Nickel oxide nanoparticles film produced by dead biomass of filamentous fungus

Marcia Regina Salvadori¹, Cláudio Augusto Oller Nascimento² & Benedito Corrêa¹

¹Department of Microbiology, Biomedical Institute II, University of São Paulo, São Paulo, 05508000, Brazil, ²Department of Chemical Engineering, Polytechnic, University of São Paulo, São Paulo, 05508000, Brazil.

The synthesis of nickel oxide nanoparticles in film form using dead biomass of the filamentous fungus Aspergillus aculeatus as reducing agent represents an environmentally friendly nanotechnological innovation. The optimal conditions and the capacity of dead biomass to uptake and produce nanoparticles were evaluated by analyzing the biosorption of nickel by the fungus. The structural characteristics of the film-forming nickel oxide nanoparticles were analyzed by scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS), X-ray photoelectron spectroscopy (XPS), transmission electron microscopy (TEM), and atomic force microscopy (AFM). These techniques showed that the nickel oxide nanoparticles had a size of about 5.89 nm and were involved in a protein matrix which probably permitted their organization in film form. The production and uptake of nickel oxide nanoparticles organized in film form by dead fungal biomass bring us closer to sustainable strategies for the biosynthesis of metal oxide nanoparticles.

Nickel oxide is an important transition metal which has been extensively studied because of its technological applications in magnetic recording media, as a catalyst, and in the medical field. The metal oxide can assume a variety of structural geometries with an electronic structure that can exhibit metallic and semiconductor characteristics, conferring different chemical and physical properties¹.

Nanomaterials have been widely studied over the past decade due their application in different fields such as photoelectric materials, recording media, catalysts, sensors, ceramic materials, and others². In this respect, nickel oxide nanoparticles (NiO NPs) exhibit particular catalytic³ anomalous electronic⁴,⁵ and magnetic⁶,⁷ properties. Nanoparticles of NiO with the following shapes have been synthesized: nanotubes, nanobelts, nanorods, hollow spheres, and hexagonal flakes⁸,⁹.

Nanotechnology in biology has drawn increasing attention due to its cutting-edge nature and the use of the nanoparticles (NPs) produced in industrial, biomedical and electronic applications such as catalysts¹⁰, in cancer detection¹¹, and as conductors in transistors¹². Biological methods for NPs synthesis can overcome many of the harmful effects of chemical and physical methods, permitting the synthesis of nanomaterials at mild pH, pressure and temperature and at a substantially lower cost.

Studies in the literature have reported the use of dead fungal biomass for the synthesis of transition metal NPs, such as the extracellular synthesis of copper NPs by Hypocrea lixii¹² and the intracellular synthesis of copper NPs by the yeast Rhodotorula mucilaginosa¹³. This study reports for the first time the synthesis and uptake of NiO NPs organized in film form by dead fungal biomass of the filamentous fungus Aspergillus aculeatus (A. aculeatus). This natural production of NPs is a unique environmentally friendly process.
Results
The filamentous fungus *A. aculeatus* was isolated from the area of a copper mine in the Amazon region. Analysis of minimum inhibitory concentrations revealed that the fungus exhibited a high tolerance to nickel (up to 1473 mg/L). Dead, dried and live biomass of *A. aculeatus* was able to produce NiO NPs from Ni (II) in aqueous solution, with a maximum uptake capacity of the metal of 19.6 mg/g, 6.2 mg/g and 5.2 mg/g, respectively. The best physicochemical conditions of nickel sorption for the three types of biomass were a contact time of 90 min, initial pH 4.0, temperature of 30°C, agitation speed of 150 rpm, initial Ni (II) concentration of 100 mg/L, and biosorbent dose of 1.0 g. The efficiency of nickel uptake was 89.02%, 53.25% and 48.26% for dead, dried and live biomass, respectively. The Langmuir model was used to describe the nickel (II) biosorption isotherm for the three types of biomass. The isotherm constants, maximum loading capacity estimated by the Langmuir model, and regression coefficients are shown in Table 1.

