Chemical-genetic profile analysis of inhibitory Nano-compounds in yeast *Saccharomyces cerevisiae*

BY

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Abstract

Nanoscience and Nanotechnology is often considered the fastest growing sector of the high tech economy, and is expected to turn into a trillion US dollar industry by 2015. This new technology offers new opportunities for the use of nanoparticles in both industry and health sectors. Because of their novel properties, engineered nanoparticles may have enhanced toxicity compared to their respective bulk material. Due to growing concerns about the safety of nanoparticles, it is essential to better investigate their effect on a living cell. Currently there is little information on the potential toxicity of metal oxide nanoparticles on human health. Here, we investigate the genetic profile of yeast non-essential gene deletion array (yGDA,~ 4700 strains) for increased sensitivity to zinc oxide and silver nanoparticles. Such profiles can be used to better understand the mode of activity of the different nanoparticles. Differential inhibitory effect of different nanoparticles for different gene deletion mutants can provide important clues into the mechanism of toxicity of these compounds. We observed that zinc oxide exhibit its activity against *Saccharomyces cerevisiae*, mainly by affecting the cytoplasmic membrane and cell wall formation/function. This was apparent from enrichments in membrane function and cell wall deficiencies among the mutant strains with increased sensitivity to zinc oxide nanoparticles. To confirm this observation we performed two secondary assays, cell wall and membrane integrity assays in both the presence and absence of zinc oxide nanoparticles. Also, a chemical genetic profile of silver nanoparticles using yGDA reveals that cellular roles of most susceptible mutants to silver nanoparticles may involve in vesicular transport, cellular respiration, and DNA repair, mainly nucleotide excision repair pathways. This was apparent from analysing the pathway of most susceptible mutant strains with increased sensitivity to silver nanoparticles.

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Special thanks to my parents for their understanding, patience and support. I dedicate this work to my father, Iraj Ghiyasvand, who always encourage and support me in academia.

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Abbreviations

Term	Definition
ATP	Adenosine triphosphate
B.subtilis	*Bacillus subtilis*
°C	Temperature in degree Celsius
CBMN	Cytokinesis blocked micronucleus
cDNA(s)	Complementary DNA(s)
CFU/ml	Colony forming unit per milliliter
Cy	Cyanine
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
ELISA	Enzyme linked immunosorbent assay
EST(s)	Expressed sequence tag(s)
ET-743	Ecteinascidin-743
G2	The gap between DNA synthesis and mitosis
G2-M	DNA damage checkpoint
GDA	Gene deletion array
In vitro	An experiment conducted outside of a living organism, usually in a test tube
In vivo	An experiment using a whole, living organism
mg	Milligram
µg	Microgram
MicroARCS	Micro arrayed compound screening
ml	Milliliter
µl	Microliter
M phase	Mitotic phase
nm	Nanometer
NAT	Nourseothricin
NP(s)	Nanoparticle(s)
PCR	Polymerase chain reaction
rDNA	Recombinant DNA
RNA(s)	Ribonucleic acid(s)
ROS	Reactive Oxygen Species
S.cerevisiae	*Saccharomyces cerevisiae*
SCGE	Single cell gel electrophoresis
SGD	Saccharomyces Genome Database
SMMs	Small molecules microarrays
S phase	Synthesis phase

Abbreviations

Term	Definition
Sub-inhibitory	A concentration less than an inhibitory level
TEM	Transmission electron microscopy
UV	Ultraviolet
WT	Wild Type
yGDA	Yeast gene deletion array
YPD	Yeast extract peptone dextrose

Chapter 1

Introduction

Abstract:

Nanoscience and Nanotechnology is often considered the fastest growing sector of the high tech economy. This new technology offers new opportunities for the use of nanoparticles in both industry and health sectors. Because of their novel properties, engineered nanoparticles may have enhanced toxicity compared to their respective bulk material. Due to growing concerns about the safety of nanoparticles, it is essential to better investigate their effect on a living cell. Currently there is little information on the potential toxicity of metal oxide nanoparticles on human health. Here, we investigate the genetic profile of yeast non-essential gene deletion array (yGDA,~ 4700 strains) for increased sensitivity to zinc oxide and silver nanoparticles. Such profiles can be used to better understand the mode of activity of the different nanoparticles.

1.1 Nanotechnology

Nanoscience and nanotechnologies encompass a novel area or technology for the 21st century that can initiate a new industrial revolution and advance health and manufacturing technologies. This new technology is considered the fastest growing sector of the high tech economy, and is expected to turn into a trillion US dollar industry by the year 2015. Nanotechnology partakes in the fabrication and application of nanomaterial (NM). It offers new opportunities for the use of NMs in both industry and health sectors (Schwegmann *et al.*, 2010). Nanotechnology is employed to modify compounds at the nano-scale in addition to producing materials with novel properties. Since NMs often have novel properties, nanotechnology has been considered as a possible source of concern in many countries (Schwegmann *et al.*, 2010). Decreasing the size of particular materials to the nano-scale may cause fundamental changes in the structural and physicochemical properties of these materials, hence generating novel

biological effects. Because of their novel properties, these engineered NMs may have enhanced toxicity compared to their respective bulk compounds (Kasemets *et al.*, 2009).

Nanotechnology refers to the production and application of a diverse array of NMs, including nano objects and nanoparticles (NPs) which often have novel qualities that distinguish them from their corresponding bulk compounds. Nanomaterials have one dimension less than 100 nanometer (nm), whereas nano objects have two dimensions less than 100 nm, and nanoparticles have three dimension less than 100 nm (Stone *et al.*, 2010). Due to the rapid growth of nanotechnology, predominantly nanoparticles in a variety of consumer products, it is crucial to acquire more information regarding their potential impacts on human and environmental health.

1.1.1 Nanoparticles

Nanoparticles are defined as particles less than 100 nm in size. They are deemed as the fundamental building blocks of nanotechnology. Nanoparticles are different from their respective bulk compounds, exhibiting novel properties such as small size, shape and chemical composition (Wang *et al.*, 2009). Nanoparticles have contributed to the application of many sectors such as medicine, pharmacy, industry and cosmetics. For instance, copper oxide is used in industry both as a catalyst and as a pigment in ceramics. Other noteworthy cases are titanium dioxide and zinc oxide which encompass ultraviolet (UV) blocking properties, hence making them ideal for uses in sunscreens (Kasemets *et al.*, 2009). Additionally zinc oxide is recognized as an active element in toothpaste and other beauty products such as baby cream which helps ease diaper rash

and skin irritations caused by burns. Moreover, some of these nanoparticles, such as iron oxide, are employed in medicine as drug delivery agents (Kasemets *et al.,* 2009). As well, silver nanoparticles have been used in various applications such as in medicine as an antimicrobial agent in dental material, and in industries in textile fabrics, and for water treatment (Rai *et al.,* 2009). Altogether, in recent year's nanomaterial have been used extensively in countless applications, thus increasing the public exposure to these materials. For that reason gathering statistics on their toxicity and safety is difficult.

1.1.2 Nanoparticle's toxicity

Promising developments of nanotechnologies in medical diagnostics and photodynamic therapy, cosmetics, and food packaging industries may require health and environmental risk evaluation. Because of their minute size, nanoparticles cover a larger surface atom proportion than volume compared to their respective bulk compounds. It is acknowledged, therefore, that nanoparticles may become more reactive and exhibit greater biological effects than larger particles or their bulk chemical constituents (Berardis *et al.,* 2010). For this reason, supplementary information concerning their safety and potential hazard is necessary.

A few toxicological studies have suggested that the biological activity of nanoparticles may depend on their surface chemistry; in particular, transition metals may be released from the surface of the particles and contribute to the production of reactive oxygen species (ROS) via Fenton reaction. Thus, based on previous research,

ROS production is thought to be a primary cause of nanoparticle toxicity (Pulskamp *et al.*, 2007).

The Fenton reaction is one of the most powerful oxidizing reactions, which involves hydrogen peroxide and a ferrous iron catalyst (Eqn. 1). In this reaction, the peroxide is broken down into a hydroxide ion and a hydroxyl free radical (which is the primary oxidizing species) and can be used to oxidize and break apart organic molecules.

Eq. 1 $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^- + HO^*$ (Pulskamp *et al.*, 2007)

ROS is a collective term which is used by biologists to include oxygen radicals such as superoxide, hydroxyl, peroxyl and alkoxyl and certain non-radicals (Wiseman and Halliwell, 1996). These are either direct oxidizing agents or are easily converted into radicals such as singlet oxygen and hydrogen peroxides. Due to the presence of unpaired valence shell electrons, ROS are highly reactive. ROS can be formed by few mechanisms, including by interaction of ionizing radiation with biological molecules, or as a by-product of cellular respiration. ROS can react with almost all biological macromolecules including lipids, proteins, nucleic acids and carbohydrates. They can damage and interfere with the cell structures and function; most importantly the mitochondrial membranes which are directly exposed to the superoxide anions produced during cellular respiration (Wiseman and Halliwell, 1996). Therefore, at high levels, ROS can lead to impaired physiological function through cellular damage to DNA, proteins, lipids, and other macromolecules. Consequently, they can lead to certain

human conditions such as cancers, neurodegenerative disorders and cardiovascular disease.

A second common focus of nanoparticle toxicity is the release of toxic ions. Since nanoparticles have larger surface to volume ratio compared to their respective bulk compound; this may affect in thermodynamic properties of this materials by changing their melting point hundreds of degrees lower than the bulk compounds. The discharge of toxic ions may occur when the thermodynamic properties of a material favors particle dissolution in a suspending medium. Several studies suggest that small size and large surface area to volume ratio of nanoparticles which is contributed to produce ROS, as well as, ions arising from nanoparticles may play an important role in their toxicity (Berardis *et al.*, 2010).

Another commonly accepted hypothesis on the mechanism of nanoparticle and cell interactions is the disruption of well-structured electronic configurations in the shrinkage in size of the material (Nel *et al.*, 2006). Because decreasing the size of particular materials at nano scale may disrupt structure of electronic configurations by leaving them with an unpaired electron on their valance shell. Thus, the electrons captured on the surface of nanoparticles may cause the formation of a superoxide such as $O_2^{\cdot-}$, which in turn may lead to ROS accumulation and oxidative stress. The role of the nanoparticles surface in the cytotoxic effect and oxidative stress induction is still under debate (Nel *et al.*, 2006).

1.2 High throughput screening methods to study the biological properties of nanoparticles

1.2.1 Chemical microarrays

In recent years, finding drug candidates via screening large numbers of chemicals against the new targets, using low cost and efficient protocols has become one of the most challenging tasks. The new development of chemical compound microarrays may overcome these tasks and further advance the high throughput synthesis (Horiuchi and Ma, 2006). In addition, microarray technology may allow scientists to analyse millions of chemicals with multiple biological targets within a reasonable time. This new technology may be used for both homogenous and heterogeneous assays with any chemical compounds, including nano compounds. In this way, an interaction between a nano compound of interest and different molecular targets may provide information about the activity of that nano compound.

For example, a chemical compound microarray may incorporate a collection of organic compounds which are spotted in a small volume on a solid surface, such as glass or plastic. Then, the immobilized compounds will be exposed to a specific molecule, which could be a nano compound. Upon binding of an organic compound in the array and the target molecule, an interaction occurs which is followed by the detection of the interacting compound. In such approaches detection is often a rate limiting factor, although detection via a microarray scanner for antibody-conjugated nano compound is possible (Arruebo *et al.*, 2009).

There are three different forms of chemical compound microarrays. These three different forms are Small molecules microarrays, Microarrayed compound screening, and Solution phase chemical microarrays.

In small molecule microarrays (SMMs), organic compounds are covalently immobilized on the solid surface by diverse linking techniques. This original type of microarray technology was developed in the late 1990s, and was greatly advanced by different immobilisation techniques. The essential requirement for this form of microarray is the presence of specific reactive groups such as thiol group on both the library of the compound and the microarray surface. This method is principally used for probing partners or identifying targets (Figure 1). However, this method has some limitations such as, the length and flexibility of linkers, compound binding orientation, spatial hindrance, and microarray surface properties may also affect the target binding. Moreover, specific chemistry for coupling may be considered as one of the major disadvantages of this method (Dickopf *et al.,* 2004; Kuruvilla *et al.,* 2002; Horiuchi and Ma, 2006).

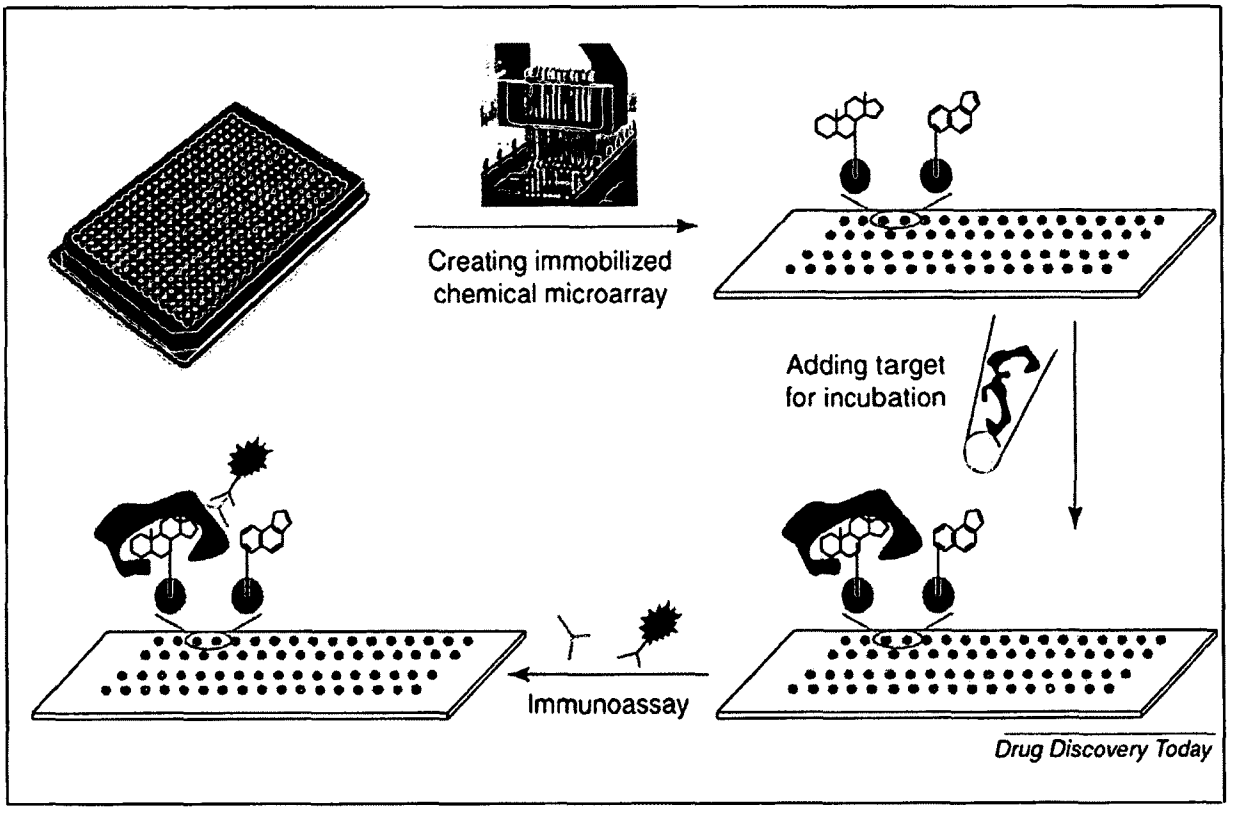


Figure 1. Small molecule microarray. Chemical compounds synthesized with the same linking functional group are arrayed and covalently immobilized on the surface of microarray chips using a standard microarrayer. Several washing steps are used to eliminate non-specific and weak binding (Figure is duplicated from Horiuchi and Ma, 2006 with permission).

To process any chemical library and avoid the limitation of immobilization, a new form of chemical microarray has been generated. Microarrayed compound screening (MicroARCS) or dry chemical microarray is another type of chemical compound microarray in which the arrays and dry organic molecules are dissolved in Dimethyl sulfoxide (DMSO) on polystyrene sheets. The advantage of this method is that ARCS can deposit any chemical compound library in a dry form onto a polystyrene sheet; then further biochemical assays can be performed in an aqueous environment. In this method, agarose gels containing biological targets are placed on top of the compound sheet. Then the compounds will be diffuse into the gel and may interact with the target. The biological reaction is then initiated by placing a second gel containing substrate on top of the target gel to allow for detection or target-compound interactions (Figure 2). ARCS have been used to test many targets such as enzymes and receptors. However, the rate of solubilisation and diffusion of different classes of dry compounds is considered as one of the major downfalls of this approach. It complicates the dynamic range of arrayed compounds versus bio-available compounds (David *et al.*, 2002; Horiuchi and Ma, 2006).

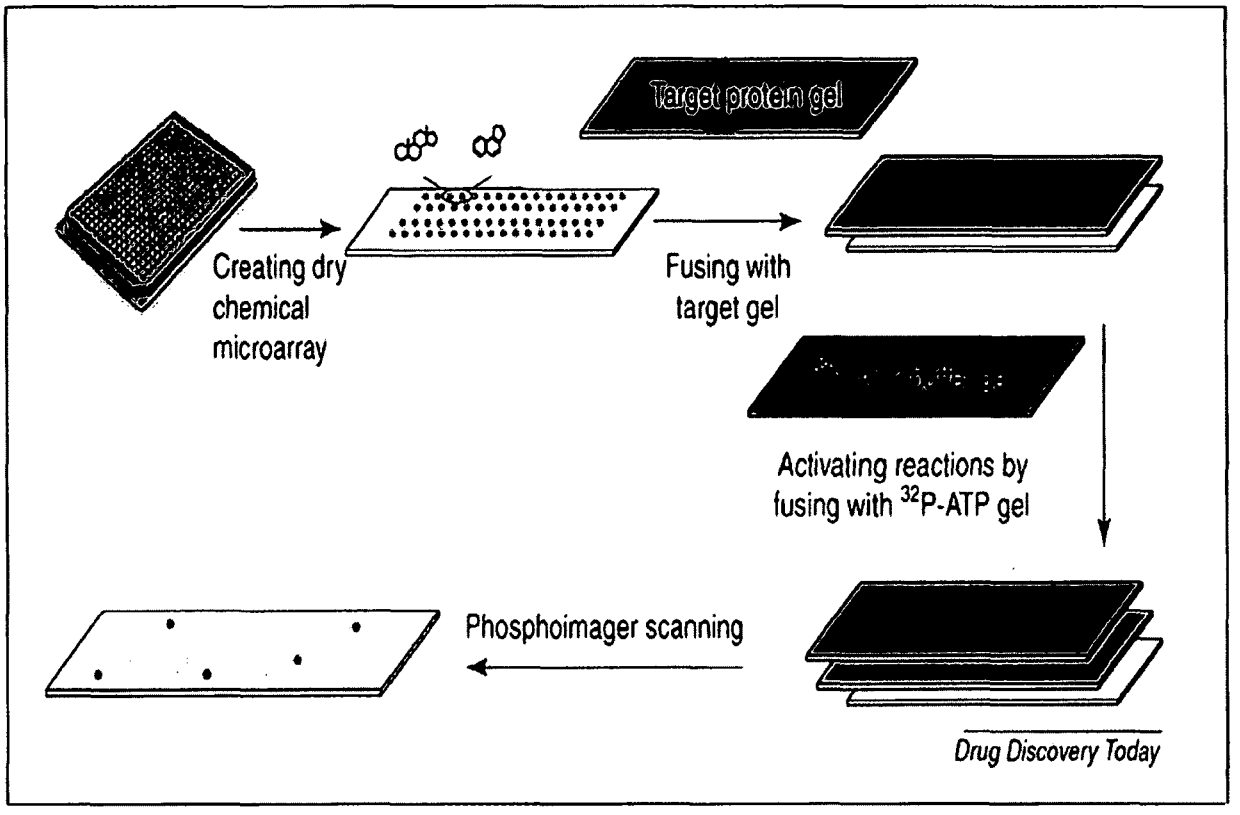


Figure 2. Dry chemical microarray. Chemical compounds are arrayed and dried on polystyrene sheets that have the same footprint as a 384-well plate. An agarose gel embedded with enzyme is applied to the surface of the array. After a short incubation, a second gel containing substrate is applied to initiate the biological reaction and the final reactions are detected using a standard phosphorimager. This method is used to detect target-compound interaction (e.g. enzyme and substrate) (Figure is duplicated from Horiuchi and Ma, 2006 with permission).

