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Genotoxicity of gold nanoparticles functionalized with indolicidin towards *Saccharomyces cerevisiae*

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ABSTRACT

The toxic effects of gold nanoparticles surface-functionalized with the antimicrobial peptide indolicidin (AuNPs-indolicidin) towards the yeast *Saccharomyces cerevisiae*, one of the major eukaryotic model organisms, have been evaluated. Growth and survival, genotoxicity, as measured by comet assay, and expression of the YCA1, an apoptosis indicating gene, following 72 hr exposure of yeast to AuNPs-indolicidin, and to AuNPs and indolicidin alone have been examined. The gold nanoparticles exerted toxicity with DNA damage, accompanied by reactive oxygen species production (ROS), but they do not inhibit yeast growth and viability. Genotoxicity was less pronounced for surface-functionalized nanoparticles, showing that *S. cerevisiae* is quite resistant to the complex AuNPs-indolicidin. A progressive reduction of the genotoxic effect was observed along 72 hr exposure, presumably due to the activation of DNA repair mechanisms. These findings suggest the occurrence of a physiological protective response of *S. cerevisiae* towards nanoparticles, thereby providing useful information to the assessment of the environmental impact of metal nanoparticles.

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Introduction

The increased use of engineered nanoparticles (NPs) in commercial products (cosmetics, electronics, paints, medical devices, food, packaging, catalysts, antimicrobial fabrics, water treatment membranes) (Aitken et al., 2006; Handy et al., 2008; Karnik et al., 2005; Roco, 2003; Savolainen et al., 2010) unavoidably leads to their significant accumulation in the earth with great concerns regarding the possible adverse effects on environment as well as on human health. Therefore, assessment of NP toxicity is needed before any application and it is strictly necessary when NPs are used in the biomedical field as drug or gene delivery systems or for other

therapeutic purposes. In particular, gold nanoparticles (AuNPs) have received significant attention because of their unique physico-chemical properties that make them well suited for biomedical applications (Levy et al., 2010; Ma et al., 2011).

A number of studies have evaluated the toxicity of a variety of AuNPs with different sizes and coatings (Alkilany and Murphy, 2010; Murphy et al., 2008), showing that AuNPs are mainly inert, though some authors report about their cytotoxicity (Pan et al., 2014; Tiedemann et al., 2014). Toxicity tests were mainly performed on freshwater algae (Renault et al., 2008), daphnias (Galdiero et al., 2015; Li et al., 2010), zebrafish embryos (Browning et al., 2009) and adult zebrafish (Geffroy et al., 2012).

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However, results are often contradictory even when using the same bioindicator (Asharani et al., 2011), due to the variety of nanoparticles examined and the multiplicity of the experimental approaches (Harper et al., 2008).

The antimicrobial peptide indolicidin is a representative of the cathelicidin host defence peptides (HDP), and was chosen because of its many activities such as antibacterial, antifungal, antiparasitic, antiviral, immunomodulator and inhibitor of aminoglycoside antibiotic resistance enzymes (Kovacs-Nolan et al., 2009; Sur et al., 2015). However, indolicidin may be degraded by proteases thus its therapeutic use may be greatly strengthened following conjugation with nanoparticles.

In this work, we conjugated indolicidin to 5 nm-diameter AuNPs in the presence of citrate buffer (Turkevich et al., 1951). The conjugation to AuNPs may allow the nanosystem to enter cells using the endocytotic mechanism in a manner dependent on its size and shape, and to reach its subcellular target; this conjugation may allow to control the specificity for the target, stability and release of the drug from the nanoparticle. The toxicity of AuNPs-complexes originates from two features: interactions with the negatively charged cell and subcellular membranes, and ability to disrupt the membranes and escape from vesicles.

Multiple organisms are used in ecotoxicology studies of engineered nanomaterials including bacteria, fungi, algae and crustaceans, whereas scarce studies report on the use of the yeast *Saccharomyces cerevisiae*. On the contrary, yeast is frequently used in toxicology evaluations of chemicals such as heavy metals, anti-cancer drugs, and herbicides (Buschini et al., 2003; Cabral et al., 2003; Schmitt et al., 2004). *S. cerevisiae*, one of the major eukaryotic model organisms, is largely used in molecular and cell biology studies since its cellular structure and organization is similar to that of higher organisms, with the advantages of a shorter generation time, easier manipulation and well-established cultivation techniques. Therefore, *S. cerevisiae* could also provide clues to understand nanotoxicity in mammalian cells and environmental organisms: not only the yeast cellular machinery and functional organization have many similarities with those of higher eukaryotes, but also the oxidative stress response and the family of drug resistance pumps are also present. However, the sensitivities of individual yeast cells to oxidative stressors are heterogeneous and fluctuate during yeast metabolic oscillations but their major consequences are apoptosis (Madeo et al., 2002).

