPs and fullerene in membranes was also reported (47, 107). ROS can disrupt membranes too, via oxidizing lipids or proteins in membrane. Cell membrane damage by direct contact of CNTs is proposed as the main mechanism for CNT cytotoxicity.

Damage to nucleic acid

Some NMs have characteristics of interacting with DNA, and they can be used to label or cleave DNA. For example, QDs have been used for imaging applications via tagging DNA, and they were also found to nick DNA (21, 23, 32). ROS induced by NPs can damage DNA, such as by TiO_2 and ZnO NPs (22).

Conclusion

The NM toxicity studies require multidisciplinary collaboration of physicist, chemist, microbiologist and other experts. On one hand, when microbiologists focus on antibacterial activity of NMs, they may neglect the characterization of NPs. However, it is essential to characterize the physical or physicochemical properties of NMs before any toxicity studies, since their features are likely critical to any toxicity. Therefore, the measurement of the NP properties should be the first step for studying their toxicity.

The main characteristic of NPs is their size, which may extend down to individual atoms or molecules, and much smaller than cells. The small size can influence other physicochemical properties of materials and increases the opportunity of uptake and interaction with or within bacteria. Surface area is another important factor in toxic effects. Normally when the size decreases, the surface area increases and there are more reactive groups. The surface group is dependent on the chemical composition and surface structure of NPs, such as electronic properties and coating, which determines the solubility of NPs, hydrophobic or hydrophilic, lipophobic or lipophilic, or active or

passive. The other main properties of NPs that may affect their toxicity include agglomeration state, shape, crystal structure, chemical composition, surface chemistry, surface charge and porosity (88). Standard and characterized NPs are critical for NP toxicity studies, as well are standardized and validated test methods.

On the other hand, physicists and chemists should use more relevant biological methods to explore toxicity study at molecular level. Simple viability tests and growth inhibition are not very helpful to answer the questions about mechanisms of NP toxicity. Use of genetics and proteomics can supply more details about bacterial reaction to NPs, and reveal increasing complex that can be useful in refining experiments for exploration of NP fate, bioavailability and effect.

There are still many unexplored areas for NP toxicity studies. For instance, there are few anaerobic studies, although it was found that fullerenes have no significant effect on anaerobic microbial communities (87). How microbial communities react to NMs still remains unknown, and it is very difficult to estimate the consequences of wide environmental impact during the exposure to NMs. Finally, it is critical to improve our understanding of the interaction between NPs and microorganisms.

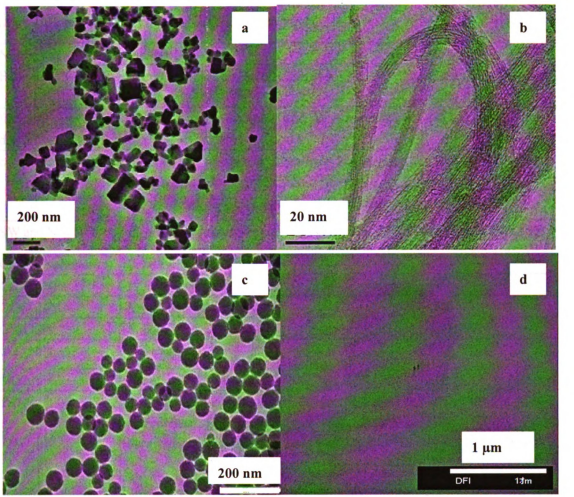

Figure 1.1 images of NPs. (a) TEM image of a fullerene (28).(b) TEM image of a SWCMT (49). (C) TEM image of SiO_2 NPs. (b) TEM image of internalized ZnO NPs in *E. sibiricum*.

Table 1.1 Examples of NMs showing antibacterial activity

NMs	Microorganism	Reference
Fullerenes	*E. coli, B. subtilis,* and *P. putida*	(94), (64), (63), (28), (26),
fullerene derivatives	*E. coli, S. typhimurium,* and *M. tubercolosis*	(6), (11), (68), (69)
carboxyfullerene	*E. coli, S. pyogenes, Staphylococcus* spp., *Streptococcus* spp., *E. faecalis, P. aeruginosa, S. typhi,* and *K. pneumoniae*	(107), (109), (108)
SW and MWNTs	*E. coli, M. lysodeikticus,* and *S. aureus*	(85), (49), (51), (50)
Carbon Nanohorn	*E. coli*	(77)
Carbon NPs	*K. pneumoniae*	(78)
QDs	*E. coli* and *B. subtilis*	(55)
Gold NPs	*E. coli*	(30), (117)
CuO NPs	*V. fischeri*	(80), (36)
SiO2	*E. coli* and *B. subilis*	(1)
TiO_2	*E. coli, M. luteus, B. subtilis, Streptococci* spp., *S. marcescens, Enterococcus* spp., *Saccharomyces Serevisiae, S. aureus, E. faecalis,* and *V. fischeri*	(95), (94), (9), (116), (76), (118), (29), (104), (1), (16), (7), (96), (72), (39), (124), (36)
N-doped TiO_2	*E. coli*	(60), (61)
Ag-coated TiO_2	*M. lylae*	(123)
Silver	*E. coli, P. aeruginosa, V. cholera,* and *S. typhi*	(102), (79), (90)
N-doped ZrO_2	*E. coli*	(61)
ZnO	*S. agalactiae, S. aureus, V. fischeri, B. subilis,* and *S. typhimurium*	(98), (92), (1), (122), (36), (97), (83), (120), (43), (80)
MgO	*B. subtilis, E. coli, S. aureus, B. megaterium,* and *S. typhimurium*	(98), (42), (41), (103), (97), (65)
CaO	*B. subtilis, E. coli, S. typhimurium,* and *S. aureus*	(98), (97)
CeO_2	*E. coli*	(105)
Ag/Al_2O_3	*E. coli*	(15)
Fe_2O_3	*E. coli*	(5)
Silica, silica/iron oxide	*E. coli*	(117)
Polyethylenimine NPs	*Streptococcus mutans*	(8)

Table 1.1 cont

Ag-loaded polystyrene NPs	*K. pneumonia* and *S. aureus*	(89)

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CHAPTER 2

TOXICITY OF OXIDE NANOPARTICLES ON *ESCHERICHIA COLI* AND *EXIGUOBACTERIUM SIBIRICUM*

Abstract

NPs are being used in a variety of areas, including cosmetics, pigments, water purification, environmental remediation and drug delivery. However, their toxicological and environmental effects have not been thoroughly investigated. In our studies, zinc oxide (ZnO) and titanium oxide (TiO_2) NPs were found to decrease cell viability of *Escherichia coli* strain K12 (Gram-negative) and *Exiguobacterium sibiricum* strain 255-15 (Gram-positive) in a dose-dependent manner, while silicon oxide (SiO_2) and iron oxide (Fe_3O_4) NPs did not inhibit the microbial growth after a 12 h exposure. Five nanometer TiO_2 NPs were more toxic to both *E. coli* and *E. sibiricum* than 150 nm TiO_2 NPs, which confirmed that size is one of the most important factors for NP toxicity. ZnO NPs displayed more toxicity to *E. coli* compared to *E. sibiricum*, while TiO_2 NPs were more toxic to *E. sibiricum* than to *E. coli*. Zn^{2+} ion dissolved from ZnO NPs in both LB and TSB medium, and reached saturation of approximately 100- 110 ppm. *E. coli* was more vulnerable to the toxicity of Zn^{2+} than *E. sibiricum*. Transmission electron microscopy (TEM) and energy dispersive spectroscopy (EDS), showed that SiO_2 and TiO_2 NPs were internalized by *E. sibiricum* but not by *E. coli*, and ZnO NPs were found in both. We concluded that size and chemical composition play a key role in NP toxicity, and that solubility of metal ions from NPs could also attribute to the toxicity of NPs. These results also show that different bacteria have different tolerance and internalization of NPs.

Introduction

NMs are defined by the U.S. National Nanotechnology Initiative as materials that have at least one dimension in the 1 to 100 nm range (34). Nanotechnology has become one of the rapidly developing fields with the potential to revolutionize medical science, biology and engineering. Meanwhile, the concerns about the toxic and environmental impacts of exposure to these NMs have been increasing and starting to attract public and governmental attention. The critical risk assessment issues about NMs include toxicology, exposure assessment, environmental and biological fate, persistence, sustainability, transportation and transformation. Moreover, a number of studies suggest that NMs might cause damage, such as lung cancer, silicosis and some autoimmune diseases (9, 10, 13, 20, 37, 42).

NPs, the type most important in NMs, are applied in environmental remediation, catalysis, drug delivery, cosmetics, water purification and lubrication (2, 14, 18, 33, 49). SiO_2, TiO_2 and ZnO NPs were the main NPs test in this study. SiO_2 is used as an additive in food, drugs, cosmetics and printer toners, as well as it is a good semiconductor and an electrical insulator. TiO_2 is used as a photocatalyst for disinfection of bacteria and a number of the papers explored the mechanism (3, 7, 27, 29, 38, 40, 44). It is also employed as a pigment in paints and as a sunscreen product for cosmetics. ZnO is widely used for application as pigments, semiconductors, and as a sunscreen. It was estimated that globally 1000 tons of NPs were used for sunscreen products during 2003 and 2004 and more than 2500 tons in 2008 (4). Because of their numerous applications, NPs may

enter the environment during their life cycle and end up as environmental contaminants in water, air or soil. However, very few studies on NPs about their environmental and toxicological effects on the microorganisms have been conducted, and their mechanism of toxicity is still unknown. The main properties of NPs that may affect the toxicity include size distribution, agglomeration state, shape, crystal structure, chemical composition, surface area, surface chemistry, surface charge and porosity (34).

We evaluated the effects of SiO_2, Fe_3O_4, TiO_2 and ZnO NPs on *E. coli* K12 and *E. sibiricum* representative Gram–negative and Gram-positive bacteria, and explored if the size and composition of these NPs influenced the degree of toxicity. Moreover, we investigated the dissolution of ZnO NPs, and compared the toxicity of Zn^{2+} ion with that of the NP suspension. TEM and EDS were employed to study the internalization of NPs and the morphological changes of these bacteria after the exposure to NPs.

Materials and Methods

Reagents and bacterial culture. *E. coli* K12 was grown aerobically in Luria-Bertani (LB) liquid broth at 37 °C and 200 rpm for 12 h. *E. sibiricum* was cultured in tryptic soy broth (TSB) medium at 30 °C and 150 rpm for 12 h. Solid medium for *E. coli* or *E. sibiricum* was as above with 1.5% agar (Difco). The bacteria were in stationary phase when the NPs were added, as well as after 12 h exposure. All the cultures with NPs were grown under the same conditions.

Preparation of NP suspensions. SiO_2 (10 nm and 1 μm), Fe_3O_4 (50 nm and 1 μm),

TiO_2 (5 nm, 150 nm and 5 μm) and ZnO (60 nm and 1 μm) were purchased from Sigma-Aldrich (St. Louis, MO). Sixty namometer SiO_2 was synthesized by reverse microemulsion (45, 50). NPs stock solutions of 100 g/L were prepared in deionized and distilled water.

Assessment of NP toxicity to bacteria. *E. coli* K12 and *E. sibiricum* were cultured as above for 12 h with NPs at 0, 10, 50, 100, 500, 1000, 2000, and 5000 ppm. Cultures were then diluted and spread onto LB or trypic soy agar (TSA) plates, and colonies were counted after growth over-night. All the cultures were grown in triplicate.

Evaluation of Zn^{2+} toxicity in ZnO NP medium. Different concentrations of 60 nm and 1 μm ZnO NPs were mixed in TSB and LB for 12 h (50 ppm, 200 ppm, 1000 ppm and 5000 ppm). After 20 min of centrifugation at 20,000×g, the supernatants were collected, diluted and measured with atomic absorption spectrometry. Zinc standard solution was purchased from Fischer Scientific Co. (Pittsburgh, PA) and diluted to construct a standard curve for Zn^{2+} concentration. *E. coli* K12 and *E. sibiricum* were cultured in triplicate in each of three concentrations of $ZnCl_2$ (0.5 mM, 1.0 mM and 2.0 mM). Culture viability was measured by plate counts and all the samples were prepared in triplicate.

Toxicity of NPs versus soluble metals from NPs. LB or TSB medium containing possible metal impurities from TiO_2 or ZnO NPs was prepared by making suspension of NPs with concentrations of 1000, 2000, and 5000 ppm for 10 nm, 150 nm and 5 μm TiO_2 NPs, and, 100, 200, 500, 1000, 2000, and 5000 ppm for 60 nm ZnO NPs. After leaving

the LB suspension at 30 °C and TSB suspension at 37 °C for 12 h, the NPs were removed by centrifuging the suspension at 20,000×g for 20 min. The supernatant was collected and used to culture *E. coli* and *E. sibiricum* respectively for 12 h, and the microbial growth was evaluated by plate counting method.

Morphological studies

E. coli K12 and *E. sibiricum* with 1000 ppm of NPs (60 nm SiO_2, 5 nm TiO_2 and 60 nm ZnO) were cultured in LB or TSB medium overnight at 30 °C or 37 °C respectively. After culturing, the mixture of cells and NPs in each sample was collected by centrifugation at 12,000 × g for 30 min. These samples were fixed with 2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M cacodylate buffer and then 1% osmium tetroxide in cacodylate buffer, dehydrated in serial acetone buffer (from 30% to 100%), and infiltrated with acetone: Poly/Bed 812 buffer for TEM. The samples were cut by MTX ultramicrotome into 70-100 nm thick sections. All the sections were collected on copper grids, and a JEOL 2200FS Transmission Electron Microscope was used for the EDS-TEM detection.

Results

Antibacterial activity of ZnO and TiO_2 NPs. *E. coli* and *E. sibiricum* was exposed to two different sizes of ZnO NPs for 12 h to evaluate their effect on microbial growth. As illustrated in Figure 2.1, the toxicity of ZnO NPs to *E. coli* and *E. sibiricum* rose with the increasing doses. ZnO NPs significantly inhibited the growth of *E. coli* when the

concentration was higher than 100 ppm (Figure 2.1(a)). The 60 nm ZnO NPs were more toxic to *E. coli* than the 1 μm ones. In the case of *E. sibiricum,* a gradual increase in growth inhibition was observed with increasing doses of ZnO NPs, while *E. sibiricum* showed more tolerance to ZnO NPs (Figure 2.1(b)). In addition, the 60 nm ZnO NPs displayed less toxicity to *E. sibiricum* than the 1 μm NPs, which was different from the size-effect of ZnO NPs on *E. coli.*

Similar to the results with ZnO NPs, all three TiO_2 NPs showed a gradual increase in toxicity to both *E. coli* and *E. sibiricum* as the doses increased (Figure 2.2). Moreover, *E. sibiricum* was more vulnerable to TiO_2 NPs than *E. coli*, which was contrary to the toxicity of ZnO NPs to *E. coli* and *E. sibiricum*. In the case of *E. coli*, both 5 nm and 150 nm NPs significantly inhibited growth of *E. coli* at 2000 and 5000 ppm, with 5 nm NPs being more toxic than the 150 nm ones (Figure 2.2(a)). The 5 μm TiO_2 NPs did not display any toxicity even at 5000 ppm. As illustrated in Figure 2.2(b), the growth of *E. sibiricum* was inhibited significantly by all the three different size TiO_2 NPs, even for 5 μm NPs when their concentrations were higher than 1000 ppm. The 5 nm NPs strongly inhibited the growth of *E. sibiricum* at concentrations above 500 ppm while the 150 nm TiO_2 NPs were intermediate in their inhibition of the growth.

Zn^{2+} toxicity in ZnO NP solution. Zn^{2+} ion concentration in LB or TSB solution of ZnO NPs was measured with atomic absorption spectrometry, and the results demonstrated that the concentrations of Zn^{2+} ion rose with the increasing ZnO NP concentrations (Table 2.1). The concentrations of Zn^{2+} ion dissolved from the same

concentration of 60 nm and 1 μm ZnO NPs were similar, which suggested that the size of NPs did not influence the dissolution of ZnO NPs much. Additionally, there was no significant difference in Zn^{2+} ion concentrations between the same concentration of ZnO NPs in LB and TSB broth. In the ZnO NP suspension of 50 ppm, the concentration of Zn^{2+} ion was approximately 40 ppm in either TSB or LB broth, which meant that most of ZnO NPs were dissolved in the solution. The concentration of Zn^{2+} was saturated at 100-110 ppm in TSB or LB medium, which occurred when the concentration of NPs was higher than 200 ppm.

In Table 2.2, the viability of *E. coli* or *E. sibiricum* decreased with increasing concentrations of $ZnCl_2$ in broth, with *E. coli* being more vulnerable of the two strains. The growth of these two strains to Zn^{2+} ions suggested that the most of overall ZnO NP toxicity came from the dissolved Zn^{2+}. The ZnO NPs, however, still played an important role in the toxicity for the solutions containing high concentrations of ZnO NPs, since the concentration of Zn^{2+} reached the saturation of 110 ppm.

Toxicity of NPs versus soluble metals from NPs. The toxicity of TiO_2 or ZnO NPs was compared to that of possibly released metal ion Ti^{4+} or Zn^{2+} from the same concentration of suspension. Since no toxicity in the TiO_2 NP suspension lower than 1000 ppm was found for either *E. coli* or *E. sibiricum*, only the suspensions > 1000 ppm were tested. The soluble fraction of 10 nm, 150 nm and 5 μm TiO_2 NPs showed little effect on the growth of *E. coli* (Figure 2.5(a)). The reason for this is probably that the TiO_2 NPs is highly insoluble, so the concentration of Ti^{4+} ion is not high enough to cause damage to *E.*

coli. Another reason may be that membrane of *E. coli* is not that permeable to TiO_2 NPs compared to *E. sibiricum*. In the case of *E. sibiricum* (Figure 2.5(b)), the biggest difference between the suspension and the soluble metal was seen at 5 μm TiO_2 NPs: no toxicity was found in soluble fractions while the viability of *E. sibiricum* in suspension of 2000 ppm and 5000 ppm 5 μm TiO_2 NPs was accordingly 74 % and 20 %.

Compared to TiO_2 NPs, the soluble fraction in ZnO NP suspension played a more important role in the microbial growth. For *E. coli*, the suspension of ZnO NPs was 10 to 40 fold more toxic than the soluble fraction, when the ZnO NP concentrations ranged from 100 to 200 ppm (Figure 2.6(a)). Furthermore, there was no toxicological difference on the microbial growth between the suspension and the soluble fraction at the concentration of ZnO NPs higher than 500 ppm. Compared to *E. coli*, *E. sibiricum* was less vulnerable to Zn^{2+} ion or ZnO NPs. Contrary to the results with *E. coli*, there was almost no significant toxic difference on the growth of *E. sibiricm* between the ZnO NP suspension and the soluble fraction, when the ZnO NP concentration is no higher than 1000 ppm (Figure 2.6(b)). At high concentrations of ZnO NPs (2000 and 5000 ppm), however, the soluble fraction was 3 to 6 fold less toxic than the whole suspension.

It is well known that low concentration of Zn^{2+} is safe or even beneficial for the bacteria, by providing stability to the membrane for example. For *E. coli*, the concentration of Zn^{2+} was unsaturated and not that high in solutions containing low concentrations of ZnO NPs, so ZnO NPs in the suspension could raise the toxicity and played a predominant role on the inhibition of the microbial growth. Furthermore, when

the concentration of Zn^{2+} reached saturation at the high concentrations of ZnO NP suspension, the soluble fraction dominated the toxicological effects. Compared to *E. coli*, *E. sibiricum* was more tolerant to the toxicity of Zn^{2+}. That is why the increasing concentrations of ZnO NPs in the suspension still could enhance the inhibition to the growth of *E. sibiricum* when the concentration of Zn^{2+} was saturated.

Morphological studies. TEM was applied to visualize the morphological changes in *E. coli* and *E. sibiricum* exposed to 1000 ppm NPs in LB or TSB medium for 12 h. EDS was performed for the validation of NPs, because osmium tetroxide, used to fix lipid in the membrane, gave some confusing black spots inside cells. The 60 nm SiO_2 NPs were found in *E. sibiricum* not in *E. coli*, which meant that it was easier for SiO_2 NPs to go through the membrane of *E. sibiricum* than that of *E. coli* (Figure 2.7(a)). The EDS results revealed that Silicon element appeared along the cross-section line and there was an increase in the distribution of both silicon and oxygen elements along the line, which confirmed the internalization of SiO_2 NPs (Figure 2.7(b) and (c)). Furthermore, the width of the silicon peak was similar to the 60 nm size of NPs.

Similar to the results with ZnO NPs, 5 nm TiO_2 NPs was found inside *E. sibiricum* cells but none in *E. coli* (Figure 2.8(a)). This is consistent with the toxicological results that TiO_2 NPs showed more toxic to *E. sibiricum* than to *E. coli*. The EDS results confirmed the presence of elemental titanium along the cross-section line that corresponds on the thin section samples. The width of the titanium peak was similar to the size of the NPs in the cell (Figure 2.8(b)).

Sixty nanometer ZnO NPs were found inside both *E. coli* and *E.sibiricum* cells (Figure 2.9(a) and (c)), which suggested that the ZnO could pass the both of membranes. Lots of dead cells with broken membranes and leaking cytoplasm were also found under TEM. The EDS data confirmed that the region along the cross-section line contained elemental zinc. The distribution of zinc displayed a peak whose width was similar in size to ZnO NPs (Figure 2.9(b) and (d) dark spot). Together these data validated that the dark spot is a ZnO NP. Similar internalization results was published by Brayner et al. (5).

Discussion

The chemical composition of NPs is one of the most important factors determining biological response, and causes their toxicity. As illustrated in Figure 2.1 and 2.2, ZnO NPs and TiO_2 NPs were toxic to both *E. coli* and *E. sibiricum*, while SiO_2 and Fe_3O_4 NPs were not generally. Only in the case of *E. sibiricum*, did 500 ppm 10 nm SiO2 NPs inhibit growth, and then only after the exposure time was increased to 48 h. The composition of NPs also influences the possible mechanisms of NP toxicity. For instance, ZnO NPs are slightly soluble (5.4mg/L), and the released Zn^{2+} ion plays a key role in ZnO NP toxicity. In the case of TiO_2 NPs, the ROS leads to oxidation of membrane and proteins, and hinders nutrient uptake and respiration, resulting cell death. Recently several papers show the toxicity of SiO_2 NPs to *E. coli*, *B. subtilis* and human cells (1, 21, 24), and some groups reported that no cytotoxicity was found for iron oxide NPs, similar to our results (15, 16, 19, 23, 36). All these data demonstrate that chemical

composition is one of the physicochemical properties important to NP toxicity.

