Fungal Sticky Porous Cell Wall Evolved for Efficient Uptake and Export

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Abstract

Observing release of large extracellular vesicles (EVs) from yeast cell raised the question about size and flexibility of cell wall pores. To estimate the approximate size of pores, internalization of nanoparticles (NPs) with different sizes, into the intact yeast cell was examined. *Candida tropicalis* was cultured in N-acetylglucosamine-yeast extract broth (NYB) and examined every 12 hr by light microscopy for observing release of EVs. Yeast culture in NYB was treated with Fluorescein isothiocyanate (FITC) -labelled NPs; gold (45, 70 and 100 nm), albumin (100 nm) and Fluospheres (1000 and 2000 nm). Fluorescence microscope was used for recording internalization of NPs with different concentrations (0.1-10%) after few seconds to 120 min of treatment. Release of EVs with different size and morphology from yeast mostly occurred at 36 hr. Best NPs’ concentration was 0.1% and internalization occurred within few seconds after treatment. Positively charged 45 nm gold NPs internalized into yeast cell and accumulated in the vacuole and 100 nm gold NPs destroyed the yeast cell. Gold NPs with 70 nm size and 100 nm negatively charged albumin NPs were internalized into the vacuole of a few yeast cells. Inert Fluospheres were first degraded outside the yeast’s cell and then absorbed into yeast’s cell and finally reached the vacuole. Release of large EVs from yeast indicated flexibility of cell wall pores and EVs for remodelling. Furthermore, physicochemical properties of internalizing NPs and those of cell wall determined uptake of NPs by yeast cell.

Introduction

Fungal cell wall was first described as a rigid basket-like scaffold around the cell that confers resistance to hydrostatic pressure exerted by cell cytoplasm and membrane. However, results of further investigations on fungal cell wall architecture and composition revealed a complex structure with unique physicochemical properties that could have evolved for much more sophisticated functions (Ahmadjian 1993). Recent studies indicate that fungal cell wall is one of the most complex and highly regulated structures in microbial world that plays an absolutely fundamental role in fungal nutrient acquisition and adaptation to a versatile range of habitats. These could not have been fulfilled without cell wall properties such as negative charge for absorption of cations (Sen et al. 2011), hydrophobicity for adhesion to surfaces (Hall and Gow 2013), flexible viscoelasticity for rapid remodelling into hyphae or bud (Walker et al. 2018) and adjusting porosity to limit the entry of large particles (De Nobel et al. 1989) while facilitating vesicular transport (Casadevall et al. 2009).

Fungal cell wall is a two-layered structure. The inner cell wall composed of β-glucans and chitin, confers strength and physical shape to the wall. The outer cell wall, in *Candida albicans*, is made of a fibrillar layer of highly glycosylated mannoproteins that determine physical properties of the cell wall such as negative charge and viscoelastic properties (Liu et al. 2012) as well as hydrophobicity, porosity, adhesiveness and immunologic characteristic (Gow et al. 2017). Mannoproteins in *Candida* also protect fungal cell against host enzymes and antimicrobials, macrophage phagocytosis and immune response to underlying β-1, 3-glucan layer (Erwig and Gow 2016). Associated with major components of fungal cell
wall are secretory hydrolytic enzymes; phospholipases, proteinases, lipases, esterases and hemolysin that are involved in degradation of nutrients (Pandey et al. 2018).

Results of several studies suggest that fungal cell wall despite acting as a rigid scaffold for protection of protoplasm, could have been specialized for facilitating transport into and out of the fungal cell. Fungi absorb small molecules that freely pass the cell wall and reach the cell membrane. Fungi also use their secretory enzymatic cocktail to digest large nutrients into smaller molecules that are then absorbed into the cell. On the other hand, export of fungal products across the cell wall is facilitated by vesicular structures that traverse the cell wall pores (Casadevall et al. 2009).

Although many details about fungal cell wall pores have remained unknown, it has been suggested that flexibility of cell wall for adjusting the pore diameter is part of the control system for in and out cellular traffic in fungi. On the one hand, the size of cell wall pores in *Saccharomyces cerevisiae* has been measured between 200 and 400 nm (de Souza Pereira and Geibel 1999). On the other hand, the size of extracellular vesicles (EVs) that could traverse the cell wall has been estimated as 60-300 nm in *Cryptococcus neoformans* (Rodrigues et al. 2007), 50-500 nm in *S. cerevisiae* (Oliveira et al. 2010) and 50–800 nm in *C. albicans* (Heydari et al. 2020). By observing that the size of fungal exported vesicles often exceeded the exclusion size estimated for cell wall permeability, it was suggested that vesicles with lipid bilayer structure can compress to traverse the cell wall pores smaller than their diameter (Casadevall et al. 2009). These data indicate that size and flexibility and probably other properties of cell wall pores may be involved in traffic across the fungal cell wall (Walker et al. 2018).

