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Comparative study of the toxicity of synthetic nanoparticles to yeast *Saccharomyces cerevisiae*

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Abstract

Conclusive scientific knowledge is still lacking for mechanisms and modes of action of manufactured nanomaterials in biological systems. The yeast *Saccharomyces cerevisiae* is a robust cellular model at the frontline of toxicology research that may provide mechanistic information about the interaction of nanoparticles with cells.The major objective of this thesis was to evaluate the toxicity of CuO-NPs by using the yeast *S. cerevisiae* as a model. Both parent *S. cerevisiae* strain BY4741 (wild-type) and the Cu stress response-deficient single deletion mutant BY4741_Δcup2 (*cup2Δ*), were exposed to CuO-NPs having as reference cells exposed to the ionic Cu^{2+} form ($CuSO_4$). The yeast strains response to CuO-NPs was assessed by studying cell growth and cell viability with colony-forming units (CFU) analysis, protein oxidation with immunoassay to detect free thiol groups and Cu distribution and accumulation profiles in cells with nuclear microscopy modalities. Results showed that the wild-type strain was more vulnerable to CuO-NPs than to Cu equivalent concentrations of $CuSO_4$, whereas the mutant *cup2Δ* showed a similar response to both compounds. CuO-NPs were also able to induce oxidative damage to proteins in both strains, suggesting that CuO-NPs action may be similar to Cu^{2+} form. Both wild-type and *cup2Δ* cells accumulated more Cu when exposed to CuO-NPs and the Cu profiles along cell depth confirmed the internalization of Cu-derived from CuO-NPs. This preliminary study highlighted the potential of *S. cerevisiae* as a model to study nanoparticles toxicity and the adequacy of screening methodologies developed. The identification of mechanisms that act in the detoxification of CuO-NPs allied to the visualization of Cu distribution inside whole yeast cells are unique outputs of this work, that are of the utmost importance to plan future studies.

Introduction

Nowadays the exponential production of nanomaterials specially the increase of manufactured nanoparticles (NPs) on quotidian uses as well as in industrial and scientific applications[1–4] had led to an increase in the concern about their short- and long-term effects in the environment, 'non-target' organisms and human health [3,5]. Physico-chemical properties of NPs including size, shape, surface charge and surface chemistry have been identified as strongly modulating the cellular uptake efficiency[6]. The foundation behind NPs cellular effects relate to increased reactivity resulting from their small size and the large number of surface atoms compared to their bulk counterparts. NPs transformations may modify their ability to translocate cell membranes, enhance electron transfer, bind of molecular species and interfere with the cell enzymatic machinery, which in turn influence the cell stability. However, how different NPs interact with cells and modulate their response are challenging issues, which require the development of efficient models and screening methods.

The yeast *Saccharomyces cerevisiae* is a robust eukaryotic cellular model at the frontline of toxicology research. The detailed functional and genomic characterization

positioned it as a promising model to assess toxicological responses and mechanistic information about the interaction of nanoparticles with cells. *S. cerevisiae* shows several advantages, such as, the similarity with higher-level organisms[2,7], the availability of systematic genome-wide mutant collections[7], catalogued response to numerous environmental stressors, including tolerance to shortage or excess levels of essential metals, such as Fe, Cu and Zn [8,9], the short generation time and undemanding laboratory procedures[2].

The aim of this work was to assess the toxicity of copper oxide nanoparticles (CuO-NPs) by using the yeast *S. cerevisiae* as a model. The rationale behind these approach stems from the increasing uses of CuO-NPs and their potential toxic effects due to interference in cellular permeability and redox balance, among others[5,10,11]. CuO-NPs currently have multiple applications from textiles to electronics and so far conclusive scientific knowledge about mechanisms and modes of action in biological systems are lacking.

Both parent *S. cerevisiae* strain BY4741 (wild-type) and the single deletion mutant BY4741_Δcup2 (*cup2Δ*), which associates to copper detoxification mechanisms were selected to study CuO-NPs toxicity. Yeast cultures were exposed to CuO-NPs having as reference, cells exposed to the ionic form Cu^{2+} ($CuSO_4$). To achieve this objective multiple approaches were used. Biological assays were used to assess cell growth and measure protein oxidative damage and advanced high-resolution nuclear microscopy techniques provided quantitative information for Cu distribution and accumulation in whole S. cerevisiae cells.

.Materials and methods

Copper solutions

A solution of CuO-NPs (CuO 99+%, 30-50 nm) with a relation of 0.2248 gCu/gNPs were purchase from Nano Amourphous, were used in this study. The CuO-NPs stock solutions (67.5 mgCu/mL) were prepared in double distilled water with 18 MΩ.cm resistivity (MilliQ, Millipore), sonicated in an ultrasonic bath, for 30 min (VWR B-3001 Leuven, Malaysia) and stored in the dark at room temperature. CuO-NPs intermediary solutions whenever needed were prepared in Teflon container and also prepared with double distilled MilliQ water, in order to avoid contaminations.