The solution-phase chemical microarray is another type of chemical microarray in which glycerol is used as an anti-evaporating reagent and mixed with chemical compounds to be arrayed on the microarray surface. To activate this solution-phase chemical microarray, an aerosol deposition technology is used in order to convert biological targets into a fine mist and cover the top of the whole microarray simultaneously. Reaction Biology corporation has licensed and developed the solution-phase chemical microarray technology into a commercial product tagged the "DiscoveryDot" chemical microarray drug screening platform (Ma *et al.,* 2005; Horiuchi and Ma, 2006). During the DiscoveryDot process, both the chemical compounds and targets are in solution; thus, all existing chemical libraries can be screened through this method (Figure 3). Because this solution-phase platform mimics the conventional large volume well-based system, the environment and mechanism of biochemical reactions on this microarray are very similar to the well-based reactions; however with a higher efficiency for profiling multiple bio-targets. Further improvements in area such as precision of the microarray, the development of multi-functional detectors, and multi task data analysis software are needed in order to take full advantage of this technology (Horiuchi and Ma, 2006).

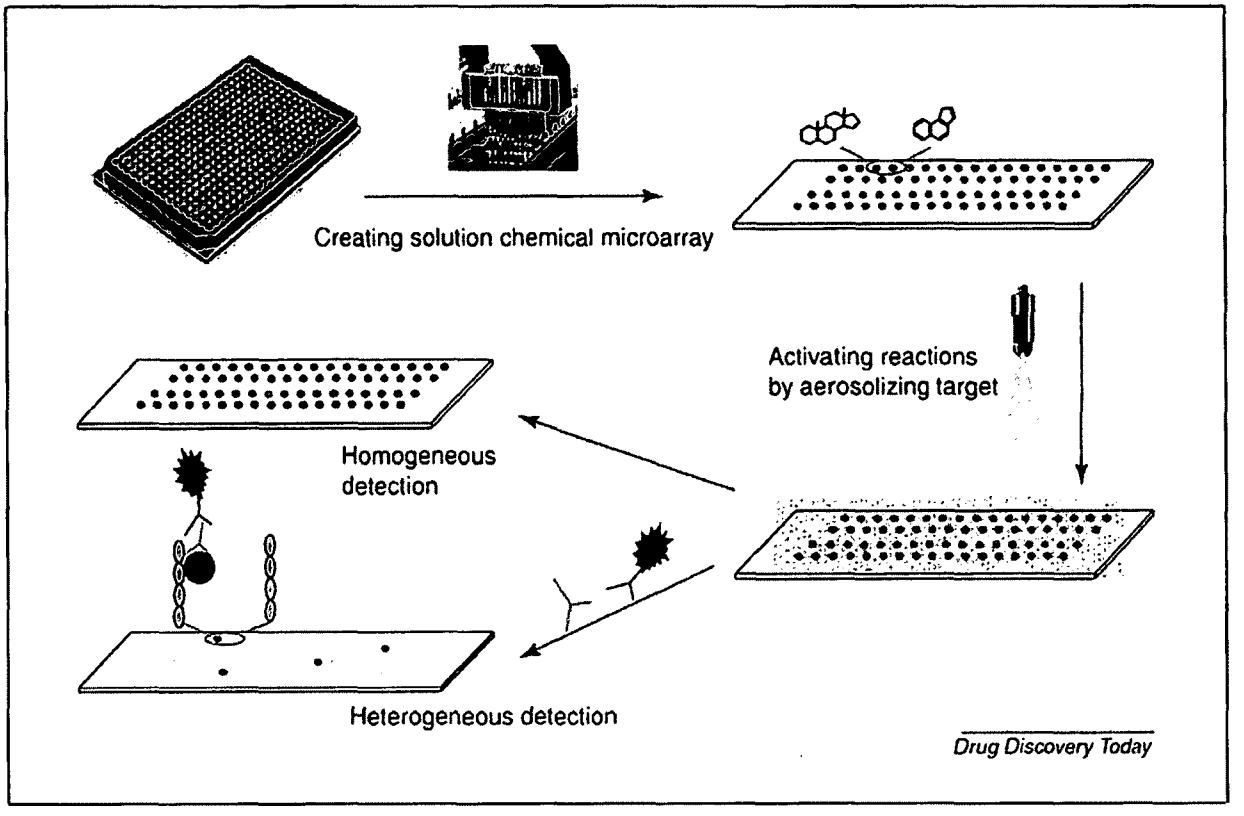


Figure 3. Solution-phase chemical microarray. Existing chemical compounds in assay buffer containing dimethyl sulfoxide (DMSO) and 10% glycerol are arrayed on the surface of the microarray. The compounds are always in solution without any chemical linking to the microarray. For homogeneous reactions, the biological target and substrate are added into each reaction dot by aerosol deposition technology, and, in the case of kinases, the reactions are initiated by spraying on ATP. The reaction products are detected by a laser scanner or imager. For heterogeneous ELISA-based reactions, such as kinase assays using whole proteins as substrates, the substrate is immobilized on the microarray surface first, before compounds are microarrayed. The kinase is then sprayed onto the array and the reactions initiated by spraying on ATP. The reactions are then detected by conventional ELISA protocols (Figure is duplicated from Horiuchi and Ma, 2006 with permission).

1.2.2 Gene expression microarrays

Gene expression microarray analysis refers to the study of correlations between the expressions of various genes in response to a specific condition. In this context, specific conditions include various chemical and physical environmental conditions, genotypic and phenotypic differences and physiological conditions. For example, it is expected that genes which are responsible to protect a cell under cold or freezing conditions (cold-shock genes) may be up-regulated when cells are challenged with cold and similarly down-regulated when the inducing condition (cold) is removed. Various bioactive compounds may also trigger the up regulation and down regulation of various genes within a cell. Such alterations in gene expression may also be used to study the activity of bioactive compounds (Kerr *et al.*, 2000; Schena, 1996).

In general, there are two types of gene expression array; in the first, the oligonucleotides are synthesised on the array using photolithographic techniques; in the second, the plasmids or polymerase chain reaction (PCR) products are printed robotically onto nitrocellulose, nylon, glass or any solid support. The cDNAs used in microarrays can be either fully sequenced clones, expressed sequence tags (ESTs), or chosen cDNAs from a library of interest. Gene expression microarray can be used to monitor the pattern of expression for different genes in many organisms including fungi and yeast (Schena, 1996). Prior to hybridization to the microarray, two ribonucleic acids (RNAs) isolated from two populations of cells, such as control and experimental treatment will be labelled by two fluorochromes, Cyanine (Cy) 5 and Cy3, respectively. After several washes, hybridization may be detected by phosphor-imaging to quantify the intensity of the two fluorochromes. Such data can then be analyzed and genes with

similar expression patterns will often cluster in similar groups. The clustering of genes with similar expression patterns may provide more information regarding the treatment condition that is investigated (Schena, 1996).

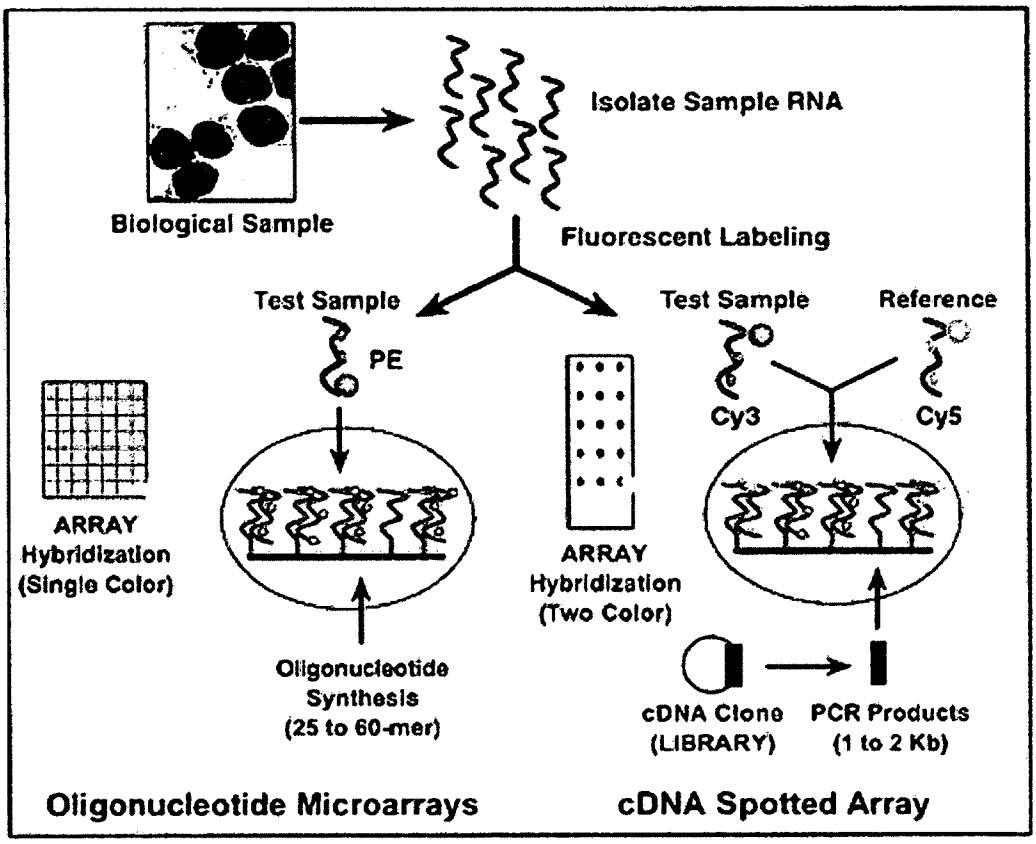


Figure 4. Oligonucleotide versus cDNA microarrays. Oligonucleotide microarrays: direct synthesis or deposition of oligonucleotides onto solid surface and single-color readout of gene expression from a test sample. cDNA microarrays: deposition of polymerase chain reaction products from cDNA libraries onto a solid surface (Figure is duplicated from Ramaswamy and Golub 2002 with permission).

Like all techniques, gene expression microarray analysis also has limitations. For example, hierarchical clustering of gene expression patterns has turned out to be a very challenging task (Clarke *et al.*, 2004). Also, powerful mathematical approaches may be required in order to discriminate subtle differences in gene expression. Thus, more advanced methods and software are compulsory for analysis of microarray results (Clarke *et al.*, 2004). Another major limitation with gene expression microarray analysis is the high rates of false positive and false negative signals that can occur with this technique. Finally, the abundance of mRNA (measured in gene expression microarrays) may not be a true representator of gene activity, where, for example, protein modification might be the regulatory step. Despite its limitations, microarray allow for the simultaneous studying of a large number of genes compared to other methods such as Northern and Southern Blotting (Nannini *et al.*, 2009).

In pharmacological studies, gene expression microarrays have been extensively used to study the effect of different compounds in large scale genome scale expression of genes. Such a profiling may enable the discovery of pharmacodynamics markers in response to a drug, and also may provide information regarding the molecular mechanism of action of drugs. The expression profiling used to study the molecular mode of action of drugs may be applied in clinical trials to help and predict which specific patient will most likely benefit from specific drugs. Therefore, the mode of action of drugs and quantitative information from gene expression studies play an important role in diagnosing diseases and facilitating drug development and also can create databases regarding biological processes and pathways (Clarke *et al.*, 2004). Methodological advancement obtained from such analysis provides scientist with an

excellent opportunity to study the mode of activity of bioactive compounds, such as nano compounds, that may not necessarily be used for pharmacological studies.

An example of the use of gene expression array analysis for investigation of the activity of a toxic compound comes from the study by Zhou et al (2002). The authors investigated the gene expression pattern of HCT116 colon carcinoma cell and the effect of its transcriptional response to two different concentrations of the DNA damaging agent, camptothecin (Zhou *et al.,* 2002). Zhou and his colleagues (2002) observed that a lower concentration of this drug may cause a reversible G2 arrest of the cell cycle, while a higher concentration may cause an irreversible arrest in the G2 phase of cell cycle (Zhou *et al.,* 2002). They observed that a group of genes known as DNA damage inducible genes and those that are associated with cell cycle arrest and apoptosis are up-regulated only at high concentrations of the drug. Also, they observed that some of the genes in this group are transcriptionally activated by the p53 DNA damage response pathway. Their results suggest that there is a different response to high and low DNA damage; such that, the mild DNA damage resulted in a reversible G2 arrest, while a high or extensive DNA damage resulted in a permanent G2 arrest (Zhou *et al.*, 2002).

Recently natural products and their derivatives have been increasingly mentioned and discussed due to their potential to act as alternatives to pharmaceuticals. To study their mode of activities, gene expression profiling has been utilized. For example, gene expression profiling was used in another study to determine the mode of action of ecteinascidin-743 (ET-743) which is measured as a DNA binding alkaloid natural product obtained from a marine tunicate (Friedman *et al.,* 2002). Their results from gene

expression profiling revealed that the ET-743 may inhibit the expression of some genes by acting on their promoters and thus reduce their transcription (Friedman *et al.*, 2002).

Taken together the gene expression profiling continues to serve as a solid approach to study mode of activity of bioactive compounds. A wealth of information generated from such studies may allow scientists to predict the mechanism of action of target compounds.

1.2.3 Chemical genetics approaches

In general, chemical genetic/genomics profiling uses gene deletion sets, prepared by knockdown or knockout techniques to generate a sensitivity profile for different gene knockouts against a target chemical. Then, the functions of genes that, when detected, result in high or low sensitivity. Also, this information may disclose what variety of pathways are disrupted by the target chemical (Sleno and Emili, 2008).

Due to the availability of genome-wide gene deletion sets for *Saccharomyces cerevisiae*, this yeast has traditionally been the most commonly used organism for chemical genetic profiling analysis. The chemical sensitivity profiling of yeast gene deletion arrays can be studied by three complementary high throughput approaches. In the first approach, deletion mutants can be grown in liquid cultures individually and their growth rates can be monitored and measured using spectrophotometry. The growth rate of mutant strains in the presence and absence of a compound may then be used to determine strain sensitivity (Engler *et al.*, 1999). A disadvantage of this technique is that it is generally time consuming and labour intensive. Warringer et al. (2003) used this

method in order to determine the sensitivity of strains in the yeast haploid deletion collection under saline conditions. They identified that around 500 gene deletion strains were sensitive to salt (Warringer *et al.*, 2003).

In the second approach, a pool of tagged deletion strains is grown in the presence and absence of the target compound. This approach is based on synthetic lethality deletion analysis on microarray. By using microarray methodology, the relative growth and sensitivity of each strain is measured relative to each other (Ooi *et al.*, 2003). In addition to the speed associated with this technique, a key advantage of tagged gene deletion library is that all yeast strains are in competition with each other and are present at the same time. Those that grow at a reasonable rate out-perform the growth of sensitive strains and eventually take over the mixed culture. Consequently, the growth dynamics of the culture can be investigated at different time intervals providing fast reliable information about the mixed culture (Parsons *et al.*, 2004; Ooi *et al.*, 2003).

The tagged microarray profiling approaches may also be used to investigate the interrelated cellular processes and interactions among complex pathways and genes. It can reveal how cells partition essential functions into multiple pathways. Ooi and his colleagues (2003) used genetic interaction analysis (combining two gene deletions and profiling growth fitness of double mutants) by microarray to determine and identify their redundant and novel biological functions of two 3′→5′ DNA helicase genes, SGS1 and SRS2 from yeast haploid deletion mutants array with molecular bar code(Ooi *et al.*, 2003). On the basis of the genetic interaction profiles for their target genes, they concluded that SRS2 may have a role in processing damaged replication forks, but SGA1 has a role in recombinant DNA (rDNA) replication, and lagging strand synthesis

(Ooi *et al.,* 2003). Moreover, their results suggested that these two genes, SGS1 and SRS2, may function with other gene, MRC1 in parallel pathways in order to transduce the DNA replication stress signal to general DNA damage checkpoint pathways (Ooi *et al.,* 2003).

In the third approach, colonies of yeast gene deletion mutant strains are arrayed on solid media in the presence and absence of the target compounds. In this case, the growth rates of individual colonies are determined by their colony size relative to controls (Parsons *et al.,* 2004). Parsons and his colleagues (2004) used drug hypersensitivity of the yeast *S. cerevisiae* gene deletion array to determine and identify the mechanism of action of twelve compounds (Parsons *et al.,* 2004). Their results show that some set of genes are involved in multiple chemical-genetic interaction profiles and therefore, they contribute to general multiple drug resistance. However, some of the genes generate compound specific profiles. Therefore, they concluded that such a chemical genetic profiling provides an opportunity to better understand and identify the mode and mechanism of action of different compounds.

Another study used a similar approach to identify deletion strains that were hypersensitive to iron. Colonies of yeast gene deletion strains were grown in both the presence and absence of iron (Kaplan *et al.,* 2004). Their results showed that some of the sensitive strains were deleted for genes either involved in the iron transport system or in the assembly of the iron transport system. However, some of the novel genes that were identified were involved in the metabolism of iron illustrating identification of multiple pathways using the method (Kaplan *et al.,* 2004).

In addition, other studies used colony size measurements to investigate the chemical genetic profile of five inhibitory compounds including cycloheximide, 3-amino-1,2,4-triazole, paromomycin, streptomycin and neomycin using the *S. cerevisiae* haploid gene deletion array. Their results indicate that these drugs may be involved in the process of protein biosynthesis (Alamgir *et al.,* 2010). Moreover, Galvan et al. (2008) used colony size reduction to also investigate antifungal and antioxidant activities of chimaphilin, a compound from the phytomedicine pipsissewa, using yeast haploid gene deletion array. Their observations and analyses from the cellular role of deleted genes in the most susceptible mutants reveal that chimaphilin may be engaged in cell wall biogenesis and transcription pathways (Galvan *et al.,* 2008). Moreover, gene expression profiling was used to infer the mode of action of *Echinacea,* which is considered as a traditional treatment for reducing the symptoms of colds and flu and also for treating fungal and bacterial infection (Mir-Rashed *et al.,* 2010). Their results from gene deletion array showed that *Echinacea* extracts may affect the fungal cell wall (Mir-Rashed *et al.,* 2010).