In the present study, examination of wet mount preparations from liquid culture of *Candida tropicalis* by light microscopy showed release of large vesicles, some carrying a visible dense cargo. These observations raised the question about the size and flexibility of cell wall pores that allowed the exit of vesicles. To estimate the approximate size of cell wall pores, internalization of nanoparticles (NPs) with different diameters, into the intact yeast cell was examined. Fluorescein isothiocyanate (FITC)-labelled gold NPs with the size of 45, 70 and 100 nm and FITC-labeled albumin NPs with the size of 100 nm were prepared. Furthermore, two commercially-manufactured Fluospheres with the size of 1000 and 2000 nm were purchased. Fluorescence microscope was used for recording internalization of NPs with different concentrations (0.1-10%) after few seconds to 120 min of treatment.

**Materials And Methods**

**Yeast strain- Release of extracellular vesicles from yeast**

One yeast isolate from the culture of gastric biopsy of a dyspeptic patient on brucella blood agar was used in this study. The isolated yeast was purified by subculturing a single colony of yeast on homemade yeast extract- glucose-chloramphenicol (YGC) agar containing yeast extract (5%), glucose (0.5%) and chloramphenicol (0.1%) (pH 7), several times. Yeast isolate produced turquoise blue colonies on CHROMagar (CHROMagar, France) and was identified as *Candida tropicalis*. Purified yeast was cultured in N-acetylglucoseamine and yeast extract broth (NYB) composed of 20 g/L N-acetylglucoseamine
(Sigma, USA) and 5 g/L yeast extract (Pronadisa, Spain) (pH 7) and incubated at 30 °C for 24-48 hr. Wet mounts were prepared from yeast culture every 12 hr and examined by light microscopy for observing release of extracellular vesicles.

**Preparation of FITC-labelled nanoparticles**

Nanoparticles (NPs) used in this study included home-made gold particles (45, 70 and 100 nm) with irregular shape and positive charge, albumin (Bio Basic, D0024) (100 nm) particles with spherical shape and negative charge and commercially purchased inert spherical Fluospheres (Thermosher Scientific, USA). Gold NPs were made with high (95%) purity using chemical reduction method according to Frens et. al (Frens 1973). Briefly, gold NPs of 45, 70 and 100 nm size were made by adding 0.9 mL, 0.6 mL and 0.4 mL of 1% tri-sodium citrate solution to 45, 70 and 100 mL of boiling 1% chloroauric acid, respectively until colour change to dark grey showed formation of gold NPs. FITC labelling was done by drop wise adding FITC (0.4 mg in 8 mL ethanol) to each gold NPs solution until appearance of orange colour. Excess ethanol was evaporated while shaking (200 rpm) at room temperature in dark for 1 hr. Labelled particles were harvested by centrifugation (6000 rpm) for 5 min and washed twice with 1X phosphate-buffered saline (PBS) (Jazayeri et al. 2018; Aghaie et al. 2019).

Albumin NPs were made according to Zhang et al. (Zhang et al.). Albumin NPs of 100 nm size were made by dissolving 10 mg of serum albumin powder in 1 mL of sterilized distilled water. FITC labelling was done by drop wise adding FITC (0.4 mg in 8 mL ethanol) to albumin solution until grey precipitate of FITC-labelled albumin formed. To fix the label, FITC-albumin was treated with 25 μL of 0.25% glutaraldehyde for 30 min. Excess ethanol was evaporated while shaking (200 rpm) at room temperature in dark for 1 hr. Finally, albumin NPs were harvested by centrifugation (15000 rpm) for 5 min and washed twice with 1X PBS. NPs with the size of 1000 and 2000 nm were purchased in their labelled forms with commercial name of Fluospheres. The manufacturer described Fluospheres as polysterene particles covered with carboxylic acid. Fluospheres were washed in Tris-NaCl (15 mM NaCl in 50 mM Tris-Cl, pH 7.6) before use (Lim et al. 2012).

