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Challenges posed by extracellular vesicles from eukaryotic microbes

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Abstract

Extracellular vesicles (EV) produced by eukaryotic microbes play an important role during infection. EV release is thought to benefit microbial invasion by delivering a high concentration of virulence factors to distal host cells or to the cytoplasm of a host cell. EV can significantly impact the outcome of host-pathogen interaction in a cargo-dependent manner. Release of EV from eukaryotic microbes poses unique challenges when compared to their bacterial or archaeal counterparts. Firstly, the membrane-bound organelles within eukaryotes facilitate multiple mechanisms of vesicle generation. Secondly, the fungal cell wall poses a unique barrier between the vesicle release site at the plasma membrane and its destined extracellular environment. This review focuses on these eukaryotic-specific aspects of vesicle synthesis and release.

It is now broadly accepted that extracellular vesicles (EV) are released by microbes of all types. EV have been characterized from bacterial, archaeal, and eukaryotic microbes, and found to be important in a number of biologically relevant contexts, including virulence and pathogenesis [1,2]. While most research has focused on their biological significance, the mechanisms for EV synthesis, cargo loading, and transport to the extracellular space remain enigmatic, particularly for eukaryotic and Gram-positive microbes. Among EV-producing microbes, the best-studied are the Gram-negative bacteria, in which the outer membrane provides both origin location and resources for EV synthesis, and these studies have served as a model for other bacteria [3]. However, the huge morphological differences between bacteria and protozoans or fungi make extrapolating findings from one system to the others difficult. Eukaryotic microbes such as *Saccharomyces cerevisiae* and *Cryptococcus neoformans* were first proposed to release secretory vesicles several decades ago [4-6], based on ultrastructural studies. These findings were not followed up largely because it was believed that the fungal cell wall would preclude release of vesicles to the extracellular space and because of concern that they could be culture artifacts arising from self-assembly of lipids. In 2007, EV were shown to be released by *C. neoformans* to the extracellular environment and subsequently were associated with a variety of other fungi [7,8]. The observation that EV could be produced by fungi despite their cell wall led to a search for these structures in Gram-positive bacteria and found several EV-producing species [9,10]. In 2008, proteomics suggested an EV secretory system in *Leishmania donovani*, which was

later confirmed to be its primary means of protein secretion [11,12]. Through the reinvigorated interest in fungal and protozoan EV in the last decade, coupled with advances in proteomic and lipidomic techniques, a more finely detailed role of EV release has begun to emerge.

The role of microbial-released EV in eukaryotic microbes is presumably similar to that in bacterial systems, namely the delivery of cargo such as enzymes and virulence factors in a concentrated form to mediate effects on the extracellular space. EV delivery solves the problem of dilution that inevitably follows release of any molecule at the cell membrane. Since volume increases with the third power of the distance from the membrane, the concentration of products released at the cell surface drops rapidly as a function of distance from the cell. Furthermore, fungi degrade complex biological structures such as cellulose and lignin and it is believed that efficient digestion requires a combination of enzymes [13,14]. Hence, EV allow concentrated delivery of cargo that can be used to digest targets and obtain nutrition. Cargo such as virulence factors can be concentrated for delivery to host cells, giving these vesicles a “virulence bag” function [10,15,16]. Vesicles can be delivered to distal host cells to deliver a larger impact than diffused soluble virulence factors. Phagocytic cells can internalize EV, which then influence innate immune response in a cargo-specific manner [17,18]. Intracellular pathogens can deliver their payload, including virulence factors working in tandem, to a single cell, where the cargo are immediately delivered to the host cell cytoplasm. Such direct cytoplasmic delivery was observed in vesicle-like structures protruding from *Trypanosoma cruzi* inside HeLa cells [19] and *L. donovani* inside macrophage cell lines [11]. One of the fascinating aspects of EV is that all have similar dimensions irrespective of type of cell of origin, on the order of 30 – 500 nm [7,19-21].

What differentiates eukaryotic microbial EV from their bacterial counterparts? Two distinct characteristics are important to keep in mind: (1) Eukaryotic microbes have multiple membrane sources that can serve as EV points of origin, while bacteria are limited to their envelope membranes and (2) some eukaryotic microbes, such as the fungi, have an additional barrier in the form of a cell wall that must be crossed before the EV can be released to the extracellular environment. This review will address these characteristics and their implications for eukaryotic EV export.