A copper sulfate ($CuSO_4$) solution was used as the equivalent salt of CuO-NPs (Cu^{2+}), serving as a positive control to experiments with NPs. The $CuSO_4$ solution with a concentration of 160 gCu/L, was made through the addition of Cupric Sulfate $CuSO_4 \cdot 5H_2O$ (Sigma) and deionized water. An intermediary solution of $CuSO_4$ (16 gCu/L) was used for the preparation of the solutions used on the assay.

Strains and growth conditions

The parental *Saccharomyces cerevisiae* strain BY4741 (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0) and the derived single deletion mutant BY4741_Δ*cup2*, used in this work, were obtained from the EUROSCARF collection. Minimal growth medium MM4 used contains (per litre): 1.7 g yeast nitrogen base without amino acids or $(NH_4)_2SO_4$ (Panreac), 20 g glucose (Merck), 2.65 g $(NH_4)_2SO_4$ (Panreac), 20 mg histidine (Merck), 20 mg methionine (Merck), 60 mg leucine (Sigma) and 20 mg uracil (Sigma). Solid media were obtained by the addition of 20 g/L agar (Iberagar). The growth media pH was adjusted to 4.5) using HCl or NaOH. *S. cerevisiae* strains were maintained at -80 ºC in appropriate media supplemented with 30 % glycerol (v/v). Yeast strains were streaked onto agarized plates, cultivated at 30 ºC until growth in the plates was observed and then kept at 4 ºC until further use.

Cu exposure assays

Exposure assays were performed in liquid culture medium MM4 medium until reaching an optic density at 600nm (OD_{600nm}) corresponding to the exponential phase (OD600nm of 1.5 ± 0.1). Then cells were re-inoculated by filtration (Membrane filters white, 0.2 µm, Whatman™, ME24/21ST), at an OD600 nm= 0.5 ± 0.05, in 50 ml of fresh medium.

Exposure conditions

Cultures were supplemented with different concentrations (ranging from 6 to 100 mgCu/L) of CuO-NPs. Cells were grown at 30ºC with orbital agitation (250 rpm), and growth was followed by measuring culture OD600nm during batch cultivation period up to 72 h. Viability was determined by colony-forming units (CFU) counts repeatedly assessed during 3 days of incubation

at 30 ºC on MM4 agar plates. Viability assays were performed using cell aliquots taken throughout growth. 100 µl from 1/10 serial dilutions were inoculated in MM4 agar plates.

The protocol was also performed in cells supplemented with $CuSO_4$, with a range of concentrations of 0.06 to 200mg Cu/L.

Cells cultures (wild type and mutant strains) growing in pristine MM4 culture medium (without CuO-NPs or $CuSO_4$ supplementation) were taken as controls.

CuO-NPs characterization using Dynamic Light Scattering (DLS)

Malvern Zetasizer Nano-ZS instrument (Malvern Instruments Ltd, England), with temperature control, was used to determine the particle size distributions. The equipment uses the Dispersion Technology Software (DTS) (V4.20) for data collection and analysis. The mean particle diameter is calculated by the software from the particle distributions measured through the Z-Average (intensity harmonic for the average particle diameter) and Intensity Mean (diameter class average, most representative in terms of intensity); polystyrene standard spheres of 0.4 µm and 0.69 µm diameter (Bangs Laboratories, Inc.) were used to validate conditions of analysis. Agglomeration indexes of CuO-NPs in biological media were calculated through changes in Z-Average values over time. CuO-NPs size distribution remained stable for 8h.

Determination of protein free thiol groups

Protein thiol labelling and detection was based on the methodology proposed by Kim et al. [12]. The method allows the detection of proteins that contain sensitive cysteine residues, providing the identification of proteins that are oxidized in response to a variety of extracellular agents.

Yeast cells were harvested at exponential phase of growth (6 h) by centrifugation at 6000 rpm, 4 °C during 5min. Cell pellets were resuspended in 500 µL Tris buffer (Tris-HCl 10 mM; pH 6.5 adjusted with NaOH 1 M) containing protease inhibitors (10 mg/mL leupeptine; 1 mg/mL pepstatine A; 20 mg/mL aprotinine; 2 mg/mL trypsin/quimotrypsin inhibitor; 1.5 mg/mL benzamidine; 1 mM PMSF, all obtained from Sigma) and Maleimide– PEG2–biotin (Pierce), added to a final concentration 100 times higher than the estimated protein concentration. Cell lysates were incubated for 45 min at room temperature to allow alkylation by the maleimide–PEG2–biotin reagent of the nonoxidized protein thiols available at pH 6.5. The labeling reaction was quenched by the addition of dithiotreitol (DTT, Sigma) to a final concentration of 20 mM and the mixture was centrifuged at 5,500×*g* for 10 min. The resulting supernatant was collected and its protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Samples containing equal protein amounts (5 mg) were subjected to SDS-PAGE (10% polyacrylamide), transferred to nitrocellulose sheets, and incubated with horseradish peroxidase-conjugated streptavidin (Sigma-Aldrich, St. Louis, MO, USA) 1:100,000. The detection of protein thiol bound maleimide–PEG2–biotin was achieved through chemoluminescence using the ECL Western blot kit (Amersham).