The NiO NPs (produced extracellularly by the dead fungal biomass) were characterized by morphological and structural analysis. The Figs. 1a and 1b shows the SEM photomicrographs before and after the synthesis of NiO NPs by dead fungal biomass, respectively. Modification of the fungal surface occurred as a result of the formation of a film coating the surface of the fungal biomass after binding to the NiO NPs (Fig. 1b). The EDS spectra in Figs. 1c and 1d show the region of the mycelium before and after nickel exposure, respectively. Fig. 1d shows the signals of nickel in the fungus. Signals of C, N and O were also observed, indicating the possible presence of proteins as a capping material on the surface of the NPs. This protein matrix may be responsible for the stabilization (capping material) and organization of the NPs in the form of a film on the biomass surface. Figs. 1e and 1f shows the images of the live fungal biomass before and after synthesis of NiO NPs, respectively. The Figs. 2a and 2b shows the two-dimensional (2D) images of dead *A. aculeatus* biomass in the absence and presence of NPs, respectively. Three-dimensional (3D) images of dead biomass impregnated or not with NPs are shown in Figs. 2c and 2d, respectively. The formation of a smooth film of NiO NPs coating the surface of dead fungal biomass can be seen in both AFM images (2D and 3D) in which the biomass was impregnated with NPs. The Figs. 2e and 2f shows the AFM images (2D) of the live biomass without and with presence of the NPs, respectively and 3D Figs. 2g and 2h of the live biomass without and impregnated with NPs, respectively.

The Figs. 3 shows the XPS spectrum of the Ni (2p3/2) core level of the NiO NPs forming a film on the surface of *A. aculeatus*. The peak at 854.1 eV corresponds to the Ni 2p3/2 level of Ni (0) and is characteristic of NiO. The peak at 852.2 eV, which would be characteristic of the Ni 2p3/2 level of Ni (0) would be the major binding energies at 532.6 eV and 400 eV (data not shown) respectively observed confirming the presence of proteins involving NiO NPs. This results corroborated with the observed in EDS spectra, which suggests the possibility of these agents acting as capping agents that would be associated with the organization of the NiO NPs in the film form. The Fig. 4 shows the TEM image of the NiO NPs synthesized extracellularly by the dead biomass of *A. aculeatus* in the cell wall surface showing an average diameter of 5.89 nm and NPs predominantly spherical.

### Table 1 | Adsorption constants from simulation with Langmuir model

| Type of biomass | qm (mg/g) | b (L/mg) | R² |
|-----------------|-----------|----------|----|
| Live            | 5.2       | 0.013    | 0.987 |
| Dried           | 6.2       | 0.017    | 0.989 |
| Dead            | 19.6      | 0.024    | 0.996 |

Discussion
The fungi has the capacity to survive metals toxicity by means of mechanisms produced in direct response to metal species concerned. The *A. aculeatus* presented a tolerance to the nickel metal transition (up to 1473 mg/L) due to the mechanism biosorption through fungal cell wall. The fungal biomass walls are formed of chitin, chitosan, glucan, lipid, phospholipids, which contain carboxyl groups, amino groups phosphates, lipids, melanin, sulphates and hydroxides, these functional groups are the sites sorption of the metals. Dead biomass exhibited the highest capacity to produce NiO NPs from Ni (II) through biosorption. The present results showed that dead *A. aculeatus* biomass had a higher adsorption capacity (19.6 mg/g) than those described for other known biosorbents, such as *Trichoderma harzianum* (11.77 mg/g), *Rhizopus arrhizus* (9.28 mg/g), *Aspergillus terreus* (7.86 mg/g), *Aspergillus niger* (7.69 mg/g), *Aspergillus flavus* (7.5 mg/g), *Alternaria alternata* (7.37 mg/g), *Cunninghamella echinulata* (4.69 mg/g), and macro fungi *Lactarius salmonicolor* (14.90 mg/g). Studies suggest that, at higher concentrations, Ni ions interact with cellular components such as organic acids, nucleotides, amino acids, phospholipids, etc resulting in the disturbance of physiological and biochemical processes. There are few studies reporting the use of dead fungal biomass to produce metal NPs. However, dead biomass has advantages such as its limited toxicity, the possibility of storage for a prolonged period of time, and the fact that it does not require growth media and nutrients for its maintenance. Morphological analysis of the NiO film formed by NiO NPs on the fungal surface by SEM and AFM provided cross-sectional images of the sample (2D SEM image in Fig. 1b and 2D and 3D AFM images in Figs. 2b and 2d). Both techniques revealed the smooth characteristic of the film of NiO NPs. There are no reports in the literature on NiO NPs produced by dead fungal biomass forming this film structure. EDS analysis showed elements that may be derived from biomolecules, such as proteins, provided by the fungal biomass surrounding the NiO NPs. This analysis was confirmed by the XPS results. Fungal cell proteins were possibly released during the autoclaving process and bound on the cell surface. Proteins probably act as a capping agent in the formation of NiO NPs, constituting a protein matrix around the NiO NPs that possibly confers their arrangement in film form. However, the type and mechanism of action of the proteins involved in these processes remain to be studied.

