Chapter 12

Analysis of Yeast Extracellular Vesicles

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Abstract

Extracellular vesicles (EV) are important carriers of biologically active components in a number of organisms, including fungal cells. Experimental characterization of fungal EVs suggested that these membranous compartments are likely involved in the regulation of several biological events. In fungal pathogens, these events include mechanisms of disease progression and/or control, suggesting potential targets for therapeutic intervention or disease prophylaxis. In this manuscript we describe methods that have been used in the last 10 years for the characterization of EVs produced by yeast forms of several fungal species. Experimental approaches detailed in this chapter include ultracentrifugation methods for EV fractionation, chromatographic approaches for analysis of EV lipids, microscopy techniques for analysis of both intracellular and extracellular vesicular compartments, interaction of EVs with host cells, and physical chemical analysis of EVs by dynamic light scattering.

Key words Fungi, Yeast, Extracellular vesicles, *Cryptococcus*, *Candida*, *Saccharomyces*

1 Introduction

Organisms of the three domains of life shed extracellular vesicles (EVs) into their microenvironment [1]. During the last decade, it has been demonstrated that EVs play fundamental roles in cancer, infectious diseases, and neurodegenerative disorders, suggesting potential targets for diagnosis, prognosis, and therapeutic intervention in different syndromes [2]. EVs are spherical bilayered compartments ranging in diameter from 20 to 1000 nm [1]. Due to their complex molecular composition, characterization of EVs in different biological systems requires a combination of several experimental approaches, including centrifugation-based protocols, microscopy techniques, chromatographic analysis, proteomics, glycomics, lipidomics, and nucleic acid sequencing, among others.

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In the fungi, EVs were firstly described in the yeast pathogen *Cryptococcus neoformans* [3]. Since then, EVs have been characterized in yeast forms a number of fungal species, including *Candida albicans* [4–7], *C. parapsilosis* [8], *Histoplasma capsulatum* [8], *Malassezia sympodialis* [9], *Paracoccidioides brasiliensis* [7, 10–13], *Saccharomyces cerevisiae* [14], and *Sporothrix schenckii* [8]. Remarkably, studies with *Alternaria infectoria* provided the only experimental evidence demonstrating that filamentous fungal forms also produce EVs [15].

Fundamental biological properties have been suggested for most of the fungal species listed above [3, 4, 7–10, 12, 13, 16–19]. However, many questions about fungal EVs remain still unanswered. In a context of high biological importance combined with still obscure properties, it is clear that improving the methods for analysis of fungal EVs will be determinant for advancement of the field. In this manuscript, we describe protocols that have been used by our group for the analysis of EVs produced by yeast forms of the pathogens *C. neoformans* and *C. albicans* and by the model organism *S. cerevisiae*.

2 Materials

Prepare all solutions with ultrapure water (18.2 MΩ cm at 25 °C) and analytical grade reagents.

2.1 Media for Storage and Growth of Yeast Cultures

1. Media: Yeast cells can be stored in standard media used for fungal growth, including Sabouraud's medium and brain-heart infusion (BHI).
2. Sabouraud's broth (2% dextrose and 1% peptone): Weight d-glucose (20 g) and meat peptone (10 g) and make up to 1000 ml with water. Sterilize through autoclaving.
3. BHI medium: Dissolve 37 g of BHI in 1000 ml water. Sterilize through autoclaving.
4. Solid media: Prepare Sabouraud's or BHI broth as described above and supplement with 2% agar (20 g per liter) (*see* **Note 1**).
5. Minimal medium (MM): 15 mM d-Glucose, 10 mM $MgSO_4$, 29.4 mM KH_2PO_4, 13 mM glycine, 3 μM thiamine-HCl. Dissolve d-glucose (2.7 g), $MgSO_4$ (1.2 g), KH_2PO_4 (4 g), glycine (1 g), and 10 μl of thiamine solution in 800 ml water. Stir, adjust pH to 5.5, and make up to 1000 ml with water in a volumetric cylinder.
6. 0.3 M Thiamine solution: Dissolve 100 mg of the vitamin in 1 ml of water and filter through sterile 0.22 μm membranes. Add 10 μl of the thiamine stock solution for each liter of MM.

2.2 EV Isolation

1. Phosphate-buffered saline 0.01 M (PBS): Dissolve 0.26 g KH_2PO_4, 1.25 g K_2HPO_4, and 8.71 g NaCl in a beaker containing 700 ml of water. Stir, adjust pH to 7.4, and make up to 1000 ml with water in a volumetric cylinder. Filter through sterile 0.22 μm membranes. Store at 4 °C.
2. Membranes for filtration and ultrafiltration, respectively: 0.4 μm Polycarbonate membrane and 100 kDa polyethersulfone membrane for stirred ultrafiltration cells.
3. Density gradient: Stock solution of 60% iodixanol in water. Gradient fractions (6%, 7.2%, 8.4%, 9.6%, 10.8%, 12%, 13.2%, 14.4%, 15.6%, 16.8%, 18%) are diluted in water.