In this thesis, colony size measurement is considered as an established method to determine gene deletion sensitivity profile to target nano compounds. For this purpose, the relative colony sizes (average growth) on plates treated with sub-inhibitory concentrations of nano compounds of interest were compared with those grown under a control condition. Drug sensitivity spot test analysis is used to investigate the accuracy of our large scale screen.

1.3 Background and focus

Genes which are involved in similar pathways often genetically interact with each other. Thus, based on the hypothesis that many eukaryotic pathways are functionally redundant we can study functional associations by investigating the interactions they make with each other (Tong *et al.*, 2001). Due to functional redundancy of many pathways in eukaryotes deletion of a non-essential gene is often tolerated with little phenotypic consequence. However, inactivation of a second functionally related gene by inhibitory compounds can cause sickness or even lethality. Therefore sickness of double mutants or "synthetic lethality" has been used as a method to examine and reveal mode of activity of inhibitory compounds (Parsons *et al.*, 2004; Alamgir *et al.*, 2008; Galvan *et al.*, 2008). In this way the hypersensitive deletion strains for genes with known functions are used to form a genetic profile for the activity of the target compounds.

Because of its simple genetics, ease of manipulation, and evolutionary conserved pathways, the yeast *S. cerevisiae* has emerged as a model organism to study functional genomics. In the late 1990s, a collection of yeast non-essential gene deletion strains was completed by systematically deleting 4700 open reading frames selected as putative genes by initial bioinformatics analysis of the *S. cerevisiae* genome (Kelly *et al.*, 2001). To produce gene knockouts, homologous recombination using a PCR method was used. Plasmid containing NAT resistance marker genes was isolated. By knowing the regions of upstream and downstream of the gene of interest, a set of oligonucleotides are designed to amplify a fragment to replace the gene of interest (Winzeler *et al.*, 1999). Therefore, gene knockout collection carries a selectable marker in place of the mutated gene in "a" yeast mating type. In our laboratory, this haploid non-essential yeast gene deletion collection was prepared and arrayed on a single 384-spots format

generating 16 plates to cover the entire genome. The same collection has been used in numerous chemical genomics investigations (Parsons *et al.*, 2004; Alamgir *et al.*, 2008; Galvan *et al.*, 2008, Alamgir *et al.*, 2010). Each colony in this array represents a deletion mutant strain for a specific non-essential gene. Sensitivity is measured by the growth inhibition of colonies. Quantification of growth reduction is measured in an automated format using pixel counts of images obtained from colonies (Memarian *et al.*, 2007; Parsons *et al.*, 2004). Sensitivity of different gene deletions to an inhibitory compound is used to generate chemical genetics profile for each treatment. Genes that when deleted result in increased sensitivity to target compounds are clustered based on the cellular process in which they participate. The profiles obtained in this manner are used to predict a general mode of activity for the inhibitory effect of compounds in yeast. Focused follow up analysis on the basis of these profiles can provide further evidence for the mode of activity of target compounds (Galvan *et al.*, 2008).

1.4 Objective

Because of their chemistry and small size, nanoparticles are quickly distributed throughout the environment through their use in many industrial products. Due to growing concerns about the safety of nanoparticles, it is essential to better understand their effect on a living cell. Currently there is little information on the potential toxicity of metal oxide nanoparticles. Here, we investigated the effect of metal oxide nanoparticles using a yeast gene deletion array. We target zinc oxide, and silver nanoparticles. These compounds were selected due to their extensive use in industry. We propose to determine the chemical genetic profile of these compounds. Such profiles can be used to better understand the mode of activity of the examined nanoparticles. Differential inhibitory effects of different nanoparticles for different gene deletion mutants can provide important clues into the mechanism of toxicity of these compounds. In this study, colony size reduction will be used as the endpoint to screen and analyze the sensitivity of yeast gene deletion arrays to different nano compounds. First, we find the sub-inhibitory concentrations of each nano compounds. Then, we apply a large-scale chemical genetic profile analysis to identify yeast deletion strains that show increased sensitivity to our target nano compounds, zinc oxide and silver nanoparticles. Also, the drug sensitivity spot test analysis will be performed for each nano compound in order to confirm the sensitivity of selected mutants. Follow up analysis will confirm the predicted mode of activity of the target compounds.

Chapter 2

Chemical-Genetic Profile Analysis of ZnO and Ag nanoparticles in Yeast *Saccharomyces cerevisiae*

Abstract

Recent years have seen an exponential growth in the application of nanoparticles in different industries. Due to growing concerns about the safety of nanoparticles, it is essential to better investigate their effects on a living cell. Here, we investigate the genetic profile of the yeast non-essential gene deletion array (yGDA,~4700 strains) for increased sensitivity to zinc oxide and silver nanoparticles. Such profiles can be used to better understand the mode of action of the nanoparticles. Multifarious inhibitory effects of various nanoparticles for different gene deletion mutants can provide important clues with regards to these compounds mechanism of toxicity. Although the role of nanoparticle toxicity is still controversial, our results from chemical genetic analysis reveal that zinc oxide may exhibit its activity against *Saccharomyces cerevisiae* mainly by affecting the cytoplasmic membrane and cell wall formation/function. This was apparent from enrichments in membrane function and cell wall deficiencies among the mutant strains with increased sensitivity to zinc oxide nanoparticles. To confirm this observation, we performed two secondary assays to substantiate the disruption of cell wall and membrane in the presence and absence of zinc oxide nanoparticles. Also, our chemical genetic profile of silver nanoparticles indicates that cellular roles of most mutant strains susceptible to silver nanoparticles involve vesicular transport, cellular respiration, and DNA repair (mainly nucleotide excision repair) pathways.

2.1 Introduction

Nanoscience and nanotechnology is often considered the fastest growing sector of the high tech economy and is expected to turn into a trillion US dollar industry by 2015. Nanotechnology refers to the production and application of nanoparticles (NPs) which have novel properties that differ from their corresponding bulk compounds. This new technology offers new opportunities for the use of NPs in both industry and health sectors (Schwegmann *et al.,* 2010). Nanoparticles have been used widely in many practices, such as medicine, pharmacy, industry and cosmetics. For instance, titanium dioxide and zinc oxide are used in sunscreens for their UV blocking properties. Zinc oxide is also used in toothpaste and other beauty products such as baby cream to reduce diaper rash and skin irritations from burns. Moreover, silver nanoparticles have been used in various applications such as in medicine as an antimicrobial agent, in dental materials, in industries in textile fabrics, and for water treatment (Rai *et al.,* 2009). Because of their novel properties, these engineered nanoparticles may exhibit enhanced toxicity compared to their respective bulk compounds (Kasemets *et al.,* 2009). Therefore, there is an urgent need to acquire more information regarding the toxicity and safety of nanoparticles.

The budding yeast, *S. cerevisiae* with a short generation time, inexpensive cultivation, fully sequenced genome, and 30% homology to human genome has been used as a unicellular eukaryotic model organism in many genomic and drug discovery studies. In this study, we use *S. cerevisiae* gene deletion array as a model platform for a large-scale functional genomics investigation of the toxic effects of zinc oxide and silver nanoparticles (Alamgir *et al.,* 2010).

In theory, the presence of redundant pathways may compensate for genetic inactivation of a single pathway, with no phenotypic changes. Therefore, increased sensitivity of gene deletion mutant strains to inhibitory compounds has been used as an approach to study gene functions and identify pathways that buffer the cell against the activity of these compounds. The inactivity of a second functionally overlapping pathway, one that is perturbed by a nano compound, may cause a "double hit" effect. This may result in phenotypic changes and provoke reduction in the rate of growth or bring about a sick/sensitive phenotype (Alamgir *et al.,* 2010).

In general, chemical sensitivity profiling of yeast gene deletion arrays can be studied by three complementary high throughput approaches: 1) in the first approach, deletion mutants can be grown in liquid cultures individually and their growth rates can be monitored and measured using spectrophotometry. The growth rate of mutant strains in the presence and absence of a nano compound may then be used to determine strain sensitivity (Engler *et al.,* 1999). 2) In the second approach, a pool of tagged deletion strains is grown in the presence and absence of the target nano compound. Using microarray methodology, the relative growth and sensitivity of each strain is measured comparatively (Ooi *et al.,* 2003). 3) In the third approach, colonies of yeast gene deletion mutant strains are arrayed on solid media in the presence and absence of the target nano compounds. In this case, the growth rates of individual colonies are determined by their colony size relative to controls (Parsons *et al.,* 2004).

Due to increased interest in nano compounds as potential agents in medicine and industry, here we focus on studying the toxicity of two commonly used nano compounds, zinc oxide and silver. For this purpose yeast genetic profile analysis is utilized. Colony size measurement is used as an established method to determine chemical sensitivity in a high

throughput format followed by sensitivity confirmation using small scale spot test analysis (Memarian *et al.,* 2007; Parsons *et al.,* 2004). Sensitivity profiles obtained from zinc oxide and silver nanoparticles are used to elucidate the mode of activity of these nano compounds.

2.2 Results

2.2.1 Characterization of nanoparticles

Nano compounds may change their size through aggregation when placed under different experimental condition (Fadeel and Bennett, 2010). Consequently we characterized the size of our target nano compounds under the experimental conditions specific to this study. Transmission electron microscopy (TEM) showed that the silver nanoparticles exist in a sphere shape and hold a size range of 6-9 nm (Figure 5). Furthermore, the hydrodynamic diameter of the silver nanoparticles was measured using Dynamic Light Scattering (DLS), and was found to be 9 nm (Figure 6). The hydrodynamic diameter of the sonicated zinc oxide nanoparticles was estimated at 278 nm (Figure 7) suggesting some aggregation. We purchased nano scale zinc metal oxide with a size between 50 to70 nm from Sigma-Aldrich. However, when we characterized zinc oxide in solution, we found that its size is 278 nm. Therefore, we suggested that the large size of nano-like zinc oxide particles is due to their tendency to aggregate in a solution (Fadeel and Bennett, 2010).

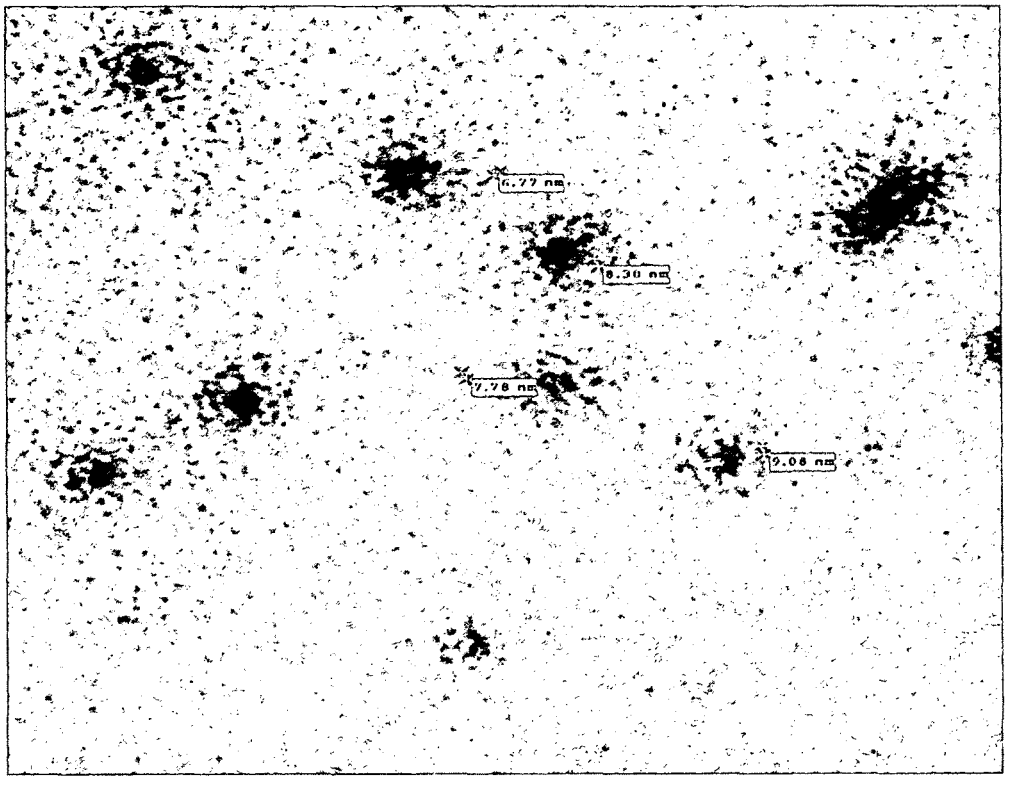


Figure 5: Transmission electron microscopy (TEM) of silver nanoparticles. The image shows the morphology and size of silver nanoparticles stock solution.

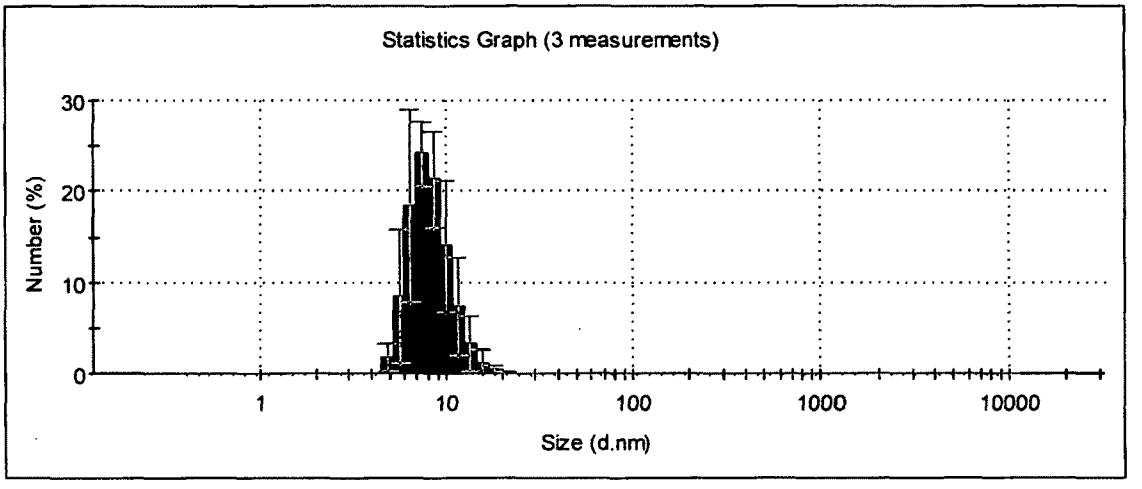


Figure 6: Dynamic light scattering (DLS) evaluation of silver nanoparticles stock solution, 10X dilution in YPD. The values are expressed as mean ± standard deviation of triplicates. It shows that the average size of silver nanoparticles is 9 nm.

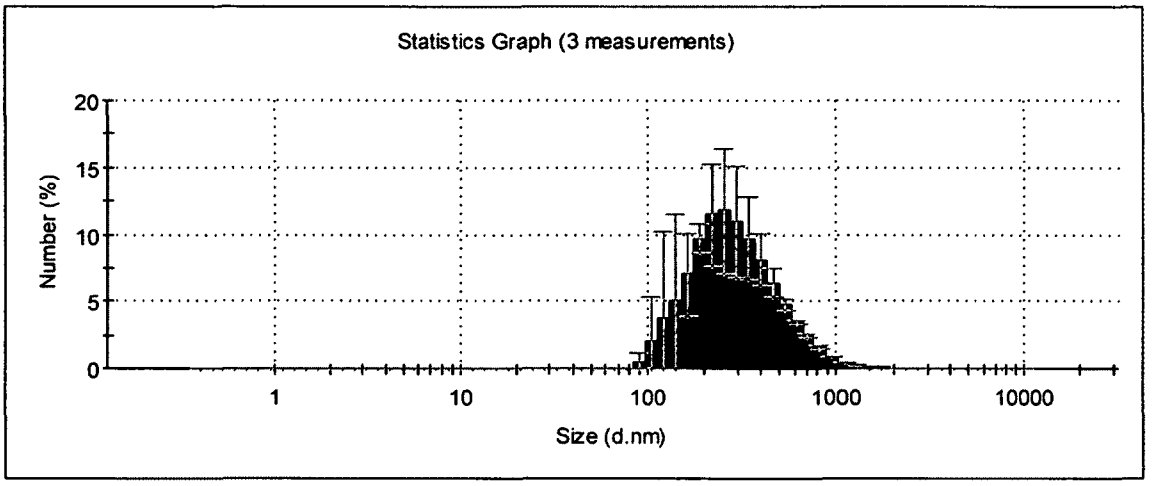


Figure 7: Dynamic light scattering (DLS) evaluation of zinc oxide nanoparticles (10X) in suspending Medium (ethanol). The values are expressed as mean ± standard deviation of triplicates. It shows that the average size of zinc oxide nanoparticles is about 278 nm. The large size of nanoparticles may be due to their tendency to agglomerate in this medium.

2.2.2 High throughput phenotypic screening (GDA analysis)

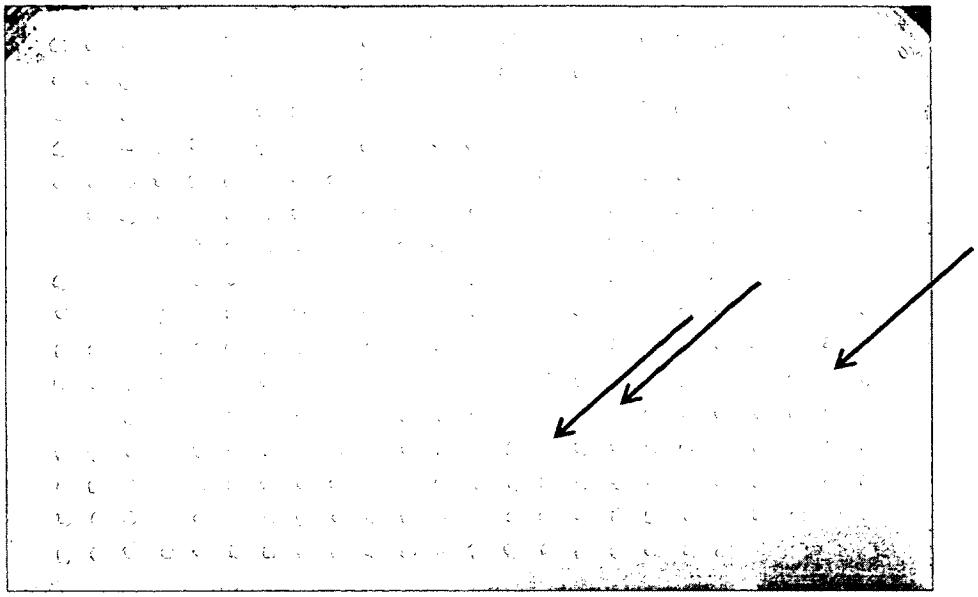
We determined that the minimum inhibitory concentrations (MIC_{100}) for both zinc oxide and silver nanoparticles were 1.5 milligram per millilitre (mg/ml) and 0.12 mg/ml, respectively. MIC_{100} was defined as the lowest concentration that resulted in complete inhibition of visible growth of *S. cerevisiae* strain S288C using a broth microdilution assay (CLSI, 2008).

