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Cytotoxicity and colloidal behavior of polystyrene latex nanoparticles toward filamentous fungi in isotonic solutions

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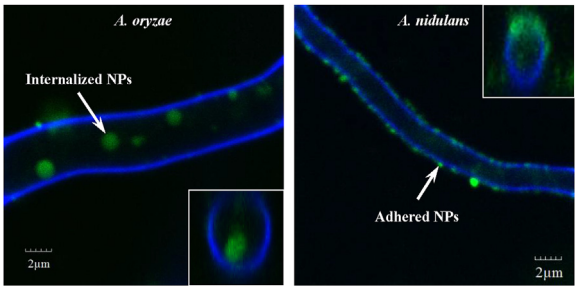
HIGHLIGHTS

- *Aspergillus oryzae* entrapped positively charged PSL NPs in 154 mM NaCl, but not *Aspergillus nidulans*.
- *A. oryzae* had soft cell walls compared with *A. nidulans*.
- Positively charged PSL NPs showed high toxicity in 292 mM sucrose.
- Agar cultivated cells were hydrophobic compared with liquid cultivated cells.
- Hydrophobin on agar cultivated cells inhibited the uptake of PSL NPs.

GRAPHICAL ABSTRACT



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ABSTRACT

The effects of surface physicochemical properties of functionalized polystyrene latex (PSL) nanoparticles (NPs) and model filamentous fungi *Aspergillus oryzae* and *Aspergillus nidulans* cultivated in different environment (aqueous and atmospheric environment) on the colloidal behavior and cytotoxicity were investigated in different isotonic solutions (154 mM NaCl and 292 mM sucrose). When the liquid cultivated fungal cells were exposed to positively charged PSL NPs in 154 mM NaCl solution, the NPs were taken into *A. oryzae*, but not *A. nidulans*. Atomic force microscopy revealed that the uptake of NPs was more readily through the cell wall of *A. oryzae* because of its relatively softer cell wall compared with *A. nidulans*. In contrast, the positively charged PSL NPs entirely covered the liquid cultivated fungal cell surfaces and induced cell death in 292 mM sucrose solution because of the stronger electrostatic attractive force between the cells and NPs compared with in 154 mM NaCl. When the agar cultivated fungal cells were exposed to the positively charged PSL NPs, both fungal cells did not take the NPs inside the cells. Contact angle measurement revealed that the hydrophobin on the agar cultivated cell surfaces inhibited the uptake of NPs because of its relatively more hydrophobic cell surface compared with the liquid cultivated cells.

1. Introduction

Endocytosis is a cellular process whereby eukaryote cells engulf substances from the outside by invaginating the cell membrane. Numerous examples of the safe endocytic uptake of engineered

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nanoparticles (NPs) into animal cells have been reported in conjunction with drug and gene delivery systems (Chithrani and Chan, 2007; Faklaris et al., 2009; Kostarelos et al., 2007; Lin et al., 2010; Tasis et al., 2006; Wang et al., 2012). Animal cells do not have cell walls, whereas eukaryote cells except animal cells are surrounded by a rigid cell wall. Because of the presence of cell walls, the endocytic uptake of NPs into biological cells possessing cell walls is still poorly understood.

Fungi, which have cell walls, include some ecologically and economically important organisms. However, fungal pathogens cause serious plant and animal diseases. In humans, fungal diseases are difficult to treat because fungi are more closely related to animals than other organisms (Shalchian-Tabrizi et al., 2008). In plants, fungal pathogens cause many of the most serious crop diseases (Tucker and Talbot, 2001). Fungal cells are classified into two groups, single-celled organisms like yeast and multicellular organisms like filamentous fungi. Yeast is widely used as a model unicellular eukaryote (Schwegmann et al., 2010). A few studies investigating the potential impact of NPs including metal oxides, silver, and fullerene found that NPs exhibit little or no toxicity toward yeast (García-Saucedo et al., 2011; Hadduck et al., 2010; Kasemets et al., 2009; Lee et al., 2009; Schwegmann et al., 2010). Prescianotto-Baschong and Riezman reported that positively charged gold NPs were taken into yeast spheroplasts whose cell walls had been almost completely removed (Prescianotto-Baschong and Riezman, 1998). Interestingly, the authors demonstrated that positively charged polystyrene latex (PSL) NPs (diameter $\leq$100 nm) were taken into the budding yeast *Saccharomyces cerevisiae* still possessing their cell walls in a high ionic strength aqueous solution (Miyazaki et al., 2015, 2014; Nomura et al., 2013). To our knowledge, the endocytic uptake of NPs into filamentous fungi responsible for plant and animal diseases has not been demonstrated. If polymer particles can be delivered into pathogenic fungal cells, the potential use of NPs as carriers of drugs to protect and treat fungal diseases in plant and animal may be possible.