Quantitation of Cu in yeast cells with Nuclear Microscopy

Sample preparation

An aliquot of 5 µl of the cell suspension was rapidly deposited on a 1.5 µm polycarbonate foil, quench-frozen at – 80 °C, and finally, cells were allowed to dry at – 25 °C in a cryostat. Cell quality and localization in the target for analysis was checked under

Distribution and depth profile of Cu in yeast cells

The nuclear microscopy technique is installed at the Van der Graaff accelerator of the Centro Tecnológico e Nuclear of IST. Analyses were carried out in vacuum using a proton beam of 2 MeV energy with a current of 100 pA. The lateral resolution under this irradiation conditions were typically ≤ 3 µm.

The techniques used were PIXE for Cu and other essential elemental detection, RBS for matrix composition and cell thickness determination and STIM for cell morphology identification. The normalization of the PIXE through RBS allows quantitative measurements of elemental concentrations. Concentrations were expressed in mg/kg of dry weight.

Maps of the scanned cells were generated assigning the various detector signals to a digital X–Y positional coordinate. The relative amount measured is represented by a colour gradient. The size of the micro-areas scanned was between 26 µm x 26 µm and 106 µm x 106 µm. Point analyses across cells rendering concentration profiles were produced. Acquisition and processing of data were performed using OMDAQ and DAN32 programs, respectively. [13,14]

Determining NPs depth profile in cells

To investigate the internalization of the CuO-NPs by the *S. cerevisiae* strains, the depth profiling capabilities of the RBS technique were explored. A proton beam of 2.0 MeV was used. The methodology consisted of dividing the barrier on RBS spectra, which correspond to copper, in energy intervals to estimate the amount of surface and internal Cu on the yeast cells. The cell thickness was assumed to be of 1 μm. The thickness interval corresponding to the backscattered energies between Ca and Cu was used. The depth resolution for protons was found to be of the order of 150-200 nm, which is enough to discriminate between the surface of the cell and the interior of the cell. The methodology can be applied safely as there are no other elements present in cell which can significantly interfere with the Cu barrier, having into account the realistic limit of 1 μm thickness for cells.

Statistical analysis

The cell elemental concentration data was obtained from serial points taken across cells. The elemental concentrations in control cells and cells exposed to Cu, either $CuSO_4$ and CuO-NPs were summarized as median and 25% and 75% interquartiles (IQ). The elemental concentrations in the different experiments using *S. cerevisiae* cells (wild type and the mutants) non-exposed and exposed to Cu were compared applying Kruskal-Wallis non-parametric test. Tests were considered significant when $p \leq 0.05$. Statistical analyses were performed with SPSS® Software version 22 (IBM Corp.).

Results

Copper susceptibility of *Saccharomyces cerevisiae* strains

Estimation of growth inhibitory Cu2+ concentration

Exposure tests of $CuSO_4$ in *S. cerevisiae* wild-type and single gene deletion mutant, *cup2Δ* had the purpose to estimate inhibitory Cu concentration levels of yeast growth that can be taken into account in the tests with Cu nanoparticles.

Concentrations of CuSO4 below 40 mgCu/L did not cause significant growth alterations. Above this concentration level a progressive inhibition of cell growth was detected up to 28 h.

Figure 1 illustrates the growth changes at tolerance limit of Cu^{2+} concentrations evaluated using CFU.

A delay in CFU increases during the first 10 h of growth was observed for 40 mgCu/L $CuSO_4$ exposure condition. However, after 24 h incubation, the CFU concentrations reached the values of controls (Figure 1). The CFU of cultures exposed to 60 mgCu/L