A few studies have investigated the potential impact of NPs on yeast. Kasemets et al. (2009) showed that the yeast *S. cerevisiae* is relatively resistant to CuO and ZnO-NPs when compared with bacteria and algae. Lee et al. (2008) reported that *S. cerevisiae* showed a higher survival rate than *Escherichia coli* and *Bacillus subtilis* after exposure to AgNPs. Overall, the experimental results reported in these studies show a relatively high robustness of yeast towards nanoparticle exposure, but toxicity mechanisms are still poorly understood. In this preliminary study, we have investigated the potential effect of the novel AuNPs-indolicidin complex on this representative organism, and explored possible toxicity mechanisms, in order to permit safe pharmacological applications and estimate how changes on the surface of Au-NPs due to the fact that functionalization might modify nanoparticle reactivity.

Considering that exposure may not produce evident cytotoxic effects on a robust cell such as yeast, we have focused on the possible genotoxic effect of the AuNPs-indolicidin complex, evaluated by the alkaline comet assay, during a long-term (72 hr) exposure and compared to that exerted by the AuNPs and indolicidin alone.

The yeast comet assay is a fast and sensitive technique to measure oxidative DNA damage, Deoxyribonucleic acid (DNA) damage repair, and the genotoxic or protective effects of chemicals (Azevedo et al., 2011; Oliveira and Johansson, 2012). Although several protocols exist for preparing slides, lysing cells, performing electrophoresis and staining slides, results are remarkably similar for mammalian cells using most of the published methods (Olive and Banath, 2006).

S. cerevisiae cells turned out to be more sensitive than mammalian cells to the action of different substances like as methyl methane sulfonate and hydrogen peroxide (Miloshev et al., 2002). The higher sensitivity of *S. cerevisiae* cells towards γ -irradiation (Nemavarkar et al., 2004), oxidative damage during replicative ageing (Grzelak et al., 2006), and Cr-(III)-organic compounds (Chatterjee and Luo, 2010) revealed by the comet Assay was confirmed (Azevedo et al., 2011; Hrenović et al., 2010; Lah et al., 2004). Currently the technique was upgraded in order to obtain higher sensitivity and reproducibility of the results (Peycheva et al., 2009).

In this work, the genotoxic effect has been related to a general oxidative stress response, evidenced by reactive-oxygen-species (ROS) production, and also to the expression of the apoptosis-indicating gene YCA1 (Madeo et al., 2002).

1. Materials and methods

1.1. Peptide synthesis

The peptide was synthesized using standard solid-phase-9-fluorenylmethoxycarbonyl (Fmoc) method as previously reported (Galdiero et al., 2003). Briefly, the peptide was obtained using a MBHA (0.6 mmol/g, Sigma Aldrich, Italy) resin by consecutive deprotection and coupling steps. Peptide deprotection was performed with a solution of 30% piperidine (analytical grade, Sigma Aldrich, Italy) in Dimethylformamide (DMF, Labscan Ltd., Dublin, Ireland), 5 min twice. The coupling was conducted with 2 equivalents of amino acid in presence of 2 equivalent of PyBop (0.5 mol/L, Sigma Aldrich, Italy, in DCM) and 4 equivalent of *N,N*-Diisopropylethylamine (DIPEA, Sigma Aldrich, Italy,) (2 mol/L in NMP) for 40 min. The peptide was cleaved from the resin and deprotected by treatment with a solution of trifluoroacetic acid (Sigma Aldrich, Italy, analytical grade) and scavengers for 300 min. trifluoroacetic acid (TFA) was concentrated and peptide was precipitated in cold ethylic ether (Sigma Aldrich, Italy, analytical grade). Analysis of the crude was performed by LC-MS using a gradient of acetonitrile for HPLC (high performance liquid chromatography)-SUPER GRADIENT (0.1% TFA) in water (0.1% TFA) from 5% to 70% for 15 min. Purification was performed by preparative RP(reversed phase)-HPLC using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 5% to 70% for 20 min. Purified peptide was obtained with good yields (30%–40%). Peptide sequence: Ac-CILPWKWPWWPWRR-CONH₂.