The entire collection of the haploid yeast gene deletion array (~4700) was screened for increased sensitivity to sub-inhibitory concentration of zinc oxide (1mg/ml) and silver (0.095 mg/ml) nanoparticles. Examples of hypersensitive deletion mutants to zinc oxide and silver nanoparticle are shown in table 1 and 2. Colony size measurement is used in determining the sensitivity of each strain to zinc oxide and silver nanoparticles. The relative colony growth (the average growth on the plate) on the treated plates with sub-inhibitory concentrations of zinc oxide and silver nanoparticles are compared to control plates without any nano compound.

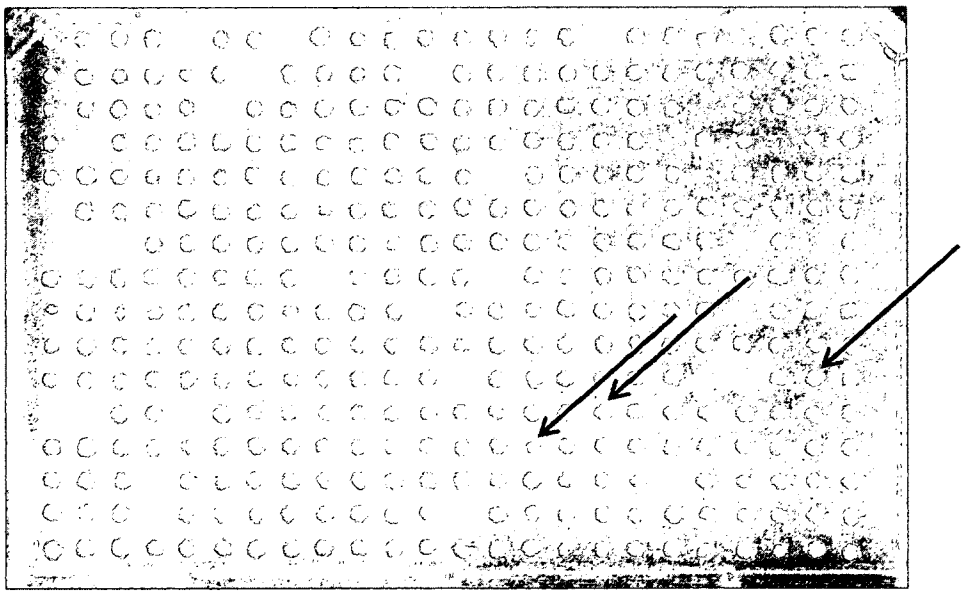
Figure 8 shows one of the yeast haploid gene deletion plates in the presence and absence of sub-inhibitory concentrations of zinc oxide nanoparticles. Determination of suitable sub-inhibitory concentration was done empirically using a plate coated with 384 mutants. Various concentrations (0.1–1.2 mg/ml) of zinc oxide were used. Ideally we targeted a concentration by which approximately 5-10% of the strains showed increased sensitivity. Concentrations less than 1mg/ml did not result in any noteworthy shifts while the higher concentrations prompted a systematic growth reduction. Therefore, we have determined the sub-inhibitory concentration of 1 mg/ml for zinc

oxide nanoparticles would be suitable for our large scale screen (Figure 8a). We compared the growth rates of individual colonies in the presence of zinc oxide nanoparticles to growth rates of individual colonies in controls with no nano compound (Figure 8b).

For silver nanoparticles, different concentrations from 0.003 mg/ml to 0.1 mg/ml were tested; we determined a suitable sub-inhibitory concentration for our large scale screening of silver nanoparticles to be 0.095 mg/ml (Figure 9a). Less than this concentration did not provoke any striking reduction; concentrations higher than 0.095 mg/ml evoked a systematic reduction. As above, the growth rate of individual colonies was estimated based on colony size relative to the controls (Figure 9b).

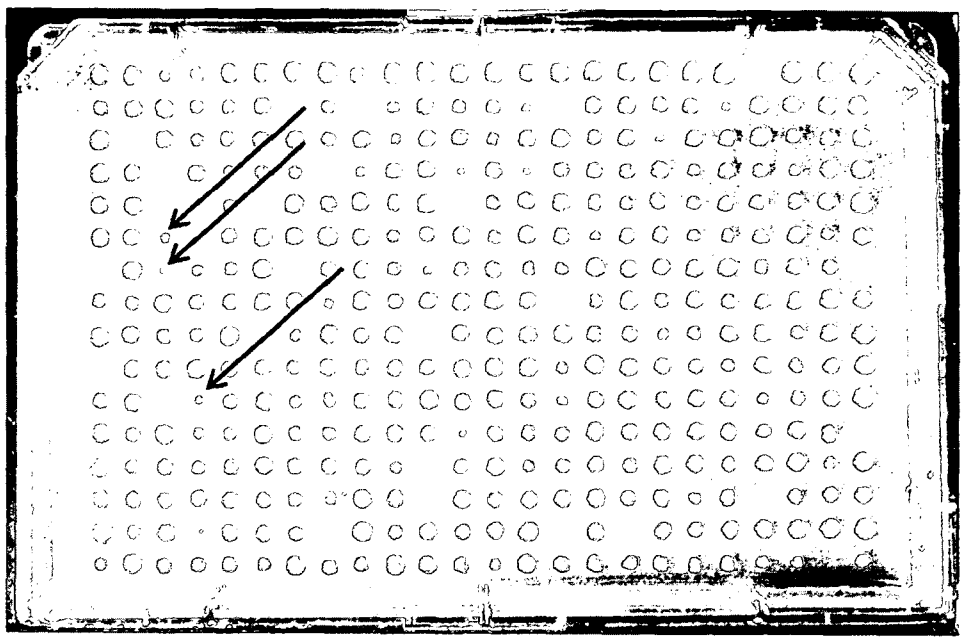


(a) 1mg/ml of zinc oxide nanoparticles

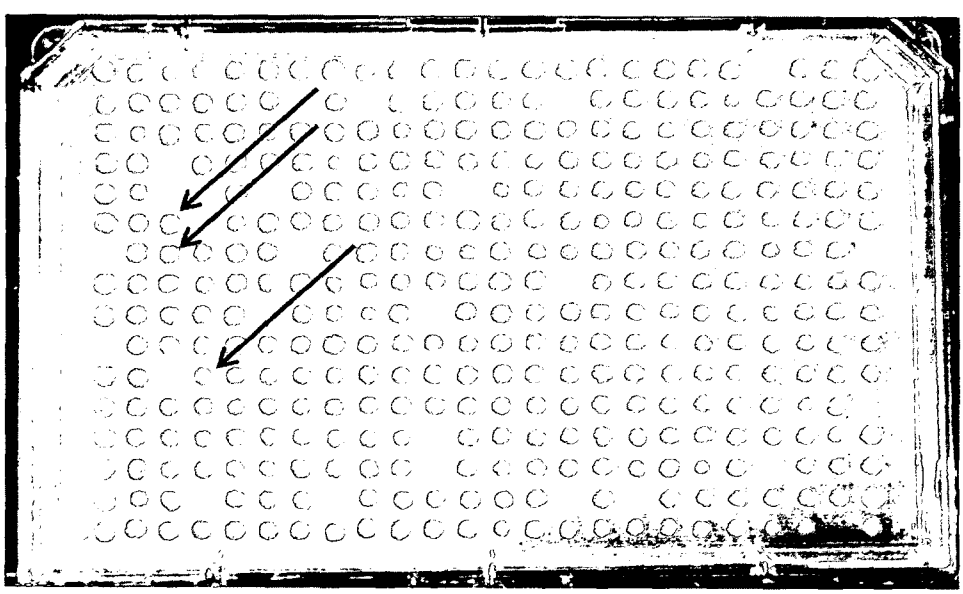


(b) Control (absence of zinc oxide nanoparticles)

Figure 8: An example of yeast gene deletion array when exposed to zinc oxide nanoparticles. (a) Yeast haploid deletion in the presence of zinc oxide nanoparticles. (b) Yeast haploid deletion in the absence of zinc oxide nanoparticles. The arrows show examples of colony size reduction in the presence of zinc oxide nanoparticles.



(a) 0.095 mg/ml of silver nanoparticles



(b) Control (absence of silver nanoparticles)

Figure 9: An example of yeast gene deletion array when exposed to silver nanoparticles. (a) Yeast haploid deletion in the presence of silver nanoparticles. (b) Yeast haploid deletion in the absence of silver nanoparticles. The arrows show examples of colony size reduction in the presence of silver nanoparticles.

The deleted genes in the super-sensitive strains were clustered separately for both zinc oxide and silver nanoparticles based on the cellular processes in which they participate (Figures 10 and 11). The profile obtained for zinc oxide nanoparticles reveal an interesting set of genes with diverse cellular functions falling into four main categories; approximately 34% of the strains sensitive to zinc oxide nanoparticles are involved in cell wall formation and function (P-value: $9.11x10^{-3}$), and 25% are associated with ion homeostasis. This is followed by approximately 21% and 20% that have either unknown or other biological functions, respectively (Figure 10)

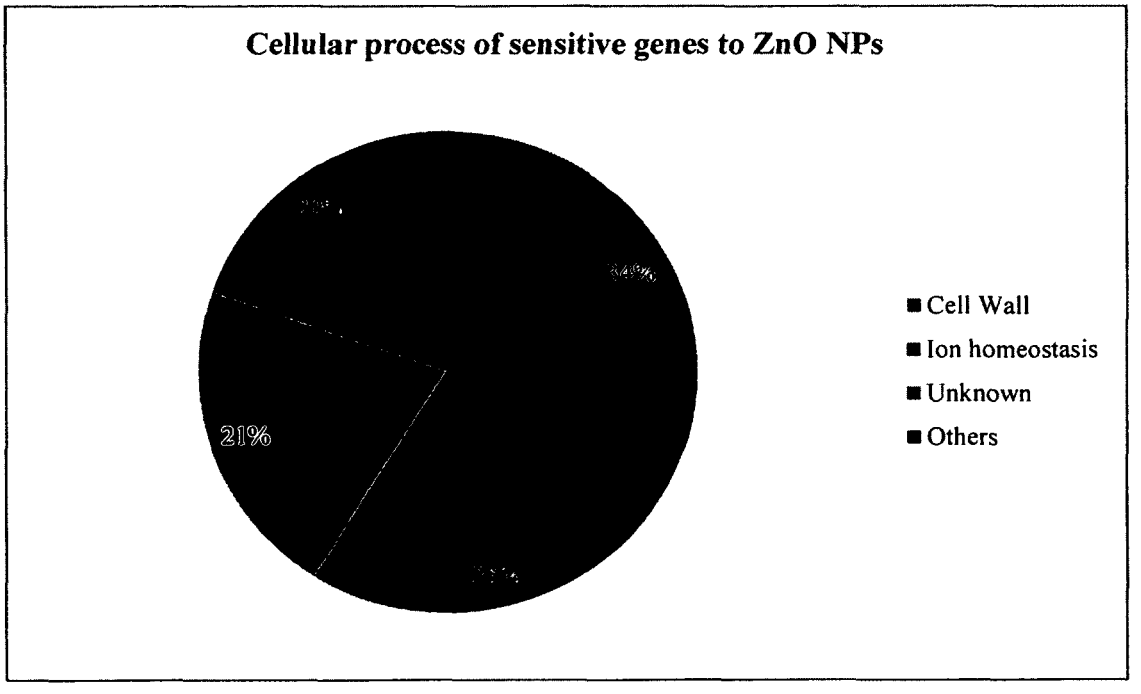


Figure 10: Clustering of the sensitive gene deletion mutants to zinc oxide nanoparticles. The zinc oxide sensitive gene deletion mutants were clustered based on their cellular process. The biggest cluster contains deletion strains for genes involved cell wall synthesis and function.

Similarly, a silver nanoparticle profile was constructed. The profile obtained for silver nanoparticles revealed at least five main categories of cellular functions. Approximately 27% of these sensitive strains are involved in vesicular transport (P-value: $5.09x10^{-3}$). Moreover, 21%, and 19% play an active role in cellular respiration and nucleotide-excision repair, respectively, and the remaining 17% and 16% of these sensitive genes have either unknown or other biological functions, respectively (Figure 11).

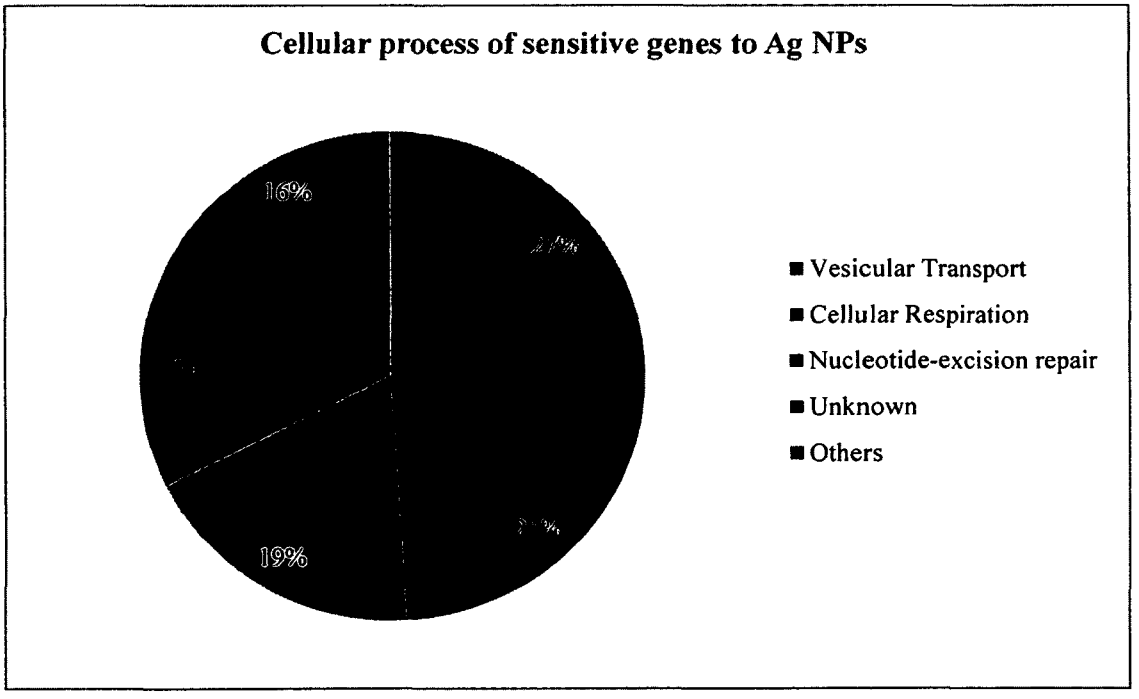
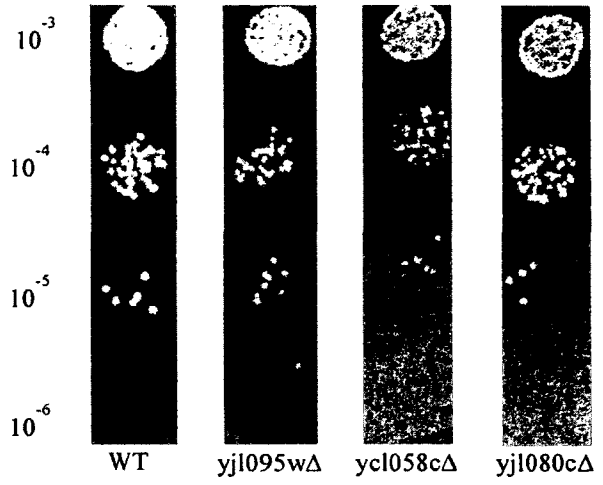


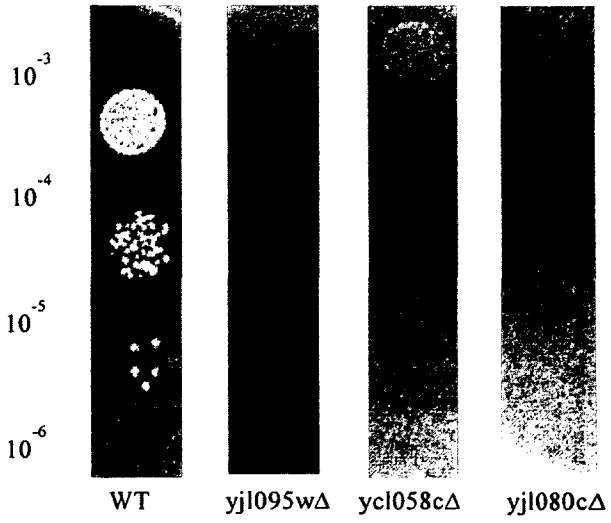
Figure 11: Clustering of the sensitive gene deletion mutants to silver nanoparticles. The silver nanoparticles sensitive gene deletion mutants were clustered based on their cellular process. The biggest cluster is for those involved in transport vesicle's form and function.

2.2.3 Drug sensitivity spot test

Drug sensitivity spot test was used in order to confirm the sensitivities of selected mutant strains, which were identified in the primary large scale screens. For this purpose, deletion strains that were super-sensitive to nanoparticles based on the GDA assays, were selected and subjected to spot test analysis in both the presence and absence of sub-inhibitory concentrations of nanoparticles (Figures 12 and 13). Figure 12a and b show examples of mutant candidates which were sensitive to zinc oxide nanoparticles based on reduction in their growth rates compared to controls (Figure 12b). Similarly, the spot tests of those mutant strains from GDA screening confirm their sensitivity to silver nanoparticles (Figure 13a and b). Control strains are used to show that the observed sensitivity is specific to super-sensitive mutants (Figures 12 and 13). Different growth rates and numbers of colonies relative to the control categories are used to determine the sensitivity of each strain. The results of our spot test analysis confirmed that the selected mutants have increased sensitivity to the target compounds.