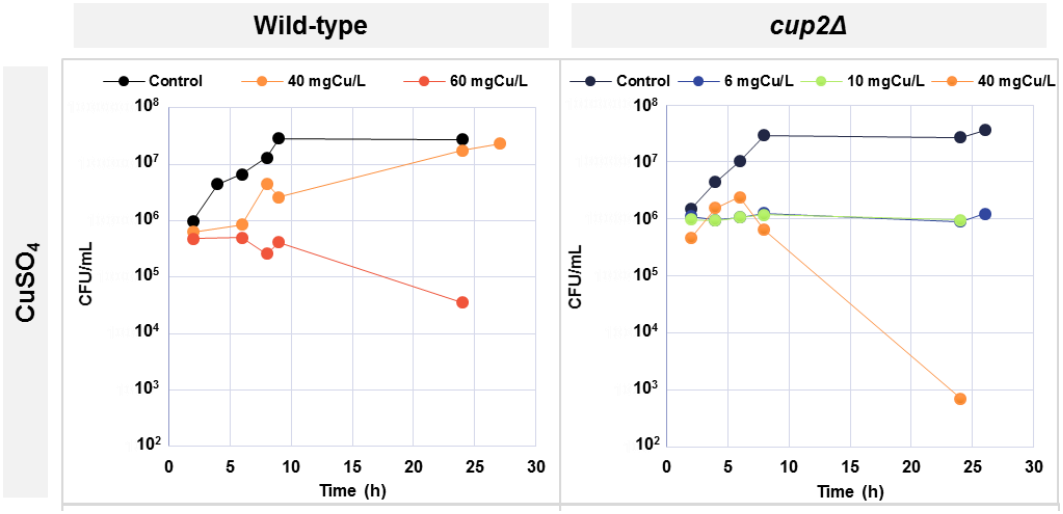

Figure 1 Colony unit formation of *Saccharomyces cerevisiae* BY4741 and BY4741_*Δcup2* in a MM4 liquid medium (pH 4.5) supplemented with different concentrations of $CuSO_4$.
Yeast viable cells in cultures supplemented with 6 to 60 mgCu/L $CuSO_4$. Control cultures were not supplemented with $CuSO_4$. The concentration of viable cells was assessed by colony forming units per millilitre of cell culture (CFU/mL).

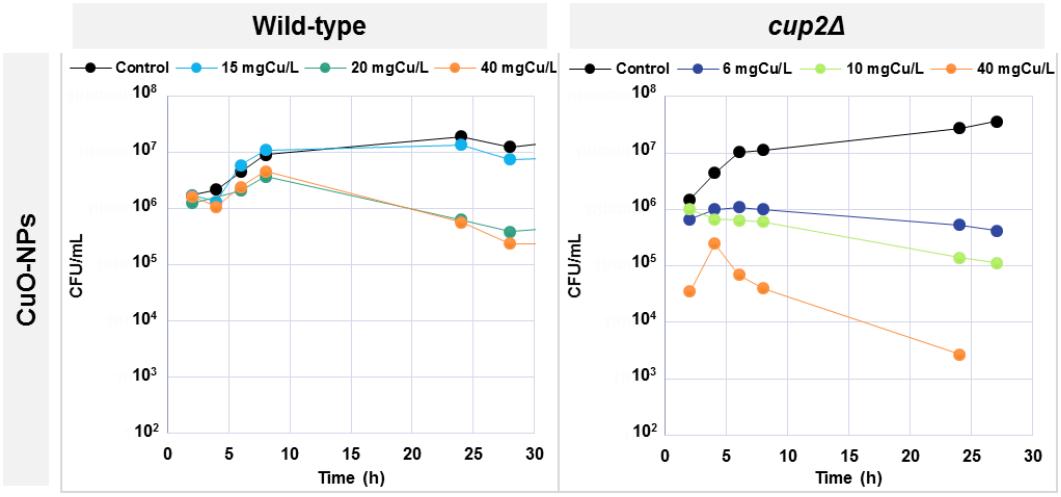

Figure 2 Colony unit formation of *Saccharomyces cerevisiae* BY4741 and BY4741_*Δcup2* in a MM4 liquid medium (pH 4.5) supplemented with different concentrations of CuO-NPs.
Yeast viable cells in cultures supplemented with 6 to 40 mgCu/L CuO-NPs. Control cultures were not supplemented with CuO-NPs. The concentration of viable cells was assessed by colony forming units per millilitre of cell culture (CFU/mL).

$CuSO_4$, remained stable for at least 8h of incubation, decreasing abruptly after 24h (Figure 1).

In *cup2Δ* cultures the CFU concentrations remained nearly constant up to 20 mgCu/L $CuSO_4$ concentrations over the incubation time. Figure 1 illustrates CFU evolution in *cup2Δ* cultures exposed to 6 10 and 40 mgCu/L $CuSO_4$. For the last concentration level the CFU drastically decreased after 8h, reaching concentrations 10,000 times lower than the controls.

The CFU assays demonstrated a higher susceptibility of the *cup2Δ* mutant for $CuSO_4$ when compared with the results obtained for the wild-type cultures. As an example, exposure to 40 mgCu/L of $CuSO_4$, yielded different outcomes. The wild type cultures even showing a slow growth increase were able to increase the CFU formation over incubation time, whereas the *cup2Δ* mutant displayed a CFU decrease for the same concentration.

The effect of CuO-NPs in S. cerevisiae strains growth

The effect of CuO-NPs in *S. cerevisiae* cultures was assessed by CFU assay.