1.2. Conjugation to AuNPs and characterization

Purified peptide was conjugated to AuNPs (Sigma-Aldrich, USA, Cod. 752,568) having a 5 nm diameter (5.47×10^{13} nanoparticles/mL) in presence of 10 mmol/L citrate buffer pH 6 at room temperature overnight as previously reported (Takatsuji et al., 2012). The resulting NPs were purified by centrifugation ($14,000 \times g$ for 30 min) and the pellet was resuspended in 2.5 mL of 5 mmol/L Hepes, 100 mmol/L NaCl and 0.05% (V/V) of Triton X-100. Fluorimetric measurements were carried out using a Varian Carry Eclipse spectrometer, to calculate the amount of peptide attached to AuNPs. The measurements were obtained exciting the samples at 280 nm and recording the emission spectrum in the range of 290 to 500 nm, using an excitation and emission slit of 5 nm. The final peptide concentration was 50 $\mu\text{mol/L}$.

Zeta potential (ZP) measurements of AuNPs and AuNPs-indolicidin were performed using a Malvern Nanosizer Nano ZS (Malvern Instruments, Worcestershire, UK) at 25°C, pH 6 at the same condition of conjugation. All measurements were performed in triplicate for each sample.

1.3. Yeast strain and cultivation conditions

S. cerevisiae BY4741 (MATa *ura3 Δ 0 leu2 Δ 0 met15 Δ 0 his3 Δ 1*) from a stock culture kept at -80°C was grown in yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% polypeptone, 2% glucose, W/V) at 28°C with shaking at 200 r/min. The cells were harvested by centrifugation ($1000 \times g$ for 5 min), washed 3 times with phosphate buffer solution (PBS, containing KH_2PO_4 0.2%; $\text{Na}_2\text{PO}_4 \cdot 12 \text{H}_2\text{O}$ 2.9%; NaCl 8%; KCl 0.2%, W/V, pH 7) and resuspended in fresh YPD medium. This cell suspension (pre-culture) was used to inoculate the cultures where toxicity of AuNPs, AuNPs-indolicidin, and indolicidin was tested.

1.4. Toxicity of AuNPs, AuNP-indolicidin, and indolicidin towards yeast cells

Toxic effect of AuNPs, AuNPs-indolicidin and indolicidin was evaluated as inhibition of growth and reduction in cell viability occurring during aerobic cultivation of yeast in the presence of the three different toxicants (exposed cultures) with respect to a control (non-exposed culture). Cultures were performed in shake flasks containing YPD medium to which the NP suspensions or the 50 $\mu\text{mol/L}$ indolicidin solution were added (75%, V/V). To avoid agglomeration or aggregation of the NPs, Tween 80 10:1 (W/W) was added to the culture medium as dispersant. Tween 80 was previously demonstrated not to have inhibitory action on *S. cerevisiae*. The cultures were inoculated with cells collected from a pre-culture in YPD, so that initial optical density of a sample measured at a wavelength of 590 nm per mL ($\text{OD}_{590}/\text{mL}$) was 0.1. The flasks were incubated at 28°C with shaking at 200 r/min.

Inhibition of growth was evaluated by determination of total biomass in exposed and non-exposed cultures after 24, 48 and 72 hr by optical density (OD_{590}). To determine cell viability, cells from exposed and non-exposed cultures were collected at 24, 48, and 72 hr, properly diluted and plated on YPD medium supplemented with 2% agar. Plates were incubated at 30°C for 72 hr and colony counting was expressed in CFU/mL.

1.5. Spheroplast preparation

Aliquots of cells ($\text{OD}_{590} = 2.0$) from exposed and non-exposed cultures were collected after 24, 48 and 72 hr by centrifugation at $1000 \times g$ for 5 min, washed with cold water, resuspended in 0.3 mL of 100 mmol/L Tris-HCl buffer pH 8, containing 5 mmol/L EDTA and 5 mmol/L dithiothreitol (Sigma-Aldrich, USA) and incubated at 28°C for 30 min with gentle agitation. Then, cells were collected ($1000 \times g$, 10 min) washed twice in 10 mmol/L Tris-HCl buffer pH 7 and resuspended in the same buffer containing 1 mol/L sorbitol and 5 mg/mL Zymolyase (Zymolyase® 20 T, Seikagaku Co., Japan). The suspensions were incubated at 28°C for 2 hr with gentle agitation, checking spheroplast formation at the optical microscope. The spheroplast suspensions were then centrifuged at $70 \times g$ for 5 min, the pellets washed three times with 1 mol/L sorbitol and resuspended in 1 mol/L sorbitol. The comet assay was carried out with the spheroplast suspensions so prepared.

