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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.

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

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(Geffroy et al., 2012). 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 heterogenous 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, 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. 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-SUPER GRADIENT (0.1% TFA) in water (0.1% TFA) from 5% to 70% for 15 min. Purification was performed by preparative RP-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-$CONH_2$.

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