Vesicle origin

Studies on model organisms such as *S. cerevisiae* and metazoan species such as *Caenhorrabditis elegans* have demonstrated two primary sources for extracellular vesicles. The first are multivesicular bodies (MVBs), which fuse with the plasma membrane to release their intraluminal vesicles to the extracellular space. The second is the plasma membrane itself, which can bud and pinch off away from the cell, forming an independent extracellular vesicle [22,23]. Although nomenclature remains field-specific, leading to discrepancies, these subpopulations will be referred to here as **exosomes** and **microvesicles**, respectively. The different mechanisms of vesiculogenesis have implications in membrane and cargo composition.

Exosome formation, composition, and release

Exosome formation relies on the formation of intracellular MVBs, which in turn relies on the endosomal sorting complex required for transport (ESCRT) protein complex [24]. Alternative mechanisms for MVB formation are described in mammalian cells [25], and may play a yet-unknown role in eukaryotic microbes. Evidence for ESCRT protein involvement in exosome formation was first suggested by proteomic analyses, which identified ESCRT members as EV-associated proteins [16,26]. Maturing endosomes, or MVBs, can be trafficked to the lysosome for degradation, but can also be steered toward the plasma membrane, where MVB fusion with the plasma membrane results in release of intraluminal vesicles (ILV) (Figure 1). This process was confirmed to play a role in eukaryotic microbial EV when mutations in ESCRT-related genes of *S. cerevisiae* decreased EV output compared to wild type [23]. Interestingly, the absence of ESCRT machinery does not drastically effect the remaining *S. cerevisiae* EV protein composition [23]. Visual confirmation of this process for *C. neoformans* and *T. cruzi* [19,27] was later made via electron microscopy. Ubiquitin machinery, known to stimulate formation of ILV within an MVB, has also been found associated with EV [11,16,27], and therefore may play a broader biological role than solely lysosomal targeted vesicle cargo selection.

Most work on eukaryotic microbial EV has centered on entire EV populations, without separating exosomes from microvesicles. These studies have found phospholipids such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine to be large constituents of EV membranes across a variety of species, including *P. brasiliensis*, *C. neoformans*, and *Histoplasma capsulatum* [7,28-30]. In *P. brasiliensis*, lipidomics comparing fatty acid and sterol incorporation of two separate isolates revealed similarities in composition but differences in proportions between whole cell lipid extracts and EV extracts [28]. This supports the idea that EV originate from specific organelles, rather than nonspecific membrane sources. Sterol composition varies between fungal species, with brassicasterol, ergosterol, and lanosterol dominating in both *P. brasiliensis* cells and EV and ergosterol and obtusifoliol dominating in *C. neoformans*. In *C. neoformans*, the cell-surface glycosphingolipid glucosylceramide is also incorporated in EV membrane composition [29,31], revealing a new role for this lipid virulence factor.

Two subpopulations of EV have been identified in *T. cruzi* through differential centrifugation. Trypanosomal exosomes are released near the flagellar pocket, a location with few subpellicular microtubules relative to the rest of the *T. cruzi* cell body [19], and are smaller in size than trypanosomal microvesicles. This is in contrast to the fungi *C. neoformans* and *S. cerevisiae*, which on average release larger vesicles as exosomes than microvesicles [23,27].

Microvesicle formation, composition, and release

Microvesicles, also called ectosomes [19] form directly from the plasma membrane (Figure 1). This is generally thought to involve the plasma membrane budding away from the cell in a “reverse endocytosis”-like event, as imaged in *L. donovani* [12]. Formation of microvesicles may also require the ESCRT protein complex, which can be redirected to the plasma membrane to form the protrusion away from the plasma membrane. An additional

mechanism in *C. neoformans* forms microvesicles through plasma membrane invagination and subsequent fusion, resulting in a “scooped out” section of plasma membrane independent of the originating cells [32] (Figure 1). The suggestion that vesicles form by inverted macropinocytosis provides a mechanism outside the ESCRT protein complex function. Vesicle production was also altered by knockdown of the secretory gene *SEC6* in *C. neoformans*, where it participates as part of the exocyst complex [33,34]. These redundancies could help explain why a true EV-null strain has proven difficult to identify by genetic means, as the mechanisms for generating and releasing these EV are likely multiple and redundant and possibly independent.