1.6. Comet assay

The alkaline comet assay was performed as described by Miloshev et al. (2002) modified as follows: 20 μL of spheroplast suspension was mixed with 1% of low melting-point agarose (LMA; Sigma-Aldrich, USA) and spread into a slide containing a layer of 1% normal melting-point agarose (NMA; Sigma-Aldrich, USA). A third layer of 1% LMA was spread in the slide. The slides were rinsed for 20 min in an alkaline buffer (30 mmol/L NaOH, 10 mmol/L EDTA and 10 mmol/L Tris-HCl pH > 12; Sigma-Aldrich, USA) to unwind DNA and after subjected to electrophoresis in the same buffer for 20 min at 20 V, 300 mA. After the electrophoresis the gels were neutralized two times for 5 min each with 0.4 mol/L Tris-HCl pH 7.5. Slides were stained with 10 $\mu\text{g/mL}$ DAPI (Sigma-Aldrich, USA) and observed under fluorescent microscope (Leica DMLB microscope with digital camera Leica DFC340FX, Nussloch, Germany). Two slides were prepared for each sample considering a minimum of 50 randomly selected nuclei for each slide. Quantitative assessment of DNA damage in selected nuclei was performed using Comet Score 1.5 Image Analysis (TriTek Corporation, Sumerduck, Virginia, USA) software, which computes the integrated intensity profile for each nucleus. The yeast comets were analysed by calculating the tail moment ($\text{TM} = \text{tail length} \times \text{DNA\% in the tail} / 100$).

1.7. ROS detection

The intracellular ROS generation was detected using 2',7'-dichlorofluorescein diacetate (DCFH-DA) staining. ROS generation was measured in both control and AuNPs, AuNPs-indolicidin and indolicidin-treated cells after 24, 48, 72 hr of incubation according to the method of Wang and Joseph (1999) with minor modifications. Cell suspensions ($\text{OD}_{590} = 1.0$) were mixed with 10 μg DCFH-DA (Sigma-Aldrich, USA) dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA). After incubation at 30°C with shaking for 60 min, cells were harvested, washed and resuspended in PBS.

The fluorescence intensity of DCFH-DA dye was measured using fluorescence spectroscopy with excitation and emission

wavelengths of 488 and 525 nm, respectively. The increase in fluorescence intensity yielded the ROS quantity.

1.8. Enzymatic activity assays

Aliquots of cells from exposed and non-exposed cultures corresponding to $OD_{590} = 1.0$ were harvested, washed in PBS and resuspended in 0.2 mL of lysis buffer (50 mmol/L phosphate buffer, pH 7, containing, 1 mmol/L EDTA and 1 mmol/L henylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, USA) and broken with glass beads (diameter 400 μ m). The cell lysate was centrifuged (1000 $\times g$, 5 min) and the supernatants used for catalase (CAT) and superoxide dismutase (SOD) determinations. Protein concentration in the lysates was quantified spectrophotometrically at 595 nm, according to the Bradford method using bovine serum albumin as standard (Bradford, 1976).

CAT activity was determined using a commercial Kit (Sigma Aldrich, USA) according to the manufacturer's protocol, and evaluated as decrease in absorbance at 240 nm due to H_2O_2 consumption. CAT specific activity was expressed as μ mol H_2O_2 consumed per min per mg of total proteins in the lysates. Superoxide dismutase (SOD) activity was determined using a SOD assay kit (WST Sigma-Aldrich, USA) according to manufacturers. The SOD activity was calculated as an inhibition activity by measuring the decrease in the colour development at 440 nm. The antioxidant enzyme activities were presented as mean \pm standard deviations from three to five replicates.

1.9. YCA1 gene expression

Total ribonucleic acid (RNA) was extracted and purified using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). The quality and amount of purified RNA was analysed spectrophotometrically with Nanodrop2000 (Thermo Scientific Inc., Waltham, MA, USA). The 1000 ng of RNA was reverse transcribed with the QuantiTect® Reverse Transcription Kit (QIAGEN, Valencia, CA, USA), used as described by the manufacturer. Afterwards, we performed a Real Time PCR using the QuantiTectSYBR Green PCR Kit (QIAGEN, Valencia, CA, USA). PCR was performed in a final volume of 25 μ L, with 100 ng of cDNA, 1 μ mol/L of each primer, 12.5 μ L of QuantiFast SYBR Green PCR Master Mix (2 \times). PCR cycling profile consisted of a cycle at 95°C for 5 min, 40 three-step cycles at 95°C for 15 sec, at 60°C for 60 sec and at 72°C for 40 sec. Quantitative RT-PCR analysis was conducted by using the $2(-\Delta\Delta C(T))$ method (Livak and Schmittgen, 2001). RT-PCR was performed in a Rotor-Gene Q cycler (QIAGEN, Valencia, CA, USA). For each RT-PCR experiment, data were normalized to the expression of the actin housekeeping gene. We analysed a fragment of 93 bp of the coding region of the YCA1 and 140 bp of the actin genes. The primers used for each gene were as follows: YCA1 forward 5'-GCAATATGCTCCACCA CCAGTCC-3', reverse 5'-TGTGCCTTTGCCTGTTCCTGTGCT-3'; ACT forward 5'-ACGTCGCCTTGGACTTCGAACA-3', reverse 5'-AGGATGGAACAAAGCTTCTGGGGC-3'. Each sample was tested and run in duplicate.