Size is also an important factor to NP toxicity. As size decreases, both surface area and surface reactive groups increase, which may enhance the NP toxicity (32, 35). We found that the smaller ZnO or TiO_2 NPs were more toxic to *E. coli* than larger NPs (Figure 2.1(a) and 2.2(a)), similar to previous findings (19, 51). There were some conflicts in the size-effect with *E. sibiricum*, since 1 μm ZnO NPs were more toxic than 60 nm ZnO NPs (Figure 2.1(b)), and 5 μm TiO_2 NPs were more toxic to *E. sibiricum* than 150 nm TiO_2 NPs (Figure 2.2(b)).

Aggregation of NPs in the medium may be one reason for these conflicts. High concentrations of salts and proteins in the medium can cause the aggregation of NPs, which increases the size the cell experienced (26). For instance, Long *et al.* found that commercial grade, nano-size TiO_2 NPs aggregated in Hank's basic salt solution and Dulbecco's modified eagle's medium, and the hydrodynamic diameter of the TiO_2 NPs increased to 2368±163 nm at 120 ppm in their studies (25). Although there is no clear consensus about the upper size limit for the NP toxicity, it was estimated that it might be between 65 nm and 200 nm (11). Additionally, there are other factors of NPs which can influence NP toxicity, such as shape and surface modification (41). So it is important for toxicology and exposure assessment of NPs to determine and characterize these parameters.

Gram-positive or Gram-negative species may show different tolerance to the toxicity of the same NPs. For example, TiO_2 NPs were more toxic to *E. sibiricum* than to *E. coli*

(Figure 2.2). Some groups reported that the TiO_2 NPs should be more toxic to Gram negative than Gram positive species, since Gram positive species have thicker cell walls and can form spores (49). *E. sibiricum* is non-spore-forming, which may be one of the reasons why TiO_2 NPs are more toxic to *E. sibiricum*. We also found that ZnO NPs were more toxic to *E. coli* compared to *E. sibiricum*. Adams *et al.* (1) found that ZnO NPs were the most toxic ones of three NMs they tested, and were more toxic to *B. subtilis*, Gram positive, than to *E. coli*, Gram negative. Other external factors, such as the initial concentration of bacteria, the physiological state of the bacteria, light intensity, pH and O_2, also can influence the toxicity of NPs (38).

The dissolution of NPs was hypothesized as one of the mechanisms by which NPs interact with cells (32). The dissolution can be influenced by the coating of NPs, pH of solution and other factors (12, 52). TiO_2 is highly insoluble (0.15 mg/L) while ZnO is slightly soluble (5.4 mg/L) (6). Even if Ti^{4+} is dissolved into solution, the reaction below usually happens:

$$Ti^{4+} + 2H_2O \rightarrow TiO_2 + 4H^+$$

Ti^{2+} is much less stable than Ti^{4+}, and it is probably oxidized by O_2 to form Ti^{4+}. The dissolution of ZnO NPs was previously reported, with the suggestion that toxicity might depend on Zn^{2+} ion in NP solution (6, 12, 17, 22). The dissolution of ZnO NPs may worsen the adverse effects on aquatic biota and cause more ecotoxicity (39). We did not find that the dissolution of ZnO NPs was size-dependent, although it had been reported that Zn^{2+} release rate of ZnO NPs was faster than that of ZnO micro-particles (52). Zinc

ions play an important role in cellular process, mostly as an enzyme cofactor (47). It has been reported that high concentrations of Zn^{2+} ion are harmful for cells by inhibiting some enzyme activity or affecting ATP (8, 28, 48). The viability results demonstrated that Zn^{2+} was more toxic to *E. coli* than *E. sibiricum*. According to the genome sequences of *E. coli* and *E. sibiricum* in the database of National Center for Biotechnology Information, there are more proteins annotated for zinc transport, efflux, or uptake in *E. coli* than in *E. sibiricum*, which may make *E. coli* more vulnerable to Zn^{2+} toxicity.

Internalization of NPs can be influenced by size and chemical composition of NPs and by types of microorganisms. For instance, 5 nm TiO_2 NPs were found in *E. sibiricum* rather than in *E. coli*, which suggests that it is easier for TiO_2 NPs to go through the membrane of *E. sibiricum* than that of *E. coli*. This could be one of the reasons why TiO_2 NPs were more toxic to *E. sibiricum* than *E. coli*, since the internalized NPs may cause direct damage and induce serious toxicity. TiO_2 NPs can decompose and peroxidate membranes, which is the explanation for the killing effect by TiO_2 photocatalysis (27, 40, 44). In the ZnO NP samples, many dead cells with damaged membranes and leaking intercellular contents were noted, also observed in a previous study (5). Furthermore, we investigated the morphology of *E. coli* and *E.sibiricum* exposed to 50 nm Fe_3O_4 NPs and 5 μm TiO_2 particles by TEM, and found that both *E. coli* and *E. sibiricum* could internalize Fe_3O_4 NPs, although Fe_3O_4 NPs were non-toxic. No 5 μm TiO_2 particles were found inside these microorganisms. The largest TiO_2 particles found inside the cells were around 150 nm. Other NPs, such as CeO_2 and silver, were previously found internalized

in *E. coli* or incorporated into the membrane, as well as interfacial charge transfer and the interaction between the TiO_2 NPs and membrane of *E. coli* (30, 43, 46). There is no clear explanation, however, about the mechanisms of internalization. The contact between NPs and bacteria was enhanced by the repulsive force generated between negatively charged bacterial cells and positively charged NPs (31).

In conclusion, we demonstrated that ZnO and TiO_2 NPs were toxic to *E. coli* and *E. sibiricum* in a dose-dependent manner, while SiO_2 and Fe_3O_4 NPs did not inhibit their growth during a 12 h exposure. Among these NPs, ZnO NPs were the most toxic ones for either of these two microorganisms. ZnO was more toxic to *E. coli* than to *E. sibiricum* with the toxicity mostly due to the Zn^{2+} ion dissolved from the NP, while TiO_2 was more toxic to *E. sibiricum* than to *E. coli*. By TEM-EDS, we found SiO_2 and TiO_2 NPs internalized only in *E. sibiricum* not in *E. coli*, while ZnO NPs appeared inside both of these bacteria.

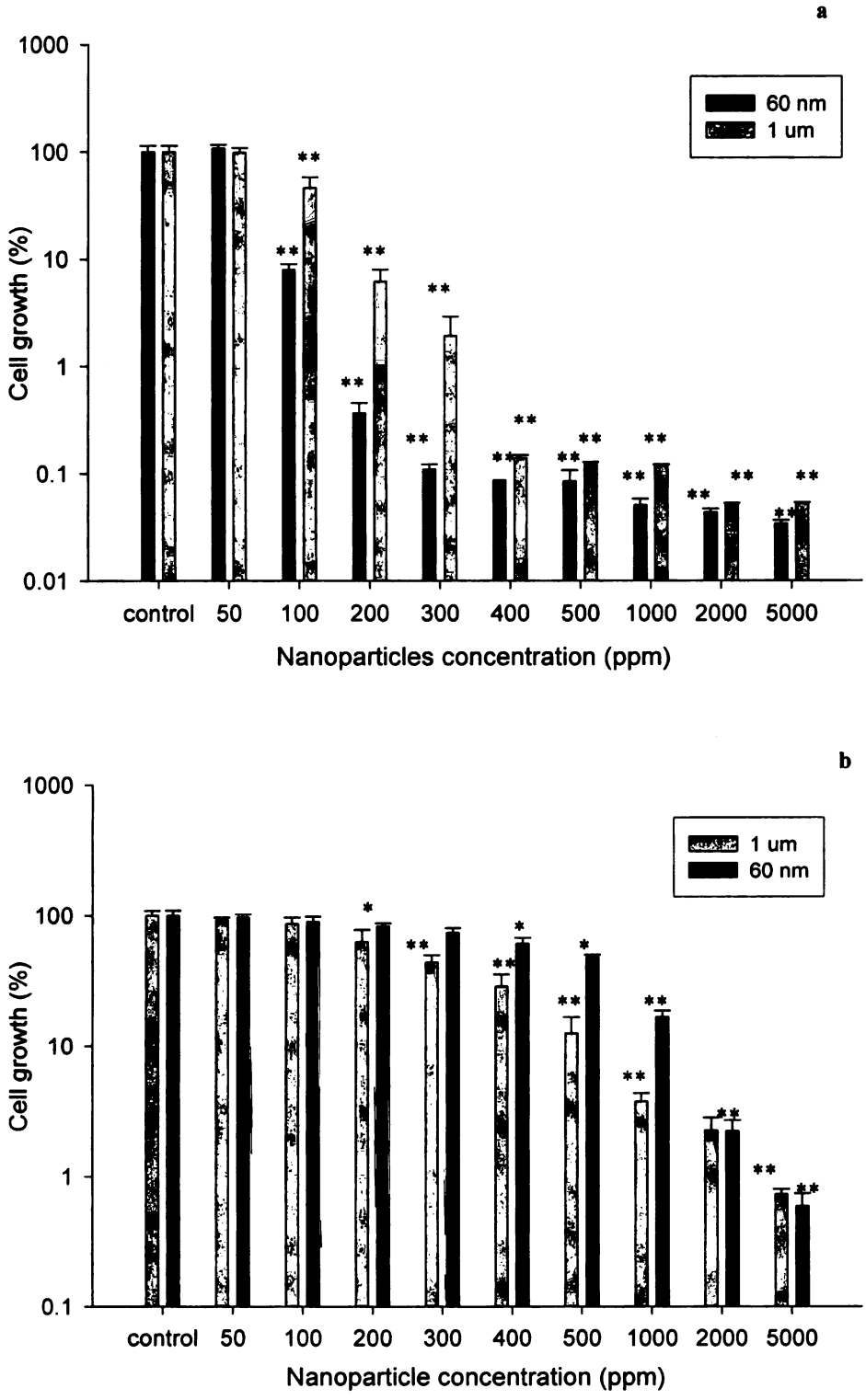


Figure 2.1 (a) The effect of ZnO NPs on *E. coli.* (b) The effect of ZnO NPs on *E. sibiricumi.* *$p<0.05$ versus control cells;**$p<0.005$ versus control.

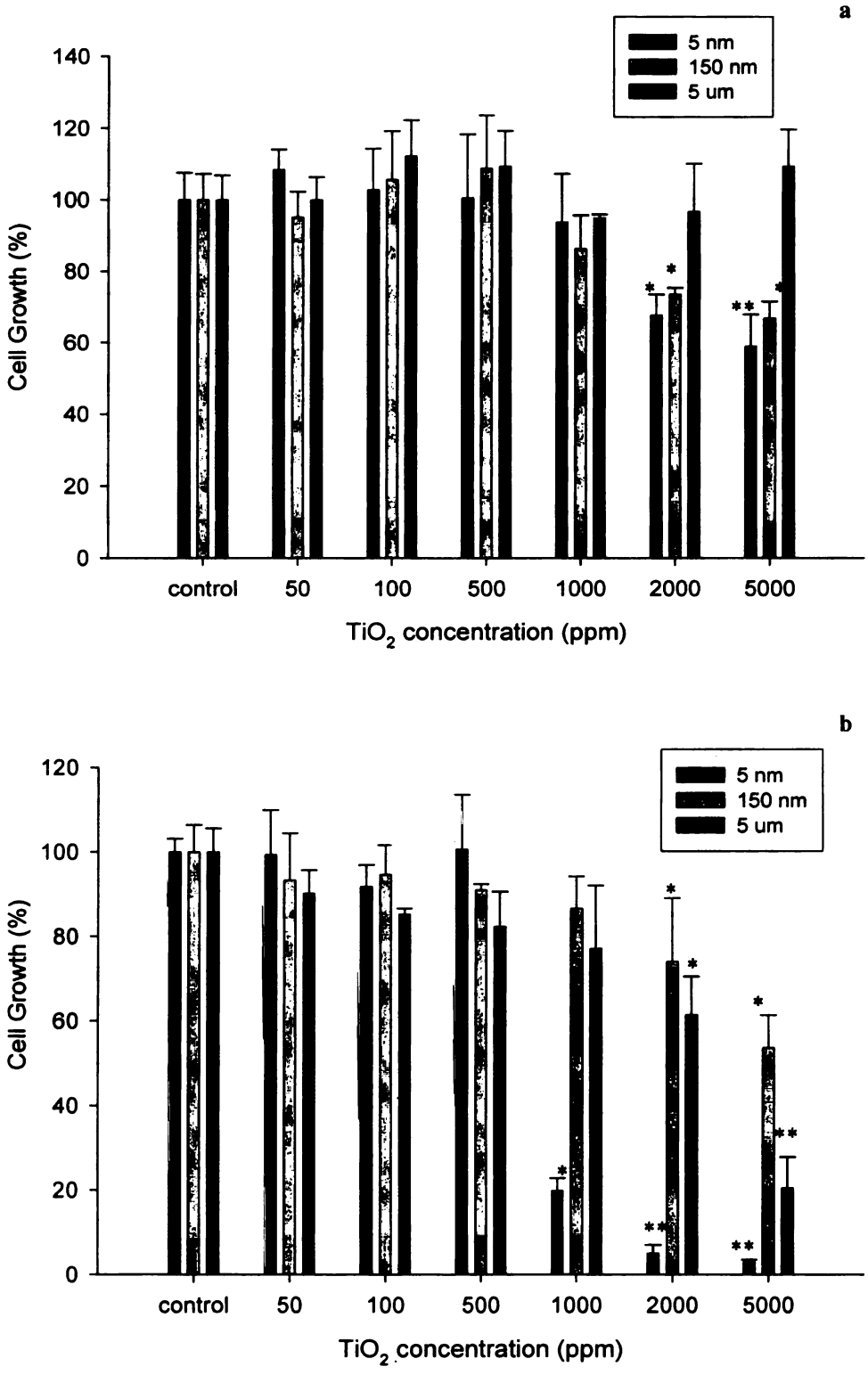


Figure 2.2 (a) The effect of TiO_2 NPs on *E. coli*. (b) The effect of TiO_2 NPs on *E. sibiricum*. *$p<0.05$ versus control cells;**$p<0.005$ versus control.

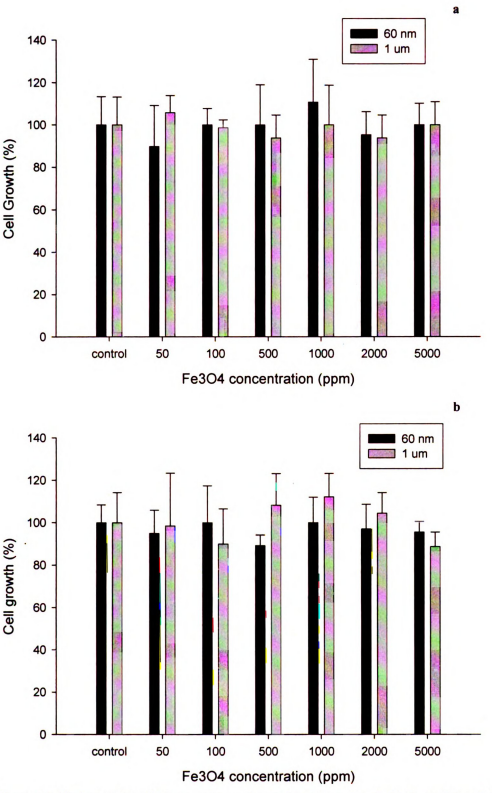
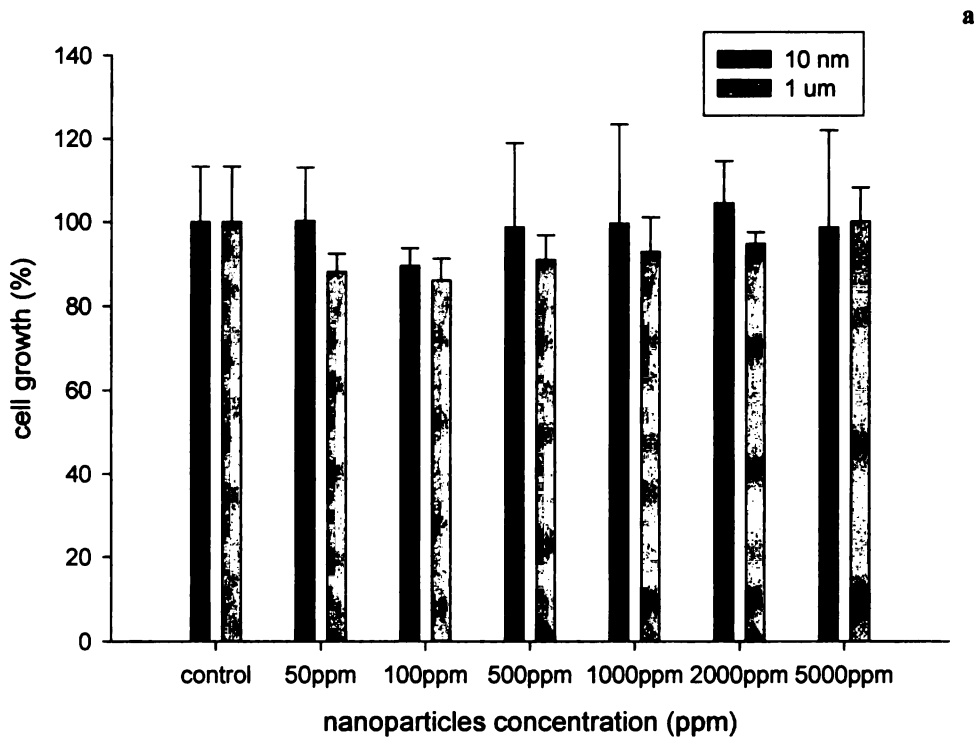


Figure 2.3 (a) the toxicity of 60 nm and 1 μm iron oxide NPs to *E coli*, (b) the toxicity of 60 nm and 1 μm iron oxide NPs on *E. sibiricum*.



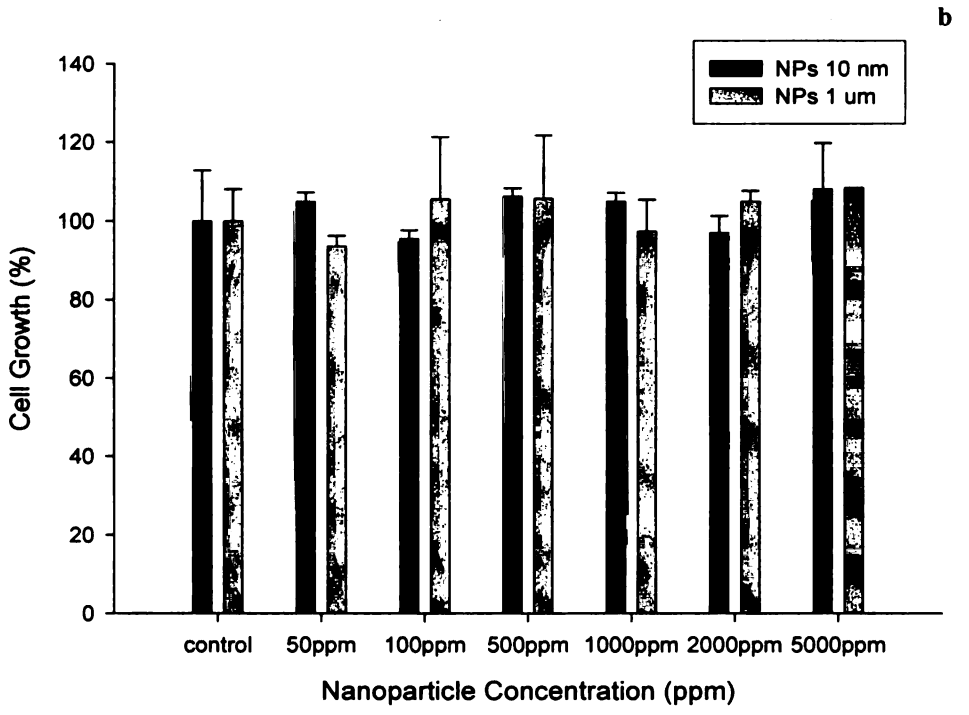


Figure 2.4 (a) the influence of SiO_2 NPs (10 nm and 1 μm) on the growth of *E. coli*, (b) the influence of SiO_2 NPs (10 nm and 1 μm) on the growth of *E. sibiricum*.