Because of their putative plasma membrane origin, microvesicle membranes closely reflect the exterior of the originating cell. In *T. cruzi*, microvesicles contain surface glycoproteins and an inner plasma membrane leaf protein that is not found in *T. cruzi* exosomes [19]. Surprisingly, the *T. cruzi* microvesicle population contained more cytoplasmic cargo than the exosome population, suggesting two different mechanisms for cargo loading in this organism.

Architectural challenges of vesicle release

Among eukaryotic microbes, fungal cells pose the unique problem of a thick cell wall that EV must cross to reach the extracellular environment. This cell wall is composed of a crosslinked mesh of beta-glucans, chitin, and mannoproteins. The pore size of the *S. cerevisiae* cell wall varies by strain from 50 – 200 nm, but can change based on cell wall remodeling enzymes, extracellular pH, culture growth phase, and accumulation of deposits such as melanin under distinct conditions [35]. The puzzle of how EV, which can be as large as 500 nm [29], cross this mesh remains largely unanswered, although these extremely large EV are relatively rare in vesicle populations, and their formation from fusion of smaller vesicles has not been ruled out. Three nonexclusive hypotheses can explain how vesicles cross the cell wall: (a) channels exists in the cell wall through which vesicles are guided to the extracellular environment, (b) cell wall-remodeling enzymes temporarily alter the cell wall as vesicles are released, and (c) the cell wall is unchanged and vesicles are forced through small pores by yet to be characterized mechanical or other physical forces (Figure 2).

Evidence to support these non-mutually exclusive hypotheses is scattered in the literature. Movement of the cell wall was observed ten years ago in *S. cerevisiae* [36], and found to be consistent with the speed of kinesins and other microtubule motor proteins. EV proteomics reveals actin, tubulin, and kinesins associated with EV from *C. neoformans*, *H. capsulatum*, and *P. brasiliensis* [7,16,26,27]. One explanation could be the presence of cytoskeletal-like channels that exist to help guide EV through the cell wall to the extracellular environment. Ultrastructural studies have yet to discern obvious channels surrounding EV in the cell wall, which would strengthen the argument for their existence. Alternatively, cytoskeletal proteins could function to guide EV to the proper site of release, or propel them through dense cell wall matter similar to *Listeria monocytogenes* usurping of cytoskeletal actin [37,38].

Several observations suggest cell wall remodeling may occur to facilitate vesicle release. First, cell wall pore size is affected by stressful conditions. *S. cerevisiae* cell wall pore size doubles from 200 nm to 400 nm under oxidative stress [35], but decreases as cells enter stationary phase [39]. These alterations may be enough to allow release of even the larger double-membrane EV, but also may limit the growth phase for optimal EV release. Second, proteomics have identified several enzymes involved in cell-wall remodeling that are associated with EV preparations. These include both cell-wall degrading machinery, such as amylase and endochitinase, and cell-wall synthesis machinery, such as glucanosyltransferase and chitin synthase proteins. These cell-wall metabolism enzymes have been identified in *S. cerevisiae*, *H. capsulatum*, *C. neoformans*, and *P. brasiliensis*, suggesting a pan-fungal association of cell wall machinery with EV [7,16,23]. In fact, proteomics on EV from the Gram-positive bacterium *Staphylococcus aureus* also reveals the presence of cell wall-altering enzymes [40], suggesting cell wall manipulation may be a common mechanism to overcome the cell wall obstacle for many microbial species. Alternative hypotheses, such as recruitment of EV to the site of cell-wall insult, must be ruled out before these enzymes can be determined to play a role in EV release via cell wall remodeling.