Two biological replicates were performed and the statistically significant expression changes were calculated using one-way ANOVA. The level of significance was also

determined by the Bonferroni method comparing all groups versus the control.

2. Results

Indolicidin was covalently conjugated to AuNPs using thiol chemistry, as described elsewhere (Takatsuji et al., 2012). The conjugation reaction was followed by fluorescence spectroscopy. As shown in Fig. 1, the amount of peptide bound is more than 90%. Moreover, the zeta potential of AuNPs functionalized is -15.3 mV with a standard deviation of 0.6, and that of AuNPs is -22.1 mV with a standard deviation of 1.3. This result shows that the presence of the peptide on the surface does not significantly change the charge of the nanoparticles, which still show a little tendency towards aggregation.

The yeast growth in nutrient medium and in the presence of AuNPs, AuNPs-indolicidin and indolicidin, was spectrophotometrically determined at 590 nm, after 24, 48, 72 hr of incubation. Cell viability was also tested in relation to the untreated sample. Results are reported in Fig. 2. Results showed no growth inhibition after administration of AuNPs, AuNPs-indolicidin and indolicidin, and also cell viability was not influenced by the toxicants. Only after 72 hr of incubation, we noticed a slight decrease of viability in all the samples, which could be attributed to the physiological ageing of cultures.

Genotoxicity of AuNPs, AuNPs-indolicidin and indolicidin towards *S. cerevisiae* was evaluated after 24, 48, and 72 hr of incubation by comet assay. Results are reported in Fig. 3. DNA damage was measured as TM. We noticed that treatment with AuNPs, AuNP-indolicidin and indolicidin, after 24 hr, induced a consistent increase of DNA damage compared to the control. The increase is more evident when we treated yeast cells with indolicidin alone, whereas when cells were exposed to the functionalized complex AuNP-indolicidin, the genotoxic damage was less pronounced. When incubation time reached 48 and 72 hr, a progressive decrease of DNA damage in exposed yeast was observed, maybe due to the activation of natural

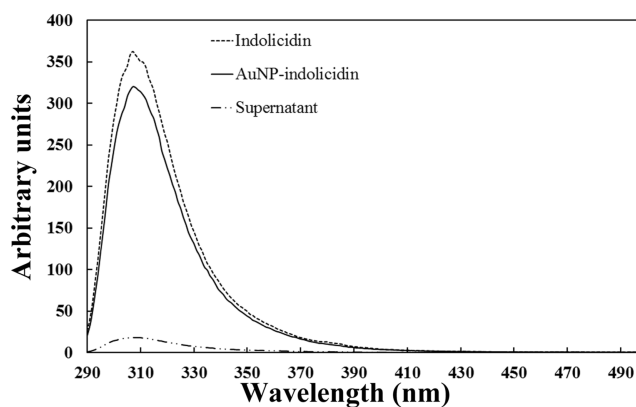


Fig. 1 – Fluorescence emission of free indolicidin, AuNPs and of the complex AuNPs-indolicidin. Also fluorescence emission of the supernatant after the purification of the complex by centrifugation is reported (for details see Materials and Methods). AuNPs: gold nanoparticles.