Table 2.1 The concentration of Zn^{2+} ion dissolved in LB or TSB from various concentrations of ZnO NPs

ZnO NPs Conc (ppm)	Zn^{2+} Conc in LB (ppm)		Zn^{2+} Conc in TSB (ppm)	
	60 nm	1μm	60 nm	1μm
0	1.2±0.0	1.2±0.0	0.7±0.0	0.7±0.0
50	38.0±2.8	37.0±1.4	40.0±0.0	38.5±2.1
200	90.0±0.0	85.0±7.1	110.0±0.0	100.0±0.0
1000	100.0±0.0	100.0±0.0	105.0±7.1	100.0±0.0
5000	115.0±7.1	100.0±0.0	110.0±0.0	100.0±0.0

Table 2.2 Viability of *E. coli* and *E. sibiricum* in various concentrations of $ZnCl_2$ solution

$ZnCl_2$ Conc (ppm)	Viability (%)	
	E. coli	*E. sibiricum*
Control	100.0±10.3	100.0±2.0
32.5	87.3±10.4	113.9±4.6
65	68.2±4.2	77.3±7.8
130	<0.01	16.4±3.9

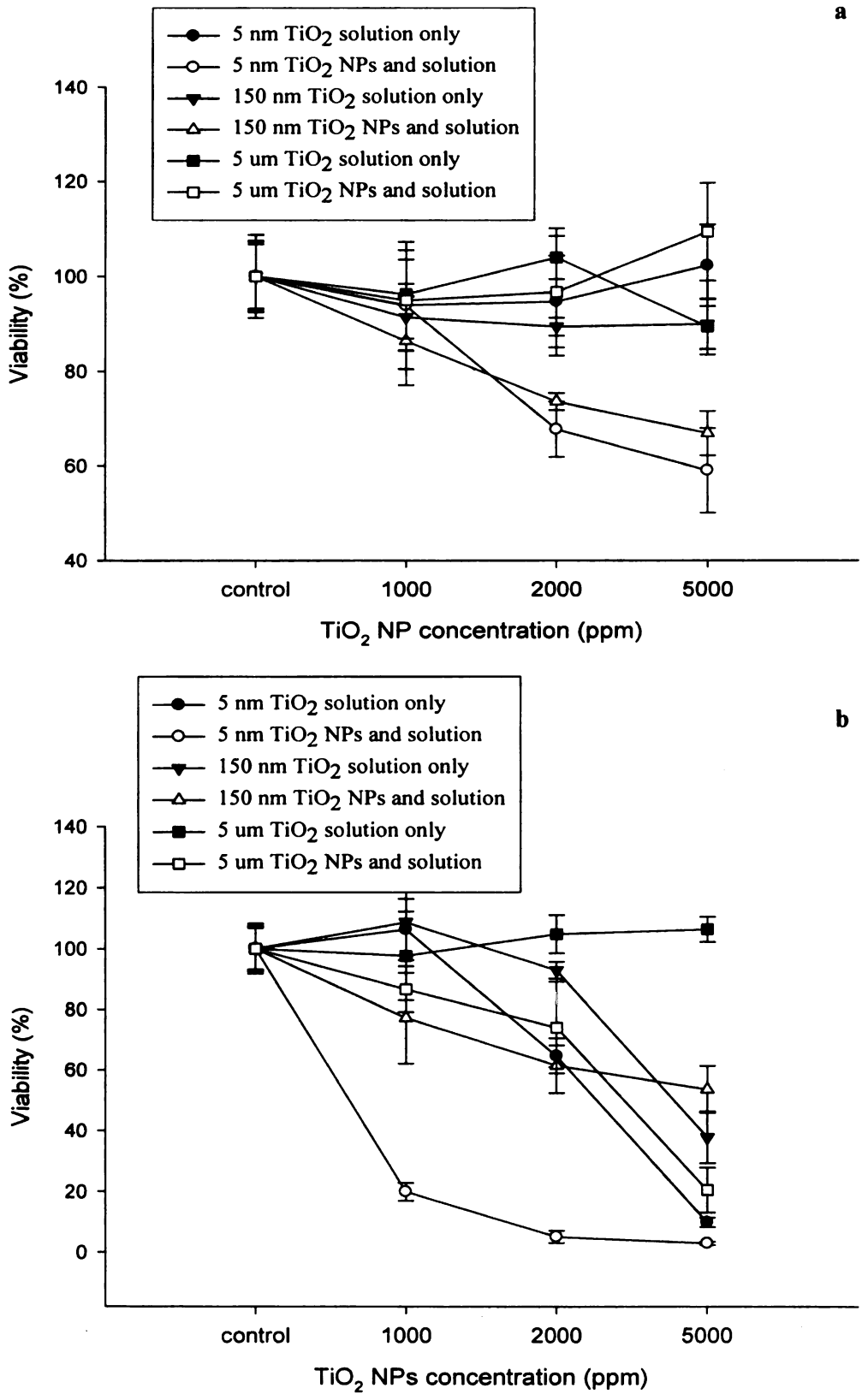


Figure 2.5 (a) The toxicity of TiO_2 NP suspension and soluble fraction on *E. coli*. (b) The toxicity of TiO_2 NP suspension and soluble fraction on *E. sibiricum*.

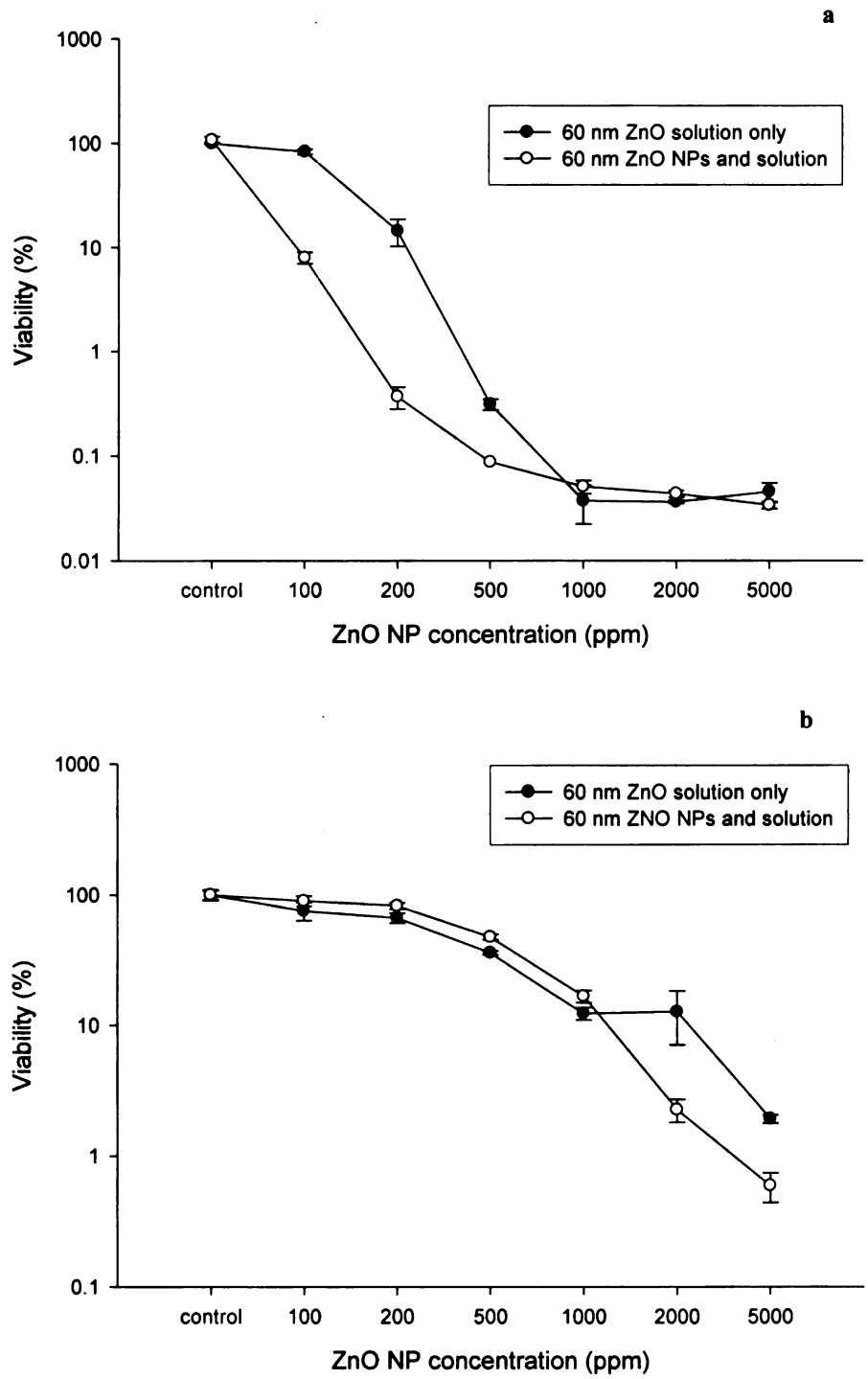


Figure 2.6 (a) The toxicity of ZnO NP suspension and soluble fraction on *E. coli*. (b) The toxicity of ZnO NP suspension and soluble fraction on *E. sibiricum*.

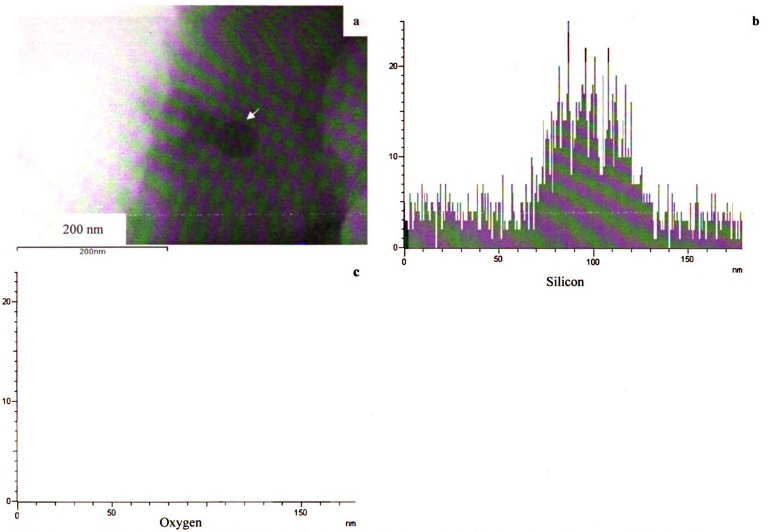


Figure 2.7. Distribution of SiO_2 in *E.sibiricum*. (a) TEM image of a SiO_2 in *E.sibiricumi*. (b) Distribution of elemental silicon. (c) Distribution of oxygen.

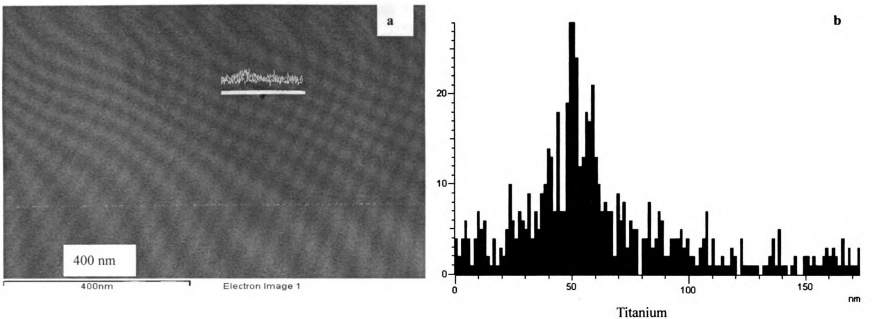


Figure 2.8. Distribution of TiO_2 in *E. sibiricum* samples. (a) TEM image of a TiO_2 NP inside *E. sibiricum* cells. (b) Distribution of elemental titanium along the cross-section line on samples. The peak displayed the increase of Ti.

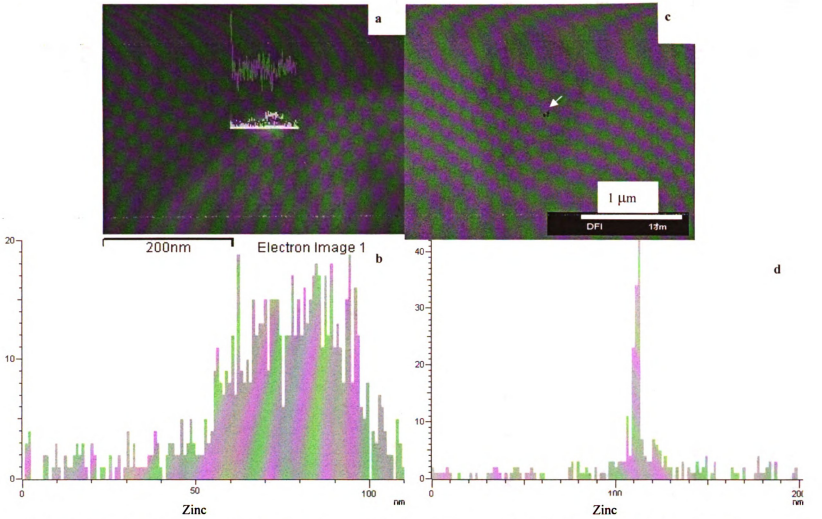


Figure 2.9. Distribution of ZnO in *E. coli* (a-b) and *E. sibiricum* samples (c-d). (a) TEM image of a ZnO NP in *E. coli*. (b) Distribution of zinc element along the line about the Sample of *E. coli*. (c) TEM image of a ZnO in *E. sibiricum*. (d) Distribution of zinc element.

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CHAPTER 3

INDUCTION OF INTRACELLULAR REACTIVE OXYGEN SPECIES BY NANOPARTICLES AND THE MEASUREMENT OF GENOTOXICITY BY COMET ASSAY

Abstract

With the rapid development of nanotechnology, various NMs have been applied in diverse areas, including cosmetics, water purification, material science, electrical devices and drug delivery. The biosafety of NPs has begun to attract attention with regard to their toxicological and environmental effects. I found that zinc oxide (ZnO) and titanium oxide (TiO_2) NPs induced reactive oxygen species (ROS) in *Escherichia coli* strain K12 (Gram-negative) and *Exiguobacterium sibiricum* strain 255-15 (Gram-positive) in a dose-dependent manner. A 12 h exposure to silicon oxide (SiO_2) NPs induced ROS in *E. sibiricum*, but not in *E. coli* except for 5000 ppm SiO_2 NPs. There were more ROS induced by TiO_2 NPs and SiO_2 NPs in *E. sibiricum* than in *E. coli*, while ZnO NPs induced more ROS in *E. coli* than in *E. sibiricum*, which is consistent with our viability results. The 5 nm TiO_2 NPs caused more ROS induction than 150 nm TiO_2 NPs, which confirmed that size is one of the important factors in NP toxicity. Both TiO_2 NPs and ZnO NPs displayed genotoxicity as measured by comet assay, and TiO_2 NPs were more genotoxic than ZnO NPs. During 3 h exposure, 10 ppm TiO_2 and 1 ppm ZnO NPs caused significant DNA damage in lymphocytes.

Introduction

The development in NMs and nanotechnology offers various new opportunities in diverse fields, such as electronics, medicines, environmental remediation and food. In 2005, more than $10 billion were spent on the nanotechnology development internationally (21). However, the environmental exposure to the NPs may have some adverse effect on microorganisms, although NPs have existed in the nature for eons (31). Few studies about the influence of NP on public health and environment are available, and there is no clear understanding of the mechanisms of NP toxicity.

Recently there have been a few reports on the antimicrobial activity of NPs and interaction between NPs and microorganisms (7, 8, 18, 34, 35, 42). The induction of ROS by NPs is considered as one of the possible mechanisms for NP toxicity. In 1997, Rahman et al. found the increasing induction of ROS by nano-sized TiO_2 in human and rat alveolar macrophages (36). Meanwhile, TiO_2 NPs were toxic and caused oxidative stress in different cell types, including bacteria and mammal cells (2, 20, 26, 39, 50-52). The induction of ROS by ZnO NPs has also been hypothesized as an explanation for their toxicity, though the mechanisms for their toxicity are not yet well established (38).

NPs may cause genotoxicity to different cells, since the ROS can enter cells and oxidize membranes, and further damage the DNA, lipids and proteins. There have been a few papers reporting DNA lesions caused by ROS induction from TiO_2 NPs (16, 20, 24, 27, 50). The alkaline comet assay is a well-established method to detect and measure DNA damage, since it is sensitive, rapid, flexible, cheap and easy to apply (45-47). This

technology has been used in different fields, including radiation biology, genotoxicity, clinical research, and genetic ecotoxicology (3, 12, 14).

In this study, we investigated the ROS induction by SiO_2, TiO_2 and ZnO NPs in *E. coli* and *E. sibiricum*. I also suggested a possible mechanism of ROS induction by TiO_2 NPs. The comet assay was performed to evaluate the genotoxicity of TiO_2 and ZnO NPs, and Olive tail moment, tail DNA and tail length were measured to quantify DNA damage in lymphocytes.

Materials and Methods

Reagents and preparation of NP suspensions. RPMI-1640 medium and 2', 7'-dichlorofluorescin diacetate (DCFH-DA) was obtained from Invitrogen (Carlsbad, CA). Normal melting agarose (NMA), low melting point agarose (LMPA), Histopaque, Phosphate Buttered Saline (PBS), SiO_2 (10 nm), TiO_2 (5 nm and 150 nm) and ZnO (60 nm) were purchased from Sigma-Aldrich (St. Louis, MO). One hundred g/L NPs stock solutions were prepared in distilled and deionized water.

Bacterial culture and cell culture. *E. coli* K12 was grown aerobically in Luria-Bertani (LB) liquid broth at 37 °C and 200 rpm for 12 h. *E. sibiricum* was cultured in TSB medium at 30 °C and 150 rpm for 12 h. Solid medium for *E. coli* or *E. sibiricum* was as above with 1.5% agar (Difco) added. All the cultures with NPs were grown under the same conditions.

Intracellular ROS measurement. The intracellular ROS was measured by using

DCFH-DA as described previously (25, 49). Ten mM of DCFH-DA stock solution in DMSO was diluted with PBS buffer into 20 μM of working solution. *E. coli* K12 or *E. sibiricum* was exposed to different concentrations of NPs in 2 ml of liquid media for 24 h. Cells were collected by centrifugation in 1.8 ml of liquid medium, washed twice with water and then incubated in 1.8 ml of DCFH-DA working solution at 37 °C or 30 °C for 1 h. Samples were washed twice with 1× PBS, and their fluorescence was measured at 485-nm excitation and 520-nm emission with SpectraMax M2 spectrometer (Molecular Devices, Sunnyvale, CA). Blank samples (only microorganisms) and control samples (only NPs) were treated in the same way. Viability was measured by plate counting. All the data are the mean of triplicate samples.

Alkaline comet assay. Fresh Heparinized venous blood was used for separation of lymphocytes. Blood (100 μl) was diluted with RPMI-1640 medium (1 ml) and the dilution was layered over 200 μl Histopaque buffer. After centrifuged at 800×*g* for 20 min, the buffy coat from the medium/Histopaque interface was collected. After washed once with RPMI-1640 and centrifuged at 250×*g* for 10 min, the pellet was resuspended in 1 ml RPMI-1640. The lymphocyte suspensions were exposed to various concentrations of TiO_2 NPs or ZnO NPs (1 ppb, 10 ppb, 100 ppb, 1 ppm, 10 ppm, and 100 ppm) for 3 h or 6 h at 37 °C, described by Tice *et al.* (46). The positive control groups received 2 mM ethyl methanesulphonate (EMS) for 3 h exposure or 1 mM for 6 h exposure. After the treatment, the lymphocytes were centrifuged at 500×g for 5 min and resuspended in 110 μl PBS solution. The Trypan blue dye viability test was applied

before and after the NP exposure, and the viability was essential for all the experiments. The lymphocyte suspension (100 μl) was mixed with 1% LMPA (100 μl) at 37 °C, and 80 μl of mixture was added onto one of the duplicate microscope slides (coated with 1% NMP and dried in advance), and covered with a cover-slip. After 5 min solidification on ice, the cover-slip was slid off, and 0.5% LMA (90 μl) was spread as a third layer with a similar procedure. After the removal of the cover-slip, the slides were dipped into freshly made, cold-lysing solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris (pH 10), with 1% Triton X-100 added just before use) for a minimum of 2 h at 4 °C. All the slides were closely placed in a horizontal electrophoresis box containing freshly-prepared cold electrophoresis buffer (1 mM EDTA and 300 mM NaOH, pH >13) for 20 min to allow for unwinding of the DNA, and subsequently electrophoresis was performed at 24 V and 300 mA for 30 min. After that, the slides were washed three times (5 min each) with neutralization buffer (400 mM Tris, pH 7.5) and stained with 1 μg/ml ethidium bromide (80 μl) for 5 min. The slides were then soaked in cold distilled water for 5 min, covered with cover-slips, and then stored at 4 °C in a humidified slide box (no more than 2 days). The observation was made at 400×g magnification with a Komet 3.1 Image Analysis System (Kinetic Imaging, Liverpool, U.K.), and the DNA damage parameters, including olive tail moment, tail DNA and tail length, were measured (17). One hundred total cells on duplicate slides were analyzed in each of three independent experiments. The data were analyzed with one-way analysis of variance (ANOVA), and $p < 0.05$ was considered significant compared with the respective controls.

Results and Discussion

Cellular oxidative stress level. DCFH-DA can react with ROS and produce fluorescence. Intracellular oxidative stress increased as NP concentrations increased during the 24 h exposure (Figure 3.1). SiO_2 NPs slightly induced ROS in *E. coli* only at 5000 ppm in Figure 3.1(a). For TiO_2 NPs, ROS was induced when the concentrations were higher than 100 ppm, which meant that TiO_2 NPs induced more ROS in *E. coli* than SiO_2 NPs. Furthermore, 5 nm TiO_2 caused more oxidative damage in *E. coli* than 100 nm TiO_2, which is consistent with the size-effect found in our viability studies. In the case of *E. sibiricum*, the ROS increased when the concentrations of SiO_2 NPs were higher than 500 ppm, and ROS was induced in *E. sibiricum* at TiO_2 NP concentrations above 100 ppm (Figure 3.1(b)). Similar to the results with *E. coli*, 5 nm TiO_2 NPs induced more ROS in *E. sibiricum* than the 150 nm NPs. Hence, SiO_2 and TiO_2 NPs induced more ROS in *E. sibiricum* than in *E. coli*, which might be one of the reasons why the growth of *E. sibiricum* was more inhibited by the same NPs than *E. coli* in our previous studies.

ZnO NPs were the most toxic of these three test NPs, and they caused the most serious inhibition on the microbial growth and induced the most ROS in both *E. coli* and *E. sibiricum*. As illustrated in Figure 3.1(c), the ROS in both *E. coli* and *E. sibiricum* began to increase at 50 ppm of ZnO NPs. ZnO NPs induced more ROS in *E. coli* than in *E. sibiricum*, which was consistent with our viability test.

Our ROS induction data also proved the size-dependent effect of NPs, and were

consistent with our previous viability test. The 5 nm TiO_2 NPs induced more ROS in either *E. sibiricum* or *E. coli* than the same concentration of 150 nm TiO_2 NPs. Other groups also have reported the size effect on ROS induction by ZnO NPs or silver NPs (11, 33). The ROS formed on the surface of TiO_2 NPs was also reported to depend on particle size, aggregation of particles and other factors (1). Since the lifetime of hydroxyl radicals is extremely short (10^{-9} s), the direct contact between NPs and membranes seems essential for an effect of NPs on bacteria (19, 22, 44). Although Gram-negative bacteria usually have thinner cell walls than Gram positive bacteria, it seems that there was no significant difference in their membrane permeability to ROS.