There are likely to be a variety of fates for EV crossing the fungal cell wall. Lipidomics of *P. brasiliensis* EV and cell wall extracts show a similar lipid composition [28,41,42]. This suggests some vesicles may persist or linger in the cell wall, either intact or as fragments after breaking while in the wall. Bursting EV within the cell wall may serve to deliver some EV cargo, such as alpha-galactosyl residues, to the wall [8]. Bursting vesicles may also account for some of the lipids observed within the cell wall itself [42,43]. An experimental approach to study cell wall transit is the use of melanized cells to influence cell wall permeability, rigidity, and vesicle release. Melanin is produced by a number of fungi and is known to accumulate in the cell wall. In fact, melanin has been proposed to be synthesized in vesicles that are then trapped in the cell wall [21]. Cell wall melanization decreases the cell wall pore size, charge, and hydrophobicity [44,45]. Melanization also correlates with increased numbers of vesicle-like structures between the plasma membrane and the cell wall [27]. These structures may represent “trapped” vesicles unable to traverse the less porous cell wall, indicating a potential new tool to track vesicle production and the initial interactions with the cell wall.

EV cargo influences

EV secrete a variety of cargo, and proteomics of *P. brasiliensis* revealed a similar number of EV-released protein cargo (205) as soluble-released proteins (260). More EV-released proteins than soluble-released proteins lacked predicted secretory signals (39%, compared to 31%) [26], while 98% of the *L. donovani* secreted proteins lack classical secretion signals despite exosomes comprising 52% of the protein secretome [11,12]. These data are consistent with EV as a nonconventional means of protein secretion, with a yet-unknown signal for incorporation as EV cargo.

The idea that environmental stimuli influences microbial secretion is well established and temperature, CO_2 concentration, iron concentration, and immune cell presence can each change microbial secretomes. It follows that a host environment can also affect EV secretion

and cargo composition. Individual variables such as temperature and pH can affect EV release and cargo selection in *Leishmania donovani* [11], with an increase in pH correlating to an increase in virulence factor EF-1α incorporation. Antibody binding was shown to have an active effect on *C. neoformans* secretion, although the differences in radiolabeled lipid secretion was not measurable [46].

Understanding genetic control of vesicle release and cargo selection has only recently begun. Knockdown of *SEC6* in *C. neoformans* decreases the number of EV, suggesting a role of exocyst in EV release [33]. Cargo selection studies have shown the *C. neoformans apt1* flippase mutant releases EV lacking normal polysaccharide cargo [47], while both *C. neoformans* and *S. cerevisiae* GRASP protein mutants have shown that post-Golgi secretion events are required for the release and inclusion of polysaccharide into EV [23,48]. In *L. donovani*, EV from strains lacking the zinc-dependent metalloprotease *GP63* have markedly different protein cargo composition and induce a greater *in vivo* inflammatory response than wild-type EV [49]. Learning more about genetic regulation of vesicle cargo will help answer the question of how cargo is selected for inclusion and how these cargo influence disease outcome.

Major unsolved problems in EV biology

The past decade has seen an upsurge in EV-focused studies, resulting in great strides toward understanding the protein and lipid composition of EV, the inducing conditions for EV synthesis, and the role in pathogenesis of EV. Yet many questions remain, such as how EV vesicle cargo is selected and incorporated or what molecular machinery is required to generate and release EV. The role of EV in eukaryotic microbiology is an exciting frontier with important implications in the host-pathogen interaction dynamic.

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Highlights

- Eukaryotic microbes face unique challenges in releasing extracellular vesicles
- Eukaryotic microbes release exosomes and microvesicles via distinct mechanisms
- Nonexclusive hypotheses for vesicle transit through the cell wall are proposed
- Genetic regulation of vesicle cargo selection and release remains an important focus