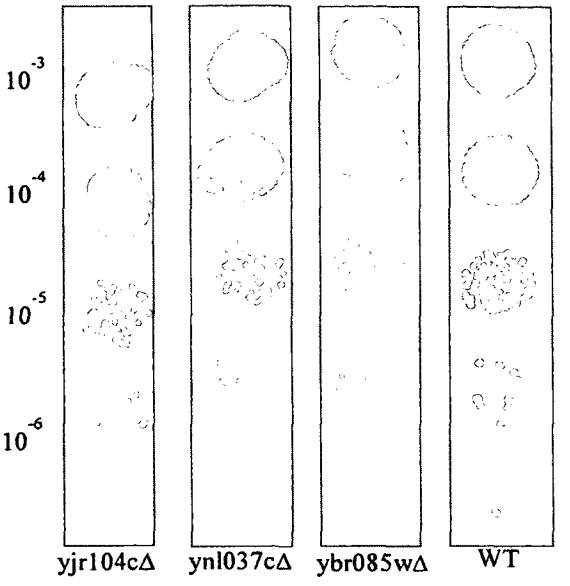


(a) Control

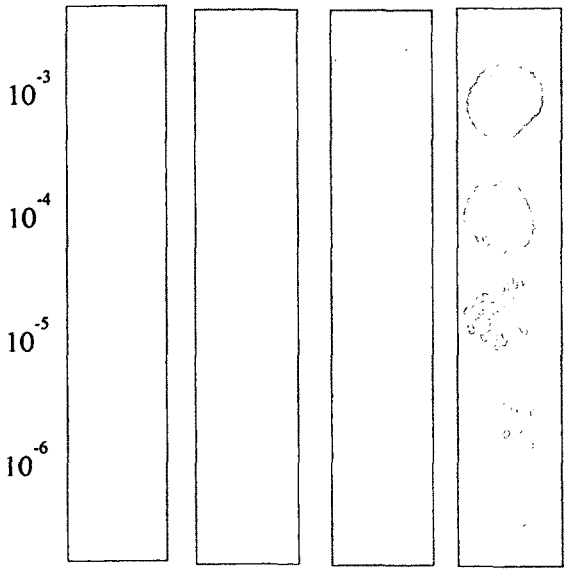


(b) 1 mg/ml of zinc oxide nanoparticles

Figure 12: Spot Test analysis of zinc oxide nanoparticles. Wild type (WT) or gene deletion mutant strains (yjl095wΔ, ycl058cΔ, and yjl080cΔ) were serially diluted, 10^{-3} to 10^{-6} (CFU/ml), and then spotted on solid medium with sub-inhibitory concentration (1 mg/ml) of zinc oxide nanoparticles; and also without any zinc oxide (control). The plates were incubated at 30°C for 1-2 days. a) Control; dilution series of mutant yeast cells were spotted on medium without zinc oxide nanoparticles. b) Dilution series of mutant yeast cells were spotted on medium with 1mg/ml of zinc oxide nanoparticles. Deletion mutant strains confer increased sensitivity to zinc oxide nanoparticles.



(a) Control

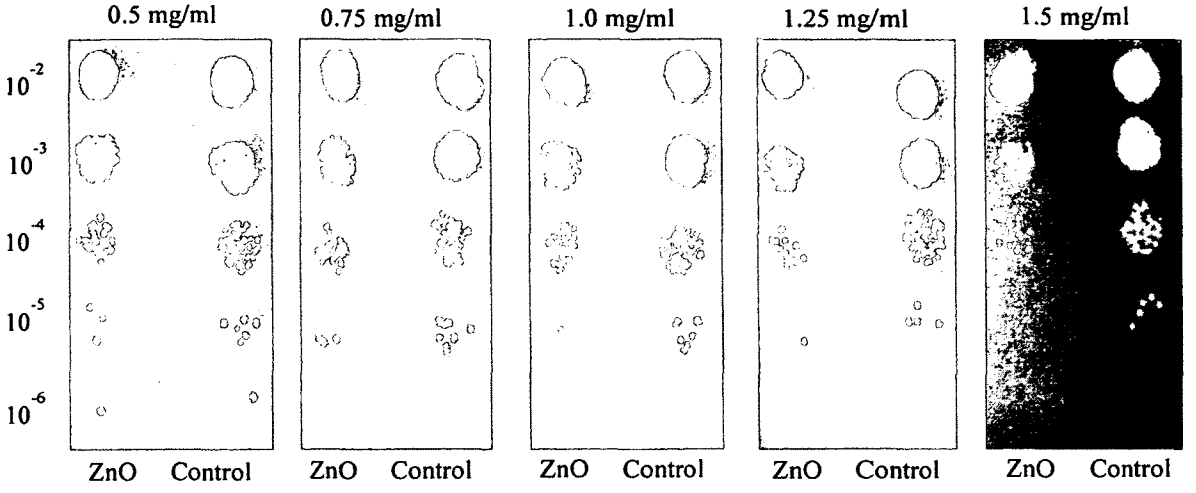


(b) 0.095mg/ml of silver nanoparticles

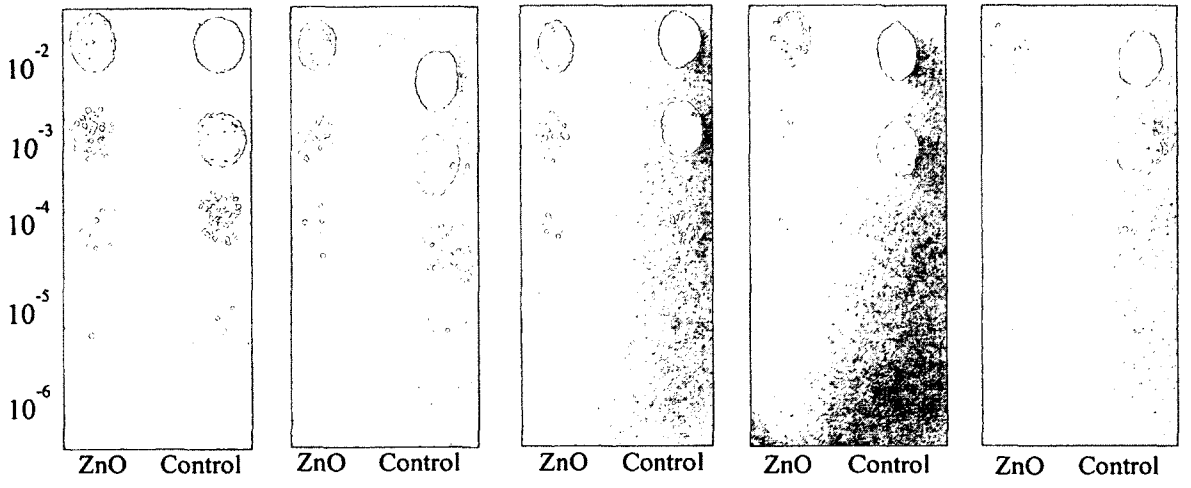
Figure 13: Spot Test analysis of silver nanoparticles. Wild type (WT) or gene deletion mutant strains (yjr104cΔ, ynl037cΔ, and ybr085wΔ) were serially diluted from 10^{-3} to 10^{-6} (CFU/ml), and then spotted on solid medium with sub-inhibitory concentration (0.095 mg/ml) of silver nanoparticles; and also without any silver (control). The plates were incubated at 30°C for 1-2 days. a) Control; dilution series of mutant yeast cells were spotted on medium without silver nanoparticles. b) Dilution series of mutant yeast cells were spotted on medium with 0.095 mg/ml of silver nanoparticles. Deletion mutant strains confer increased sensitivity to silver nanoparticles.

2.2.4 Cell wall integrity assay using sonication

The cell wall integrity assay was applied to test the effect of zinc oxide nanoparticles on yeast cell wall. For this purpose, different concentrations of zinc oxide nanoparticles, below and above the sub-inhibitory concentration (1 mg/ml) such as, 0.5 mg/ml, 0.75 mg/ml, 1mg/ml, 1.25 mg/ml, and 1.5 mg/ml and the corresponding controls with no zinc oxide were used. Sonication was used as a physical stress to disrupt the cell wall and test the effect of zinc oxide nanoparticles on yeast cell wall. It is expected that yeast cells that are treated with a cell wall inhibitory compound are more sensitive to physical disruption of the cell wall (sonication). Following sonication, drop plate assays using 10 fold dilution series (10^{-2}-10^{-6}) were used in order to estimate cell viability for each treatment (Figure 14b). As well, the same procedure was undertaken without sonication, in order to compare the effect of zinc oxide nanoparticles on yeast cell wall when the cell wall is not mildly disrupted by physical stress (Figure 14a). Each experiment was repeated three times and the average colony forming unit per millilitre (CFU/ml) for each concentration was used to show the frequencies of live cells in both the absence and presence of the target nanoparticle.



(a) Without sonication



(b) With sonication

Figure 14: Cell wall integrity assay using sonication. Yeast strain S228C was grown overnight in YPD medium to mid-log phase and adjusted to approximately 10^4cells/ml. Two ml of the culture were transferred into each of 10 disposable culture tubes to obtain five tubes of cultures with concentrations of zinc oxide nanoparticles of 0.5mg/ml, 0.75mg/ml, 1mg/ml, 1.25mg/ml and 1.5mg/ml, and five tubes of cultures with equivalent volume of carrier solvent (ethanol), without any nanoparticles used as controls. Then, culture tubes were incubated overnight in gentle shaking at 30°C. The optical density was adjusted for all 10 tubes to 10^7 cells/ml. Sonication was used as a treatment to disrupt the cell wall. Following the sonication, drop plate assays using 10 fold dilution series (10^{-2} to 10^{-6}) was used in order to estimate cell viability for each concentration. Also, same procedure was used without cell disruption, with no sonication, in order to compare the effect of zinc oxide nanoparticles on yeast cell wall. (a) Drop plate assays using 10 fold dilution series (10^{-2} to 10^{-6}) in the absence of sonication. (b) Drop plate assays using 10 fold dilution series (10^{-2} to 10^{-6}) in the presence of sonication.

Based on our results from the primary screening, zinc oxide nanoparticles may interfere with yeast cell wall formation/function. This was apparent in that the largest number of mutant strains with increased sensitivity to zinc oxide nanoparticles had deletions in genes involved in cell wall functions. To confirm this observation we used wild type yeast and sonication as a means of physical stress to test whether or not zinc oxide nanoparticles have the ability to interfere with cell wall formation and function. Different concentrations above and below the sub-inhibitory concentration of zinc oxide nanoparticles were tested (Figure 14). Also, the same experiment was used without any physical stress. As we described, following the sonication, drop plate assays using 10 fold dilution series (10^{-2} to 10^{-6}) was used in order to estimate cell viability for each concentration.

We also used the number of colony forming units (CFU) per millilitre (ml) of 10^{-4} dilution of S288C yeast in the absent of zinc oxide nanoparticles (control) and in the presence of zinc oxide nanoparticles at various concentrations ranging from 0.5 mg/ml to1.50 mg/ml with and without physical stress. The graphs illustrate the average number of CFU/ml of 10^{-4} dilution in both the presence and absence of zinc oxide nanoparticles and its controls, with and without sonication (Figures 15 and 17). The number of CFU/ml in the presence of both zinc oxide nanoparticles and sonication was decreased compared to the number of CFU/ml in the presence of only zinc oxide nanoparticles and the absence of sonication (Figures 15 and 17). Moreover, these results illustrate that as the concentration of zinc oxide nanoparticles increases in the presence of sonication, the number of CFU/ml declines. This was observed through comparing the number of CFU/ml of the lowest concentration of zinc oxide nanoparticles (0.5 mg/ml) to the highest concentration of zinc

oxide nanoparticles (1.50 mg/ml) in the presence of both zinc oxide nanoparticles and sonication (Figures 15 and 17). As seen in Figures 16 and 17, the addition of sub-inhibitory concentrations (1.25-1.5 mg/ml) of zinc oxide to the yeast cell cultures resulted in a reduction of colony forming units per milliliter (CFU/ml) in a dose dependent manner. For example, noticeably reduction in colony forming unit was observed when zinc oxide was in sub-inhibitory concentration (1 mg/ml) compared to the untreated control (Figure 16b). Also, normalized ratio of ZnO nanoparticles with sonication over ZnO nanoparticles without sonication at various concentrations compared to their respective controls with/without sonication was shown in Figure 18.

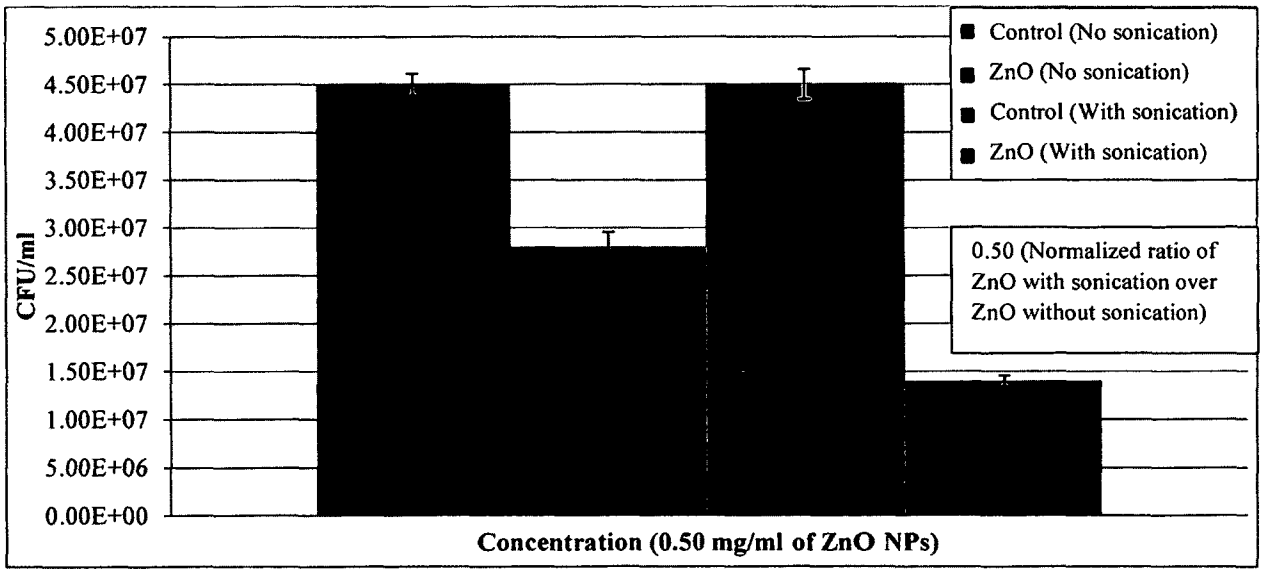
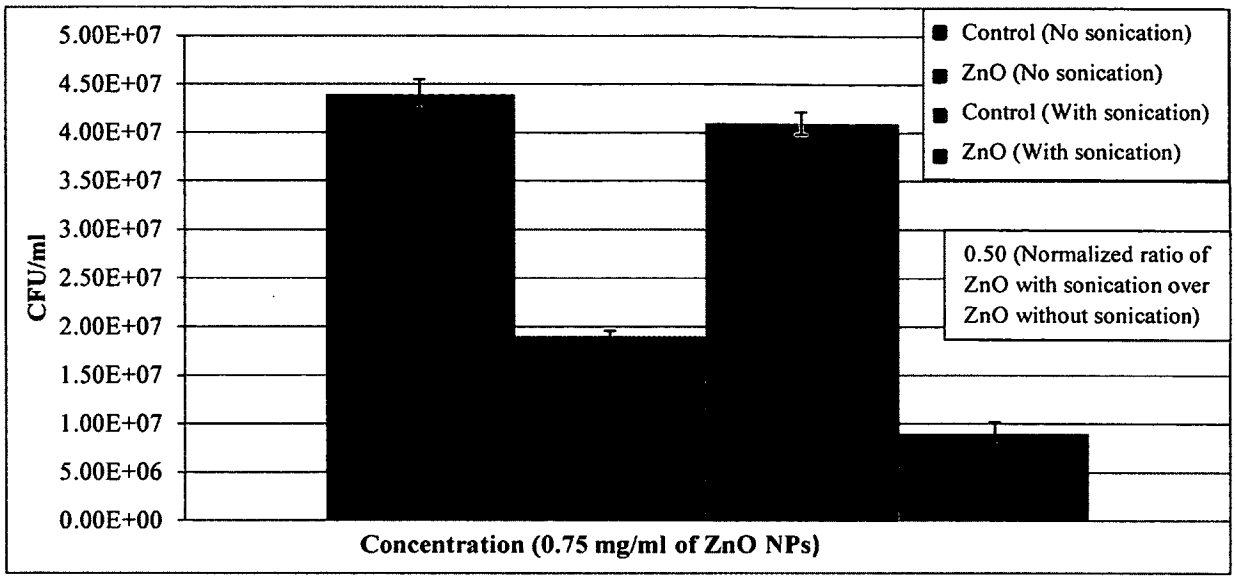
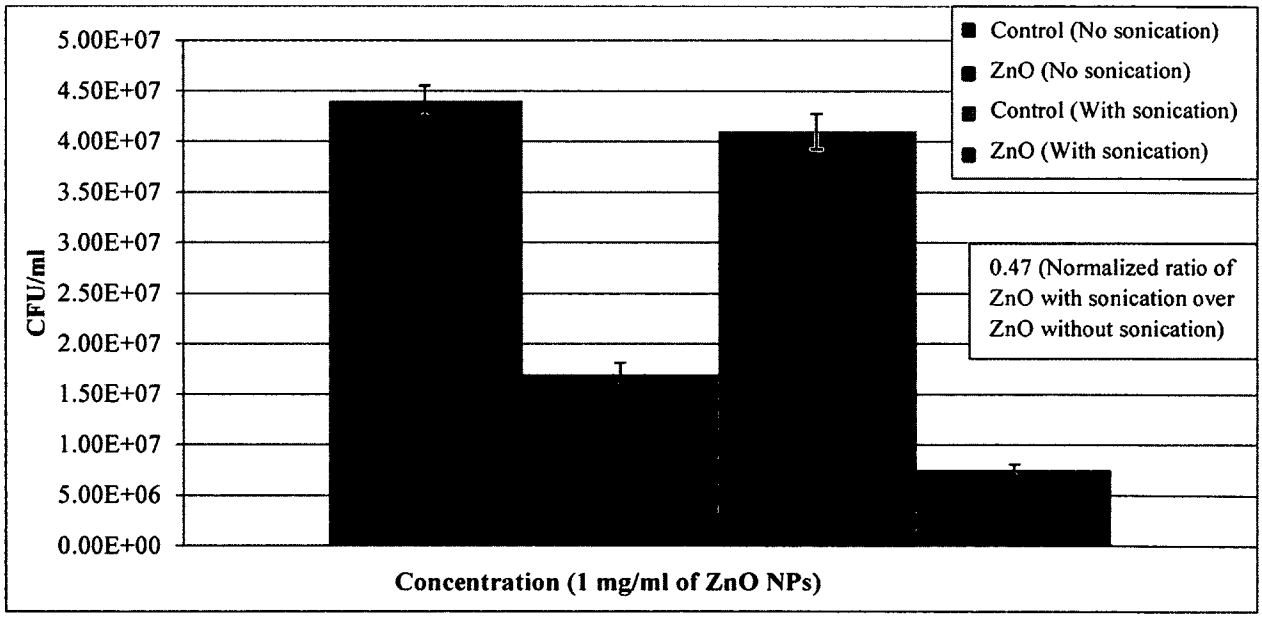


Figure 15: CFU/ml of S288C when exposed to 0.5 mg/ml of ZnO NPs. The number of colony forming units (CFU) per millilitre (ml) of 10^{-4} dilution factor of S288C yeast in the absent of zinc oxide nanoparticles (control) and in the presence of zinc oxide nanoparticles at the concentration of 0.5 mg/ml with and without sonication.