The concentration of viable cell from cultures exposed to 10, 15, 20 and 40 mgCu/L CuO-NPs were assessed by the CFU assay for 30 h. Up to 15 mgCu/L growth did not significantly differ from controls. Above this CuO-NPs concentration level a decrease in the number of viable cells was observed up to 28h (Figure 2).

The analysis of the CFU numbers in cultures of *cup2Δ* mutant exposed to CuO-NPs, showed that the CFU concentrations decreased with incubation time and inhibition

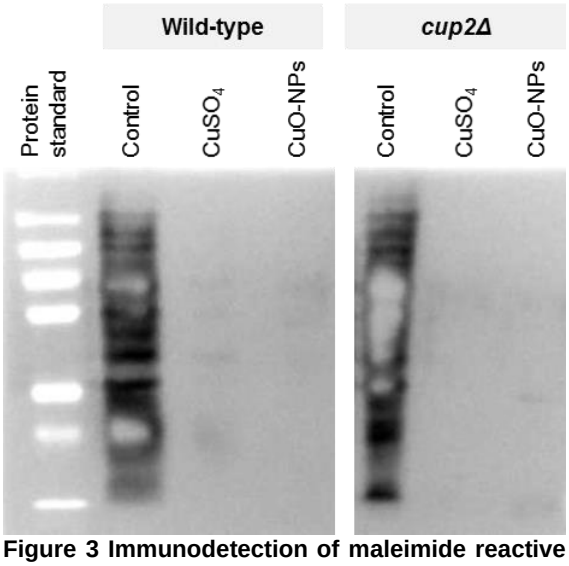

Figure 3 Immunodetection of maleimide reactive molecules expressing protein thiol reactive groups in *S. cerevisiae* wild type and cup2Δ exposed to CuO-NPs and $CuSO_4$.
Labeled protein extracts were obtained from WT cells and *cup2Δ* cells after cultivation for 6 h, exposure to 40 mgCu/L of $CuSO_4$ and 40 mgCu/L CuO-NPs. Controls were obtain from no supplemented cells. The white spots visible in the controls are due to the formation of air bubbles in sample.

is CuO-NPs concentration dependent (Figure 2).

The wild-type cultures were more susceptible to CuO-NPs than $CuSO_4$, in equivalent Cu concentrations. Whereas yeast cultures were able to endure exposures of 40 mgCu/L $CuSO_4$ when exposed to the equivalent-Cu concentration of CuO-NPs, a sharp decrease of viable cells were observed after 8h incubation. Furthermore, this pattern was displayed for Cu concentration levels as low as 20 mgCu/L CuO-NPs.

The results obtained with *cup2Δ* mutant strain showed similar susceptibility for both CuO-NPs and $CuSO_4$, suggesting that CuO-NPs mode of action may be similar to Cu^{2+} form.

Effect of CuO-NPs in protein oxidation

The effect of CuO-NPs on protein oxidation was assessed to inspect possible mechanisms of interaction of CuO-NPs with cells that may justify the detrimental outcomes observed on yeast growth. The sulfydryl-reactive agent maleimide–PEG2–biotin (MPB) was used to assess the reduction of thiolate anions in proteins as a measure of the oxidative damage.

The yeast strains exposed to CuO-NPs were studied against control cultures (non-supplemented) and positive controls supplemented with equivalent concentrations of Cu ($CuSO_4$). The results obtained for the presence of protein thiol groups were similar in both *S. cerevisiae* cells, wild-type and