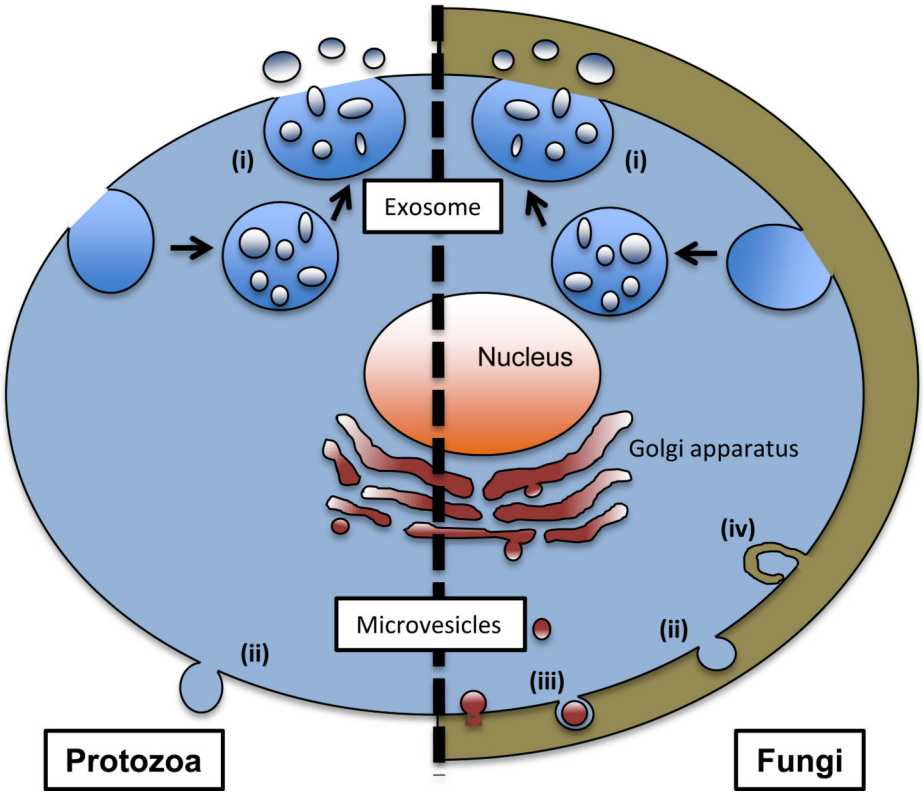

Figure 1.

Vesicle exit from eukaryotic microbes. Both protozoa (left) and fungi (right) generate extracellular vesicles by multiple means: (i) Exosomes exit via multivesicular body fusion with the plasma membrane while (ii) microvesicles exit by plasma membrane budding away from the cytoplasm. In fungi, there is evidence for (iii) golgi involvement in vesicle production and (iv) “scooping out” of cytoplasm by inverted macropinocytosis.

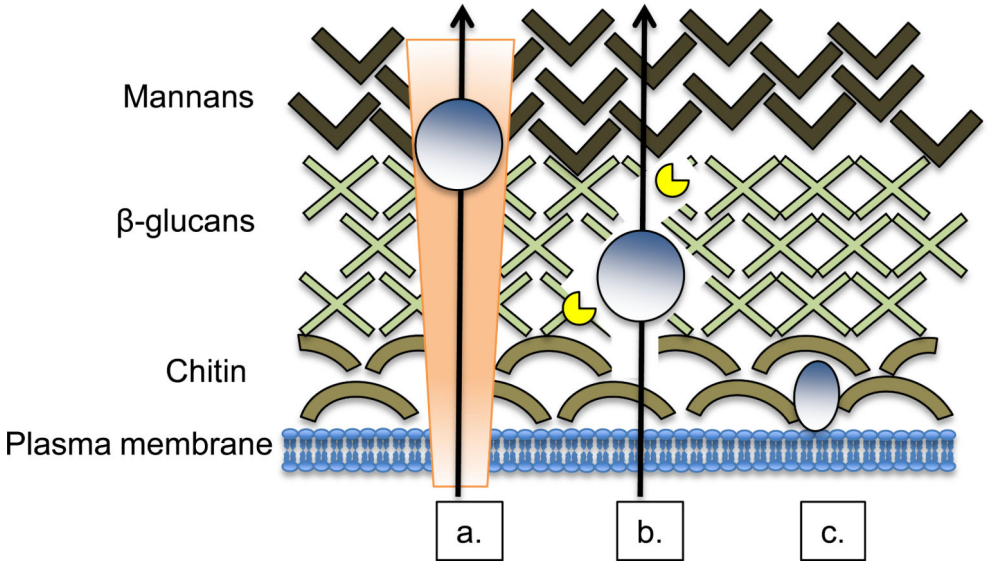

Figure 2.

Three nonexclusive models of vesicle exit through the fungal cell wall: (a) Channels guide vesicles through the wall toward the extracellular environment; (b) cell wall remodeling enzymes increase pore size to facilitate vesicle release; (c) vesicles move into the wall due to still-uncharacterized physical or mechanical forces.