Photocatalytic TiO_2 chemistry. The reactions below describe the possible mechanisms of ROS induced by TiO_2 NPs (9, 10, 53). In the first equation, a hole (e^-)/electron (h^+) in the valence band and conduction band is generated and induced by light (λ<390 nm) and TiO_2 NPs. The e^- is absorbed onto the surface of TiO_2 NPs and available for electron transfer, which can reduce O_2 to O_2^- (reaction 2), and produce H_2O_2 by further reducing O_2^- (reaction 3). The OH radicals can be produced by the reaction of superoxide with H_2O_2 (reaction 4) and reduction of H_2O_2 by e^- (reaction 5). The OH radicals can also be formed by the reactions of H_2O or OH^- with h^+ and electron abstraction from absorbed oxidizable species by h^+ (reaction 6 and 7). H_2O_2 can be produced by either a reductive pathway (reaction 3) or an oxidative pathway (reaction 8), while it can be decomposed into OH radicals by induction of light (reaction 9).

$$TiO_2 + h\nu \rightarrow TiO_2\,(e^- + h^+) \qquad (1)$$

$$e^- + O_2 \rightarrow O_2^- \quad (2)$$

$$O_2^- + 2H^+ + e^- \rightarrow H_2O_2 \quad (3)$$

$$H_2O_2 + O_2^- \rightarrow \bullet OH + OH^- + O_2 \quad (4)$$

$$e^- + H_2O_2 \rightarrow \bullet OH + OH^- \quad (5)$$

$$h^+ + OH^- \rightarrow \bullet OH \quad (6)$$

$$h^+ + H_2O \rightarrow \bullet OH + H^+ \quad (7)$$

$$2h^+ + 2H_2O \rightarrow 2H^+ + H_2O_2 \quad (8)$$

$$h\nu + H_2O_2 \rightarrow 2\bullet OH \quad (9)$$

An interfacial charge transfer occurred between TiO_2 NPs and the membrane of *E. coli* (32). The reaction 10 and 11 exhibit the surface charge trapping on TiO_2 NPs. During the process of interfacial charge transfer, h^+ reacts with peptidoglycan (PGN) and lipo-polysaccharides (LPS) on microbial cell wall membranes, such as *E. coli* (reaction 12). As well, h^+ can be scavenged by PE, LPS or *E. coli* during surface oxidation (reaction 13).

$$e^- + Ti(IV)O\text{-}H \rightarrow Ti(III)O\text{-}H^- \quad (10)$$

$$h^+ + Ti(IV)O\text{-}H \rightarrow Ti(IV)O\bullet\text{-}H^+ \quad (11)$$

$$h^+ + RH \rightarrow R\bullet + H^+ \quad (12)$$

$$Ti(IV)O\bullet\text{-}H^+ + RH \rightarrow OH + R\bullet + H^+ \quad (13)$$

ROS induction can result in other intracellular damage and disorder of cellular activities. Matsunaga *et al.* reported that TiO_2 could oxidize CoA and cause cell death (28, 29). The cell membrane may be oxidized prior to the oxidation of CoA (13, 30, 43). Saito

et al. found rapid leakage of K^+ ions and slow release of proteins and RNA, and they explained that the cell death was due to a significant disorder in cell permeability (5, 37).

Genotoxicity of TiO_2 and ZnO NPs. Exposure to TiO_2 and ZnO NPs caused significant DNA damage in lymphocytes. Lymphocytes treated with different concentration of TiO_2 NPs for 6 h, and the clear DNA tails appeared at concentrations greater than 1 ppm. As shown in Table 3.1, the tail length, DNA tail and Olive tail moment increased with the increasing TiO_2 NP concentration for both 3 h and 6 h exposures, which meant that DNA damage aggravated as the NP concentration increased. The lymphocytes displayed significant DNA damage in 3 h exposure when the TiO_2 NP concentrations were greater than 1 ppm. During the 6 h exposure, the tail length, the tail DNA and the Olive tail moment demonstrated significant difference at concentrations above 100 ppb, compared to the negative control. There is no significant difference between 3 h and 6 h exposures.

In the case of ZnO NP treatment, the enhancement of tail length, DNA tail and olive tail moment was demonstrated as the concentration of ZnO NPs increased. The concentrations of ZnO NPs, at which significant DNA damage started, were 10 ppm for 3 h exposure and 1 ppm for 6 h exposure. These results were ten times higher than those for TiO_2 NPs. There is no clear explanation about genotoxicity caused by NPs, although one of the hypotheses is ROS induction (8, 16, 19, 20, 35, 40, 43, 48).

The results suggest that TiO_2 NPs caused more genotoxicity than ZnO NPs, consistent with other reports (23). The explanations are:

(a) Dissolution of ZnO NPs. Our previous studies have shown that ZnO dissolves in LB and TSA medium to form Zn^{2+} ion, which is not genotoxic. Actually low concentrations of Zn^{2+} ion can stabilize membrane structures and prevent ROS production (6, 41). The saturation concentration of Zn^{2+} ion in the solution of ZnO NPs is approximately 100-110 ppm. The dissolution of ZnO NPs may reduce the genotoxicity of ZnO NPs.

(b) Size difference between TiO_2 NPs and ZnO NPs. The size of TiO_2 NPs was 5 nm, while the size of ZnO NPs was 60 nm. For the same mass, TiO_2 NPs provide more surface area and hence supply more surface reactive sites than ZnO NPs. There may be more TiO_2 NPs absorbed to the lymphocyte membrane than ZnO NPs due to their smaller size. Moreover, it is probably easier for the much smaller TiO_2 NPs to enter the membranes of lymphocytes than for the larger ZnO NPs.

Induction of ROS has been hypothesized as one of possible mechanisms for genotoxicity. As shown in Figure 3.1, ZnO NPs induced much more ROS than TiO_2 NPs. When the concentrations of NPs were lower than 100 ppm, however, there was not much difference between the ROS induced by ZnO NPs and by TiO_2 NPs, especially in *E. sibiricum*. Fullerenes can cause DNA damage, and it is much more genotoxic than ZnO NPs and TiO_2 NPs (15). The reason may be that fullerenes can disperse more evenly in solution and not aggregate as easily as ZnO NPs and TiO_2 NPs. In addition, Silica NPs was reported to caused no genotoxicity by the comet assay (4).

In conclusion, the ROS induction by NPs was found in both *E.coli* and *E. sibiricum*.

SiO_2 NPs could induce ROS in *E. sibiricum* when the concentration was greater than 500 ppm, while they did not induce ROS in *E. coli* except at 5000 ppm. TiO_2 NPs induced more ROS in *E. sibiricum* than in *E. coli*, which is consistent with our previous viability tests. The 5 nm TiO_2 NPs induced more ROS than 150 nm TiO_2 NPs in either *E. sibiricum* or *E. coli*. According to the measurement of Olive tail moment, tail DNA and tail length in comet assay, 1 ppm ZnO NPs in 6 h exposure and 10 ppm ZnO NPs in 3 h exposure caused significant DNA damage in lymphocytes. The 100 ppb TiO_2 NPs for 6 h exposure and 1 ppm TiO_2 NPs for 3 h exposure damaged DNA significantly, which demonstrated that TiO_2 NPs are more genotoxic than ZnO NPs for lymphocytes.

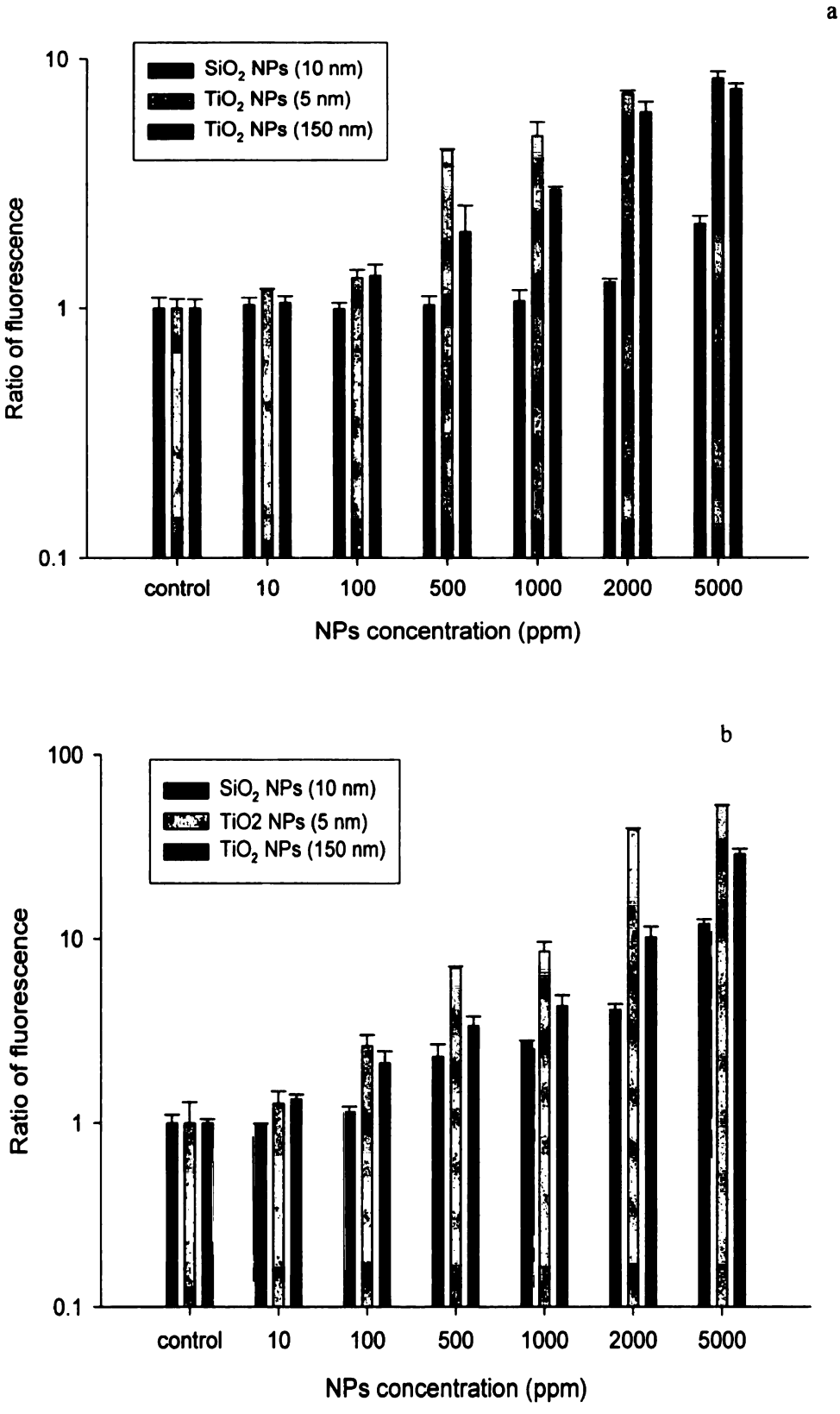

Figure 3.1. (a) The production of ROS by SiO_2 and TiO_2 NPs in *E.coli*. (b) The production of ROS by SiO_2 and TiO_2 NPs in *E.sibiricum*.

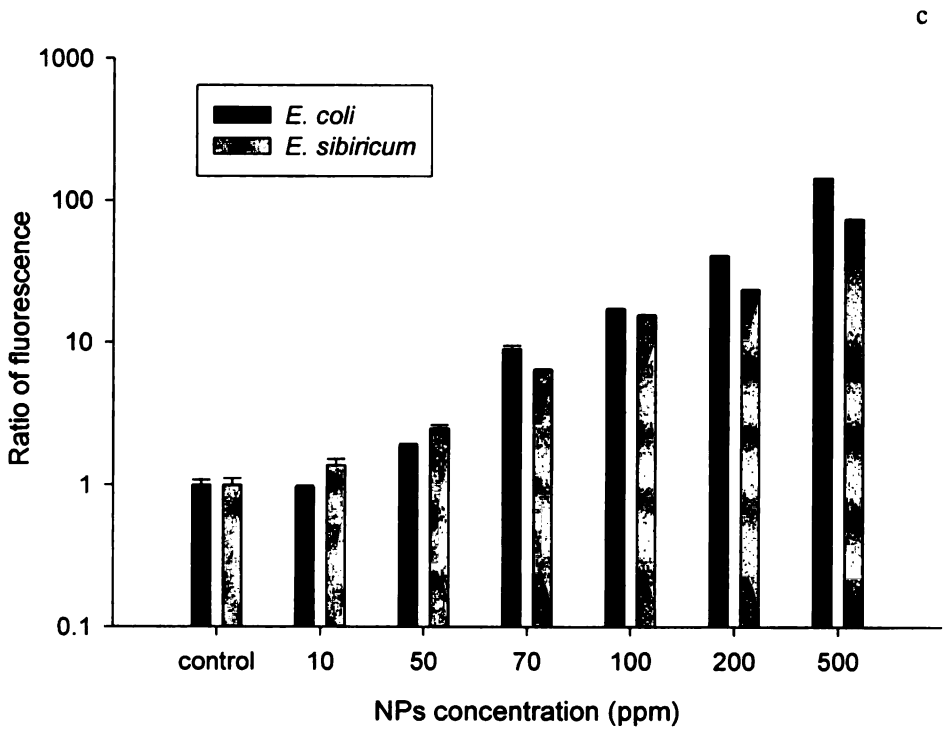

Figure 3.1 cont. (c) The production of ROS in *E. coli* and *E. sibiricum* by ZnO NPs.

Table 3.1. Measurement of tail length (μm), tail DNA (%), Olive tail moment (arbitrary units) by TiO_2 NPs in comet assay

	Tail length (μm)		Tail DNA (%)		Olive tail moment	
	3 h	6 h	3 h	6 h	3 h	6 h
1 ppb	7.77±0.45[a]	7.70±1.57[a]	2.55±0.32[a]	2.92±0.23[a]	0.38±0.03[a]	0.41±0.17[a]
10 ppb	8.58±1.09[a]	9.58±1.36[a]	2.60±0.42[a]	3.38±1.67[a]	0.38±0.04[a]	0.49±0.24[a]
100 ppb	10.85±1.31[a]	11.06±1.07[b]	3.80±0.48[a]	4.00±1.94[b]	0.54±0.05[a]	0.57±0.28[b]
1 ppm	10.94±0.26[c]	16.02±0.13[c]	4.34±0.62[c]	6.46±1.58[c]	0.59±0.05[c]	0.91±0.20[c]
10 ppm	17.58±2.94[c]	19.37±1.98[c]	7.56±1.61[c]	7.67±1.65[c]	1.19±0.38[c]	1.18±0.33[c]
100 ppm	21.85±0.58[c]	26.06±2.35[c]	9.32±0.38[c]	10.86±0.07[c]	1.59±0.19[c]	1.96±0.21[c]
Negative	7.47±0.80	7.77±1.08	2.97±0.89	2.67±0.96	0.41±0.09	0.37±0.14
Positive	53.79±9.58[c]	60.25±2.62[c]	51.28±7.48[c]	57.35±3.89[c]	15.00±3.64[c]	18.25±3.16[c]

[a] Not significantly different from Negative control; [b] $p<0.05$; [c] $p<0.01$ compared to negative control.

Table 3.2. Measurement of tail length (μm), tail DNA (%), Olive tail moment (arbitrary units) by ZnO NPs in comet assay

	Tail length (μm)		Tail DNA (%)		Olive tail moment	
	3 h	**6 h**	**3 h**	**6 h**	**3 h**	**6 h**
1 ppb	7.77 ± 0.40^{a}	9.02 ± 1.04^{a}	3.11 ± 0.42^{a}	3.59 ± 0.56^{a}	0.45 ± 0.01^{a}	0.52 ± 0.07^{a}
10 ppb	9.03 ± 1.39^{a}	9.17 ± 1.48^{a}	3.12 ± 0.52^{a}	3.72 ± 0.57^{a}	0.47 ± 0.04^{a}	0.54 ± 0.07^{a}
100 ppb	10.56 ± 1.16^{a}	11.96 ± 1.17^{a}	4.19 ± 0.35^{a}	4.97 ± 0.99^{b}	0.64 ± 0.18^{a}	0.73 ± 0.24^{a}
1 ppm	10.68 ± 0.83^{a}	12.15 ± 0.91^{c}	4.75 ± 0.68^{a}	5.79 ± 0.41^{c}	0.67 ± 0.20^{a}	0.77 ± 0.08^{c}
10 ppm	11.77 ± 1.54^{b}	15.71 ± 1.41^{c}	6.30 ± 0.88^{c}	6.45 ± 1.08^{c}	0.91 ± 0.03^{c}	1.01 ± 0.23^{c}
100 ppm	18.29 ± 1.32^{c}	18.25 ± 1.20^{c}	7.98 ± 1.39^{c}	8.63 ± 1.22^{c}	1.30 ± 0.24^{c}	1.32 ± 0.46^{c}
Negative	8.41 ± 1.23	9.14 ± 1.38	2.85 ± 0.35	3.00 ± 0.17	0.42 ± 0.03	0.43 ± 0.03
Positive	53.79 ± 9.58^{c}	60.25 ± 2.62^{c}	51.28 ± 7.48^{c}	57.35 ± 3.89^{c}	15.00 ± 3.64^{c}	18.25 ± 3.16^{c}

^{a} Not significantly different from Negative control; ^{b} $p<0.05$; ^{c} $p<0.01$ compared to negative control.

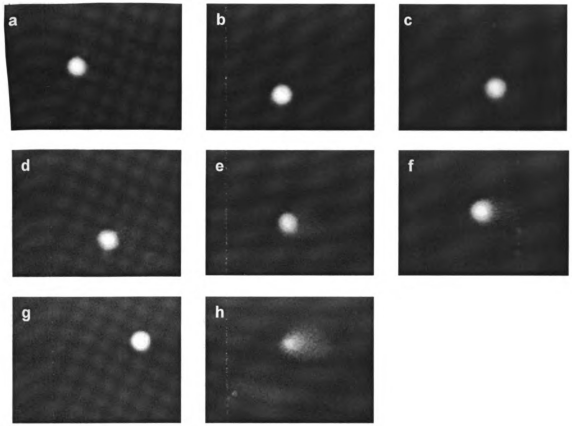

Figure 3.2. Human lymphocytes treated with TiO_2 showing genotoxicity (magnification 400): (a, b, c, d, e, and f) nuclei from TiO_2-treated 6 h (1 ppb, 10 ppb, 100 ppb, 1 ppm, 10 ppm and 100 ppm) human lymphocytes showing DNA damage; (g) nucleus from an untreated human lymphocyte (negative control); (h) nucleus from an ethyl methanesulfonate (2 mM; positive control) treated human lymphocyte showing DNA damage.

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CHAPTER 4

THE INFLUENCES OF OXIDE NANOPARTICLES ON GENE EXPRESSION OF *ESCHERICHIA COLI* K12

Abstract

SiO_2, TiO_2, and ZnO NPs are used in various fields as support or catalysts. Our previous toxicity data demonstrated their antibacterial activities. In this study, we explored their influence on the gene expression of *E. coli*, which generally increased from SiO_2 to TiO_2 to ZnO NPs. In SiO_2 and ZnO NPs treatments, the number of repressed genes was higher than that of the induced ones, while more genes were induced than the repressed in TiO_2 exposure. The transporters for amino acid and carbohydrate were down-regulated, while the transporter for iron or a few other metal ions were up-regulated. Moreover, stress-related genes and DNA repair genes were generally induced. This may be due to a number of reasons including ROS induction, damage to membrane, proteins or DNA, alteration of membrane permeability and fluidity, attack to iron-sulfur cluster and nick on DNA. It was also demonstrated that ZnO NPs influenced microbial respiration. The microarray data demonstrated that NPs regulated the genes in different categories, and *E. coli* adjusted their metabolic pathways and defense mechanisms during the exposure to NPs.

Introduction

Nanotechnology, defined by The Royal Society, is "the design, characterization, production and application of structures, devices and systems by controlling shape and size at nanometer scale" (29). The growth in nanotechnology offers numerous technological advances and industrial development opportunities, and it is estimated that the global market for nanotechnology grow to about $1 trillion by 2015. NMs, one proportion of nanotechnology, possess enhanced and unique mechanical, optical and electrical characteristics, which broaden their application in diverse fields, such as environmental remediation, cosmetics, medicine and catalysis. Although the number of studies addressing to the toxicity of NPs is increasing, there are many unknowns about the environmental impacts of NPs and the mechanisms of NP toxicity (70, 81).

The toxicity of NPs to microorganisms is an important concern for evaluating the environmental impact of NPs. Microorganisms play a fundamental role in the environment, and supply key environmental services in maintaining healthy ecosystems. Studying toxicity of NPs on microorganisms is beneficial for us to evaluate the environmental impacts of NPs and explore the nanotoxicology on animals and human. Possible explanations for NP toxicity include induction of ROS, disruption of membrane, releases of hazardous constituents, and damage to DNA (52).

SiO_2, TiO_2, and ZnO NPs are popular NPs, and used for cosmetics, food, painting and electrical devices. Several papers have been published about their antibacterial activities (3, 7, 32, 33, 80). In this study, *E. coli* K12 is chosen as the test species to

explore gene expression influenced by SiO_2, TiO_2, and ZnO NPs. *E. coli* K12, Gram-negative, facultative anaerobic and non-spore forming, is one of the most studied bacteria in the field of microbiology. Microarray technique was applied to explore the gene expression of *E. coli* in the NP exposure. Microarray data were validated by quantitative PCR for a few target genes. We categorized the influenced genes, and also analyzed and explored the pathways and the transcriptional units that are regulated by NPs.