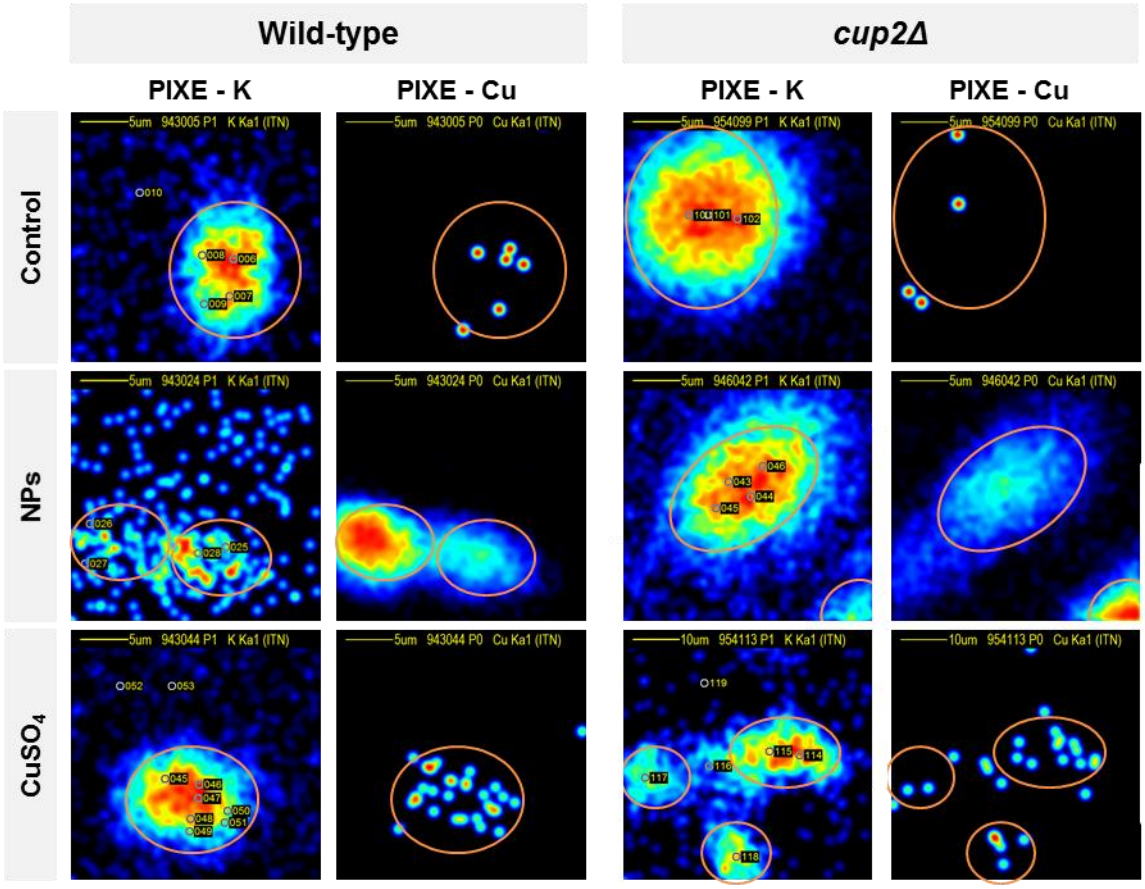

Figure 4 Nuclear Microscopy images of *S. cerevisiae* wild-type and *cup2Δ* cells.
Images of K and Cu, from *S. cerevisiae* wild-type cells and *cup2Δ* cells are displayed. Top panel: without Cu supplementation – Control; Middle panel: exposed to 40mgCu/L CuO-NPs; Low panel: exposed to 40mgCu/L $CuSO_4$. The images were obtained with PIXE technique using protons at 2.0 MeV as projectile. The dots displayed in the K maps indicated the regions used for the quantitative elemental determination.

cup2Δ. Cell extracts harvested after 6 h of exposure to both CuO-NPs and $CuSO_4$ Cu-equivalent concentration of 40 mgCu/L, showed almost undetectable MPB labeling (Figure 3).

This result strongly suggests that both $CuSO_4$ solution and CuO-NPs suspensions at the concentration examined induced large cysteine oxidation, either in wild-type or in *cup2Δ* cells.

Cu imaging and accumulation in *Saccharomyces cerevisiae* cells

Individual *S. cerevisiae* cells (wild-type and *cup2Δ*) were analysed with nuclear microscopy, in order to identify differences in intracellular Cu distribution. Typical images of Cu distribution in *S. cerevisiae* cells can be depicted in Figure 4. The distribution maps of K, a physiological important element, were also represented to help defining the cell boundaries.

The images of cells of both *S. cerevisiae* strains supplemented with CuO-NPs, showed significantly higher Cu levels than control cells. Comparing the Cu distribution maps, it can also be inferred that the cells exposed to CuO-NPs accumulated more Cu than cells exposed to the equivalent Cu concentration delivered by $CuSO_4$. For 40mgCu/L exposure conditions, the Cu concentration in cells exposed to CuO-NPs were of 263577±13693 mg/kg and of 1119±89 mg/kg in CuSO4 assays.

It is also possible to infer that in cells exposed to CuO-NPs, the Cu concentration in cells significantly increased as a function of exposure concentration (3044 mg/kg in cells expose to 15 mgCu/L; 130433 mg/kg in cells exposed to 20mgCu/L; and 263577 mg/kg in cells exposed to 40mgCu/L; $p<0.05$). This is not observable in cells exposed to $CuSO_4$, where the Cu concentration in cells exposed to 40 mgCu/L and 60 mgCu/L of $CuSO_4$ did not significantly changed (1119±89 mg/kg and 1524±240 mg/kg, respectively).

The Cu concentration in cells exposed to CuO-NPs decreased with exposure time, i.e., the Cu concentration in cells diminished after 24 h of exposure when compared to cells exposed for 6h to the same Cu concentration. This may indicate loss of cell integrity, as viability studies described previously suggested.