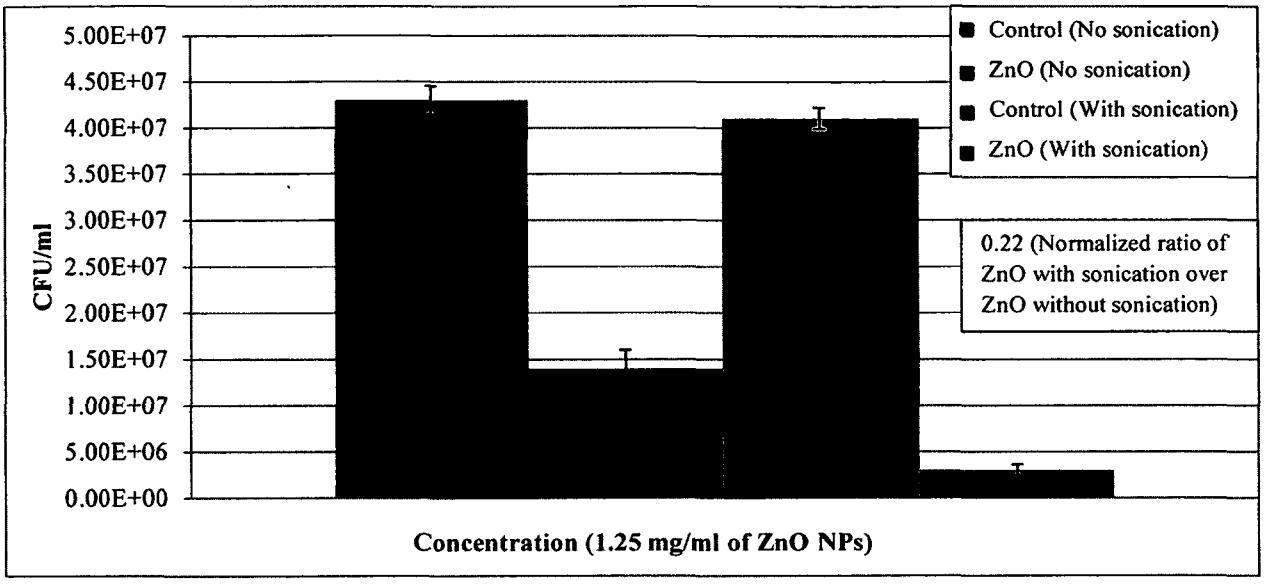
(a)



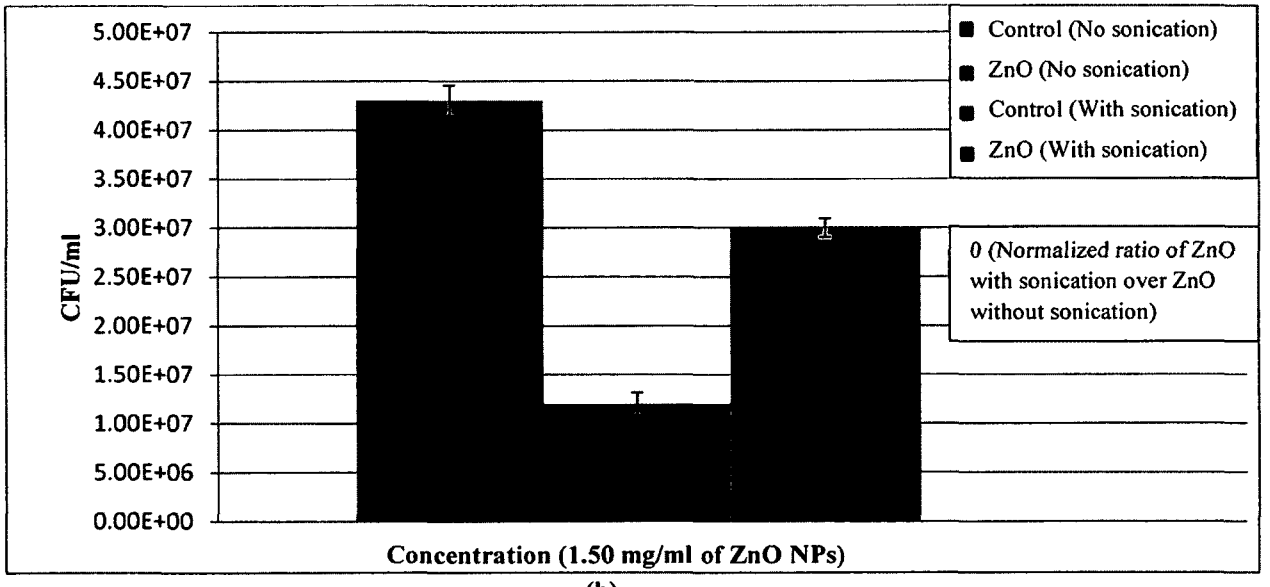
(b)

Figure 16: (a) CFU/ml of S288C when exposed to 0.75 mg/ml of ZnO NPs. The purple and red bars illustrate the presence of zinc oxide nanoparticles at the concentration of 0.75 mg/ml with and without sonication respectively. Blue and green bars shows the absent of zinc oxide nanoparticles (control) without and with sonication.

(b) CFU/ml of S288C when exposed to 1 mg/ml of ZnO NPs. The number of colony forming units (CFU) per millilitre (ml) of 10^{-4} dilution factor of S288C yeas were shown for four cases of controls and ZnO with and without sonication at the concentration of 1 mg/ml of zinc oxide nanoparticles.



(a)



(b)

Figure 17: (a) CFU/ml of 10^{-4} factor of S288C when exposed to 1.25 mg/ml of ZnO NPs. The purple and red bars illustrate the presence of zinc oxide nanoparticles at the concentration of 1.25 mg/ml with and without sonication respectively. Blue and green bars shows the absent of zinc oxide nanoparticles (control) without and with sonication.
(b) CFU/ml of S288C when exposed to 1.5 mg/ml of ZnO NPs. The red bar illustrates the presence of zinc oxide nanoparticles at the concentration of 1.50 mg/ml without sonication. Blue and green bars shows the absent of zinc oxide nanoparticles (control) without and with sonication. However, in the presence of zinc oxide nanoparticles at the concentration of 1.50 mg/ml with sonication, no colonies were formed.

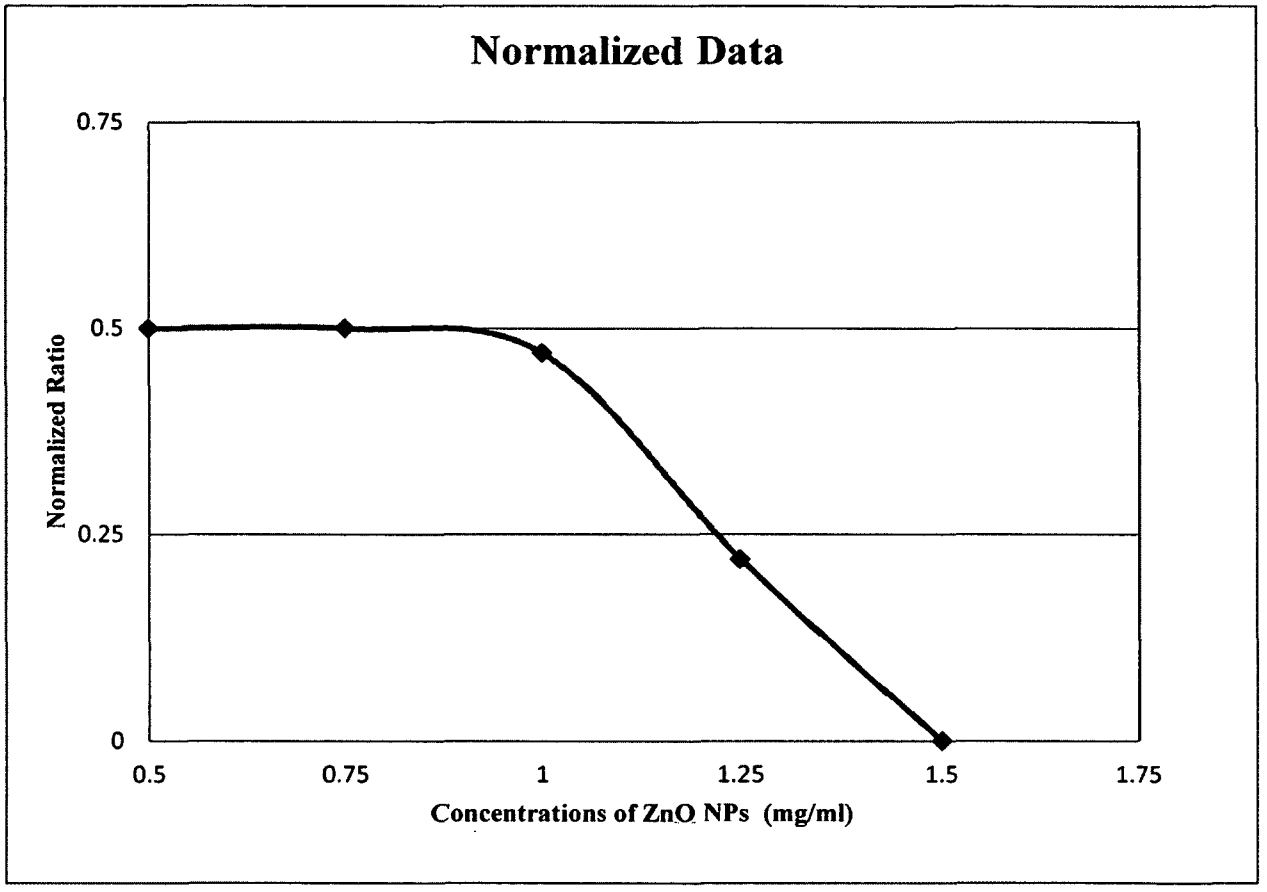


Figure 18: Normalized data of CFU/ml of 10^{-4} factor of S288C when exposed to different concentrations of ZnO NPs. Normalized ratio of ZnO NPs with sonication over ZnO NPs without sonication at various concentrations with respect to their controls with/without sonication.

2.2.5 Vital staining using trypan blue

Another assay that was employed to test the effect of zinc oxide nanoparticles on yeast cells was the trypan blue assay. For this assay, we used trypan blue dye as a vital stain to measure cell viability in both the presence and absence (control) of zinc oxide nanoparticles (Figure 19). The number of cells in both the presence and absence of zinc oxide nanoparticles at various concentrations such as 0.1 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 1.25 mg/ml, and 1.50 mg/ml were measured by using a hemacytometer. The numbers of intact (unstained) and damaged (stained) cells were counted separately for each concentration and its corresponding controls. The percentage of intact cells for each different concentration was calculated based on the formula explained in the method section, and presented in figure 19. The results from Figure 19 illustrate that as the concentrations of zinc oxide nanoparticles increases the percentage of intact cells decreased compared to controls. For instance, the percentage of intact cells at the 1.50 mg/ml of zinc oxide nanoparticles was 78.4%, while the percentage of intact cells at 1 mg/ml and 1.25 mg/ml of zinc oxide nanoparticles were 84.7% and 82.87%, respectively. This reduction reveals that as the concentration of zinc oxide nanoparticles increases, the number of live cells decreases.

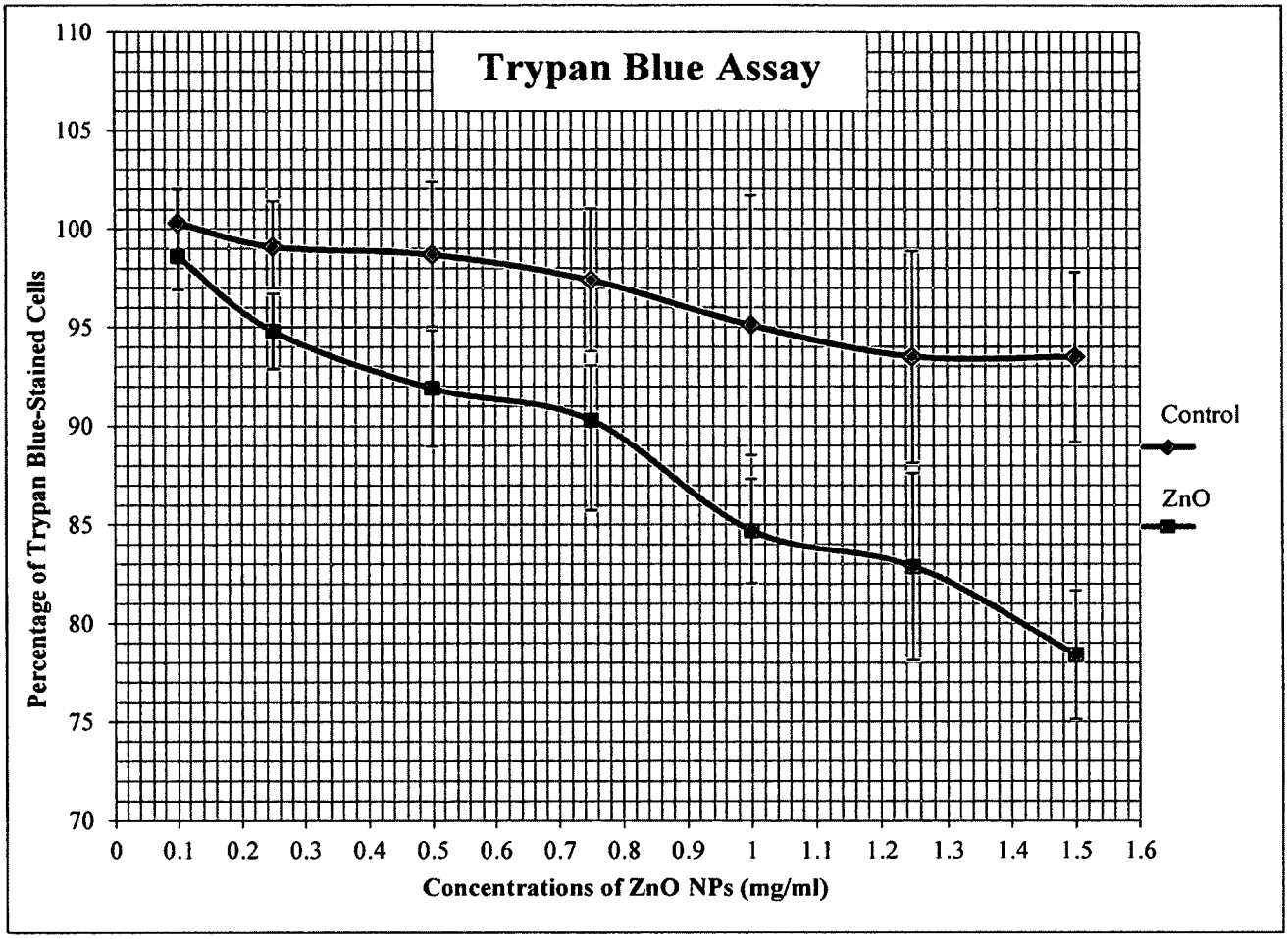


Figure 19: Vital staining using trypan blue. The percentage of cells stained with trypan blue for five different concentrations of zinc oxide nanoparticles. Two millilitres of 10^4 cells/ml of S288C yeast in a mid-log phase was transferred into 10 different test tubes, with concentrations of ZnO nanoparticles of 0.1mg/ml, 0.25mg/ml, 0.5mg/ml, 0.75mg/ml, 1mg/ml, 1.25mg/ml and 1.5mg/ml, and their corresponding controls (ethanol). Then, culture tubes were incubated for 1-2 hours with gentle shaking at 30°C. The optical density was adjusted for all 10 tubes to 10^6- 10^7 cells/ ml. A 1:1dilution of the suspension using 0.4% trypan blue solution was prepared for all 10 tubes. By using the hemacytometer the percentage of cells stained blue was measured for each five different concentrations of ZnO nanoparticles and their corresponding controls under the microscope.

Table 1: Examples of deletion mutants identified as most sensitive to zinc oxide nanoparticles.

Gene Name	Function	GDA % growth reduction
Cell wall		
BCK1	Mitogen-activated protein (MAP) kinase kinase kinase which controls cell integrity	96.9
BUD18	Protein involved in synthesis of ergosterol	83.4
SPC72	cellular component movement	78.9
HOM6	small molecule biosynthetic process	89.1
DFG5	cell wall biogenesis	90.58
SLT2	cell wall integrity	94.7
Ion Homeostasis		
GEF1	plasma membrane, and involved in cation homeostasis	84.6
FTR1	ion transmembrane transporter activity	79.5
CCC2	Cu (+2)-transporting P-type ATPase	74.2
FYV5	Ion Homeostasis	77.1
NHA1	Na+/H+ antiporter involved in sodium and potassium efflux through the plasma membrane	72.9
SPF1	P-type ATPase, ion transporter of the ER membrane involved in ER function and Ca2+ homeostasis	74
Others		
BUD32	telomere maintenance and transcription	70.1
SCP160	Protein involved in control of mitotic chromosome transmission	73.9
MET18	Protein involved in nucleotide excision repair and transcription by RNA polymerase II	77.3
RNR3	Ribonucleotide reductase (ribonucleoside-diphosphate reductase) large regulatory subunit	80.2
Unknown		
YLL005C	Protein of unknown function	79.1
PKR1	Protein of unknown function	80.7
YBR246W	Protein of unknown function	86.4
YHL023C	Protein of unknown function	75.3

Table 2: Example of deletion mutants identified as most sensitive to silver nanoparticles.