Materials and Methods

Reagents and preparation of NP suspensions. Random hexamer primers, 100 mM nucleotide set, RNaseOUT, salmon sperm DNA, superscriptase II RT, formamide, 10% sodium dodecyl sulfate, 20x Saline-Sodium Citrate (SSC), and 0.5 M ethylenediaminetetraacetic acid (EDTA) were obtained from Invitrogen Co. (Carlsbad, CA). Bovine serum albumin (BSA), dimethyl sulfoxide, sodium hydroxide, 1 M hydrochloric acid and isopropanol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). RNAlater, SlideHyb buffer and aminoallyl-modified dUTP were obtained from Ambion Inc. (Austin, TX). Lysozyme, DNase I, Tris-Cl EDTA (TE) buffer, RNeasy mini kit, Quiaquick PCR purification kit and RNA protect bacteria reagents were purchased from Qiagen Co (Valencia, CA). SiO_2 (10 nm), TiO_2 (5 nm) and ZnO (60 nm) were purchased from Sigma-Aldrich (St. Louis, MO). One hundred g/L NPs stock solutions were prepared in deionized and distilled water. Phosphate wash buffer (100 ml)

for cDNA purification was made of 0.5 ml K_2HPO_4 (1 M, pH 8.5), 84.25 ml ethanol and 15.25 ml H_2O and phosphate elution buffer (100 ml) contains 0.4 ml K_2HPO_4 (1 M, pH 8.5) and 99.6 ml H_2O. All solutions were autoclaved and stored at room temperature

Cell growth, harvest and RNA extraction. From the freezer stock of *E. coli* streak plates were prepared with LB medium and grown at 37 °C overnight. One colony on the plate was transferred to the tubes with 5 ml LB broth. The NP stock solution was added to each tube with 10 ml LB broth to result in final NP concentrations of 1000 ppm for SiO_2 NPs, 1000 ppm for TiO_2 NPs and 50 ppm for ZnO NPs. The samples were inoculated and incubated until mid-log growth phase of *E. coli* ($0.5<OD_{600}<1$). According to the protocol of RNAprotect bacteria reagent, 1 ml culture and 2 ml RNAprotect were mixed by vortexing for 5 s and incubated for 5 min at room temperature. The pellet was collected by centrifuging for 10 min at 5000xg, and supernatant was decanted. The 200 µl lysozyme in TE buffer (1 mg/ml) was added to the pellet, mixed by vortexing for 10 s, and incubated at room temperature for 5 min on a shaker. Buffer RLT (700 µl) in RNeasy mini kit was applied and vortexed vigorously. After 2 min centrifugation at maximum speed and removal of the NP sediment, the supernatant was mixed with 500 µl ethanol by shaking vigorously. The lysate was transferred to RNeasy Mini spin column as per the procedure described in RNeasy mini kit, and centrifuged for 14 s at >8000x g. DNase digestion was applied to the column by buffer RW1 washing, DNase I solution washing (10 µl) and incubating, and then buffer RW1 re-washing. The RNA was purified as per the protocol of RNeasy mini kit and the

final concentration of RNA was measured by Nanodrop from Thermo Scientific Co. (Wilmington, DE). RNA from control samples was processed in a similar way as NP samples. All experimental and control samples were prepared in triplicate.

cDNA synthesis and dye-labeling. For each sample, 5 μg of total RNA was used to synthesize cDNA and two RNA populations of each sample were needed for dye-swapping. RNA mixture contained 5 μg RNA, 5 μl random hexamer primers (3 mM), Rnase-free water in the final volume of 16.8 μl. The RNA mixture was incubated at 70°C for 10 min and then cooled on ice for another 10 min. The RT-PCR reaction had a 30 μl volume and contained 6 μl 5x first strand synthesis buffer, 3 μl DTT (0.1 M), 1 μl RNaseOUT, 1.2 μl 25x dNTPs (7.5 mM aa-dUTP, 5 mM dTTP, 12.5 mM dCTP, 12.5 mM dGTP and 12.5 mM dATP), 2 μl Superscript II RT and 16.8 μl RNA mixture. The RT-PCR reaction was incubated at 42°C overnight, and then 10 μl NaOH (1 M) and 10 μl EDTA (0.5 M) were added to the reaction. After incubating at 65°C for 15 min, 10 μl HCl was added to neutralize the pH. The cDNA was purified according to the protocol of Qiagen purification kit with some modification. The cDNA mixture was mixed with 300 μl PB buffer, and transferred to a Quiaquick column. After 1 min centrifuging at 17900x g, the collection tube was emptied and the column was washed with 750 μl phosphate wash buffer twice and centrifuged for 1 min. After 1 min additional centrifuging, the column was transferred to a new tube, and then eluted twice by incubating the column with 30 μl phosphate elution buffer for 1 min and centrifuging for 1 min at maximum speed. The cDNA sample was dried using speedvac from Global Medical Instrumentation

Inc. (Ramsey, MN). The aa-dUTP cDNA was resuspended in 4.5 µl sodium carbonate (0.1 M, pH 9.0) and mixed with 4.5 µl Cy5 or Cy3. After incubating 2 h at room temperature in the dark, 35 µl NaOAc (100 mM, pH 5.2) was added and the dye-coupled cDNA was purified as described in the protocol of Qiaquick purification kit. The concentrations of cDNA and dye were measured by Nanodrop from Thermo Scientific Co. (Wilmington, DE).

Hybridization. The glass-slide arrays were cross-linked at 6000 µJ UV and water bath was pre-warmed to 42°C. The slides were placed in pre-hybridization buffer containing 5x SSC, 0.1% SDS and 0.1 mg/ml BSA at 42°C for 90 min. After washed twice with 0.1x SSC for 5 min and twice with water for 0.5 min, the slides were dried by centrifuging at 1600 rpm for 3 min and transferred to dry and clean tubes covered with foil. The cover-slips were washed with 2% SDS, water and ethanol, followed by additional washing twice with water. Then, the cover-slips were dried with filtered pressured air and placed in clean tubes covered with foil. The labeled DNA of the control sample was re-suspended in 4 µl EDTA, and heated at 95°C for 30 s, and then spun down. The other differently labeled DNA from NP-treated sample was suspended in the same 4 µl EDTA, which was heated at 95°C for 5 min and spun down. The dry slide-array was placed in a clean and dry hybridization chamber and coverslip was placed on the slide. The 40 µl SlideHyb buffer, which was preheated to 68°C, was added to the sample and mixed by pipetting (preventing bubbles). All the hybridization solution was loaded on the slide, and 25 µl water was added in each well in the hybridization chamber. The chamber

was placed in water-bath at 42°C for 18 h. For each NP treatment, six hybridizations from three biological replicates and two technical replicates (dye-swapping) were performed.

Washing, scanning and data analysis. After 18 h, the chamber was removed from waterbath and dried with paper towel before open. Wash Buffer 1, Wash Buffer 2 and slide staining jars were prewarmed at 37 °C. The slide was taken out with forceps, washed once with Wash Buffer 1 (2x SSC and 0.5% SDS) to remove the coverslip on belly dancer for 5 min, then twice with Wash Buffer 2 (0.1x SSC and 0.1% SDS) on belly dancer for 5 min, and twice with Wash Buffer 3 (0.1x SSC) for 2.5 min. The slide was dried by centrifuging at 1800 rpm for 3 min and transferred to dry tubes.

Axon Genepix 4000B laser scanner was used for slide scanning and Genepix 3.0 software was used to produce a raw data set. The scanner was turned on and warmed up for 15 min while the computer was off. The slide was placed face-down, and PMT and power % were adjusted to optimal by verifying histogram. The resolution to 5 pixels per μm and scanning area was selected. Then the interested area was scanned and the image was saved. GENESPRING 6.0 software (Silicon Genetics, Redwood City, CA) was used for data analysis. The data was normalized both per chip and per gene (Lowess method), and dye-swapping. Only the spots with more than 55% of pixel greater than background plus 2x standard deviation in either Cy5 or Cy3 channel were used for analysis. Genes that showed a statistically significant change in gene expression ($p>0.05$) and a greater than or equal to 2-fold change in magnitude were regarded as significant.

Validation of micoarray data by quantitative PCR. For control samples and

ZnO treated samples, 5 μg of total RNA was used to synthesize cDNA. RNA mixture contained 5 μg RNA, 5 μl random hexamer primers (3 mM), Rnase-free water in the final volume of 16.8 μl. The RNA mixture was incubated at 70°C for 10 min and then cooled on ice for another 10 min. The reverse transcriptase PCR reaction had a 30 μl volume and contained 6 μl 5x first strand synthesis buffer, 3 μl DTT (0.1 M), 1 μl RNaseOUT, 1.2 μl 25x dNTPs (12.5 mM dTTP, 12.5 mM dCTP, 12.5 mM dGTP and 12.5 mM dATP), 2 μl Superscript II RT and 16.8 μl RNA mixture. The RT-PCR reaction was incubated at 42°C overnight, and then 10 μl NaOH (1 M) and 10 μl EDTA (0.5 M) were added to the reaction. After incubating at 65 °C for 15 min, ten μl HCl was added to neutralize the pH. cDNA was purified according to the protocol of Qiagen purification kit with some modification. The cDNA mixture was mixed with 300 μl PB buffer, and transferred to a Quiaquick column. After 1 min centrifuging at 17900x g, the collection tube was emptied and the column was washed by 750 μl phosphate wash buffer twice and centrifuged for 1 min as above. After 1 min additional centrifuging, the column was transferred to a new tube, and then eluted twice by incubating the column with 30μl phosphate elution buffer for 1 min and centrifuging for 1 min at maximum speed. For quantitative PCR, the forward primers and the reverse primers were designed for ten target genes and one reference gene (Table 4.1). The real time PCR reaction had a final volume of 15 μl and contained 7.5 μl PCR green master mix, 0.9 μl primer mixture (5 μM for forward primer and reverse primer respectively), 5.6 μl PCR water and 1 μl DNA template. Real time PCR was performed on ABI Prism 7900HT Sequence Detection System and the cycling

parameters were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The dissociation curves were run for product identification. Real time PCR was done on ABI Prism 7900HT Sequence Detection System from Applied Biosystems (Foster City, CA). Comparative C_T Method ($\Delta\Delta C_T$) was applied for quantitative PCR analysis. The gene expression fold is equal to $2^{\Delta\Delta CT}$ and $\Delta\Delta C_T = \Delta C_T$ test sample $-\Delta C_T$ control sample.

Results

Global gene expression profiles under NP exposure. In the treatments of SiO_2 and ZnO NPs, a greater number (279 for ZnO and 52 for SiO_2) of genes were repressed than the induced (197 for ZnO and 2 for SiO_2). For TiO_2 NPs, there were more up-regulated genes (28 genes) compared to the number of repressed genes (11 genes). The genetic response to 100 ppm ZnO NPs was with more than 8.0% of the genome expressing differentially whereas only approximately 1% of genome showed differential expression for both 1000 ppm TiO_2 NPs and 1000 ppm SiO_2 NPs respectively. In our previous studies, *E. coli* K12 was more tolerant to TiO_2 and SiO_2 NPs, but more vulnerable to ZnO NPs, compared to *E. sibiricum*. During the 1000 ppm TiO_2 NPs exposure, there were 289 down-regulated genes and 197 up-regulated genes in *E. sibiricum*, while 11 induced genes and 28 repressed genes were found in *E. coli* at the same concentration of TiO_2 NPs. Not many genes were common among these three different treatments. According to

the 1 way-ANOVA analysis, there were 499 genes expressing differently in these three NP treatments.

Gene expression profile following ZnO treatment. In the ZnO treatment, globally the expression fold of 5,969 genes ranges from 0.02 to 12.81, while there were 279 down-regulated genes and 197 up-regulate genes. In the treatment of ZnO NPs, there were more repressed and induced genes than in the treatment of SiO_2 or TiO_2 NPs.

The up-regulated genes in ZnO treatment. The categories "transportation" (19.7%), "transcription" (12.6%) and "translation" (10.1%) were the biggest ones for the up-regulated genes in ZnO treatment. The transporters genes included those for carbohydrate, amino acid, ion, proton, and heme, such as gene *glpF, rhaT, tsp,* and *nikD* (9, 79, 91, 97). Induction of some transporter genes was the response to Zn^{2+} in ZnO NP suspension. For instance, the induced gene *zntA* (3.8 fold), coding zinc, cobalt and lead efflux system, was found to be activated by an increased concentration of Zn(II) and Cd(II), showing greater induction by Cd than Zn (85). Gene *znuA*, coding subunit of ABC transporter, was also induced by Zn^{2+} ions (2.1 fold) (100). Moreover, gene *srlA* (3.5 fold), coding glucitol/sorbitol-specific enzyme IIC component of PTS, was up-regulated, and its expression was reported to be elevated under basic conditions and addition of Zn to culture grown in minimal medium (58, 65, 87). The aerobic/anaerobic conditions in ZnO NP suspension also affected the expression of a few transporter genes. For example, gene *ulaA*, coding L-ascorbate transporting phosphotransferase system, was up-regulated

(2.6 fold), which is required for the ability to utilize L-ascorbate as the carbon source under anaerobic growth conditions (105). It was also found that induced gene *nark* (4.6 fold), coding Nark MFS nitrate/nitrite antiporter in anaerobic nitrate respiration, was regulated by nitrate and oxygen (53). Moreover, a threonine sugar transport protein (STP) transporter, coded by gene *tdcC*, was induced (2.1 fold), and its expression was reported under anaerobic conditions (86).

The aerobic/anaerobic conditions in ZnO suspension also influenced the expression of some other genes, especially for those involved in both anaerobic and aerobic respiration. Both *cyoB* and *cyoD* were up-regulated (2.6 and 2.1 fold respectively), coding subunits of cytochrome *bo* terminal oxidase in aerobic respiration. The expression of gene *aldA* was also induced (3.0 fold), which codes subunit of aldehyde dehydrogenase A. Aldehyde dehydrogenase A was expected to be only present under aerobic conditions and be regulated by catabolite repression, because previous work showed that it was repressed under anaerobic conditions and induced by the carbon source (30, 75). Meanwhile, gene *sdhC* was also up-regulated (2.4 fold), which codes succinate dehydrogenase membrane protein in aerobic respiration, as a large subunit of cytochrome b_{556}. Gene *ndh* is another example, and it codes NADH:ubiquinone oxidoreductase II in aerobic respiratory chain (2.0 fold) (69, 73). Genes involved in anaerobic respiration were also induced, such as genes *narG* and *narH* (2.3 and 2.1 fold), coding subunits of nitrate reductase A in anaerobic respiration. Nitrate reductase A functions as an electron acceptor and benefits proton motive force (11, 93). These

suggested that both anaerobic and aerobic respiration is important for survival in ZnO NP solution.

In the category "transcription", Induction of some genes were due to the stress caused by ZnO NPs. TreR transcriptional repressor, coded by gene *treR*, was induced by 2.4 fold, and *treR* can de-repress operons related to carbohydrate transport and catabolism under osmotic stress (44). The induced gene *yiiA* (2.9 fold), coding CpxR transcriptional dual regulator, can sense a variety of stress and activate some stress-combative genes, such as those involved in drug efflux (42). In the presence of zinc, gene *zraS* (2.5 fold) was induced, responding to environmental signals. Meanwhile, gene *asr* (3.3 fold) was also up-regulated, which responds to zinc and plays a role in survival under acid conditions (58, 84). Furthermore, induced gene *bhsA* (2.2 fold) codes protein involved in stress resistance, as well as *phoH*, responding to stress, such as starvation (2.1 fold) (28, 65, 104). All of these demonstrated that *E. coli* responded to stress resulting from ZnO NP by inducing some gene expression.

Genes about cell structure and division were also induced. For example, gene *ydaD* (2.3 fold) implicated in cell division and cycling was up-regulated, whose over-expression inhibits cell division and leads to morphological defects (21). Gene *ycfJ* was also induced, which involves in flagellar synthesis, swarming, and cell elongation (26). Moreover, induced gene *mukB*, coding cell division protein, involves in chromosome segregation and condensation, and also functions in cell division and cycle. The induction of these genes is probably due to the slow growing rate of bacteria, caused

by ZnO NPs. The categories about metabolism and biosynthesis of carbohydrate, lipid, amino acid or nucleotide were also important in the ZnO NP treatment. For instance, gene *sucB* and *sucD* were up-regulated, functioning in tricarboxylic acid cycle. The changes in these categories are probably due to the ROS induction by ZnO NPs, since ROS can oxidize amino acid and DNA, and also interrupt nutrient uptake.

Since ZnO NPs can cause genotoxicity, the genes related to DNA repair and RNA modification were found to be up-regulated. Up-regulation of gene *dpiA* can induce some genes involving citrate fermentation and SOS response, or repress some transcriptional regulator. The subunit of DNA gyrase, coded by induced gene *gyrB*, involves in DNA modification and replication, as well as transcription. Meanwhile, gene *recC*, involved in DNA repair and essential for recombination and conjugation as well as repair of double-strand breaks, was also up-regulated (22). Moreover, gene *mcrC* which codes for subunit of 5-methylcytosine restriction system and defends cell against foreign DNA, was induced. Genes involving in detoxification was also up-regulated. For instance, induction of gene *dsdA*, coding D-serine deaminase, serves in a catabolic function and detoxification, since D-serine is still a good nitrogen source and moderate carbon source. Moreover, gene *frmA*, coding formaldehyde dehydrogenase, was also induced, which can detoxify exogenous formaldehyde and be induced by formaldehyde (38, 40).

Not only single genes, quite a few operons in different categories was also found to be induced by ZnO NPs, for example, the transcriptional unit *rplNXE-rpsNH-rplFR*

-rpsE-rpmD-rplO-secY-rpmJ and *rpsJ-rplCDWB-rpsS-rplV-rpsC-rplP-rpmC-rpsQ*. These two big operons encode a serial of 50S and 30S ribosomal subunit proteins. The transcription unit *rpsLG-fusA-tufA* is another induced operon in the category "translation", containing genes *tufA* (elongation factor Tu), *fusA* (elongation factor G) and *rpsG* (30S ribosomal subunit protein S7), and it was estimated that elongation factor Tu was the most abundant protein in *E. coli* and maximally expressed during stress response (68). Furthermore, both of the transcriptional unit *glpABC* and *glpTQ* were induced, coding glycerol-3-phosphate dehydrogenase under anaerobic conditions and glycerol-3-P MFS transporter respectively, which are important for utilization of glycerol (99). The intracellular level of glycerol-3-phosphate is important for biosynthesis of phospholipids. Genes *narG* (α subunit of nitrate reductase A) and *narH* (β subunit of nitrate reductase A), in the operon *narGHJI*, was up-regulated, constituting catalytic dimmer of nitrate reductase A in anaerobic respiration.

A few operons in the category "energy production" were also induced by ZnO NPs. In the transcriptional unit *fdnGHI*, *fdnG* (α subunit of formate dehydrogenase N), *fdnH* (β subunit of formate dehydrogenase N) and *fdnI* (γ subunit of formate dehydrogenase N) exhibited induction, and their expression have been found to be induced by nitrate and anaerobiosis (35, 92). The formate dehydrogenase N is beneficial to generate the proton motive force and allow generation of the ubiquitous energy carrier ATP by ATP synthase. One ATP synthase, coded by the transcription unit *atpBEFHAGDC*, was induced, proved

by induction of gene *atpC* (ε subunit of ATP synthase), *atpD* (β subunit of ATP synthase), *atpG* (γ subunit of ATP synthase), *atpA* (α subunit of ATP synthase), *atpH* (δ subunit of ATP synthase), and *atpF* (b subunit of ATP synthase), and this ATP synthase can transport proton and synthesize energy. Moreover, in the transcriptional unit *napFDAGHBC-ccmABCDEFGH*, *ccmA* (subunits of CcmABCDE protoheme IX ABC transporter), *ccmB* (subunits of CcmABCDE protoheme IX ABC transporter), and *ccmG* (CcmEFGH holocytochrome c synthetase), and *napC* (cytochrome c protein), *napH* (ferredoxin-type protein), *napG* (ferredoxin-type protein), and *napA* (large subunit of periplasmic nitrate reductase), were induced, which were required for electron transfer from ubiquinol (12, 13, 31). Many genes transporting proton were up-regulated during ZnO exposure, involved in maintaining the proton motive force and generate energy.

Down-regulated gene profile in the exposure to ZnO NPs.

The categories "transportation" (19.0%) and "hypothetical proteins" (10.8%) had the highest fraction of down-regulated genes. For instance, the gene *manX*, *manY* and *manZ* in category "transportation" were repressed, which construct mannose PTS permease and transport exogenous hexoses (76). The transcriptional unit *gatYZABCD* was also down-regulated, coding tagatose-1,6-biphosphate aldolase (GatYZ), galactitol-1-1phosphate dehydrogenase (GatD), and galactitol PTS permease (GatABC) that take up and transport exogenous galactitol. A few genes related to the metabolism of carbohydrate and amino acid (totally 11.1%) were down-regulated too, maybe due to the

hindrance of their uptake. Moreover, other categories of genes that were down-regulated included "transcription" (6.8%), "response to stress" (5.0%), "cell division and motion" (3.9%), "nucleotide biosynthesis" (3.6%), "DNA repair" (2.9%), RNA modification (2.9%) and "signal transduction" (2.9%).

The genes related to anaerobic respiration were also found to be down-regulated. On one hand, some of genes were repressed by the presence of oxygen. For example, the transcription unit *hyaABCDEF* was repressed, which codes hydrogenase 1. This enzyme consists of HyaA (small subunit of hydrogenase 1), HyaB (large subunit of hydrogenase 1), HyaC (b-type cytochrome subunit of hydrogenase 1), HyaD (protein involved in processing of HyaA and HyaB proteins), HyaE (protein involved in processing of HyaA and HyaB proteins), HyaF (protein involved in nickel incorporation into hydrogenase 1 proteins), and it is tolerant to oxygen and provide complementary redox properties to the cell (57). The *hya* operon was found to be induced under anaerobic conditions and by the presence of formate, or induced under acidic conditions, but repressed by nitrate (51, 78). Meanwhile, gene *fdhF*, coding formate dehydrogenase, was also down-regulated, and it was found to be repressed by nitrate, nitrite and the presence of oxygen (1). On the other hand, our results also displayed the repression of genes that was reported in anaerobic conditions. For instance, the repressed gene *aldA* codes NAD-linked aldehyde dehydrogenase A, and its repression occurs under anaerobic conditions via ArcA (59). Under anaerobic conditions, protein ArcA also mediates the repression of L-lactate

dehydrogenase, coded by gene *lldD*, which functions in aerobic respiration and anaerobic nitrate respiration (47). Repressed gene *poxB* codes pyruvae oxidase, which is responsible for pyruvate metabolism under aerobic conditions, and its expression decreases under anaerobic conditions (2, 20). Aerobic/anaerobic conditions in ZnO NP suspension exhibited down-regulation of some operons. In the transcriptional unit ynfEFGH-dmsD, gene *ynfE* (oxidoreductase subunit paralog of DmsA), *ynfF* (oxidoreductase subunit), *ynfG* (oxidoreductase, Fe-S subunit paralog of DmsB), *ynfH* (DMSO reductase anchor subunit, paralog of DmsC) and *dmsD* (DMS reductase maturation protein) were repressed, which can utilize dimethyl sulfoxide as a terminal oxidant under anaerobic conditions.