The electron micrograph reveals a NiO NPs profile produced by a green process using dead fungal biomass with average diameter of 5.89 nm. Similar results have been reported in previous studies producing Ag and others NPs, but using live fungal biomass. In summary, the present study explored for the first time the production and uptake of NiO NPs produced by dead biomass of *A. aculeatus*. These NPs formed a film on the biomass surface. This natural method provides low-cost, rapid and economically friendly process for the formation and uptake of NiO NPs. In a next step, we intend to characterize the biomolecules that are involved in the production of NiO NPs and the arrangement of these NPs in the formation of the film.

Methods
Growth and maintenance of the organism. *A. aculeatus* was isolated from the water collected in a copper wastewater pond at the Sossego mine, located in Carajás, Pará, Brazilian Amazonia region (06° 26′ South and 50° 4′ West longitude). The fungus was maintained and activated on Sabouraud Dextrose Agar (SDA) (Oxoid, England).

Analysis of nickel (II) tolerance. The tolerance of the isolated fungus to nickel was determined as the minimum inhibitory concentration (MIC) by the spot plate method. SDA plates containing different nickel concentrations (50 to 2000 mg/L) were prepared and inocula of the fungus were spotted onto the metal and control.
plates (plate without metal). The plates were incubated at 25°C for 5 days. The MIC is defined as the lowest concentration of metal that inhibits visible growth of the isolate.

Preparation of the adsorbate solutions. All chemicals used in the present study were of analytical grade and were used without further purification. All dilutions were prepared in double-deionized water (Milli-Q Millipore 18.2 MΩ cm−1 resistivity).

Figure 1 | SEM images of the surface of dead and live A. aculeatus biomass, and EDS spectra of the dead biomass. (a) SEM micrograph before the adsorption of nickel by dead biomass, (b) SEM micrograph showing the formation of a film coating the surface of the dead fungal biomass after binding to the NiO NPs. (c) EDS spectrum before exposure of dead biomass to the nickel solution, (d) EDS spectrum after exposure of the dead biomass to the metal, confirming the presence of nickel, (e) SEM micrograph before adsorption of nickel by live biomass and (f) SEM micrograph showing the live fungal biomass, after binding to the NiO NPs.
Figure 2 | AFM images of the surface of dead and live *A. aculeatus* biomass. (a) AFM image (2D) of the dead fungal biomass without NiO NPs, (b) AFM image (2D) of the dead fungal biomass impregnated with NiO NPs, showing the formation of a film coating the surface of the dead fungal biomass, (c) AFM image (3D) showing the dead fungal biomass without NiO NPs, (d) AFM image (3D) showing the dead fungal biomass after impregnation with NiO NPs which form a film on the surface of the dead biomass, (e) AFM image (2D) showing the live fungal biomass without NiO NPs, (f) AFM image (2D) showing the live fungal biomass impregnated with NiO NPs, (g) AFM image (3D) showing the live fungal biomass without NiO NPs and (h) AFM image (3D) showing the live fungal biomass after impregnation with NiO NPs.
The nickel stock solution was prepared by dissolving NiCl₂·6H₂O (Carlo Erba, Italy) in double-deionized water. The working solutions were prepared by diluting this stock solution.