Gene Name	Function	GDA % growth reduction
Vesicular Transport		
LAA1	AP-1 accessory protein	99.4
ENT3	Protein containing an N-terminal epsin-like domain involved in clathrin recruitment	87.7
APL2	involved in clathrin-dependent Golgi protein	85.1
APL1	involved in vesicle mediated transport	87.3
VBA1	Permease of basic amino acids in the vacuolar membrane	83.6
SOD1	Cytosolic copper-zinc superoxide dismutase	96.2
GRX4	Hydroperoxide and superoxide-radical responsive glutathione-dependent oxidoreductase	77.1
PEX30	Peroxisomal integral membrane protein	74.2
TIP1	member of the Srp1p/Tip1p family of serine-alanine-rich proteins	80.7
YPS6	Putative GPI-anchored aspartic protease	78.9
STL1	Glycerol proton symporter of the plasma membrane	83.6
APM4	involved in vesicle transport	75.1
YOR246C	Protein with similarity to oxidoreductases, found in lipid particles	72.9
SMI1	Protein involved in the regulation of cell wall synthesis	76.3
Cellular Respiration		
GDS1	Required for growth on glycerol as a carbon source	87.7
COQ10	Coenzyme Q (ubiquinone) binding protein	90.1
AAC3	Mitochondrial inner membrane ADP/ATP translocator	97.3
AAC1	Mitochondrial inner membrane ADP/ATP translocator	89.9
KGD1	Component of the mitochondrial alpha-ketoglutarate dehydrogenase complex	91
IDH1	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase	78.5
MRPL51	Mitochondrial ribosomal protein of the large subunit	87.5
ETR1	2-enoyl thioester reductase	75.4
MRPL28	Mitochondrial ribosomal protein of the large subunit	78.3
YLH47	Mitochondrial inner membrane protein exposed to the mitochondrial matrix	72.5

Table 2: (Continued)

Gene Name	Function	GDA % growth reduction
Nucleotide-excision repair		
DDI1	DNA damage-inducible v-SNARE binding protein	72.6
SLX4	Endonuclease involved in processing DNA during recombination and repair	80.3
Other		
IES6	Protein that associates with the INO80 chromatin remodeling complex	74.3
NPL4	Endoplasmic reticulum and nuclear membrane protein	76.2
RDR1	Transcriptional repressor involved in the control of multidrug resistance	82.1
HMO1	Chromatin associated high mobility group (HMG) family member involved in genome maintenance	70.5
Unknown		
LDB16	Protein of unknown function	88.7
YGL235W	protein of unknown function	77.9

2.3 Discussion

Decreasing the size of particular materials to the nano-scale may cause changes in the structural and physicochemical properties of these materials. Because of their novel properties, these engineered nano materials may have enhanced toxicity compared to their respective bulk compounds (Kasemets *et al.*, 2009). Due to the rapid expansion of nanotechnology and public exposure to nanoparticles in a variety of consumer products, it is essential to obtain more information regarding their potential toxicity on human and environmental health. Many studies suggested that the role of toxic metal ions may be attributed to their surface chemistry, the release of toxic ions, and the production of ROS (Pulskamp *et al.*, 2007); for the most part however, detailed mechanisms of action for different nano compounds remains unclear. Studies on the mechanism of action of nanoparticles might provide new scientific knowledge and insight regarding nanoparticle toxicity and safety.

Increased sensitivity of gene deletion mutant strains to inhibitory compounds with an unknown mode of action has been used to study pathways that buffer the cell against the activity of these compounds. Thus, using high throughput approach to investigate the chemical genetic profile analysis of inhibitory compounds such as nano compounds may lead to the identification of the overall mode of action (Alamgir *et al.*, 2008). Chemical genomics studies may also reveal possible side effects associated with the toxicity of inhibitory compound (Alamgir *et al.*, 2010). For the purposes of the current study, colony size reduction was used as an endpoint to study the sensitivity of a yeast gene deletion array to two different nano compounds (Figures 8 and 9). The entire collection of the haploid yeast gene deletion array was screened for increased sensitivity to zinc oxide and silver nanoparticles. The drug sensitivity spot test was performed to affirm the

results from the primary screening in both the presence and absence of selected nanoparticles (Figures 12 and 13).

In the present research, the DLS was used to measure the average size of silver and zinc oxide nanoparticles in an aqueous medium (YPD). This technique records the hydrodynamic size distribution of particles based on time variation of scattered light from suspended particles in a solution (media) (Fadeel and Bennett, 2010). Results from our DLS reveal that zinc oxide nanoparticles are much larger in size to those of silver nanoparticles when suspended in an aqueous solution (Figures 6 and 7). This may be due to the aggregation property specific to zinc oxide nanoparticles when placed in an aqueous solution. Thus, an increase in the mean size of zinc oxide nanoparticles compared to the powder that was purchased from Sigma is due to their affinity to aggregate in a suspending medium.

The results from our profile analysis showed that zinc oxide may exhibit its activity against *S. cerevisiae* cell wall functions. The second largest category is ion homeostasis pathway (Figure 10). This was apparent from enrichments in the membrane function and cell wall deficiencies among the mutant strains with increased sensitivity to zinc oxide nanoparticles. It is noteworthy that these two processes are actually interconnected as deficiencies in cell wall can also compromised cell membrane integrity and similarly alteration of membranes can affect cell wall integrity. Therefore one possible explanation for our current observation is that zinc oxide affects cell wall integrity through ion homeostasis deficiencies. Many studies have been conducted to investigate the underlying effect of nanoparticles on either eukaryotic or prokaryotic cells. One study regarding the toxicity of zinc oxide nanoparticles using mammalian cell cultures suggested that nanoparticles may interrupt cellular homeostasis via lysosomal and

mitochondrial damage, eventually leading to cell death (Xia *et al.,* 2008). Another study by Liu et al. (2008) surveys the toxic effect of zinc oxide on bacteria cells and reveals the damages it may cause to the bacterial cell membrane through the leakage of intracellular contents such as lipid and some proteins progressively bringing about cell death. The common assumption regarding the key factor in the toxicity of zinc oxide nanoparticles is the generation of reactive oxygen species that may damage the cell membrane and thus interact with intracellular contents (Ravishankar and Jamuna, 2011). Similarly, another study suggests that zinc oxide nanoparticles may act on bacterial cells either through promoting chemical interactions between hydrogen peroxide and membrane proteins or sponsoring chemical interactions involving unknown species, generated by zinc oxide nanoparticles and the lipid bilayer (Zhang *et al.*, 2010).

According to this literature it appears that the mode of action or toxicity by zinc oxide nanoparticle is still controversial. To confirm the results from our primary screening, we performed two secondary assays; 1) cell wall integrity assay using sonication in both the presence and absence of zinc oxide nanoparticles (Figures 14 and 18), and 2) vital staining using trypan blue dye as an exclusion stain to measure the percentage of cell viability in both the presence and absence of zinc oxide nanoparticles (Figure 19). The results from these assays support the idea that zinc oxide nanoparticles exhibit toxicity by affecting the cytoplasmic membrane and cell wall formation and function.

Limbach et al. (2007) explained that the rigid cell wall of yeasts may prevent the direct uptake of nanoparticles but, once the metal oxide nanoparticles enter into the cell, they may liberate metal ions at damaging concentrations (Limbach *et al.*, 2007). Despite the presence of an inflexible cell wall, nanoparticles may damage the cell wall and membranes via dissolved ions

and /or oxidative stress (Kasemets *et al.*, 2009). Thus, the change in membrane permeability by dissolved ions/ or oxidative stress may increase the frequency of nanoparticles penetration into the cell (Huang *et al.*, 2008). Also, it has been shown that nanoparticles entry into the bacteria *Bacillus subtilis (B. Subtilis)* and *Escherichia coli* (*E. coli*) cells was through oxidative damage to the cell membrane (Kloepfer *et al.*, 2005).

Many studies suggest that the toxic effect of nanoparticles may be due to two different actions: 1) a chemical toxicity based on chemical composition either by release of toxic ions or formation of reactive oxygen species; and, 2) a stress or stimuli caused by the surface, shape and size of the particles (Brunner *et al.*, 2006). This group studied the toxicity of soluble and insoluble nanoparticles on human and rodent cell lines; and based on their results, the toxicity of soluble nanoparticles is mainly attributed to the release of metal ions from nanoparticle dissolution (Brunner *et al.*, 2006). Thus, the mechanism of nanoparticle toxicity may be associated with the release of toxic ions and the production of ROS, which can cause damage and disorganization of the cell wall. This proves that once nanoparticles penetrate inside the cell they can cause damages to the cell wall, cell membrane, cell division and, consequently, lead to cell death.

The baker's yeast cell wall contains large quantities of negatively charged organic functional groups such as amino and carboxyl groups; in addition to amylase, protein, lipid, and chitin which make the cell wall more attracted to the cations in solution (Zhang *et al.*, 2008, Brady *et al.*, 1994). Therefore, negatively charged functional groups present in yeast cell walls may increase the electrostatic attraction to the metal cations (Xu *et al.*, 2011). This is one of the reasons for absorbing more metal cations than anions and ionic (neutral) metals. Therefore, for

cellular internalization the surface charge of the nanoparticles may play a significant role in the interaction with the oppositely charged cell membrane (Kumari *et al.*, 2010). In contrast to the zinc oxide nanoparticles with an ionic (neutral) charge, the silver nanoparticles have positively charged ions which may increase their interaction with the negatively charged organic functional groups present in yeast cell walls. Therefore, cationic nanoparticles may interact with heparin sulfate proteoglycans on the membrane surface making their way into the cells rather effortlessly in comparison to the anionic or ionic nanoparticles (Huang *et al.*, 2010).

Silver ions are highly reactive substances and many studies have shown that after binding to proteins, they may cause structural changes to the cell wall and nuclear membrane, and subsequently, causing cell death. It has been shown that the release of silver ions may increase membrane permeability by affecting the proton motive force, efflux of phosphate, and leakage of cellular contents (Jones and Hoek 2010). Moreover, many studies suggest that silver ions have a tendency to bind to phosphorus containing compounds, such as DNA and RNA and cause the inhibition of bacterial replication (Castellano *et al.*, 2007; Lansdown, 2002). Another study by Kazachenko et al. (2000) reported that silver ions may penetrate the bacterial cell walls and alter the DNA structure into a condensed form in order to react with thiol groups on proteins and interfere the DNA replication, and consequently, cause cell death. Furthermore, they suggested that silver ions may inhibit the replication process by interacting with histidine and tryptophan amino acids of replication proteins (Kazachenko *et al.*, 2000).

Our results from the primary screening (GDA analysis) revealed that the toxicity of silver nanoparticle may involve the vesicular transport, cellular respiration, and DNA repair (nucleotide excision repair) pathways (Figure 11). These were apparent from analysing the

pathways of those mutant strains which were sensitive to silver nanoparticles. Dastjerdi and Montazer (2010) explained that generally, metal ions may destroy cell membrane and decrease the enzymatic activity of microorganisms by either binding to the -SH group or thiol groups of cellular enzymes and inhibit their growth until the cell death (Dastjerdi and Montazer, 2010; Sintubin *et al.*, 2011). It has been suggested that silver may denature protein via interacting with its nucleophilic amino acid residues and also by attaching to amino, imidazole, phosphate and carboxyl groups of membranes or enzyme proteins, all of which may block cellular respiration and cause cell death via forming R-S-S-R bonds (Kumar *et al.*, 2004). Moreover, Rosenkranz and Carr (1972) explained that silver may inhibit oxidative enzymes, such as yeast alcohol dehydrogenase, and lead to the uptake of succinate by membrane vesicles and the respiratory chain. Also, it may cause metabolite efflux and may inhibit DNA replication (Rosenkranz and Carr, 1972). Thus, the mechanisms of action of silver are not fully understood and remains controversial; however, the most common proposed mechanism of silver nanoparticles in bacteria is the generation of reactive oxygen species by silver ions that may weaken cell membrane or cell wall via the leakage of cellular content, destroying the enzymes activity, and transportation of nutrients, consequently leading to the disruption of DNA replication (Jones and Hoek, 2010).

Moreover, studies regarding the mechanism of toxicity of silver nanoparticles in mammalian cells reveal that silver nanoparticles may induce apoptosis in mouse fibroblast cells by affecting the mitochondrial mediated pathways and making of ROS (Hsin *et al.*, 2008). Also, it has been shown that silver nanoparticles may cause necrosis in human colon cancer cells by effecting mitochondrial respiratory chain by way of generating ROS and reducing the synthesis of ATP which may consequently lead to DNA damage (Asharani *et al.*, 2009). The authors

measured the amount of DNA damage by using single cell gel electrophoresis (SCGE) and cytokinesis blocked micronucleus assay (CBMN) approaches. Their results show that silver nanoparticles may cause cell cycle arrest in both G_2 and M phase in order to repair damaged DNA (Asharani *et al.*, 2009).

Obtaining more information about different types of antifungal agents has recently become a concern. Regarding antifungal activity of silver nanoparticles, Kim et al. (2009) showed that silver nanoparticles may disorder the fungal cell membrane structure and inhibit the normal budding process through destruction of the membrane integrity (Kim *et al.*, 2009). And this mechanism is supported by the electrostatic attraction of positively charged silver ions to the membrane surface.

Since fungal cells may maintain their membrane potential through the establishment of multiple ion gradients across the cytoplasmic membrane. The maintenance of intracellular components, such as trehalose and glucose are essential for yeast cell viability. Trehalose may protect membrane proteins from many environmental stresses such as desiccation, heat, cold, toxic agents and also provide protection against inactivation and denaturation. Kim et al. (2009) suggest that the presence of silver nanoparticles may cause the release of several intracellular components, including the release of glucose and trehalose during membrane disruption. Moreover, they explained that the disruption in membrane permeability may also disrupt the membrane lipid bilayer and cause leakage of ions and other materials through created pores. This may lead to the dissipating of the electrical potential of the membrane. Their results from TEM analysis showed that silver nanoparticle created pits on the yeast membrane surfaces which may result in the formation of pores and cell death (Kim *et al.*, 2009; Nasrollahi *et al.*, 2011;

Ravishankar and Jamuna, 2011). Also, results from flow cytometry analysis reveal that silver nanoparticles may cause DNA damage by arresting yeast in the G_2 and M phase of the cell cycle (Kim *et al.*, 2009).

Another study that investigated the effect of silver nanoparticles on *S. cerevisiae* cell walls suggested that silver nanoparticles may alter the yeast cell wall integrity and cause the destruction of chromatin. These observations on the yeast cell walls suggest that silver nanoparticles may alter cell wall integrity by diffusing across the membranes and causing damages to the DNA and chromatin, and consequently, lead to cell death (Das and Ahmed, 2012).

2.4 Conclusion and remarks

Due to the rapid expansion of nanotechnology and the rise of public exposure to nanoparticles in a variety of consumer products, it is essential to obtain more information regarding their potential toxicity on human and environmental health. In this study we reported the potential mechanism of action of both zinc oxide and silver nanoparticles on yeast *Saccharomyces cerevisiae*. Results of two secondary assays confirmed our observation for the toxicity of zinc oxide nanoparticles on cytoplasmic membrane and cell wall formation and function. Also, according to our results from the primary screening (GDA analysis), the silver nanoparticles may affect the yeast vesicular transport, cellular respiration, and DNA repair (nucleotide excision repair) pathways. These were apparent from analysing the pathways of those mutant strains which were sensitive to silver nanoparticles. However, further studies by

means of secondary assays such as liposomal assay can be performed to confirm the effect of silver nanoparticles on vesicular transport. This may further reveal and confirm the accuracy of our observations.

2.5 Experimental procedures

2.5.1 Growth media

Standard rich, yeast extract peptone dextrose (YPD) medium was used for these experiments (Sherman *et al.*, 1986). Yeast cells were grown at 30°C for 1-2 days. For the maintenance of deletion strains carrying the $G418^R$ selectable marker, the YPD medium containing Geneticin (G418; 200μg/ml) was used. G418 was purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.5.2 Chemicals

Nano scale zinc metal oxide 50-70 nm (catalog #544906) and the bulk zinc oxide (catalog # 96479) were purchased from Sigma-Aldrich. For both nano and bulk compounds, the zinc oxide stock solution (50 mg/ml) was suspended in ethanol and ultra-sonicated for 5 minutes in order to reduce agglomeration (Vibracell, Sonics & Materials Inc., USA, Mod. No. V1A, Ser.No.V8080) as in Kasemets *et al.*, (2009). They were stored at 4°C but, before undergoing toxicity testing, metal oxide stock solutions were vigorously vortexed (Kasemets

et al., 2009). Aqueous Silver nanoparticles stock solution with the concentration of 2 mg/ml (31% silver) was purchased from Sciventions Inc.

2.5.3 Antifungal activity

Saccharomyces cerevisiae (S288C) was used in antifungal activity assays. Minimum inhibitory concentration (MIC) for each compound was measured by the lowest drug concentration that inhibits the visible growth of yeast strains on sterile 96-well microtitre plates. Plates were incubated at 30°C for 1-2 days (CLSI, 2008). In order to investigate the effects of nano compounds on the growth of yeast deletion mutants, the sub-inhibitory concentration of zinc oxide (1 mg/ml) and silver nanoparticles (0.095 mg/ml) were added to the YPD medium.

2.5.4 Characterization of nanoparticles

Dry powder of zinc oxide nanoparticles was suspended in ethanol at a concentration of 1 mg/ml and ultra-sonicated for 5 minutes in order to reduce agglomeration and form a homogeneous suspension. From silver nanoparticles stock solution, 0.095 mg/ml of silver nanoparticles was added to YPD media. Since we purchased the water suspended silver nanoparticles stock solution, no sonication was used for this solution. However, the sonicated 1 mg/ml of zinc oxide nanoparticle stock solution (10X) was used for size measurement purposes. Additionally, for silver nanoparticles solution 10X dilution was used

to measure particles size. The dynamic light scattering (DLS) (Zetasizer NanoZS, Malvern Instruments Ltd) was used in order to determine the hydrodynamic size for both silver and zinc oxide nanoparticles suspension in the culture medium. Moreover, for silver nanoparticles, transmission electron microscopy (TEM) (JOEL JEM 1230 (60 KV)) was also used to characterize the size and shape of the particles. For this purpose, a drop of silver nanoparticle stock solution, was placed onto a grid and air dried. Then, the size and shape of silver nanoparticles were observed by TEM.

2.5.5 High throughput phenotypic screening (GDA analysis)

For high throughput phenotypic screening, the approximately 4700 MATa haploid gene deletion strains of *S.cerevisiae* in the BY4742 (MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0) parental strain that were maintained in an ordered array of approximately 384 individual strains in 16 plates were used (Tong *et al.*, 2001). Various concentrations of zinc oxide nanoparticles, 0.1–1.2 mg/ml were used. Concentrations less than 1 mg/ml did not result in noticeable changes in their sensitivity to zinc oxide, and higher concentrations resulted in systematic growth reduction. Moreover, different concentrations of silver nanoparticles, from 0.003-0.1 mg/ml were tested. But, we determined the sub-inhibitory concentration of silver nanoparticles to be 0.095 mg/ml; at less than this concentration, no obvious reduction was observed, and any concentration higher than 0.095 mg/ml, resulted in a systematic reduction. The growth rates of individual colonies in the presence of nanoparticles (experimental) were compared to growth rates of individual colonies in the absence of nanoparticles (control). Gene deletion mutants were arrayed using a V&P hand-

held arrayer onto agar plates with sub-inhibitory concentrations of silver (0.095 mg/ml), and zinc oxide (1 mg/ml) nanoparticles. This method is similar to a method described by (Parson *et al.,* 2004; Sintubin *et al.*, 2011). A plate without nano compounds was used as control. Plates were incubated for 1-2 days at 30°C before; digital images of these plates were captured. The digital analysis (Growth Detector software) was used to determine colony areas as described by Memarian *et al.,* (2007). The size of each colony was compared to the average size for all colonies on both nano and control plates (Galvan *et al.*, 2008; Alamgir *et al.*, 2010). Each experiment was repeated three times. Colonies with 70% or more reduction in size in two replicates, or those with an average reduction of more than 80% in all 3 replicates were identified and classified as sensitive colonies or highly susceptible mutants, respectively. Gene ontology annotation analysis was done with online software (GeneMANIA; http://www.genemania.org/) and Saccharomyces Genome Database (SGD, Hong *et al.*, 2006) for functional profiling of highly susceptible mutants in our large-scale experiment. Then, drug sensitivity spot test was used in order to confirm the sensitivities of selected mutant strains which were identified in the primary screens.