The down-regulation of some transcriptional units was also considered as response to the stress caused by ZnO NPs. The transcriptional unit *uxaCA*, including *uxaA* (D-altronate dehydratase) and *uxaC* (D-glucuronate isomerase/D-galacturonate isomerase) was repressed. It was reported that Zn^{2+} ion could inhibit the enzyme (8). In the transcriptional unit *hdeAB-yhiD*, *hdeA* (inactive form of acid-resistance protein), *hdeB* (acid stress chaperone), *yhiD* (Mg^{2+} transport ATPase) were also down-regulated, and all of them involved acid resistance, as well as gene *slp* (starvation lipoprotein) and *hdeD* (acid-resistance membrane protein) (34, 50, 64, 89). The down-regulated transcriptional unit *gadE-mdtEF* also related to low pH, including gene *gadE* (GadE transcriptional activator), *mdtE* (subunit of MdtEF-Tolc multidrug efflux transport system) and *mdtF*

(subunit of MdtEF-Tolc multidrug efflux transport system) (64). Quite a few of down-regulated stress related genes involved in response to pH.

Even although ZnO NPs can cause genotoxicity, some genes related to DNA repair and detoxification were still down-regulated, such as gene *ruvC* (Holliday Junction nuclease). The repressed gene *uvrD*, coding DNA-dependent ATPase I and helicase II, can also protect DNA from degradation. Gene *ksgA* was downregulated, coding S-adenosylmethionine-6-N',N'-adenosyl (rRNA) dimethyltransferase, and its expression is positively regulated by growth rate (74). Meanwhile, some genes functioning in nucleotide metabolism were also down-regulated. Gene *rutC* (endoribonuclease), *rutD* (hydrolase) and *rutE* (nitroreductase) in the operon *rutABCDEFG* were repressed. These genes function in the pathway for pyrimidine degradation pathway, and the operon is found under the control of nitrogen regulatory protein (107). The transcriptional unit *xdhABC* was also down-regulated, including *xdhA*, *xdhB*, and *xdhC*, which consist of xanthine dehydrogenase, and xanthine dehydrogenase plays a role in purine salvage (101).

Some genes involved in detoxification were repressed by ZnO NPs. For example, repressed gene *yiiM* codes a protein involved in base analog detoxification (54). Moreover, in the transcriptional unit *yafQ-dinJ*, coding the YafQ-DinJ toxin-antitoxin system, YafQ functions in reducing protein synthesis and inhibiting growth, when DinJ alleviates that phenotype, acting as the antitoxin (67). Both of these two genes were down-regulated in the treatment of ZnO NPs. A few genes about cell division, cell motion

and biofilm formation were also repressed. For example, repressed gene *yihA*, coding cell division protein, is essential for normal cell division (6, 23). RNA polemrase sigma F factor coded by gene *fliA* was also down-regulated, and it is related to flagellin production, motility and biofilm formation (10, 60).

Gene expression profiles following SiO_2 treatment. In SiO_2 treatment, 5924 genes ranged in the expression fold between 0.40 and 2.05, which was much narrower than those in these other two treatments (Fig 5.1). Based on the available information in the databases of Clusters of Orthologous Groups of proteins (COGs), fifty-two down-regulated genes in the response to SiO_2 NPs were grouped into 17 functional categories, among which "transcription" (11.5%) and "Not in COGs" (30.8%) were the two of the biggest dominant categories. Other large categories included "Inorganic ion transport and metabolism" (7.7%), "coenzyme transport and metabolism" (5.8%), "Cell wall/membrane/envelope biogenesis" (5.8%), "General function" (5.8%), "Function unknown" (5.8%), "defense mechanisms" (5.8%). However, only 2 genes were down-regulated in the categories "transcription" and "function unknown".

"Transportation" is always one of the biggest categories in all the treatments, and for SiO_2 NP exposure 10 out of 52 down-regulated genes were in this category, covering transporters for carbohydrate, amino acid and metal ions, such as gene *ycfT*, *yeeO*, *yaaU*, *yiaV*, and *ycfT* (14). Gene *ycdN*, functioning as ferrous iron transporter, was repressed probably due to osmotic up-shift that may be caused by membrane damage from ROS, or

anaerobic conditions (48, 95). Meanwhile, gene *napC*, involving in transportation and anaerobic respiration, was also repressed. Gene *cyoB* is another repressed gene involved in respiration, and it codes cytochrome terminal oxidase subunit I, a terminal oxidase in the respiratory chain used under high oxygen growth conditions as a proton pump (77). Moreover, both genes *phnE* and *ccmB* are ABC transporters, functioning as the integral membrane protein and the membrane protein of type 1 cytochrome *c* biogenesis system respectively (77, 94).

"Transcription" is another big category in the treatment of SiO_2 NPs. The down-regulated gene *bglJ* was considered as a positive DNA-binding transcriptional regulator of transport and utilization of the aromatic β-glucosides arbutin and salicin. YgeK was repressed as a predicted response regulators of two-component regulatory systems (62, 103). Gene *ascG* was also down-regulated, which can repress the expression of *ascFB* involving in transport and utilization of the glucosidse suargars arbutin, salicin, and cellibiose (39).

Exposure to NPs can cause some cellular stress and damage, such as oxidative stress and genotoxicity by ROS, and they also influence the genes related to stress condition and DNA repair. For instance, gene *yodA*, which can bind divalent metal ions including cadmium, zinc, and nickel and is proposed to be a generalized stress factor, was repressed (24). Meanwhile, gene *yedQ* was also found to be down-regulated, which responds to a number of stress conditions and involve in cellulose biosynthesis (96).

Gene *xisE* was repressed, functioning as DNA recombination, when gene *cca* was also down-regulated, involving in RNA repair (56).

There were a few of transcriptional units down-regulated during the exposure to SiO_2 NPs. The transcriptional unit *nhaAR* consists of gene *nhaA*, a sodium ion/proton antiporter that uses the proton electrochemical gradient to expel sodium ions from the cytoplasm and functions primarily in the adaptation to high salinity at alkaline pH, and *nhaR*, a transcriptional activator. Moreover, *nhaA* is also believed to be responsible for adaptation to alkaline pH when sodium is available (49). In the transcriptional unit *hcaEFCBD*, *hcaE* coding α subunit of 3-phenylpropionate dioxygenase, *hcaF* coding β subunit of 3-phenylpropionate dioxygenase, and *hcaB* coding 3-phenylpropionate-2',3'-dihydrodiol dehydrogenase were repressed, functioning in utilize aromatic acids as carbon and energy sources (15).

Gene expression profile following TiO_2 treatment. Totally 5940 genes ranged in the expression fold from 0.22 to 7.25. In Figure 5.1, globally 11 down-regulated genes in the TiO_2 treatment were grouped into 7 functional categories, including "energy production and conversion" (27.3%), "coenzyme transport and metabolism" (18.2%), "general function" (18.2%), "cell motility" (9.1%), "inorganic ion transport and metabolism" (9.1%), "signal transduction mechanisms" (9.1%) and "not in COGs" (9.1%). Similarly, twenty-eight up-regulated genes were grouped into 10 functional categories. Besides "amino acid transport and metabolism" (32.1%) and "not in COGs"

(17.9%), most of induced genes belonged to the categories of "carbohydrate transport and metabolism" (10.7%), "inorganic ion transport and metabolism" (10.7%), "energy production and conversion" (7.1%), "transcription" (7.1%).

Most of repressed genes in the treatment of TiO_2 were related to transportation and cofactor synthesis. Gene *yejF* was down-regulated, coding an ATP-binding protein of a ABC transporter that transports peptides through membrane, and its expression was reported to be regulated by small RNA at the mRNA level (5, 71, 82). The repressed genes *moaA* and *moaB* were in the transcriptional unit *moaABCDE*, functioning in Mo-molybdopterin cofactor biosynthetic process (4). The gene *dmsB*, coding dimethyl sulfoxide (DMSO) reductase chain B, was also down-regulated. Moreover, one of DMSO reductase paralogues was downregulated, including gene *ynfE* (oxidoreductase subunit paralog of DmsA), *ynfF* (oxidoreductase subunit), *ynfG* (oxidoreductase, Fe-S subunit paralog of DmsB), and *ynfH* (DMSO reductase anchor subunit paralog of DmsC) in the same operon, which utilize DMSO as a terminal oxidant and function in energy production and electron transferring (61). Both the DMSO reductase and its paralogue catalyze in anaerobic electron transport chain, and they contain iron ions and molybdenum cofactor. Their down-regulation probably relate to the repression of molybdopterin biosynthesis.

Among up-regulated genes, transportation (32.1%) and amino acid biosynthesis and catabolism (42.9%) were the biggest categories. For the instance, gene *ybaE* was

up-regulated, functioning as ABC transporter. In the transcriptional unit of glucitol/sorbitol phosphotransferase system (PTS) permease, genes *srlA* (glucitol/sorbitol-specific enzyme IIC component of PTS), *srlB* (glucitol/sorbitol-specific enzyme IIA component of PTS) and *srlE* (glucitol/sorbitol-specific enzyme IIB component of PTS) were up-regulated, which involve in carbohydrate transport via glycolysis (76). It was reported that GutM and GutR are positive and negative transcriptional regulators of *srl* operon expression respectively, and the operon expression is also under the control of the cyclic AMP-cyclic AMP receptor protein (CRP) complex (102). The transcriptional unit of modABC is another induced operon, coding ModA, ModB and ModC as subunits of molybdate ABC transporter, and their expression was found to be regulated by a molybdate-dependent repressor protein (36, 37, 100).

For amino acid biosynthesis and catabolism, the transcriptional unit *gltBDF* were induced, coding a glutamate synthase that consists of the large (GltB) and small (GltD) subunits, and a protein that is implied in the self-operon regulation (GltF). It was reported that all of these three subunits were induced by exposure to TiO_2 NPs, and they involve in glutamate biosynthesis (18, 19). The activation of this operon could be due to Lrp (global modulator in response to leucine levels), IHF (global DNA bending proteins) and GadE (regulator for the resistance to low pH) (43, 98). The *thrLABC* operon codes four enzymes involved in threonine and homoserine biosynthesis, all of which were up-regulated. Moreover, the *tdcABCDEFG* operon was induced, which codes L-serine deaminase III (TdcG), endoribonuclease (TdcF), 2-ketobutyrate formate-lyase/pyruvate

formate-lyase (TdcE), propionate kinase (TdcD), threonine STP transporter (TdcC), catabolic threonine dehydratase (TdcB), and TdcA transcriptional activator (TdcA), involving amino acid catabolism, transport, and anaerobic respiration (27, 41, 72, 86, 90). This operon can be induced under anaerobiosis, but it is repressed by glucose (83). The genes *ilvL* and *nmpC* were also up-regulated, relating to amino acid biosynthesis and ion transporter activity respectively. Gene *arcB*, which functions in two-component signal transduction system and aerobic respiration, was induced. It involves in amino acid catabolism, transportation, and anaerobic respiration (27, 41, 90).

Validation of microarray data by quantitative PCR. In Table 5.1, quantitative PCR for 10 selected genes was used to test the robustness of the microarray hybridization data, which included two highly repressed genes (*yheN* and *hdeB*), two moderately repressed genes (*hyaB* and *manX*), a slightly induced gene (*mltC*), three slightly induced genes (*atpD*, *sdhC* and *mopB*), one moderately induced gene (*ibpB*) and another moderately induced gene with high variations (*glpA*). Gene *gcE* was used as a reference gene for comparative C_T method. A high correlation (R^2=0.95) was obtained between microarray analysis and quantitative PCR analysis.

Discussion

Aerobic/anaerobic transition in the NP solution. Among the influenced genes, there were quite a few of them related to aerobic/anaerobic conditions, especially in ZnO treatment. In ZnO NP treatment, most of repressed genes about aerobic/anaerobic

conditions were related to anaerobic respiration, while there were both of genes about aerobic and anaerobic respiration induced. For example, in the transcriptional unit *appCBA-yccB*, genes *appC* (subunit I of cytochrome *bd*-II terminal oxidase), *appB* (subunit II of cytochrome *bd*-II terminal oxidase) and *appA* (acid phosphatase) was down-regulated, and this operon codes cytochrome *bd*-II terminal oxidase, which was not expressed under normal conditions of growth. Meanwhile, gene *cyoA*, *cyoB* and *cyoD* in transcriptional unit *cyoABCDE* were induced, which code cytochrome *bo* terminal oxidase used under high oxygen growth conditions. Another example is both aerobic and anaerobic glycerol-3-phosphate dehydrogenases, functioning in glycerol metabolism and amino acid transport, as well as providing the proton-motive force for ATP synthesis (55). The anaerobic glycerol-3-phosphate dehydrogenase consists of GlpA (large subunit), GlpB (membrane anchor subunit) and GlpC (small subunit), and aerobic one is coded by gene *glpD*, both of which was induced by ZnO NPs. Moreover, gene *tdcB* coding catabolic threonine dehydratase was induced by ZnO NPs, and it was reported to be only expressed during anaerobic growth in the absence of glucose, suggesting the growth during a transition from aerobic to anaerobic condition. Meanwhile, gene *doc* was repressed, coding heme-regulated phosphodiesterase and acting as a direct sensor of oxygen (25, 66). Gene *fucO*, coding L-1,2-propanediol oxidoreductase, induced by growth on L-fucose under both aerobic and anaerobic conditions, but inactivation of the enzyme under aerobic conditions (16). In TiO_2 NP suspensions, anaerobic DMSO reductase and its paralogue were down-regulated while the operon *tdcABCDEFG* was

induced, which involves in L-serine degradation and anaerobic amino acid metabolism. The repression of some anaerobic pathways and the induction of some anaerobic/aerobic pathways suggest that aerobic/anaerobic transition may occur in NP suspension. It was also reported that fullerene can inhibit E. coli respiration and dioxygen consumption (63).

The function of iron during the exposure to NPs. Iron ions play an important role for microorganisms. During the exposure to NPs, they can serve as a reducing agent and repair oxidative damage. For example, gene *dps*, coding Dps complex that can sequester iron, was repressed by ZnO NPs, although it can bind to DNA and protect them from oxidative damage (106). It was reported that protein Dps is very abundant in stationary-phase and in normal starvation response, which is contrary to our conditions (45). In the category "transportation", most of repressed genes related to carbohydrate and amino acid transport, while 25% of induced transporter genes function in ion transport. In fact, ROS can attack sulfur-iron cluster , and lead to Fenton chemistry to generate more ROS (46). Many oxidoreductases, dehydrogenases, hydrogenases, oxidases, and reductases contain iron-sulfur clusters or bind iron ions, and that is why their expression was regulated by NPs. In the operon *sufABCDSE*, gene *sufB* (component of SufBCD cysteine desulfurase activator complex), *sufC* (component of SufBCD complex), *sufD* (component of SufBCD cysteine desulfurase activator complex), *sufS* (subunit of selenocysteine lyase), and *sufE* (sulfur acceptor) were repressed. This operon synthesizes the enzymes in a secondary pathway of iron-sulfur cluster assembly, which is related to intracellular availability of iron ions (88). This may be related to oxidation of

sulfur atoms by ROS (46).

The bacterial tress-related genes during NP exposures. The stress-related genes were found in both of down-regulated and up-regulated gene profiles. Some transcription regulators can sense the stress and influence the expression of other genes. There were more induced genes about response to stress than repressed ones, such as those coding starvation proteins, acid resistant proteins and heat shock proteins. Quite a few genes responding to osmotic stress were up-regulated, maybe due to ROS induction. ROS can oxidize fatty acids of phospholipids on membrane, and cause lipid peroxidation. This process can change permeability and fluidity of bacterial membrane, making bacteria more vulnerable to osmotic stress and interrupt nutrient uptake (17).

Conclusion

The influence on the gene expression of *E. coli* increased generally, from SiO_2 to TiO_2 to ZnO NPs. The number of induced and repressed genes was the highest in ZnO NPs, and for all the three treatment the number of repressed genes is higher than the repressed ones. ROS induction was evidenced by microarray data, such as the over-expression of osmotic stress genes that may be due to the alteration of permeability and fluidity caused by lipid peroxidation, and expression change of iron ion transporter led by attacking iron-sulfur cluster. Both of them result in the damage from ROS, which also hinder nutrient uptake and induce DNA pair genes. It was also demonstrated that ZnO NPs influenced the microbial respiration. The genes related to anaerobic and aerobic

respiration was induced, while only genes about anaerobic respiration were repressed. The microarray data demonstrated that NPs regulated the genes in various categories, and adjusted the pathways and the metabolisms of *E. coli*.

Table 4.1 primer design of target genes for validation.

Gene name	Former primer	Reverse primer	Fold (microarray)	Quantitative PCR
ygcE	TGCGTTATTGAAAGCCGATGGCAC	TATGCCACGTCAGGGTTAGTGTGT	0.97	1
yheN	GGCCATGAGTTAAGCAGCGTCTTT	AAATTCGTCACTTGCCGGAGAGGT	0.09	0.82
mltC	TTTGATTGCGCCGTTGCTCATCTC	TGGTATCTTTGACCCAGGCTTCGT	0.45	0.04
hyaB	TTGAACGTATCTGCGGCGTCTGTA	GATATTGGCGTTGTCCGGCACTTT	0.17	0.05
manX	ACAACAACCCGAAATATGCTGGCG	TGCCATACCACCGACGTTAACAGA	0.24	0.16
hdeB	TGGCAGCCAATGAATCCGCTAAAG	TTCATGCAGCATCCACCATGCAAC	0.09	0.01
atpD	ATCGCCATCCTGGGTATGGATGAA	AACCGGTGAATACTTCTGCCACGA	2.21	1.42
ibpB	ATATGGAAGTCTCTGGCGCAACCT	TATTTAACGCGGGACGTTCGCTGA	4.11	3.23
sdhC	ATCACCTTTGTTGCAGTGGGCATC	AAGAAGCTGCCCATAATCGCGGAA	2.38	2.06
glpA	TAACCATCTCACCGGCGAAACTCA	GCAGATCGGCATATTCGGCAATGT	6.90	6.04
mopB	GCTGGATGTGAAAGTTGGCGACAT	CCAGAATGTCGCTTTCGGACATGA	2.59	2.97

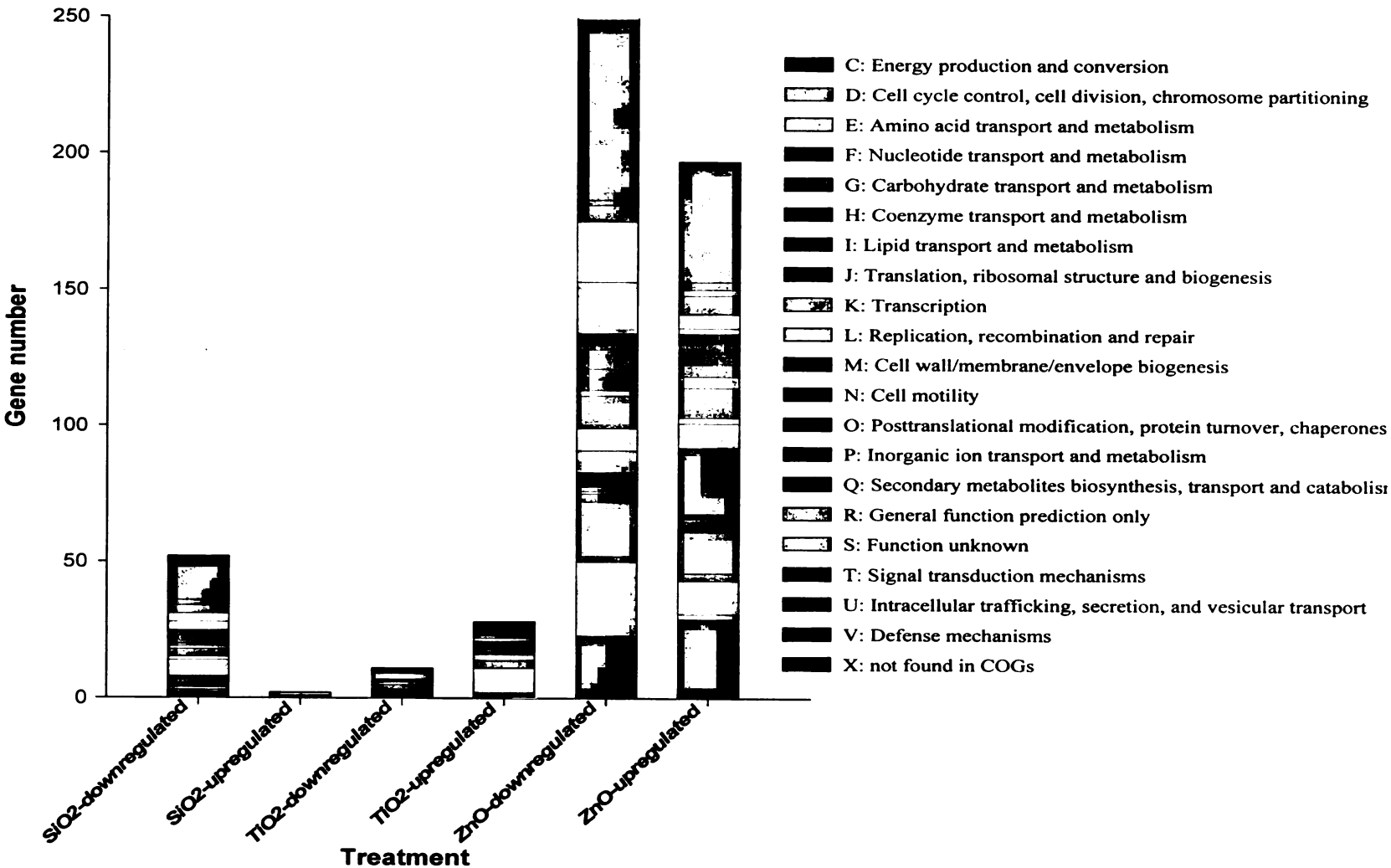

Figure 4.1 The functional categories of the gene profiles in SiO_2 NP, TiO_2 NP and ZnO NP treatments for *E. coli*.