Examining the Cu concentrations in *S. cerevisiae* BY4741_Δ*cup2* mutant cells, exposed to both CuSO4 and CuO-NPs, similar results to those observed in wild-type cells were obtained. The Cu concentration, in Δ*cup2* cells exposed to CuO-NPs was 250 times higher than in cells expose to $CuSO_4$. Also, the increase of Cu concentration in cells was dependent on CuO-NPs exposure levels.

However, the images obtained of the Cu distribution in cells and the quantitative measures of Cu in cells cannot assure whether Cu was uptake by cells or is deposited on the cell surface.

To inspect at which distance from cell surface Cu can be found, a technique with depth resolution capabilities has to be applied.

Cu depth profiles in yeast cells

In order to investigate if the CuO-NPs or Cu-derived from NPs were internalized by *S. cerevisiae* cells, the capability of RBS depth profiling was explored.

The cell was divided in three layers, i.e., “top”, “in” and “down”. The depth of each slice corresponding to the energy interval that can be discriminated between the Ca and Cu backscattered energies was found to be of the order of 150-200 nm for protons (the projectile used).

In Figure 5 images of the Cu distribution for both studied strains can be depicted for the three layers defined. The “in” layer corresponds to intracellular medium, and the Cu signal appeared to be higher inside the cell than in “top” layer. The top layer mainly correspond to cell wall, which in *S. cerevisiae* is of the order of 150 nm[15].

These results showed that in both *S. cerevisiae* strains exposed to CuO-NPs, Cu was mainly found inside the cells.

It is not noticeable an increase in the copper content in the limits of the cells indicating that the CuO-NPs do not preferentially aggregate

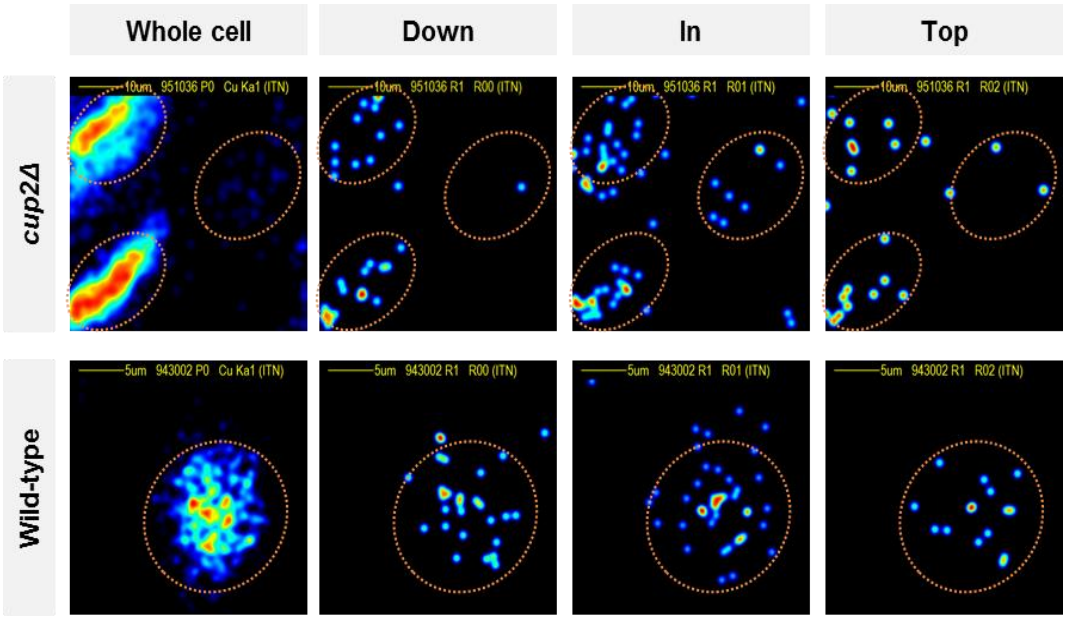

Figure 5 Imaging the Cu in *S. cerevisiae* WT and Δ*cup2* cells, and the Cu in different sections of the cell.
The images were obtain through RBS technique using protons at 2.0 MeV as projectile. Images of the copper content in *S. cerevisiae* cell after the exposure to CuO-NPs. The wild-type cells were expose to 20 mgCu/L CuO-NPs and the mutant cells (*cup2Δ*) were expose to 15 mgCu/L CuO-NPs. The images on the left display de image from the whole cell analysis. The three images from de right display the images obtained from different sections of the cell (top, in and down).

to cell wall. Instead, Cu was found to spread through the whole internal cell space.

The deepest layer ("down") may have a contribution of both backing where the cell is attached and the cell wall. Cells are attached to the polycarbonate foil (backing), which may retain CuO-NPs in culture media. Other possibility for Cu signal increment may derive of cell geometry changes. Attached cells on the foil may lose their typical spherical morphology, which may lead to a compression of cellular contents.

Therefore, the distribution images of Cu along cell depth unequivocally showed that Cu derived from CuO-NPs was internalized.