**Biomass preparation.** The fungal biomass was prepared in Sabouraud broth (SB) (Oxoid, England) and incubated at 25°C for 5 days, at 150 rpm. After incubation, the pellets were harvested and washed with double-deionized water. This preparation is referred to as live biomass. For the preparation of dead biomass, an appropriate amount of live biomass was autoclaved. The dried biomass was obtained by drying the fungal mat at 50°C until it became crispy. The dried mat was ground to obtain uniform sized particles.

**Analysis of the effects of physicochemical parameters on the adsorption efficiency of NiO NPs onto the biosorbent.** The effect of pH (2–6), temperature (20–60°C), contact time (5–300 min), initial nickel concentration (50–500 mg/L), agitation rate (50–250 rpm), and biosorbent dose (0.15–1.25 g) on the removal of nickel was analyzed. These experiments were optimized using 45 mL of a Ni (II) test solution (100 mg/L) in plastic flask. Different nickel (II) concentrations were prepared by appropriate dilution of the nickel (II) stock solution. The pH of the solution was adjusted using HCl or NaOH. The desired biomass dose was then added and the content of the flask was shaken. After shaking, the Ni (II) solution was separated from the biomass by vacuum filtration through a Millipore membrane. The metal concentration in the filtrate was determined by inductively coupled plasma-optical emission spectrometry. The efficiency (R) of metal removal was calculated using the following equation (1):

\[ R = \frac{(C_i - C_f)}{C_i} \times 100 \]  

where \( C_i \) and \( C_f \) are initial and equilibrium metal concentrations, respectively. The metal uptake capacity, \( q_u \), was calculated using the following equation (2):

\[ q_u = \frac{V(C_i - C_f)}{M} \]  

where \( q_u \) (mg/g) is the biosorption capacity of the biosorbent at any time, M (g) is the biomass dose, and V (L) is the volume of the solution.

**Sorption isotherm.** The Langmuir equilibrium model was used to fit Ni (II) biosorption isotherm experimental data, as follows:

The linearized Langmuir isotherm model according to equation (3):

\[ \frac{C_i}{q_u} = \frac{1}{q_m b} + \frac{C_i}{q_m} \]  

where \( q_m \) is the monolayer sorption capacity of the sorbent (mg/g), and \( b \) is the Langmuir sorption constant (L/mg).

**Characterization of the NiO NPs.** In this study, dead biomass of A. aculeatus was used which showed a high adsorption capacity of nickel compared to live and dried biomass. The NiO NPs were synthesized by dead biomass using the following conditions: incubation time of 90 min, initial pH 4.0, temperature of 30°C, agitation speed of 150 rpm, biosorbent dose of 1.0 g, and a solution containing 100 mg/L Ni (II). The surface morphology of the NiO film formed by NiO NPs produced by the dead and live biomass was examined by SEM (JEOL 6460 LV). The dead biomass was examined by energy dispersive spectrometer (EDS) to identify the composition of elements of the sample and the dead and live biomass by AFM Icon NanoScope V (Bruker). Transmission electron microscopy (TEM) ([JEOL-1010]) was used to observe the microstructure of the NiO NPs forming the film, such as size and shape. The chemical state of nickel in the film was analyzed by XPS. The XPS analysis was carried out at a pressure of less than 10⁻⁶ Pa using a commercial spectrometer (UNI-SPECS UHV System). The Mg Kα line was used (hv = 1253.6 eV) and the analyzer pass energy was set to 10 eV. The inelastic background of the electron core-level spectra was subtracted using Shirley’s method. The composition (at.%) of the near surface region was determined with an accuracy of ±10% from the ratio of the relative peak areas corrected by Scodell’s sensitivity factors of the corresponding elements. The spectra was fitted without placing constraints using multiple Voigt profiles.

**Figure 3** XPS spectrum of the NiO NPs that compose the film.

**Figure 4** TEM micrograph of dead A. aculeatus biomass. Section of the fungus showing extracellular localization of the NiO NPs (darkest arrow) in the cell wall (lighter arrow).

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Author contributions
M.R.S. conceived the experiments. M.R.S. designed and performed the experiments, and analysed the data. M.R.S., C.A.O.N. and B.C. discussed the data and wrote the paper.

Additional information
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