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CHAPTER 5

COMPARATIVE ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES IN *EXIGUOBACTERIUM SIBIRICUM* FOLLOWING EXPOSURE TO OXIDE NPS

Abstract

NPs are being developed for diverse applications, such as water purification, drug delivery and electronic manufacturing. However, their environmental impact and toxicological implications are still unknown. Although numerous studies are now available about their antibacterial activity, information related to their impact at the level of gene expression are only beginning to emerge. In this study, microarrays were used to study the influence of SiO_2, TiO_2, and ZnO NP on gene expression of *E. sibiricum*. The toxicity of NPs was consistent with their impact of gene expression. Both the number of repressed and induced genes generally increased in the order: SiO_2 to TiO_2 to ZnO. For all the three treatments, more genes were down-regulated genes compared to the number of up-regulated genes. The results demonstrated that they influenced expression of genes in different functional categories, including transportation, carbohydrate metabolism, lipid metabolism and DNA repair. Microbial respiration was suppressed during exposure of NPs, especially for aerobic respiration, as well as TCA cycle, oxidation of pyruvate and oxidative phosphorylation. Up-regulation of genes related to DNA damage repair and lipid or fatty acid biosynthesis, indicated that ROS induction in *E. sibiricum* was a response to NP exposure.

Introduction

Nanotechnology is defined as "the understanding and control of matter at dimensions of roughly 1 to 100 nanometers, where unique phenomena enable novel applications" by National Nanotechnology Initiative. It is an emerging science that is revolutionizing the advancements taking place in many industries e.g., electronics, food, cosmetics, and medicines (http://www.nano.gov/html/facts/whatIsNano.html). It is estimated that by 2015 the market about nanotechnology will be around $1 trillion (44). Due to the increasing production and widespread application of diverse NMs, the concerns about the exposure to human and environmental release are also widespread (19, 59). However, studies related to the impacts of NMs on environment and human health are limited. Their small size, large surface area, shape, chemical composition, charge, solubility and aggregation, increase the difficulty to study and characterize their toxicological properties.

NPs, the building blocks of NMs, have been used in diverse fields, such as environmental remediation, paint manufacturing and drug delivery. It has been reported that NPs had adverse impacts on bacteria and animals (16, 21, 31, 32, 58). However, there is little explanation about the mechanism of NP toxicity. It is hypothesized that the toxicity of NPs might be due to ROS induction (8, 44, 46, 49).

DNA microarrays consist of short oligonucleotides or PCR products attached to a solid substrate. Oligonucleotide microchips containing multiple oligonucleotides are spotted on the chip surface. Probes targeting an entire microbial genome can be easily

represented in a single array, making it feasible to perform genome-wide analysis. Since its inception more than a decade ago, DNA microchips have been used widely for gene expression study (6, 11, 51). DNA samples for analysis are labeled with fluorescent dyes and hybridized with the oligonucleotide spots on the chip. The fluorescence pattern is then recorded by a laser scanner, and then signals are quantified and analyzed using dedicated software to handle the amount of data. This technique can be extremely helpful in increasing our understanding about the effects of NP exposure to microorganisms and aiding in explaining the possible mechanisms of NP toxicity.

Exiguobacterium sibiricum strain 255-15, one member of the *Bacillaceae*, is a low G+C, Gram-positive, rod-shaped and non-spore-forming psychrotrophic bacterium (53, 62). It is wide-spread capable of living over a broad temperature range (-5 °C-40 °C) (54). The goal of this study was to explore the microbial response to different oxide NPs and possible mechanisms for their toxicity. More specifically, we wanted to evaluate the gene expression profiles of *E. sibiricum* when exposed to SiO_2, TiO_2 and ZnO NPs. Moreover, we wanted to evaluate the influences of NPs on selected pathways focusing on the ROS damage response mechanisms in *E. sibiricum*.

Materials and Methods

Reagents and preparation of NP suspensions. Random hexamer primers, 100 mM nucleotide set, RNaseOUT, salmon sperm DNA, Superscriptase II RT, formamide, 10% sodium dodecyl sulfate, 20× Saline-Sodium Citrate (SSC), and 0.5 M

ethylenediaminetetraacetic acid (EDTA) were obtained from Invitrogen Co. (Carlsbad, CA). Bovine serum albumin (BSA), dimethyl sulfoxide, sodium hydroxide, 1 M hydrochloric acid and isopropanol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). RNAlater, SlideHyb buffer and aminoallyl-modified dUTP were obtained from Ambion Inc. (Austin, TX). Lysozyme, DNase I, Tris-Cl EDTA (TE) buffer, RNeasy mini kit, Quiaquick PCR purification kit and RNA protect bacteria reagents were obtained from Qiagen Co (Valencia, CA). SiO_2 (10 nm), TiO_2 (5 nm) and ZnO (60 nm) were purchased from Sigma-Aldrich (St. Louis, MO). The 100 g/L NPs stock solutions were prepared in deionized and distilled water. Phosphate wash buffer (100 ml) for cDNA purification was made of 0.5 ml K_2HPO_4 (1 M, pH 8.5), 84.25 ml ethanol and 15.25 ml H_2O and phosphate elution buffer (100 ml) contains 0.4 ml K_2HPO_4 (1 M, pH 8.5) and 99.6 ml H_2O. All the solutions were autoclaved and stored at room temperature

Cell growth, harvest and RNA extraction. From a freezing stock of *E. sibiricum*, streak plates were prepared with TSA medium and grown at 30 °C overnight. One loop of the plate was transferred to the tubes with 5 ml TSB. The NP stock solution was added to each tube with 100 ml TSB to result in final NP concentrations of 5000 ppm for SiO_2 NPs, 1000 ppm for TiO_2 NPs and 500 ppm for ZnO NPs. The samples were inoculated and incubated until mid-log growth phase of *E. sibicirum* ($0.1<OD_{600}<0.4$). According to the protocol of RNAlater, 1 ml of RNAlater was added to 100 ml culture for RNA extraction, and incubated for 5 min at 30 °C. The cells were collected by centrifuging for 20 min at 5000× g at 4 °C, and the supernatant was decanted. The cells were resuspended

in 1 ml RNAlater, transferred to a microcentrifuge tube and pelleted at 5000× g at 4 °C for 10 min. The 100 µl lysozyme in TE buffer (3 mg/ml) was added to the cells, mixed by vortexing for 10 s, and incubated at room temperature for 30 min on a shaker. Buffer RLT (350 µl) in RNeasy mini kit was applied and vortexed vigorously for 30 s. After 2 min centrifugation at maximum speed and removal of the sediment of NPs, the supernatant was mixed with 250 µl ethanol by shaking vigorously (but not centrifuging after shaking). As described in RNeasy mini kit, the lysate was transferred to RNeasy Mini spin column, and centrifuged for 14 s at >8000× g. DNase digestion was applied to the column by buffer RW1 washing, DNase I solution washing (10 µl) incubating, and then buffer RW1 re-washing. The RNA was purified as the protocol of RNeasy mini kit and the final concentration of RNA was measured by Nanodrop from Thermo Scientific Co. (Wilmington, DE). RNA from control samples was processed in a similar way as NP samples. All experimental and control samples were prepared in triplicate.

cDNA synthesis and dye-labeling. For each sample, 5 µg of total RNA was used to synthesize cDNA and two RNA populations of each sample were prepared for dye-swapping. RNA mixture contained 5 µg RNA, 5 µl random hexamer primers (3 mM), Rnase-free water in the final volume of 16.8 µl. The RNA mixture was incubated at 70 °C for 10 min and then cooled on ice for another 10 min. The RT-PCR reaction had a 30 µl volume and contained 6 µl 5× first strand synthesis buffer, 3 µl DTT (0.1 M), 1 µl RNaseOUT, 1.2 µl 25× dNTPs (7.5 mM aa-dUTP, 5 mM dTTP, 12.5 mM dCTP, 12.5 mM dGTP and 12.5 mM dATP), 2 µl Superscript II RT and 16.8 µl RNA mixture. The

RT-PCR reaction was incubated at 42 °C overnight, and then 10 μl NaOH (1 M) and 10 μl EDTA (0.5 M) were added to the reaction. After incubating at 65 °C for 15 min, 10 μl HCl was added to neutralize the pH. The cDNA was purified according to the protocol of Qiagen purification kit with some modification. The cDNA mixture was mixed with 300 μl PB buffer, and transferred to a Quiaquick column. After 1 min centrifuging at 17,900× g, the collection tube was emptied and the column was washed by 750 μl phosphate wash buffer twice and centrifuged for 1 min as above. After 1 min additional centrifuging, the column was transferred to a new tube, and then eluted twice by incubating the column with 30 μl phosphate elution buffer for 1 min and centrifuging for 1 min at maximum speed. The cDNA sample was dried using Speedvac from Global Medical Instrumentation Inc. (Ramsey, MN). The aa-dUTP cDNA was resuspended in 4.5 μl sodium carbonate (0.1 M, pH 9.0) and mixed with 4.5 μl Cy5 or Cy3. After incubating 2 h at room temperature in the dark, thirty-five μl NaOAc (100 mM, pH 5.2) was added and the dye-coupled cDNA was purified as described in the protocol of Qiaquick purification kit. The concentrations of cDNA and dye were measured by Nanodrop from Thermo Scientific Co. (Wilmington, DE).

Hybridization. The glass-slide arrays were cross-linked at 6000 μJ UV and waterbath was prewarmed to 46 °C. The slides were placed in prehybridization buffer containing 50% formamide, 5× SSC, 0.1% SDS and 0.1 mg/ml BSA at 46 °C for 60 min. After washing three times with deionized and distilled water and once with isopropanol, the slides were dried by centrifuging at 1600 rpm for 3 min and transferred to dry and

clean tubes covered with foil. The coverslips were washed with RNase-free water and ethanol, dried by centrifuging 600 rpm and then placed in clean tubes covered with foil. The heat block was preheated to 95 °C and the hybridization oven to 60 °C. The labeled DNA of the control sample was resuspended in 35 µl hybridization buffer containing 50% formamide, 5× SSC, 0.1% SDS, 0.1 µg/µl salmon sperm DNA. The other differently labeled DNA from NP-treated sample was suspended in the same hybridization solution, which was heated at 95 °C for 5 min. The dry slide-array was placed in a clean and dry hybridization chamber and coverslip was placed on the slide. The hybridization solution was loaded on the slide, and 20 µl 5× SSC was added in each well in the hybridization chamber. The chamber was placed in waterbath at 46 °C for 16 h. For each NP treatment, six hybridizations from three biological replicates and two technical replicates (dye-swapping) were performed.

Washing, scanning and data analysis. After 16 h, the chamber was removed from waterbath and dried with paper towel before opening. Wash Buffer 1 and slide staining jars were prewarmed at 46 °C. The slide was taken out with forceps, and the coverslip was removed by immersing the array in a tube containing Wash Buffer 1 (1× SSC and 0.1% SDS). The slide was washed twice with Wash Buffer 1 (1× SSC and 0.1% SDS) for 5 min at 50 °C, then twice with Wash Buffer 2 (0.1× SSC and 0.1% SDS) for 10 min at room temperature, and four times with Wash Buffer 3 (0.1× SSC) for 1 min. The slide was dried by centrifuging at 1600 rpm for 3 min and transferred to dry tubes.

Axon Genepix 4000B laser scanner was used for slide scanning and Genepix 3.0

software was used to produce a raw data set. The scanner was turned on and warmed up for 15 min while the computer was off. The slide was placed face-down, and PMT and power was adjusted to yield optimal signals (as verified by histogram). A 5 μm pixels resolution setting was chosen for scanning. The area of interest was scanned and the image was saved. GENESPRING 6.0 software (Silicon Genetics, Redwood City, CA) was used for data analysis. The data was normalized both per chip and per gene (Lowess method), and dye-swapping. Only the spots with more than 55% of pixel greater than background plus 2× standard deviation in either Cy5 or Cy3 channel were used for analysis. Genes that showed a statistically significant change in gene expression ($p>0.05$) and a greater than or equal to 2-fold change in magnitude were regarded as significant.

Results

Global gene expression profiles under NP exposures. For all three NP exposures, the number of expressed genes exceeded the number of induced genes. The expression response to 500 ppm ZnO NPs was broader with more than 27% of the genome expressing differentially. For 1000 ppm TiO_2 NPs, 18% of genome showed differential expression response while for 5000 ppm SiO_2 NPs, it was 14%. According to our previous viability data, it could be concluded that the exposure to more toxic NPs caused more differential expression in *E. sibiricum*. Among the up-regulated genes, almost 40% of TiO_2-induced genes were also up-regulated in ZnO treatment, while only 15% of TiO_2-induced genes were up-regulated in SiO_2 treatment (Figure 5.1(a)). Meanwhile,

48% of TiO_2-repressed genes were down-regulated in ZnO treatment, whereas 27% of TiO_2-repressed genes were down-regulated in SiO_2 treatment (Figure 5.1(b)). Thus the response of *E. sibiricum* to ZnO NP and TiO_2 NP exposures was closer, compared to SiO_2 NP exposure. A total of 13 genes were induced in all the three treatment, and 38 genes were repressed by all three different NP exposures in Figure 5.1.

Gene expression profiles following SiO_2 treatment. For SiO_2 treatment, fold-expression for 2539 genes ranged between 0.01 and 11.1. Based on the available information in the databases of Kyoto Encyclopedia of Genes and Genomes, UniProt and InterPro, the set of 234 down-regulated genes in the response to SiO_2 NPs were grouped into 17 functional categories. Categories "transportation" (24.8%) and "hypothetical" (20.9%) were the two dominant categories. Others included "biosynthesis" (8.5%), "carbohydrate metabolism" (7.3%), "amino acid metabolism" (6.4%), "transcription" (5.6%), "nucleotide metabolism" (3.8%), and "energy metabolism" (3.8%). Similarly, 158 up-regulated genes were grouped into the same 17 functional categories. Besides "hypothetical" (20.9%) and "transportation" (12.7%), most of induced genes belonged to the categories of "unknown" (9.5%), "carbohydrate metabolism" (9.5%), "transcription" (7.0%), "replication and repair" (7.0%), "amino acid metabolism" (6.3%), "translation" (5.1%), "lipid metabolism" (4.4%), "biosynthesis" (4.4%) and "cellular process" (3.8%). There were more down-regulated genes than up-regulated ones in categories of "biosynthesis" and "xenobiotics degradation and metabolism", which might mean that

the bacteria closed some unnecessary pathways to save energy for survival. Meanwhile, more genes were induced compared to repressed ones in "lipid metabolism", "replication and repair" and "signal transduction", which could be related to the damage to membrane and DNA by ROS induction.

Response of "transportation" related to genes in SiO_2 NP exposure. Transportation and internalization of NPs is an important factor influencing the fate of NPs. Because NPs are generally much larger than all the transportation channels, the mechanisms about how they enter the bacterial cells require additional exploration. In all the expression profiles, "transportation" was always one of the largest groups for both of induced and repressed genes. During the exposure to SiO_2 NPs, there were 58 down-regulated genes and 20 up-regulated genes in the "transportation" category. Among the repressed genes, 41.4% belonged to ABC transporters, 6.9% related to Na^+ symporters, 5.2% functioned as PTS components, and 13.8% involved in sugar or amino acid transportation. For the induced genes in "transportation", 50.0% were ABC-transporters, 10.0% K^+ transporters and 5.0% were involved in PTS system.

ABC transporters are a family of membrane proteins that function in the ATP-dependent transportation of many compounds across cellular membranes (1, 23). ABC transporters were found in both induced and repressed genes. The down-regulated genes mainly involve in transporting sugar, amino acid and peptides, such as ABC-type sugar transport system permease components and olio-peptide ABC transporter permease.

The up-regulated ones are related to ferric and other metal ions, such as ABC-type Fe^{3+}-hydroxamate transport system components and ferric anguibactin transport system permease. Zhang *et al.* also reported over-expression of ABC transporters in mouse cells by poly(ethylene glycol)-block-polylactide NPs (66). ROS is known to oxidize fatty acids of phospholipids on membrane and cause lipid peroxidation (2). This process can change the membrane permeability and fluidity, and interrupt nutrient uptake, which may explain the response of amino acid and carbohydrate transport genes. Iron ion is another damage target for ROS, which may change the expression of metal ion ABC-transporter.

Sodium/solute symporters and Potassium (K^+) transporters were the other two groups of transporters influenced by SiO_2 NPs. Sodium/solute symporters catalyze the uptake various solutes into the microbial cells (50). Under extreme conditions, bacterial cells can use Na^+ as coupling ions to generate Na^+-electrochemical gradients by Na^+ pumps and Na^+/H^+ antiporters, and this is important for accumulation of nutrients (25, 26). K^+ ion, as a major intracellular cation, plays a fundamental role for many physiological activities, such as protein synthesis and enzyme activation (14, 29). Under some conditions, Na^+ substitutes functionally for K^+ (55). In the SiO_2 treatment, Na^+ symporters were repressed while K^+ transporters were induced. The rapid leakage of K ions in bacterial cells during NP exposure was reported by Saito *et al.*, and this may be related to the up-regulation of these K^+ transporters influenced by SiO_2 NPs (56).

Category of "carbohydrate metabolism" in SiO_2 treatment. "Carbohydrate metabolism" is another important category in the gene expression profile. It involves various biochemical processes and is helpful to understand how bacteria survive through the exposure to NPs. There were 17 repressed genes and 14 induced genes in this category. Among the down-regulated genes, 23.5% of the repressed genes were related to CoA, 23.5% to pyruvate, and 23.5% to glycosidases or glucosidases. CoA functions as an acyl group carrier and plays a fundamental role in TCA cycle as well as synthesis and oxidation of fatty acids. It was reported that NPs could cause oxidation of CoA, and decrease of CoA may influence the activity of CoA-related enzymes (37, 43). CoA is also involved in the oxidation of pyruvate, which may explain why the genes for pyruvate dehydrogenases were also down-regulated. The pyruvate dehydrogenase complex transforms pyruvate into acetyl-CoA, controls pyruvate entry into TCA cycle, and also participates in glycolysis and glucose oxidation (38). It may be concluded that SiO_2 NPs can repress the TCA cycle by oxidizing CoA and down-regulating the pyruvate dehydrogenases. The SiO_2 NPs also down-regulated the genes encoding glucosidases, glucosidase-related glycosidases and 1-phosphofructoknase. These enzymes are involved in glycolysis, generating ATP and producing pyruvate as a part of aerobic respiration.

For up-regulated genes in "carbohydrate metabolism", there was no clear pattern among them. However, more than half of them were dehydrogenases, including alcohol dehydrogenases and aldehyde dehydrogenases, which transfer protons or electrons to

acceptors in electron transport chains, usually some coenzymes such as nicotinamide adenine dinucleotide/ nicotinamide adenine dinucleotide phosphate (NAD/NADP) (60). It is well known that NAD/NADP participates in generation of ATP, which might explain how *E. sibiricum* survives the exposure to SiO_2 NPs. Most of dehydrogenases contain sulfur-iron cluster, which is another attacking target for ROS. There were some coenzyme-dependent enzymes that were up-regulated in this category, such as flavin-dependent oxidoreductases, which may also adjust the cellular redox conditions since NPs can induce intracellular ROS.

Category of "energy metabolism" in SiO_2 treatment. "Energy metabolism" is important for understanding how *E. sibiricum* produces energy and survives in the exposure to SiO_2 NPs. Almost 80% of repressed genes in this category were the subunits of cytochrome *c* oxidase or cytochrome caa3 oxidase. They function in one of electron transport chains in oxidative phosphorylation, and this process is important for cellular metabolism and energy synthesis as a part of aerobic respiration. Many enzymes involved in oxidative phosphorylation are located on membrane, which is one of the main attacking targets for ROS. ROS can also be produced during oxidative phosphorylation, such as free radicals and hydrogen peroxide, which cause some cellular damage.

Among the induced genes in this category, it seems most could serve as antioxidant preventing the damages from ROS. For example, one gene encoding a catalase which can catalyze and converse hydrogen peroxide into water and oxygen, was up-regulated (24).

Two genes related to cysteine synthesis, which is beneficial for glutathione system in detoxification metabolism, were also up-regulated (40, 41). This could be another evidence for ROS damage, since cysteinyl residues in proteins are oxidized at the presence of ROS (22).

Category of "metabolism of cofactors and vitamins" in SiO_2 treatment. It is well known that cofactors and vitamins involve many biological processes and own diverse biochemical functions, such as activation of apoenzymes and regulation of microbial growth. Some of them function as antioxidant preventing microbes from ROS damages. Factors related to ROS damage are important in determining the response to NPs. For SiO_2 NP treatment, there were 6 repressed genes and 2 induced genes. Among the down-regulated genes, half were coenzyme F390 synthetases. Coenzyme F390 system was first found in *Methanobacterium* spp., and this system can sense the intracellular reduction and oxidation potential (61). Coenzyme F390 usually is present when the cellular redox potential increases. So it may function as a signal compound, transferring information about intracellular redox potential. Two genes encoding ketopantoate reductase and thiamine monophosphate synthase respectively were repressed. The ketopantoate reductase is involved in biosynthesis of pantothenate, which is needed to form CoA and important for carbohydrate metabolism. The thiamine monophosphate synthase participates in thiamine metabolism, which relates to modification of CoA. It

seems that oxidation of CoA by NPs influences the relative pathways to other cofactors and vitamins.

In the SiO_2 treatment, a gene encoding isochorismate synthase was up-regulated. Isochorismate synthase participates in ubiquinone biosynthesis and ubiquinone, also known as coenzyme Q (CoQ), usually functions as an antioxidant. So the induction of isochorismate synthase probably benefits the detoxification and protection from oxidative damages. Another induced gene was related to nitroreductase, which is involved in the reduction of nitrogen-containing compounds, such as NADH or FMN.

