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Review

The impact of proteomics on the understanding of functions and biogenesis of fungal extracellular vesicles☆

Marcio L. Rodrigues[a,b,*], *Ernesto S. Nakayasu*[c,d], *Igor C. Almeida*[c], *Leonardo Nimrichter*[b]

[a]*Fundação Oswaldo Cruz – Fiocruz, Centro de Desenvolvimento Tecnológico em Saúde (CDTS), Rio de Janeiro, Brazil*
[b]*Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Brazil*
[c]*The Border Biomedical Research Center, Department of Biological Sciences, University of Texas at El Paso, El Paso, TX, USA*
[d]*Biological Science Division, Pacific Northwest National Laboratory, Richland, WA, USA*

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ABSTRACT

Several microbial molecules are released to the extracellular space in vesicle-like structures. In pathogenic fungi, these molecules include pigments, polysaccharides, lipids, and proteins, which traverse the cell wall in vesicles that accumulate in the extracellular space. The diverse composition of fungal extracellular vesicles (EV) is indicative of multiple mechanisms of cellular biogenesis, a hypothesis that was supported by EV proteomic studies in a set of *Saccharomyces cerevisiae* strains with defects in both conventional and unconventional secretory pathways. In the human pathogens *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Paracoccidioides brasiliensis*, extracellular vesicle proteomics revealed the presence of proteins with both immunological and pathogenic activities. In fact, fungal EV have been demonstrated to interfere with the activity of immune effector cells and to increase fungal pathogenesis. In this review, we discuss the impact of proteomics on the understanding of functions and biogenesis of fungal EV, as well as the potential role of these structures in fungal pathogenesis.
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Contents

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* *Corresponding author at:* Centro de Desenvolvimento Tecnológico em Saúde (CDTS), Fiocruz, Av. Brasil 4.365, Casa Amarela, Manguinhos, 21040-360 Rio de Janeiro, Brazil. Tel.: +55 21 95681712; fax: +55 21 22900494.
E-mail address: marciolr@cdts.fiocruz.br (M.L. Rodrigues).

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1. Introduction

In most eukaryotic cells, secretion is a sophisticated biological process that functions to traffic proteins to the plasma membrane and/or to the extracellular space [1]. Eukaryotic secretion occurs through mechanisms that are finely regulated at different cellular levels [1,2]. The literature about secretory mechanisms used by eukaryotic cells is abundant and several comprehensive reviews in this field are available [1–5].

During secretion, the plasma membrane is, in general, the final barrier to be traversed by eukaryotic molecules destined to the extracellular space. Fungal and plant cells, however, differ from most eukaryotes, since they are surrounded by a complex and very dynamic cell wall [6,7]. Extracellular molecules in plants and fungi have been first described more than fifty years ago [8] and many of them were characterized in detail during the last decades (reviewed in [9]). The observation of extracellular molecules in fungal and plant cells implies that *trans*-cell wall secretion is mandatory in these organisms. Indeed, the fact that the final step of secretion in fungi and plants is the traversal of the cell wall brings additional complexity to the secretory mechanisms used by these cells. A similar rational is valid for cell wall-containing prokaryotes. Mechanisms of trans-cell wall secretion in bacteria, however, have been extensively discussed in recent years and will not be the topic of this review [10–12]. Trans-cell wall secretion in plants is also the focus of some recent, excellent reviews [7,13,14]. In this article, we will focus on vesicular mechanisms of secretion in fungi, highlighting the contribution of proteomics to the understanding of how fungal molecules reach the extracellular space.

2. Export of macromolecules in fungi requires EV

The mechanisms by which molecules produced by fungal cells are transported across the cell wall remained unknown until very recently. The cell wall is mainly composed of polysaccharides and proteins [6], but early reports suggested that lipids could be transitory components of this cellular compartment [15,16]. Moreover, studies combining electron microscopy and the freeze-etching technique suggested the association of membrane vesicles with the cell wall of fungi four decades ago [17,18]. Approximately thirty years later, a cell-wall lipid was fully characterized in a fungal species for the first time, and its association with cell-wall vesicles was again proposed [19]. Together, these reports suggested that cell-wall lipids could be components of carrier vesicles involved in the transport of macromolecules to the extracellular space. This hypothesis, raised in many of these early reports, was experimentally confirmed only about five years ago in the human pathogen *Cryptococcus neoformans* [20]. In this organism, polysaccharide-containing vesicular structures were isolated from culture supernatants, supporting the hypothesis that vesicular cell-wall transport could represent a fungal solution for the delivery of extracellular components, as illustrated in Fig. 1. After the initial description and further confirmation of the existence extracellular vesicles (EV) in *C. neoformans* [20–31], analogous compartments were found in *Paracoccidioides brasiliensis* [32–35], *Histoplasma capsulatum* [21–23], *Sporothrix schenckii* [23], *Candida albicans* [23], *C. parapsilosis* [23], *Saccharomyces cerevisiae* [23,36,37], and *Malassezia sympodialis* [38]. Similar findings were observed for plant cells [39].

In eukaryotes, protein secretion or exocytosis follows a conventional endoplasmic reticulum-*trans*-Golgi network-plasma membrane route, where a finally coordinated network of vesicle transport promotes vesicular fusion with the plasma membrane and release of cargo to the extracellular space [1]. The characterization of fungal EV implied the existence of alternative mechanisms of secretion by which vesicles would be transported through the plasma membrane. EV can have multiple mechanisms of biogenesis [4], which usually results in compositional divergence. In this regard, recent proteomic studies suggest that fungal EV have complex and still obscure mechanisms of biogenesis that may share similarities with those described in mammalian cells [23,24,35,37]. The principal features of EV produced by fungi will be discussed in the next sections.

3. Characterization of fungal EV in different species: a great molecular diversity

In *C. neoformans*, EV were first identified with classic cell biology tools. By transmission electron microscopy, vesicles that were isolated from culture supernatants were demonstrated to contain bilayered membranes. They manifested various sizes and morphologies, including electron-dense and electron-lucid vesicles, vesicular structures with membrane-associated electron-dense regions, and vesicles containing hyper-dense structures resembling a dark pigment [20,24]. These vesicles were found to be associated with inner and external layers of the cell wall [20], suggesting an involvement in trans-cell wall traffic. Serological approaches revealed the presence of polysaccharides and proteins that were recognized by sera from human individuals infected with *C. neoformans* [20,24]. Mass spectrometry (MS) analysis of lipids revealed the presence of sterols and glucosylceramide [20], a glycolipid component of the fungal cell wall [19].

A more comprehensive characterization of *C. neoformans* EV was only achieved when vesicle samples were analyzed by proteomic-based approaches [24]. More than seventy proteins were identified in the *C. neoformans* EV. Surprisingly, most of these proteins lacked the characteristic signal peptide required for conventional secretion [24]. Protein classification revealed very diverse cellular processes; most of them were not related to conventional secretory mechanisms. This observation, which had precedents in mammalian systems [40,41], suggested that fungal EV also derived from unconventional and/or still unknown pathways of secretion.

The combined use of serology, biochemistry, and MS led to the identification of polysaccharides, phospholipids, neutral lipids, and seventy-six proteins as EV components secreted by *C. neoformans* [20–22,24–26,42]. Under conditions of higher sensitivity, however, similar samples from other species were analyzed and the results revealed an interesting complexity in the composition of fungal EV (Table 1). In the human dimorphic pathogen *H. capsulatum*, the number of proteins

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