**Internalization of NPs into yeast cell- Impact of NP concentration and exposure time**

Different concentrations of NPs (0.01, 0.1 and 1%) and times of exposure (a few seconds up to 120 min) were examined for internalization into yeast cell. Final concentrations of different NPs were obtained by adding 15 μL of the original NPs solutions (0.1, 1 and 10%) to 135 μL of fresh NYB and mixing thoroughly by pipetting. Finally, 10 μL of yeast culture (6×10⁶ cells / CFU) was inoculated into each tube. Tubes were incubated at 30 °C while shaking (250 rpm) and examined by fluorescence microscope for NPs internalization after different times. Five μL of 0.01% Evans blue was added to tubes for contrast and wet mounts were prepared in dark. For viability test, a drop of NPs treated yeast cultures was surface inoculated on brain heart infusion (BHI) agar (Pronadisa, Spain) and examined for growth up to one week of incubation at 30 °C.

**Utilization of Fluospheres as carbon source**
By observing that yeasts internalized Fluospheres by degrading them into smaller molecules, Fluospheres were added to a solid medium as the sole supplement and carbon source. A 30-μL volume of each Fluosphere original solution was added to 1.5% agar (Pronadisa, Spain) solution in distilled water which was sterilized and cooled to 45 °C. The solidified media of two Fluospheres were surface inoculated with yeast (6×10⁶ cells / CFU), incubated at 30 °C and examined for growth up to 48 hr. Culture of yeast on pure agar, without carbon source, was used as a control.

Results

Release of extracellular vesicles from yeast

Light microscopy observations on wet mounts from yeast’s liquid culture showed release of vesicles from yeast at about 36 hr. Released vesicles had different size and morphology, some carrying dense cargos (Fig. 1 top). Some of the released vesicles contained moving bodies similar to those encased in a vesicle inside the yeast cell and close to inner surface.

Internalization of FITC- NPs and Fluospheres into yeast cell and utilization of Fluospheres

Fluorescence microscopy observations showed that concentration of 0.1% was the best for both 45 and 70 nm gold NPs to internalize into the yeast cell. Internalization of gold NPs occurred within the first few seconds after treatment of yeast and did not increase by longer time of exposure (Fig. 2 top and bottom). While 45 nm gold NPs were internalized into all yeast cells, the 70 nm gold NPs were internalized into a few yeast cells. Yeasts exposed to 45 and 70 nm gold NPs retained their viability and grew on BHI agar. Gold NPs with 100 nm size destroyed the yeast cell within few seconds of exposure. Microscopic observations on FITC- albumin-treated yeasts also showed that concentration of 0.1% was the best for internalization into yeast cell. Internalization of albumin NPs occurred within the first few seconds after treatment of yeast and did not increase by longer exposure (Fig. 3). Albumin NPs were internalized into a few yeast cells. Yeasts exposed to 100 nm albumin NPs retained their viability and grew on BHI agar. Fluorescence microscopy observations showed that within the first few seconds of yeasts treatment with 0.1% concentration of 1000 and 2000 nm Fluospheres, they either remained intact and attached to the surface of yeast cells, due to their large size or degraded into smaller molecules, internalized and ended up in the vacuole. Uptake of Fluospheres occurred by all the yeast cells (Fig. 4 top and bottom). Furthermore, yeast cells assimilated the fluospheres and showed considerable growth on fluospheres agar, compared with no growth on control agar medium without carbohydrate supplement (data not shown).

Discussion

Release of extracellular vesicles from yeast

Fungi like all types of living cells release membrane-bound EVs that carry different cargos and mediate intercellular communications (Choi et al. 2015). Release of vesicles has been reported in Cryptococcus
**Estimation of cell wall pore diameter by the size of internalized NPs**

**Internalization of gold NPs:** Fluorescence microscopy observations of yeasts treated with FITC-labelled gold NPs showed that irregularly-shaped and positively-charged gold NPs with 45 and 70 nm sizes internalized into fungal cell within the first few seconds after treatment. Internalization of 45 and 70 nm NPs did not affect the yeasts viability and their culture on BHI agar was positive. However, yeast cells treated with 100 nm gold NPs were destroyed and their culture on solid medium showed no growth. Internalization of 45 nm gold NPs was observed in a considerable number of yeast cells. However, 70 nm gold NPs were observed in a fewer number of yeast cells, indicating restricted internalization due to their larger size or aggregation of positively charged NPs with cell wall components that reduced permeability. TEM observations on *S. cerevisiae* cells treated with 15-20 nm gold NPs showed decrease in gold accumulation as the concentration of gold increased, suggesting restriction of cell wall permeability to high amounts of foreign particles. It was found that internalization of gold NPs did not affect the viability of yeast as demonstrated by their growth on solid medium (Sen et al. 2011). It has been shown that bivalent cations form bridges between multiple negative charged phosphate groups in mannoproteins in yeast cell wall, reducing the cell wall permeability (Ballou 1976). Furthermore, treatment of *C. neoformans* with bivalent cations, such as Ca$^{2+}$ led to aggregation of surface polysaccharide molecules due to formation of inter- or intra-molecular bridges (Nimrichter et al. 2007), reducing fungal permeability to immunoglobulin penetration (Frases et al. 2009).