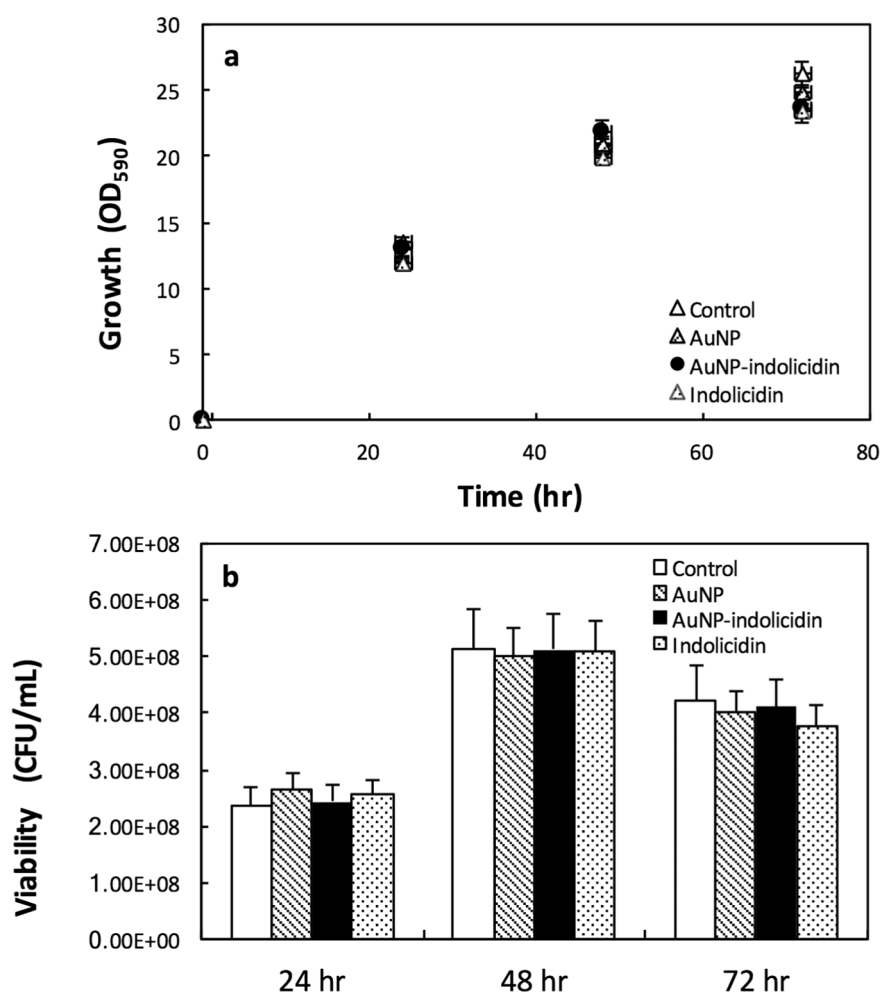


Fig. 2 – Effect of AuNPs, AuNPs-indolicidin and indolicidin on growth (a) and viability (b) during 72 hr cultivation of *Saccharomyces cerevisiae*. Data presented are the means of three independent experiments \pm standard deviations.

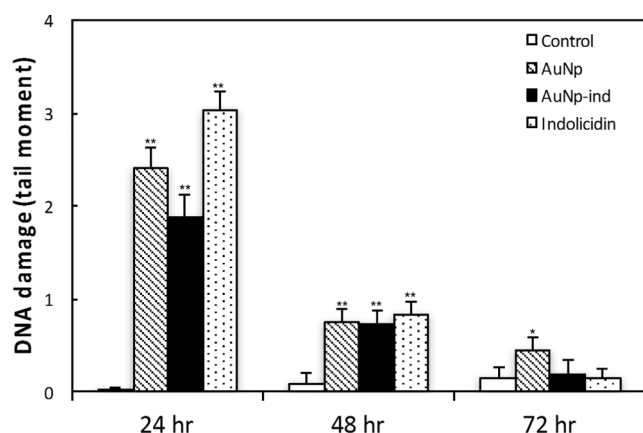


Fig. 3 – DNA damage of *Saccharomyces cerevisiae* exposed to AuNPs, AuNPs-indolicidin and indolicidin for 24 hr, 48 hr, and 72 hr. Results are expressed as median values with the standard error. Nonparametric test (Kruskal-Wallis test) was used to evaluate the difference in time-reliable cell responses for different time and treatments, made one by one population versus control. Statistically significance changes in DNA damage with controls are represented with an asterisk (** $p < 0.001$; * $p < 0.05$).

repair profiles of cells. As expected, in the control samples, TM was progressively higher at 48 and 72 hr with respect to 24 hr, as a consequence of ageing of the culture.

Further, to investigate the relation between genotoxicity and a possible oxidative stress exerted on yeast cells exposed to AuNPs, AuNPs-indolicidin and indolicidin, we monitored the ROS production and the activation of the oxidative enzymes, CAT and SOD.

Our results are reported in Fig. 4 and Table 1. The level of ROS in yeast cells was determined by DCFH-DA, which is oxidized by ROS to the fluorescent chromophore DCF (2,7-dichlorofluorescein).

We showed that exposed cells enhanced intracellular ROS level at 24 hr, whereas, we observed an evident decrease at 48 and 72 hr, in concomitance with the reduction of the genotoxic damage. As regards CAT and SOD activities the results did not show any activation in the exposed cultures. In all samples, CAT activity increased during exposure as a consequence of the ageing of the cultures.