2.5.6 Drug sensitivity spot test

To confirm the sensitivities of selected mutant strains identified in the primary screens, the drug sensitivity spot test was used similar to that explained by Jessulat *et al.*, (2008). Those mutant strains selected in the primary screens (GDA analysis) were used for the drug sensitivity test in order to confirm their sensitivity and the accuracy of the GDA results. Yeast cells were grown in liquid medium (YPD), until their growth rate had reached

a mid-log phase. Following this, each gene deletion mutant strain was serially diluted to10^{-3} -10^{-6} cells/15 µl and spotted onto a solid medium containing sub-inhibitory concentrations of the nano compounds, and without nano compounds (control). Plates were then incubated at 30°C for 1-2 days. Different growth rates and numbers of colonies relative to controls were used to determine the sensitivity of each strain.

2.5.7 Cell wall integrity assay using sonication

Yeast strain S228C was grown overnight in YPD media to mid-log phase and adjusted to approximately 10^4cells/ml. Two ml of the culture was transferred into each of 10 disposable culture tubes; five tubes of cultures with different concentrations of zinc oxide nanoparticles (0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 1.25 mg/ml and 1.5 mg/ml), and five tubes of cultures with equivalent volumes of carrier solvent (ethanol) without any nano particles served as our controls. Then, culture tubes were incubated overnight and gently shaken at 30°C. The cell density was adjusted for all 10 tubes to 10^7 cells/ ml by using optical density at 600 nm. Sonication (SONICS & MATERIALS INC. Vibra Cell, Model#VCX130, Serial No. 49913N) at 3-µm amplitude for 180 seconds was used as a treatment to disrupt the cell wall. This method is best described by (Mir-Rashed *et al.*, 2010). Following sonication, drop plate assays using 10 fold dilution series was used in order to estimate cell viability for each of the concentrations; this method is well described by (Chen *et al.,* 2003). Also, same procedure was used with no sonication in order to compare the effect of zinc oxide nanoparticles on yeast cell wall. Each experiment was repeated three times and the average

CFU/ml for each concentration was used to show the frequencies of live cells in both the absence and presence of zinc oxide nanoparticles.

2.5.8 Vital staining using trypan blue

Yeast strain S228C was grown overnight in YPD media to mid-log phase and adjusted to approximately 10^4cells/ml (Smith *et al.*, 2008). Two ml of the culture was transferred into 10 disposable culture tubes; five tubes of cultures with different concentrations of zinc oxide nanoparticles (0.1 mg/ml, 0.25 mg/ml, 0.5mg/ml, 0.75mg/ml, 1mg/ml, 1.25mg/ml and 1.5mg/ml), and five tubes of cultures with equivalent volumes of carrier solvent (ethanol) without any nano particles served as our controls. Afterwards, culture tubes were incubated for two days with gentle shaking at 30°C. The optical density was adjusted for all 10 tubes to 10^7 cells/ ml, and mixed (1: 1) with a 0.4% trypan blue solution (Freshney, 1994). By using the hemacytometer, the numbers of viable (unstained) and nonviable (stained) cells were counted separately for each different concentration and its corresponding controls under the microscope (CARL ZEISS #4649608). Each experiment was repeated three times; also, the average number of viable and nonviable cells for each concentration was used for further calculation. Then, the percentage of cell viability was measured for each of the five different concentrations of ZnO nanoparticles and their corresponding controls. The percentage of cell viability was calculated for each concentration and its corresponding controls by using this formula.

$$\textbf{Cell Viability (\%):}\ \frac{Total\ viable\ cells\ (unstained)}{Total\ cells\ (stained\ and\ unstained)} \times 100 \qquad \text{(Freshney, 1994)}$$

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Chapter 3

Discussion

3.0 Discussion

Nanotechnologies and nanoparticles have been used in many applications, such as medical diagnostics and photodynamic therapy, cosmetics, and the food packaging industries. Consequently it is essential to examine their health and environmental risks. Decreasing the size of particular materials to the nano-scale may cause changes in structural and physicochemical properties of these materials. Because of their novel properties, these engineered nano materials may have enhanced toxicity compared to their respective bulk compounds (Kasemets *et al.*, 2009). Due to the rapid expansion of nanotechnology, and public exposure to nanoparticles in a variety of consumer products, it is essential to obtain more information regarding their potential toxicity on human and environmental health. The biological mode of activity of nanoparticles remains controversial. A number of studies suggested that the toxicity of metal NPs may be attributed to their surface chemistry, the release of toxic ions, and the production of ROS such as superoxide, hydroxyl, peroxyl and alkoxyl (Pulskamp *et al.*, 2007). Additional studies on the mechanism of action of nanoparticles might provide new knowledge regarding nanoparticle toxicity.

Increased sensitivity of gene deletion mutant strains to inhibitory compounds with unknown modes of action has been used to study gene functions and identify pathways that buffer the cell against the activity of these compounds. It has also been used to study novel genetic pathways that may be affected by that compound leading to a better understanding of the chemical-genetic interaction for the target compound. Thus, using high throughput approaches to investigate the chemical genetic profile analysis of inhibitory compounds such as nano

compounds may lead to identification of the overall mode of action and possible side effects associated with toxicity of that compound (Alamgir *et al.,* 2010).

For the current study, colony size reduction was used as an endpoint to evaluate the sensitivity of a yeast gene deletion array to two different nano compounds (Figures 8 and 9). The entire collection of the haploid yeast gene deletion array was screened for increased sensitivity to zinc oxide and silver nanoparticles. To confirm the results from the primary screening, the drug sensitivity spot test in both the presence and absence of nanoparticles was used (Figures 12 and 13).

Nanoparticles size measurement can be analysed by different methods. In this research, transmission electron microscopy was used to observe a small fraction of the silver nanoparticles. However the results from transmission electron microscopy do not reflect the average particle size values in aqueous solution such as biological media (Fadeel and Bennett, 2010). For this reason, DLS was also used as a method to characterize the hydrodynamic particle size for both silver and zinc oxide nanoparticles in aqueous media. This technique obtains the hydrodynamic size distribution of particles based on time variation of scattered light from suspended particles in a solution (medium) (Fadeel and Bennett, 2010). Results from DSL revealed that the size of zinc oxide nanoparticles is much larger compared to the size of silver nanoparticles in aqueous solution (Figures 6 and 7). We purchased nano scale zinc metal oxide with a size between 50 to70 nm from Sigma-Aldrich. However, when we characterized zinc oxide by DLS, we found that its size is 278 nm. Therefore, we suggested that the large size of nano-like zinc oxide particles may be due to the tendency of zinc oxide nanoparticles to aggregate in aqueous solution (Fadeel and Bennett, 2010).

The results from our profile analysis show that zinc oxide may exhibit its activity against *S. cerevisiae* mainly by affecting ion homeostasis pathways through disruption of the cytoplasmic membrane and cell wall functions (Figure 10).

Many studies have been conducted to investigate the effect of nanoparticles on either eukaryotic or prokaryotic cells. One study regarding the toxicity of zinc oxide nanoparticles using mammalian cell cultures suggests that zinc oxide nanoparticles may disrupt cellular homeostasis and consequently may lead to cell death by causing lysosomal and mitochondrial damage (Xia *et al.,* 2008). Another study by Liu et al. (2008) revealed that zinc oxide nanoparticles may cause damage to bacterial cell membrane, and lead to leakage of intracellular contents, such as lipid and some proteins and consequently leading to the cell death (Liu *et al.*, 2008). However, Schmitt et al. (2004) explained that the toxicity of zinc oxide nanoparticles on yeast is relatively low compared to the algae and bacteria. This may be due to the existence of an important cellular defense system in *S. cerevisiae,* mainly vacuoles and zincosomes that may buffer both the excess and deficiency of zinc ions (Schmitt *et al.,* 2004). The common assumption regarding zinc oxide nanoparticles toxicity is the generation of hydrogen peroxide that may cause damage to cell membrane and thus interact with intracellular contents (Ravishankar and Jamuna, 2011). Similarly, another study suggests that the mechanism of action of zinc oxide nanoparticles on bacteria may either relate to the chemical interactions between hydrogen peroxide and membrane protein or the chemical interactions between unknown species which are generated by zinc oxide nanoparticles with the lipid bilayer (Zhang *et al.*, 2010). Based on this literature, it appears that the mode of action or toxicity by zinc oxide nanoparticles is still controversial.

To confirm the results from our primary screening, we performed two secondary assays; 1) a cell wall integrity assays using sonication in both the presence and absence of zinc oxide nanoparticles (Figures 14 and 18), and 2) a vital staining using trypan blue dye as an exclusion stain in both the presence and absence of zinc oxide nanoparticles (Figure 19). The results from these assays support the idea that zinc oxide nanoparticles exhibit toxicity by affecting cytoplasmic membrane and cell wall formation and function.

Limbach et al. (2007) explained that rigid cell walls of yeast may avoid the direct uptake of nanoparticles, but once metal oxide nanoparticles enter into the cell, they may release metal ions at damaging concentrations (Limbach *et al.*, 2007). Later it was shown that in the presence of a very rigid cell wall, nanoparticles may still cause the disruption of cell wall and membrane via dissolved ions and /or oxidative stress (Kasemets *et al.*, 2009). Thus, the change in membrane permeability by dissolved ions/ or oxidative stress may increase the entry of nanoparticles into the cell (Huang *et al.*, 2008). Also, it has been shown that nanoparticle entry into the bacteria *B. subtilis* and *E.coli* was through oxidative damage to the cell membrane, indicating that a direct relationship with cell wall may not be needed for the metal oxide effect on the cell (Kloepfer *et al.*, 2005).

Many studies suggest that the toxic effect of nanoparticles may be due to two different actions; 1) a chemical toxicity based on chemical composition either by release of toxic ions or formation of reactive oxygen species; 2) a stress or stimuli caused by the surface, shape and size of the particles (Brunner *et al.*, 2006). Studies on the toxicity of soluble and insoluble nanoparticles to a human and rodent cell lines suggests that the toxicity of soluble nanoparticles is mainly attributed to the release of metal ions from nanoparticle dissolution (Brunner *et al.*,

2006). Similarly, Franklin et al. (2007) explained that the toxicity of zinc oxide nanoparticles to a freshwater microalga may be attributed to dissolved zinc ions (Franklin *et al.*, 2007). Nevertheless, other studies suggest that both the ions and nanoparticles themselves are the source of nanoparticles toxicity, rather than one alone (Wang *et al.*, 2009). Thus, the mechanism of nanoparticles toxicity may be associated with the release of toxic ions and the production of ROS which can cause damage and disorganization of the cell wall along with other cellular components. Once nanoparticles penetrate the cells they can cause damages to the cell wall, membrane, and cell division and, consequently, lead to cell death.

The baker's yeast cell walls have large quantities of negatively charged organic functional groups including amino and carboxyle groups, in addition to amylase, protein, lipid, and chitin which make the cell wall more attracted to the cations in a solution (Zhang *et al.*, 2008, Brady *et al.*, 1994). Therefore, negatively charged functional groups in yeast cell wall may increase the electrostatic attraction to the metal cations (Xu *et al.*, 2011); this may be one of the reasons for absorbing more metal cations than anions and ionic (neutral) metals. Therefore, for cellular internalization, surface charge of the nanoparticles may play a significant role on the interaction with the oppositely charged cell membrane (Kumari *et al.*, 2010). In contrast, to zinc oxide nanoparticles that carry a neutral charge, silver nanoparticles have positively charges ions which may increase their interaction with the negatively charged organic functional groups on yeast cell walls. Therefore, cationic nanoparticles may interact with heparin sulfate proteoglycans on the membrane surface; and consequently enter to the cells more easily compared to anionic or ionic nanoparticles (Huang *et al.*, 2010).

Because of the large surface area to volume ratio of metal nanoparticles, they have been used in many applications, such as burn treatment, sunscreen lotion and as an antimicrobial agent. Recently, due to the emergence of antibiotic resistance bacteria and also limitation of the use of antibiotics, increased attention have been paid to anti-microbial activity of the silver nanoparticles (Rai *et al.*, 2009). Silver ions are highly reactive, and many studies showed that after they bind to proteins they may cause structural changes to the integrity of cell wall and nuclear membrane and, consequently, may lead to cell death. It has been shown that the release of silver ions may increase membrane permeability by effecting proton motive force, efflux of phosphate, and leakage of cellular content (Jones and Hoek 2010). Moreover, many studies suggest that silver ions have a tendency to bind to the phosphorus containing compounds, such as DNA and RNA and cause the inhibition of bacterial replication (Castellano *et al.*, 2007; Lansdown, 2002). Another study by Kazachenko et al. (2000) shows that the silver ions may enter into the bacterial cell walls and change the DNA into a condensed form in order to react with the thiol groups on proteins and interfere the DNA replication and, consequently, cause cell death. The same authors suggest that silver ions may also inhibit the replication process by interacting with histidine and tryptophan amino acids of replication proteins (Kazachenko *et al.*, 2000).

Our results from primary screening (GDA analysis) reveal that the toxicity of silver nanoparticles may involve vesicular transport, cellular respiration, and DNA repair (nucleotide excision repair) pathways (Figure 11). These were apparent from analysing the pathways of those mutant strains which were sensitive to silver nanoparticles. These results are in general agreement with some of those reported in literature. Dastjerdi and Montazer (2010) explained that generally, metal ions may destroy cell membrane and decrease the enzymatic activity of

microorganisms by either binding to the -SH group or thiol groups of cellular enzymes and inhibit their growth and causing cell death (Dastjerdi and Montazer, 2010; Sintubin *et al.*, 2011). It has been suggested that silver may denature protein via interacting with its nucleophilic amino acid residues and also by attaching to amino, imidazole, phosphate and carboxyl groups of membrane or enzyme proteins and may block cellular respiration and lead to the cell death via forming R-S-S-R bonds (Kumar *et al.*, 2004). Moreover, Rosenkranz and Carr (1972) explained that silver may inhibit oxidative enzymes, such as yeast alcohol dehydrogenase, and lead to uptake of succinate by membrane vesicles and the respiratory chain. Also, it may cause metabolite efflux and may inhibit DNA replication (Rosenkranz and Carr, 1972).

Several studies on rodents show that silver nanoparticles can accumulate inside the body and cause tissue damage to liver, lungs, and olfactory bulbs in addition to a reduction of mitochondrial function (Arora *et al.*, 2009; Stolle *et al.*, 2005; Hussain *et al.*, 2005). Their results suggest that silver nanoparticles may reduce rat lung function and produce inflammatory lesions in the lungs. Also, it has been shown that silver nanoparticles may accumulate in the olfactory bulbs and in the brain of rats. Moreover, Stolle et al. (2005) shows that silver nanoparticles may reduce mitochondrial function and increase membrane leakage of liver cells of rats, and mouse spermatogonial stem cells (Stolle *et al.*, 2005). Another study on the effect of silver nanoparticles on human fibrosarcoma and skin/carcinoma cells demonstrates that silver nanoparticles may cause oxidative stress through reducing the cellular level of glutathione and result in cellular damages and lipid peroxidation (Arora *et al.*, 2008). However, in the study by Arora et al. (2008), the concentration of silver nanoparticles which was used to induce apoptosis (0.78-1.56 μg/ml) was much smaller than the concentration of silver nanoparticles that caused necrosis (12.5 μg/ml). The authors suggested that by performing *in vivo* studies it may be

possible to define a safe range of silver nanoparticles for applications as an antimicrobial agent (Arora *et al.*, 2008).

Moreover, studies regarding the mechanism of toxicity of silver nanoparticles in mammalian cells reveal that silver nanoparticles may induce apoptosis in mouse fibroblast cells via effecting the mitochondrial mediated pathways and generation of ROS (Hsin *et al.*, 2008). Other studies showed that silver nanoparticles may cause necrosis in human colon cancer cells by effecting mitochondrial respiratory chain via generation of ROS and reduced production of ATP or ATP synthesis which may consequently lead to DNA damage (Asharani *et al.*, 2009). They measured the amount of DNA damage using SCGE and CBMN approaches. Their results show that silver nanoparticle may cause cell cycle arrest in both G_2 and M phase in order to repair damaged DNA (Asharani *et al.*, 2009).

Concerns about antifungal drug resistance have been increasing in recent years. Regarding antifungal activity of silver nanoparticles, Kim et al. (2009) shows that silver nanoparticles may disrupt fungal cell membrane structure and inhibit the normal budding process through destruction of membrane integrity (Kim *et al.*, 2009). And this mechanism is highly supported by electrostatic attraction of positively charged silver ions with the membrane surface.

Fungal cells may maintain their membrane potential by the establishment of multiple ion gradients across the cytoplasmic membrane. The maintenance of intracellular components, such as trehalose and glucose are essential for yeast cell viability. Trehalose may protect membrane proteins from many environmental stresses such as desiccation, heat, cold, toxic agents. Kim et al. (2009) suggest that the presence of silver nanoparticles may cause the release of several intracellular components, including the release of glucose and trehalose during membrane

disruption. Moreover, they explained that the disruption in membrane permeability may also disrupt membrane lipid bilayers and cause the leakage of ions and other materials creating pores. This may lead to the dissipating of the electrical potential of the membrane. Their results from TEM analysis show that silver nanoparticle may create pits on yeast membrane surfaces and result in the formation of pores, leakage and cell death (Kim *et al.*, 2009; Nasrollahi *et al.*, 2011; Ravishankar and Jamuna, 2011). Also, results from flow cytometry suggest the possibility that silver nanoparticles may cause DNA damage by arresting yeast in the G_2 and M phase of the cell cycle (Kim *et al.*, 2009).

Another study that investigates the effect of silver nanoparticles on *S. cerevisiae* cell walls suggests that silver nanoparticles may alter the yeast cell wall integrity, and affect chromatin. The authors suggest that silver nanoparticles may alter cell wall integrity by diffusing across the membranes, and cause DNA damage and destruction of chromatin and, consequently, lead to cell death (Das and Ahmed, 2012).

In the current study we used a chemical genomics approach to study toxicity of target nano particles. One deficiency of such large scale investigations is that they do not offer a detailed mode of activity. What they offer is an indication to possible mode of action. More focused investigations are needed to detect the exact cellular target(s) of the inhibitory compound of interest. Another limitation of the technique relates to its inability to detect mildly sensitive mutants that might play important roles in elucidating the activity of target compound. A third limitation is analysis of large amount of data gathered using this method. Clustering genes into distinct functional groups is often very challenging.

Conclusion and Future Direction

Due to the rapid expansion of nanotechnology, and public exposure to nanoparticles in a variety of consumer products, it is essential to obtain more information regarding their potential toxicity on human and environmental health. In this study, we reported that zinc oxide nanoparticles may cause the disruption of yeast cytoplasmic membrane and cell wall formation and function. Results of two secondary assays further support the hypothesis that zince oxide nanoparticles can affect cell wall and membrane integrity. Also, according to our results from primary screening (GDA analysis), the silver nanoparticle toxicity may affect yeast vesicular transport, cellular respiration, and DNA repair (nucleotide excision repair) pathways. These were apparent from analysing the pathways of those mutant strains which were sensitive to silver nanoparticles. However, further studies by means of secondary assays regarding the mechanism of action of silver nanoparticles are needed to confirm the accuracy of our observation.

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