The surface physicochemical properties of NPs and biological cells can affect the colloidal behaviors (diffusion, adhesion, uptake) and cytotoxicity of NPs (Nomura et al., 2013). Hydrophobins are well-known amphiphilic fungal proteins widely distributed throughout the fungal kingdom, and are not known to occur in other organisms (Wessels, 2000; Zapf et al., 2007). A model to explain the biological role of hydrophobins during the growth and development of filamentous fungi was proposed as follows (Wessels, 1996; Wösten and Scholtmeijer, 2015). Hydrophobin monomers are secreted at the tip of the growing hypha, and self-assemble at hydrophilic–hydrophobic interfaces. Self-assembly of hydrophobins changes a surface from hydrophilic to hydrophobic, or hydrophobic to hydrophilic. Based on this model, in the aqueous environment, hydrophobin monomers diffuse to the medium–air interface, and the surface of the submerged fungal cells retain their hydrophilic nature. In contrast, the hydrophobin secreted by emerging aerial hypha cannot diffuse into the atmospheric environment and assembled at the cell wall–air interface and changes the fungal cell surface from hydrophilic to hydrophobic. In this study, the effects of surface physicochemical properties of functionalized PSL NPs and model filamentous fungi *Aspergillus oryzae* and *Aspergillus nidulans* cultivated in different environment (aqueous and atmospheric environment) on the colloidal behavior and cytotoxicity of NPs were investigated in different NP exposure environment (154 mM NaCl and 292 mM sucrose solutions).

2. Materials and methods

2.1. Filamentous fungi strains and growth conditions

A. oryzae niaD300 and *A. nidulans* FGSC A4 (biA1) were used as model filamentous fungi. Conidia from *A. oryzae* and *A. nidulans* were germinated in a liquid medium or on an agar plate at 30 °C in standard minimal medium for *A. oryzae* (Yamada et al., 1997) and for *A. nidulans* (Rowlands and Turner, 1973) containing appropriate supplements, respectively. After a 24-h cultivation, the fungal cells were washed three times using the sterilized isotonic solution (154 mM NaCl aqueous solution or 292 mM sucrose aqueous solution) as a dispersion medium to remove the remainder of the medium components. The agar medium was prepared on a coverslip equipped with a glass bottom dish (D11130H, Matsunami, Kishiwada, Japan). The glass bottom dish was rinsed with deionized water and ethanol before agar plate preparation. A filter paper soaked in deionized water was set in the glass dish to prevent the agar from drying out during the cultivation.

2.2. Polystyrene latex nanoparticles

Positively charged amine-modified PSL NPs with a fluorophore (nominal diameter: 100 nm) were purchased from Sigma–Aldrich (L9904, St. Louis, MO, USA) and named PS-NH_2. Negatively charged carboxylate-modified PSL NPs with a fluorophore (nominal diameter: 100 nm) were purchased from micromod Partikeltechnologie GmbH (29-02-102, Rostock, Germany) and named PS-COOH. The PSL NPs were suspended in sterilized isotonic solution (154 mM NaCl aqueous solution or 292 mM sucrose aqueous solution) using a vortex for 10 s prior to use. The concentration of PSL NP suspension was adjusted to 40 μg/ml.

2.3. Characterization

The hydrodynamic diameter and electrophoretic mobility of the PSL NPs dispersed in the isotonic NaCl or sucrose solution were measured using a zeta potential and particle size analyzer (ELS-Z, Otsuka Electronics, Hirakata, Japan). The zeta potential of the NPs was calculated using the Smoluchowski equation for a hard particle: $u = (\varepsilon_r \varepsilon_0 \zeta)/\eta$, where u is the electrophoretic mobility, ε_r is the relative permittivity of the medium, ε_0 is the permittivity of the vacuum, ζ is the zeta potential of NP, and η is the viscosity of the medium. The zeta potential of the fungal cells was also measured using the zeta potential analyzer with a flat plate sample cell (EZ8400, Otsuka Electronics), based on the Mori-Okamoto equation (Mori and Okamoto, 1980). Fungal cells obtained after a 2-d cultivation from a dense lawn were used for zeta potential measurements after washing.

The contact angle between a fungal cell lawn and a droplet of a specified probe fluid (water, formamide, and α-bromonaphthalene) was measured using a contact angle analyzer (FTA125, First Ten Ångstroms, Portsmouth, VA, USA), following a procedure described previously in the literature (Busscher et al., 1984; Yoshihara et al., 2014). Fungal cells obtained after a 2-d cultivation from a dense lawn were used for contact angle measurements. Subsequently, the total surface tension γ^{Total}, the Lifshitz–van der Waals component γ^{LW}, and the acid–base component γ^{AB}, which can be expressed as the geometric mean of an electron-donor γ^- and an electron-acceptor γ^+, were calculated from at least five independent measured contact angles using the van Oss approach (Nomura et al., 2009; Van Oss et al., 1988). The hydrophobicity of the fungal cell surface was estimated using the change in the Gibbs free energy ΔG^{Total}, which was defined as the sum of the free energy changes in the Lifshitz–van der Waals (ΔG^{LW}) and acid-base (ΔG^{AB})