Category of "replication and repair" in SiO_2 treatment. Since the induction of ROS by NPs and their genotoxicity have been reported before, "replication and repair" category may be important in understanding how *E. sibiricum* protects itself from DNA damage by NPs. For SiO_2 NP treatment, there were 5 repressed genes and 11 induced genes in this category. Thus more DNA repair systems were activated to alleviate DNA damages. Two bacteriophage integrases and one UV-damage repair protein were down-regulated by SiO_2 NPs. It is well known that short-length UV can induce prophage-related genes. Therefore it appears that UV-related DNA repair system was repressed and not applicable for the DNA damage by ROS and NPs. The UV-damage repair protein was repressed in all the three treatments. It was reported that UV-irradiated TiO_2 had been applied as a photocatalyst for bacterial killing, and the suppression of UV-damage repair protein by NPs could worsen the bacterial survival during the

photo-killing (34, 52, 64). The induced genes included transposases, recombinases, ribonucleases, excisionases and oxidative damage protectants. Almost half of these genes were related to recombination, which may be important for DNA repair process.

Gene expression profiles following TiO_2 treatment. A total of 2681 genes showed a fold-change in the expression ranging from 0.01 to 17.31. As illustrated in Figure 5.2, the 289 down-regulated genes in the TiO_2 treatment were grouped into 17 functional categories, including "hypothetical" (25.3%), "transportation" (15.2%), "carbohydrate metabolism" (8.7%), "amino acid metabolism" (8.7%), "transcription" (7.3%), "unknown" (5.2%) and "repair and replication" (4.2%). Similarly, 197 up-regulated genes were grouped into the same 17 functional categories. Besides "transportation" (26.4%) and "hypothetical" (23.4%), most of induced genes belonged to the categories of "unknown" (5.6%), "replication and repair" (5.6%), "transcription" (5.1%), "lipid metabolism" (4.6%), "carbohydrate metabolism" (4.6%), and "nucleotide metabolism" (4.1%). Usually there were both induced and repressed genes in the same functional categories, whereas more genes were repressed in the categories of "amino acid metabolism", "carbohydrate metabolism", "transcription" and "translation", which may mean that *E. sibiricum* survived exposure to TiO_2 NPs by reducing cellular activity.

In "carbohydrate metabolism", there were 25 down-regulated genes and 9 up-regulated genes. Among the repressed genes, 5 genes were related to CoA and another 5 genes were alcohol or pyruvate dehydrogenases. In the category of "transportation", a

total of 44 genes were down-regulated and 52 genes were up-regulated. The repressed genes included ABC-transporters (43.2%), PTS components (9.1%), and Na^+ or H^+ symporters (4.5%), while the up-regulated genes covered ABC-transporters (42.3%), ferrous related transporters (11.5%), PST components (5.8%), antiporters or sympoters (5.8%), and K^+ or Na^+ transporters (3.8%). Similarly to SiO_2 treatment, TiO_2 NPs repressed a few cytochrome *c* oxidases and induced some genes encoding thioredoxin-like proteins or NADPH:quinone reductases in the "energy metabolism" category. It is well known that thioredoxin is another enzyme system functioning as an antioxidant and it can prevent *E. sibiricum* from oxidative damage by NP exposure. The NADPH:quinone reductases participates in oxidative stress resistance by acting NADPH with a quinine as an acceptor (63). In the "replication and repair" category of TiO_2 treatment, some UV-related repair genes were down-regulated, such as UV-damage repair protein and DNA photolyase. Recombinases and recombinational DNA repair proteins (23.1%) were also repressed by TiO_2 NPs. Helicases (18.2%), transposases (18.2%) and DNA-directed RNA polymerases (18.2%) was among the induced genes by TiO_2 NPs.

Gene expression profiles following ZnO treatment. In the ZnO treatment, globally the fold-change in expression of 2616 genes ranged from 0.01 to 19.9. There were 402 down-regulated genes and 365 up-regulate genes. ZnO NPs had the largest number of up- or down-regulated genes (compared to in SiO_2 and TiO_2 treatments). The down-regulated

genes can be grouped into 16 functional categories, including "hypothetical" (22.4%), "transportation" (13.4%), "carbohydrate metabolism" (12.2%), "amino acid metabolism" (9.5%), "transcription" (8.5%), "unknown" (6.7%) and "repair and replication" (4.0%) (Figure 6.2). Similarly, the up-regulated genes were grouped into the 17 functional categories, including "hypothetical" (18.4%), "transportation" (14.8%), "translation" (11.0%), "replication and repair" (9.3%), "unknown" (5.8%), "nucleotide metabolism" (5.8%), "amino acid metabolism" (5.8%), "carbohydrate metabolism" (5.2%) and "lipid metabolism" (4.4%). In ZnO treatment, more genes were repressed in the categories of "amino acid metabolism", "carbohydrate metabolism", and "transcription", while more genes were induced in the categories of "replication and repair", "lipid metabolism" and "nucleotide metabolism". It can be concluded that *E. sibiricum* reduced cellular activity to save energy and survive the exposure to ZnO NPs, and activated relative pathways to repair DNA damages by ROS induction.

There were 49 repressed genes and 19 induced genes in "carbohydrate metabolism". Similar to the treatment of SiO_2 and TiO_2 NPs, it was found that 5 repressed genes were related to CoA and another 10 repressed genes were alcohol or pyruvate dehydrogenases. In the category of "transportation", there were 54 repressed genes and 54 induced genes. ABC-transporters (38.9%), PTS components (9.3%), Na^+ or K^+ transporters (9.3%), and symporters (5.6%) were found in the repressed transportation genes while there were ABC-transporters (38.9%), ferrous and ferric transporters (9.3%), antiporters (5.6%), K^+

or Na^+ transporters (5.6%), and PST components (1.9%) among the induced genes. ZnO NPs repressed a few cytochrome c oxidases, while they also induced some coenzyme related oxidoreductases. In ZnO treatment, there were 16 repressed genes and 34 induced genes in "replication and repair" category. Thus more DNA repair mechanisms were activated to alleviate the DNA damages from ZnO NPs. Among the down-regulated genes, helicases (12.5%), DNA-directed RNA polymerases (12.5%), transposases (25%) and recombinases or recombination proteins (12.5%) contributed the majority. The up-regulated genes included helicases (8.8%), DNA-directed RNA polymerases (11.8%), transposases (5.9%) and recombinases or recombination proteins (8.8%), DNA polymerases (8.8%), nucleases (11.8%), DNA repair proteins (8.8%) and topoisomerases (5.9%).

Category of "metabolism of cofactors and vitamins" in TiO_2 and ZnO treatments. There were 9 repressed genes and 6 induced genes by TiO_2 NPs, while there were 12 repressed genes and 11 induced genes in the ZnO treatment. Similar to SiO_2 treatment, coenzyme F390 synthetase was down-regulated, which demonstrated the changes of intracellular redox potential. Dihydroneopterin aldolase, which participates in folate biosynthesis, was down-regulated in both TiO_2 and ZnO NP treatments. It is well known that folate is important and necessary for DNA replication and cell division (27). Glutamyl-tRNA reductase was another repressed gene in both of treatments, which participates in porphyrin metabolism. Porphyrin is synthesized by reaction of glycine and

succinyl-CoA from TCA cycle. It is likely that oxidation of CoA reduced the synthesis of porphyrin and repressed the activity of glutamyl-tRNA reductase. Porphyrin is contained in heme, which is one subunit of cytochrome c oxides. In TiO_2 treatment, uroporphyrinogen decarboxylase, also related to heme biosynthesis, was down-regulated (57). In ZnO treatment, the genes related to metabolism of CoQ and vitamin B_6 were repressed. Vitamin B_6 assists in the balancing between Na^+ and K^+. The repression of vitamin B_6 biosynthesis protein may be related to the rapid leakage of K^+.

In both TiO_2 and ZnO NP treatments, thiamine pyrophosphokinase, which catalyzes the reaction to produce thiamin pyrophosphate, was induced. Glycine oxidase, which participates in thiamine metabolism, was induced too. Meanwhile, two genes related to molybdopterin biosynthesis were up-regulated. Molybdopterin usually functions as a cofactor necessary for sulfite oxidases and nitrate reductases, which are very important in bacterial electron transfer chains and supply electron acceptors for anaerobic respiration (28).

Category of "xenobiotics degradation and metabolism" in SiO_2, TiO_2 and ZnO treatment. Although it is not a large category, it contained some interesting information about NP exposure, especially among the down-regulated genes. In SiO_2 treatment, one gene involved with ethyl tert-butyl ether (ETBE) degradation was down-regulated, which also happened in the treatments of TiO_2 and ZnO NPs. ETBE has been used as a fuel oxygenate in gasoline and it is toxic and relatively non-degradable (39). Oxidation of

ETBE involves electron chain transfer by a cytochrome P450 monooxygenase (3, 13). The genes related to degradation of phenylacetate or phenylacetic acid were down-regulated, including some phenylacetate-CoA oxygenase subunits that may produce some TCA cycle intermediates, and phenylacetyl-CoA ligase that activates phenylacetic acid to phenylacetyl-CoA (12, 45). In SiO_2 treatment 83.3% repressed genes were related to phenylacetate or phenylacetic acid, 50.0% in TiO_2 treatment and 66.7% in ZnO treatment. Type I pullulanase and NADH-dependent nitro/flavin oxidoreductase were induced in both TiO_2 and ZnO treatment. Type I pullulanase can specifically cleave the α-1, 6-glycosidic linkages in pullulan and branched or linear oligosaccharides (33). NADH-dependent nitro/flavin oxidoreductase is one type of nitroreductases, participating in reduction of nitrogen containing compounds (9).

Shared down-regulated and up-regulated genes in SiO_2, TiO_2 and ZnO treatments. As shown in Figure 5.3, there were 13 repressed genes and 38 induced genes that were common to all the three treatments. The categories "hypothetical" and "transportation" contained the largest number of common genes for both down-regulated and up-regulated genes. Among the down-regulated genes, the category "biosynthesis", "carbohydrate metabolism" and "xenobiotics degradation and metabolism" were important. The induced genes in the "transportation" category were associated with ABC transporters for metal ions, such as ferric or zinc, while the most of repressed genes in the same category were sugar transporters, including ABC sugar transporters and PTS

components. In "biosynthesis" category, all the repressed genes were related to polysaccharide synthesis. Among the repressed genes of "carbohydrate metabolism", nearly half were CoA-related when the other half were pyruvate-related. In the "energy metabolism" category, the repressed genes mainly function in bacterial electron transfer chain. All the down-regulated genes in the "metabolism of cofactors and vitamins" were related to F390 coenzyme, which may be influenced by the changes of intracellular redox potential, and all the repressed genes in the "xenobiotics degradation and metabolism" involve in degradation of phenylacetic acid or ETBE. The patterns in these categories were useful for choosing molecular target candidates of NP toxicity.

Discussion

The microarray data was quite consistent with the studies reported in this dissertation focusing on viability and ROS. The order for the toxicity of NPs to *E. sibiricum* was ZnO > TiO_2> SiO_2, same as ROS induction. The microarray results displayed that the *E. sibiricum* exposed to ZnO NPs possessed the most induced and repressed genes and the expression fold was the widest ranging from 0.01 to 19.9. TiO_2 NPs possessed more influence on gene expression than SiO_2 NPs. According to 1-way ANOVA analysis, there were 1,677 genes expressed differently among these three treatments.

The microarray data also indicated that NPs might suppress microbial respiration. A few genes coding for pyruvate dehydrogenase subunits were repressed by NPs. These

genes participate in oxidative decarboxylation of pyruvate, which links glycolysis and TCA cycle. The down-regulation of these genes probably reduced the synthesis of acetyl-CoA, which is very important for TCA cycle. Meanwhile, some genes encoding CoA-related enzymes were down-regulated during the exposure to NPs. It has been reported that NPs could oxidize CoA (43). In this study, several cytochrome c oxidases were repressed by NPs. Cytochrome c oxidases are involved in oxidative phosphorylation, one of the key pathways for aerobic respiration. The essential enzymes in glycolysis were not influenced, although the exposure to NPs down-regulated a couple of genes in glycolysis, not requiring oxygen. It appeared that NPs influenced the aerobic respiration more than the anaerobic respiration. Similarly, fullerenes or its derivatives have also been found to inhibit *E. coli* respiration of glucose, and also hinder dioxygen consumption and respiratory chain (35-37). During the SiO_2 treatment, there were a few alcohol and aldehyde dehydrogenase genes that were up-regulated. These genes facilitate the conversion between alcohols and aldehydes and play an important role in fermentation. Quite a few dehydrogenases and oxidoreductases were also induced by TiO_2 NPs. These included NADPH: quinone reductase and ferredoxin oxidoreductase. For the ZnO NP exposure, however, some genes encoding important enzymes in the TCA cycle, including malate dehydrogenase, Succinyl-CoA synthetase beta subunit, isocitrate dehydrogenase and pyruvate dehydrogenase E2, were induced. Some alcohol and aldehyde dehydrogenases were repressed in ZnO NP treatment.

Previously we have demonstrated that ROS was induced in *E. sibiricum* by these NPs, and gene expression supported this observation. After exposure to NPs, most of the repressed ABC transporters were related to sugars, while induced ABC transporters were mostly related to metal ions, especially for iron ions. Iron is essential for most living bacteria functioning as a component for some important molecules, such as ribonucleotide reductase and cytochromes (30, 65). One possible explanation for the up-regulation of iron transporters is that ROS, induced by NPs, attacks sulfur-iron clusters and causes release of iron ions. It may also be due to the participation of iron in some electron transfer reactions since other cellular respiration pathways were suppressed. Additional evidence is the induction of enzyme in the categories of "lipid metabolism" and "replication and repair", since ROS can cause damage to membrane and DNA. Although a few enzymes in these two categories were down-regulated, the reason may be that they participated in the inactive pathway, and furthermore the number of induced genes was larger than that of the repressed, especially in the ZnO treatment. It was also observed that the expression of many oxidoreductases was influenced by the NPs, which might be related to the intracellular changes of redox potential caused by ROS. No induction of superoxide dismutases was found in *E. sibiricum*.

The category "transportation" was always the most influenced category in all NP treatments. ABC transporters were the most influenced class of membrane transport proteins for various substances by using energy from ATP-binding and hydrolysis (4, 5,

18, 47). Some K^+ transporters were up-regulated. Leakage of K^+ occurs in bacteria in response to exposure to TiO_2 NPs, which mayb be the reason for the induction of K^+ transporters (56). K^+ ion plays an important role in maintaining cellular viability, and the accumulation of K^+ ions influences the rates of cellular growth, respiration and protein synthesis (10, 17, 20, 42). A few Na^+/solute symporters were found down-regulated while some Na^+/H^+ antiporters were up-regulated. Both Na^+ ion and H^+ ion are critical for cell bioenergetics and physiology (15, 48). The down-regulation of Na^+/solute symporters and up-regulation of Na^+/H^+ antiporters should be related to H^+ or Na^+ electrochemical gradient, which is essential for solute uptake and energy storage. The proton gradient is important during NP exposure, perhaps due to the suppression of respiration by NPs, although Na^+ ion is particularly used as coupling ions in extreme environments (7, 26).

The categories "translation" and "signal transduction" were also important in all the three treatment. Most of the influenced genes in the category "translation" coded ribosomal proteins or enzymes related to tRNA. In the "signal transduction" category, the influenced genes coded histidine kinases. Not many genes were influenced in the category "biosynthesis", and most repressed genes were related to polysaccharide biosynthesis. In other categories, such as "amino acid metabolism" and "transcription", no specific pattern was observed.

In conclusion, the microarray data demonstrated that the three types of NPs tested in this study influenced the gene expression of *E. sibiricum*. ZnO NPs were the most toxic

one. Exposure to ZnO NPs resulted in more up- and down-regulated genes than either SiO_2 or TiO_2 NPs. For all three treatments, the number of down-regulated genes was higher than the number of up-regulated genes. ROS induced by NPs caused damage to membrane, protein and DNA. The functional category "transportation" was the largest for both the induced and repressed genes in these three treatments. These included ABC transporters, PTS, exporters, and antiporters/symporters. More transporters for carbohydrate, amino acid and drugs were found in the down-regulated group than in the up-regulated group, while there were more metal ion transporters induced. For down-regulated genes, the categories "transcription", "amino acid metabolism" and "carbohydrate metabolism" were other important ones, maybe due to the slowdown activity and growth caused by NP exposure. A number of CoA-related genes, which function in TCA cycle, glycolysis, biosynthesis of fatty acids and amino acid metabolism, were also repressed. Genes related to DNA repair were induced, maybe due to genotoxicity caused by NPs. The microarray data demonstrated that NPs influenced the genes of *E. sibiricum* in a variety of functional categories.

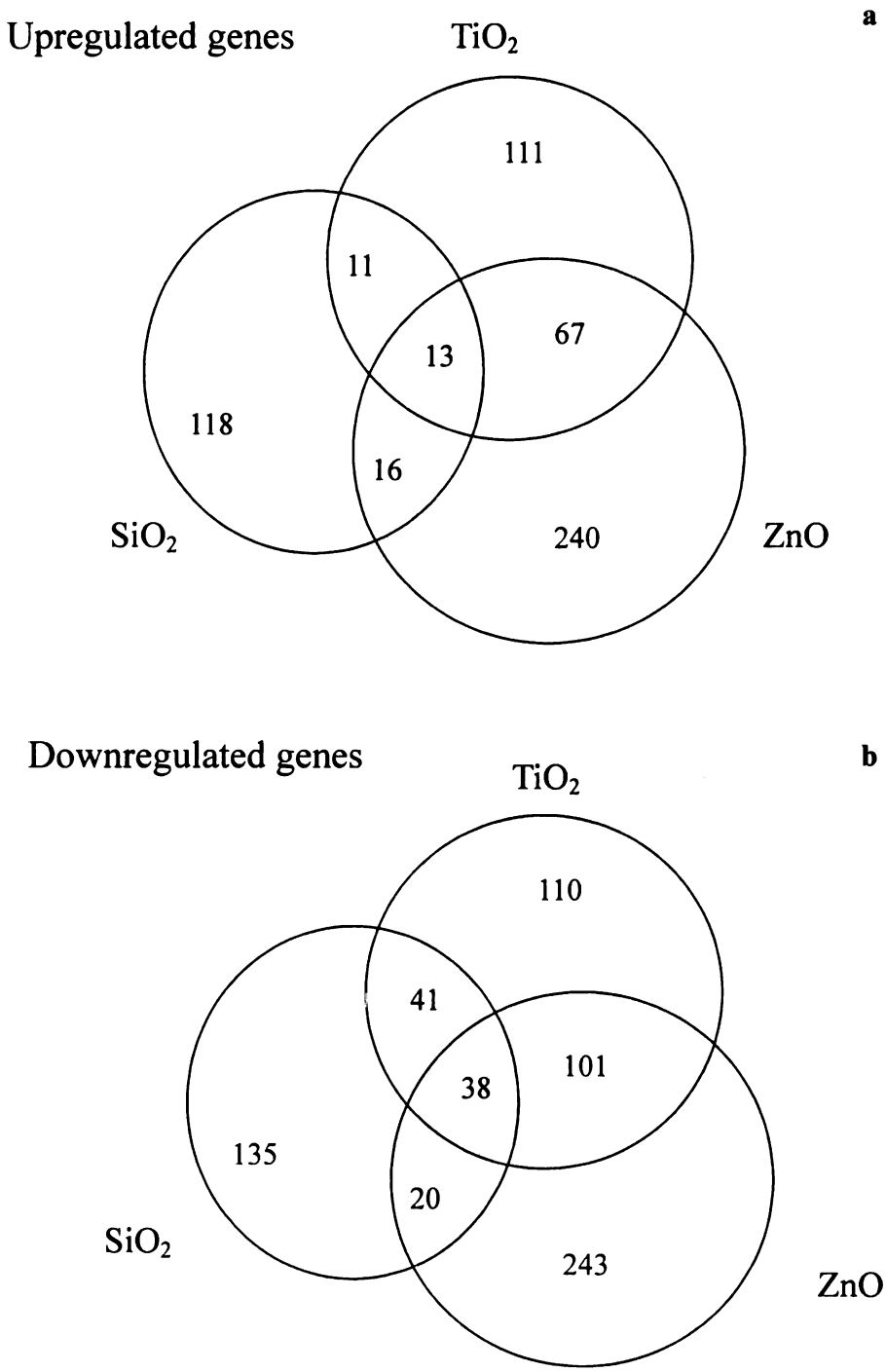

Figure 5.1 (a) The up-regulated gene profile in three different treatments. (b) The down-regulated gene profile in the three treatments.

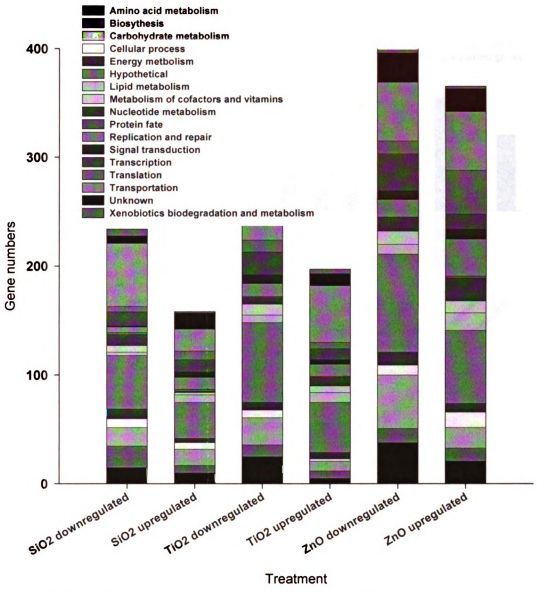

Figure 5.2 The functional categories of the gene profiles in SiO_2 NP, TiO_2 NP and ZnO NP exposures.

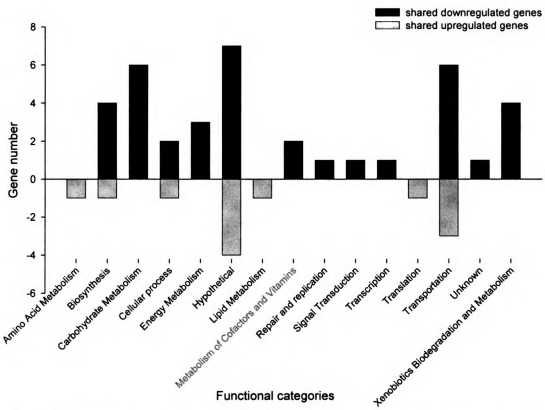

Figure 5.3 The functional categories of the shared genes among SiO_2 NP, TiO_2 NP and ZnO NP exposures.

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