To show the connection between DNA damage, ROS production and possible occurrence of apoptosis, we investigated the expression of the apoptosis-indicating gene YCA1 in our samples at 24, 48, 72 hr after exposition to AuNPs,

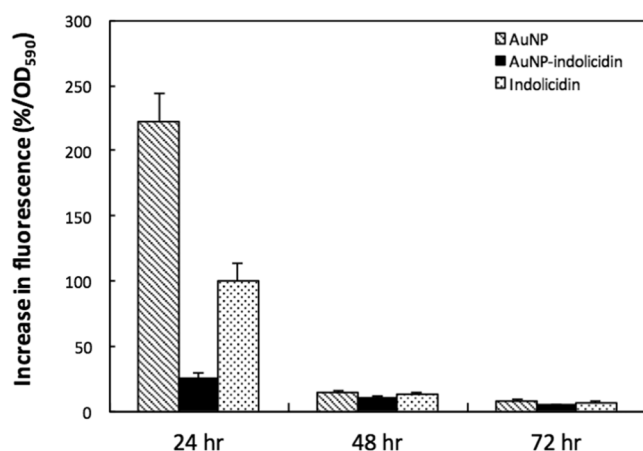


Fig. 4 – ROS production expressed as increase in dye fluorescence per OD₅₉₀ of yeast cells exposed to AuNPs, AuNPs-indolicidin and indolicidin after 24, 48, and 72 hr. Data presented are the means of three independent experiments \pm standard deviations. ROS: reactive oxygen species.

AuNPs-indolicidin and indolicidin (Fig. 5). A significant increase in YCA1 gene expression in cells exposed to AuNPs and indolicidin for 24 hr was detected; meanwhile no-effect was observed when cells were treated with AuNPs-indolicidin. The YCA1 expression at 48 and 72 hr in all the exposed samples remained stable at levels statistically not significant.

3. Discussion

The occupational and public exposure to nanomaterials is increasing dramatically and so there is an urgent need for information on toxicity and safety of manufactured nanomaterials.

To the best of our knowledge, the present paper is one of the few studies evaluating the toxicity of AuNPs on yeast cells. We examined the effect of exposure of *S. cerevisiae* to AuNPs functionalized and not with an antimicrobial peptide, focusing on their genotoxicity and showing a link between oxidative stress, genotoxicity and gene expression of YCA1, an apoptosis-indicating gene.

S. cerevisiae is a simple, yet efficient model to study the biological effects of NPs in eukaryotic cells; moreover, it is

suitable to measure biological responses for assessing nanotoxicity.

Among the ecotoxicological studies, a number of authors have used the microbial eukaryotic model *S. cerevisiae*. The use of yeast as a test organism is correlated to the fact that it is non-pathogenic, simple, easy to cultivate, has a short generation time, has a fully annotated genome and shows a strong conservation, at both metabolic and regulatory levels, with experimentally less accessible higher eukaryotes (Daniel et al., 2004). Surprisingly, the cytotoxicity of NPs to yeast is still poorly understood and a very few toxicity studies have considered the impact of NPs on *S. cerevisiae*.

Hence, the objective of this study was to apply the yeast as a tool to gain new insights into the ecotoxicological impact of NPs functionalized or not. Our data show that yeast is resistant to the effect of tested NPs, since no significant reduction on growth and cell viability was observed. Moreover, toxicity tests showed that yeast was also resistant to indolicidin alone.

ROS formation is one of the mechanisms of NPs toxicity which is largely reported to cause oxidative stress (Khalili Fard et al., 2015), and consequent damages to the proteins, cell membranes and also DNA. It is well known that the exposure of yeast cells to a variety of toxicants including NPs, is characterized by ROS production which determines an oxidative stress response (Bayat et al., 2014). In yeast, the response to ROS depends on the dose: at very low doses cells can adapt to become more resistant, at higher doses the cells activate various anti-oxidant functions, that is the complex anti-oxidant defence system which includes a wide range of metabolites, enzymes, and specific transcriptional factors (Perrone et al., 2008).

In this work, we have observed a genotoxic effect indicative of a DNA damage accompanied by ROS production in the yeast cells exposed for 24 hr to AuNPs, AuNPs-indolicidin and indolicidin. The damage was revealed by comet assay, largely employed in ecotoxicological studies. As a matter of fact, also in the case of a robust unicellular organism as yeast, in which we do not observe any evident decrease in vitality and viability after the exposure, the comet assay is a powerful tool to evidence DNA damage and DNA repair (Azevedo et al., 2011). Apparently, the DNA damage observed in our experiments is the result of ROS induction. Since both DNA damage and ROS production decrease during exposure, the anti-oxidant defence system of the cells was sufficient to face the ROS production, so that specific DNA-repair pathways may be activated.