**Internalization of albumin NPs and Fluospheres:** Fluorescence microscopy observation on yeast cells treated with negatively charged and spherical albumin NPs with 100 nm diameter, showed only a few yeasts with NPs adhered to the surface or internalized into the vacuole. This could be due to their negative charge and large size that reduced particle internalization. Internalized albumin showed no negative effect on viability of yeasts. Fluorescence microscopy observations showed that inert and spherical carboxylated polysterene Fluospheres with 1000 and 2000 nm diameters either remained intact and adhered to the surface of yeast cell, due to their large size, or degraded into smaller molecules by secretory enzymes, internalized and ended up inside the vacuole. Uptake of degraded Fluospheres occurred within few second after treatment with no negative impact on their viability. Furthermore, confluent growth of yeast on Fluospheres solid medium, showed assimilation of carboxylated polysterene as sole sources of carbon by yeast. Uptake of degraded fluospheres by all the yeast cells showed no limitation in their internalization compared with gold and albumin NPs. Internalization of inert carboxylated polysterene Fluospheres could be facilitated by their adhesion to polysaccharide networks of fungal cell wall as a nonspecific adhesive surface (Frases et al. 2009). Furthermore, having $-\text{COOH}$
functional groups may have increased the negative charge of Fluospheres, enhancing their internalization (Holzapfel 2006).

**Conclusion**

Physicochemical properties of NPs determine their interaction with yeast cell wall and internalization. Release of EVs with different size, morphology and cargo, showed flexibility of EVs and cell wall pores for readjusting. Fungi with the stationary mode of life need to act as a predator to attract and entrap any passing by particle. The cell wall by being negatively charged, hydrophobic, sticky, porous and flexible as well as benefiting from a cocktail of hydrolysing enzymes, appears to have evolved as a highly dynamic structure for efficient uptake and export. In this regard, cell wall pores and EVs play the critical roles.

**Declarations**

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**Conflicts of interest:** The authors declare that they have no conflict of interest.

**Availability of data and material:** it is not applicable to this article

**Code availability:** not applicable

**Authors’ contributions:** FS designed the study. HE performed the experiments. MHJ, AS, PS and MM coordinated the research. FS and HE analysed the data, did the literature review and wrote the manuscript text.

**Ethics approval:** This article does not contain any studies with human participants or animals.

**Consent to participate:** not applicable

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**Figures**

![Figure 1](image-url)
Release of extracellular vesicles (EVs) from yeast. Light microscopy observations showed release of EVs with different cargos. Top) Released EVs from yeast with different size and morphology. Bottom) Released EVs from yeast, some carrying moving bacterial structures (EVB). Original magnification: × 1250.

Figure 2

Fluorescence microscopy shows internalization of 45 and 70 nm FITC-gold nanoparticles (NPs) into yeast cell after few seconds of treatment. Top) Internalized 45 nm FITC-NPs accumulated inside the vacuole (V) of all yeast cells (A1-5). Bottom) Internalization of 70 nm FITC-NPs into the vacuole (V) of a few yeast cells (B1-5). Original magnification × 1000.
Figure 3

Fluorescence microscopy shows internalization of 100 nm FITC-albumin nanoparticles (NPs) into yeast cell after few seconds of treatment. Internalized 100 nm FITC-NPs accumulated inside the vacuole (V) of a few yeast cells (A1-4). Non-internalized (NI) albumin NPs remained attached to the surface (A5). Original magnification × 1000.
Figure 4

Fluorescence microscopy shows internalization of 1000 and 2000 nm Fluospheres (FLs) into yeast cell after few seconds of treatment. Top) Degraded 1000 nm FLs internalized and accumulated inside the vacuole (V) of all yeast cells (A1-5). Bottom) Degraded 2000 nm FLs internalized and accumulated inside the vacuole (V) of all yeast cells (B1-5). Non-degraded (ND) FLs remained attached to the surface (Top and Bottom). Original magnification × 1000.