Discussion

In this study we analysed toxic effect of CuO-NPs in parental *S. cerevisiae* BY4741 (wild-type) and in the derived single deletion mutant BY4741_*Δcup2* (*cup2Δ*). The most notable results obtained in this study were: i) that the yeast cells accumulate and internalize more copper when exposed to CuO-NPs than when they are exposed to equivalent concentrations of $CuSO_4$; and that ii) CuO-NPs toxicity likely use cellular mechanisms involved in Cu^{2+}/Cu^{+} detoxification.

The assays with CuO-NPs showed that wild-type cultures were more susceptible do CuO-NPs than to $CuSO_4$ in equivalent Cu concentrations. A sharp decrease of CFU was verified for Cu concentration levels as low as 20 mgCu/L CuO-NPs, after 8h incubation, whereas yeast cultures exposed to $CuSO_4$ were able to endure concentrations as high as 40 mgCu/L. Opposite *cup2Δ* cultures exposed to CuO-NPs showed similar CFU concentrations to $CuSO_4$, indicating similar susceptibility of *cup2Δ* for both Cu compounds. This suggests that detoxification mechanisms activated by CUP2 gene is also acting in the detoxification of CuO-NPs. Kasemets et al.[7] also studied the effect of CuO-NPs on S. cerevisiae strains, including Cu stress response deficient *cup2Δ* strain. The reported results corroborate our findings and the authors hypothesized that the toxic effect of CuO-NPs may act through dissolved Cu ions. The massive oxidation observed of cysteine groups of cellular proteins in both strains (wild-type and *cup2Δ*) when exposed to CuO-NPs, strengthens the view that the redox balance in yeast is affected through the

action of Cu derived from the nanomaterial. Further, since the used assay determines the proteins that were oxidized by H_2O_2[12], the results also point to the effect of CuO-NPs in H_2O_2 stress mechanism. The *S. cerevisiae* Cup1 and Crs5 metallothioneins scavenge excess Cu by tight coordination with cysteine thiolates (Festa et al[16]). Therefore, the extensive protein oxidation observed in the *cup2Δ* mutant cultures after CuO-NPs exposure clearly related the metallothioneins detoxification mechanism of Cu derived from CuO-NPs. However, only high levels of Cu were tested in this prospective study, which hindered to discriminate between $CuSO_4$ and CuO-NPs protein oxidation levels.

Yeast cells of both strains accumulated more Cu when exposed to CuO-NPs, although the cellular Cu concentration diminished after 24 h of exposure. The profile of Cu along the cell depth also demonstrated that Cu content is higher inside yeast cells than on cell surface. These results strongly suggest that CuO-NPs can be transferred to the yeast cell intracellular environment. Similar findings were described for mammalian cells. Cronholm et al[17] reported on higher uptake of CuO-NPs compared to nil or low uptake of the soluble salts. Bondarenko et al[3] suggested a CuO-NPs uptake by endocytose (Trojan horse model). These authors also hypothesized that when inside the cell, CuO-NPs may solubilize obstructing the regulator mechanisms for Cu ions in the cell (e.g., detoxification mechanisms) hindering their normal function. Yet, the mechanism by which CuO-NPs can enter in the yeast cell is unknown. The toxicity of CuO-NPs was proposed to cause damage in cellular membrane. Karlsson et al.[6] have studied the effect of the CuO-NPs in biological membranes demonstrating that CuO-NPs can interact by adsorbing on the phospholipid membrane surface, giving rise to alterations on its viscoelastic properties. These findings could explain the viability loss and the decrease of Cu content observed in yeast cultures exposed to CuO-NPs at 24h of exposure.

Conclusions and future perspectives

This study highlighted the potential of *S. cerevisiae* as a model to study CuO-NPs toxicity and the adequacy of screening methodologies developed. In this work images of the Cu distribution in whole cells and Cu depth profiles were established, demonstrating for the first time the internalization of Cu derived from CuO-NPs in *S. cerevisiae* cells wild-type and *cup2Δ* cells. It was demonstrated that CuO-NPs exposure caused protein oxidative damage, which may be due to Cu derived from CuO-NPs internalized in the cell. “How the CuO-NPs enter the cell?” and which metabolic pathways are affected by the CuO-NPs?” are two important questions that remain unanswered. However, the work carried out helped establishing strategies to study mechanisms of nanoparticle toxicity.

Some of the issues that would be important to improve are: i) the study of protein oxidation using lower CuO-NPs concentrations and smaller exposure incubation times; ii) optimize nuclear microscopy experimental conditions to enhance Cu depth profile resolution and enable 3D imaging to identify at which level the NPs act in the cell; iii) extend the study to other mutants associated to different transporters and metabolic pathways. These would enable a better understanding of uptake mechanisms and of the cell responses to CuO-NPs.

Acknowledgments

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