Table 1 – Catalase and superoxide-dismutase in lysates from yeast cells exposed and not exposed to AuNPs, AuNPs-indolicidin and indolicidin.

	Catalase activity ($\mu\text{mol/L H}_2\text{O}_2$ decomposed/min)			Superoxide-dismutase activity (% control activity)		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Control	14.7 \pm 0.7	40.1 \pm 2.4	35.3 \pm 1.7	89.5 \pm 5.0	88.0 \pm 4.8	91.3 \pm 5.3
AuNP	16.2 \pm 0.7	55.0 \pm 3.3	39.0 \pm 1.9	93.2 \pm 5.1	94.0 \pm 5.0	93.8 \pm 4.9
AuNP-indolicidin	17.1 \pm 0.8	68.8 \pm 4.1	46.6 \pm 2.3	94.3 \pm 4.8	95.0 \pm 5.5	92.9 \pm 4.8
Indolicidin	16.5 \pm 0.8	41.4 \pm 2.4	36.6 \pm 1.8	83.5 \pm 5.5	92.3 \pm 4.9	90.9 \pm 5.1

Data presented are the means of three independent experiments \pm standard deviations.

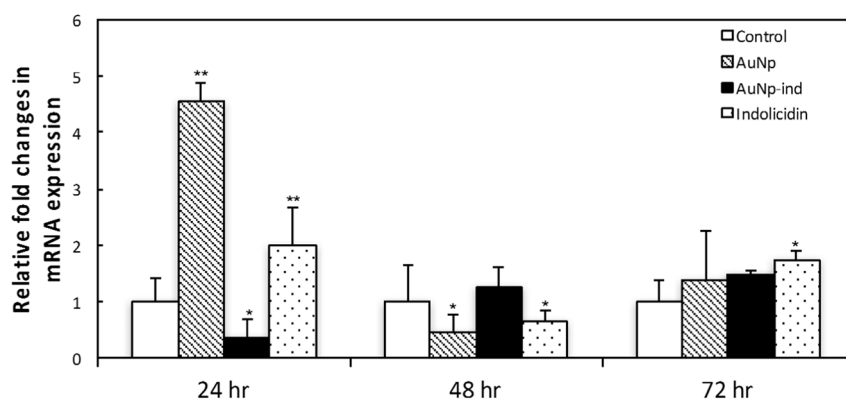


Fig. 5 – The relative expression of YCA1 gene in *Saccharomyces cerevisiae* exposed to AuNPs, AuNPs-indolicidin and indolicidin, using actin gene as internal control, after 24, 48 and 72 hr of cultivation. The relative expression levels are depicted as the mean \pm SD, calculated from two biological replicate. The relative change in gene expression was analysed using the $2^{-\Delta\Delta C(T)}$ method. Statistically significant expression changes were calculated using one-way ANOVA and the level of significance was also determined by the Bonferroni method comparing all groups versus the control. Statistically significance changes in relative expression are represented with an asterisk (* $p < 0.01$; ** $p < 0.05$).

However, DNA damage accompanied by ROS generation has been shown to lead to apoptosis in yeast (Carmona-Gutierrez et al., 2010). On the contrary, our results show no evidence of apoptotic death in the exposed cultures since cell survival and reduction of DNA damage in the exposed cultures clearly indicate that conditions are far from death-inducing. It has been outlined that in yeast, as well as in mammalian cells, only the combination of several markers such as ROS accumulation, DNA fragmentation, cell integrity and clonogenic determination of viability determines an apoptotic phenotype (Carmona-Gutierrez et al., 2010). Therefore, the higher expression of the YCA1 gene found especially in the cells exposed to AuNPs and indolicidin alone can be indicative of an initial apoptotic cascade or the activation of the Yca1p defence system, as suggested by some authors, who speculate that Yca1p may have an additional role in oxidative stress defence under non-apoptotic conditions (Perrone et al., 2008).

In conclusion, in this preliminary paper, we showed that *S. cerevisiae* is relatively resistant to AuNPs-indolicidin when compared to AuNPs and indolicidin alone. We do not provide any evidence about the mechanisms concerning the possible internalization of NPs into yeast cells. It is presumable, however, that the thick cell wall should avoid the direct uptake of the NPs, differently from other protists that have highly developed systems for internalization of NPs. Notwithstanding the cell wall, the disruption of membrane by dissolved ions or oxidative stress caused by NPs may change membrane permeability and favour their entrance inducing as a consequence ROS production and DNA damage.

The lower toxicity exhibited by the AuNPs-indolicidin complex could represent an interesting feature in the perspective of its potential use for clinical applications.

Competing financial interests

The authors declare that they have no conflict of interests.

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