2.3 Lipid and Protein Content of EVs

1. HPTLC silica gel $60F_{254}$ plate: Glass plates with 5 cm × 10 cm dimension.
2. Separation solvent: Combine hexane, diethyl ether, and glacial acetic acid to form a 40:20:1 (vol:vol:vol) mixture (*see* **Note 2**).
3. Sterol detection reagent: 0.05% $FeCl_3$, 5% acetic acid, and 5% sulfuric acid. Weight 0.05 g $FeCl_3$ and dissolve in 90 ml water in a glass beaker. Add 5 ml of glacial acetic acid. Slowly add 5 ml of concentrated sulfuric acid. Stock at room temperature and protect from light.
4. Sterol quantification: Amplex Red cholesterol assay kit (Molecular probes).
5. Dissolving buffer: Chloroform:methanol:0.75% KCl (8:4:3, v/v/v).
6. Ergosterol.
7. Protein quantification: BCA protein assay kit (Pierce).

2.4 EV Staining

1. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine solution (DiI) staining solution: Add 5 μl of stock solution (1 mM) to 995 μl of PBS. Mix well by gently pipetting (*see* **Note 3**).

2.5 Host Cells

1. Macrophage-like cultures: RAW 264.7 murine macrophages (American Type Culture Collection).
2. Sodium pyruvate.
3. L-Glutamine.
4. Gentamicin.
5. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).
6. 2-Beta-mercaptoethanol.
7. Complete Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS): Add 10 ml of filter-sterilized FCS to 90 ml of DMEM. Supplement with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mg/ml gentamicin, 10 mM HEPES, and 50 mM 2-beta-mercaptoethanol.

2.6 Transmission Electron Microscopy

1. Cellulose capillaries for high-pressure freezing (Leica Microsystems): 200 μm inner diameter, wall thickness 8 μm.
2. High-pressure freezing-carries filling: 1-Hexadecen.
3. Osmium tetroxide.
4. Acetone.
5. Glutaraldehyde.
6. Uranyl acetate.
7. Lead citrate.
8. Sodium citrate.
9. Freeze substitution medium: To prepare the osmium tetroxide stock solution, dilute 1 g of the crystal in 25 ml of pure acetone. For the working solution, mix 500 μl of 4% osmium tetroxide in acetone, 1.4 μl of 70% glutaraldehyde, and 9.6 μl of deionized water and add pure acetone to complete 1 ml. Keep the solution frozen in liquid nitrogen.
10. Epoxide resin embedding: Epon-812 or Spurr (EMS).
11. Electron microscopy copper grids: 200 mesh grid and slot grid.
12. Post-staining solutions: To prepare 5% uranyl acetate, add 2.5 g of the solid to 50 ml of distilled water. Store at room temperature protected from light; solution can be used 24 h after preparation. To prepare Reynold's solution, add 1.33 g of lead citrate, 1.76 g of sodium citrate, and 5 ml of 1 N NaOH to 30 ml of distilled water. Stir for 10 min and make up to 50 ml with water. Store at 4 °C.
13. Substitution medium: 2% Osmium tetroxide, 0.1% glutaraldehyde, and 1% of water in acetone.

2.7 Cryoultramicrotomy and Immunogold Electron Microscopy

1. 0.2 M Sodium cacodylate buffer: Add 21.4 g of the solid to 400 ml of distilled water. Adjust pH to 7.2–7.4 and add distilled water to complete 500 ml.
2. Formaldehyde.
3. Gelatin.
4. Polyvinylpyrrolidone.
5. Methylcellulose.
6. Fixation medium: Mix 4 μl of 25% glutaraldehyde, 250 μl of 16% formaldehyde, 500 μl of sodium cacodylate buffer, and 246 μl of distilled water. Adjust pH to 7.2.
7. Sample preparation and cryoprotectant solutions: For 10% gelatin, add 1 g of gelatin powder to 5 ml of warmed distilled water under shaking. After dissolution, make up to 10 ml with water. For 2.3 M sucrose/PVP solution, add 2.5 g of polyvinylpyrrolidone (PVP) to 5 ml of 2.3 M sucrose (prepared previously by adding 78.7 g of the solid to 100 ml phos-

phate buffer 0.1 M, pH 7.2). Leave the solution overnight under shaking and add 2.3 M sucrose to complete 10 ml. Store at −20 °C.

8. Thawing and staining solutions: To prepare 3% methylcellulose, add 0.3 g of methylcellulose to 10 ml of water. For staining solution, prepare a 9:1 (v/v) mixture of 3% polyvinyl alcohol (PVA) (previously prepared by adding 0.3 g of PVA to 10 ml of water) and 5% uranyl acetate (prepared as mentioned above).
9. Grid washing solution: 3 g BSA per 100 ml PBS, pH 8.0.
10. Quenching solution: Add 26 mg of NH_4Cl to 10 ml of PBS, to form 50 mM NH_4Cl.

3 Methods

3.1 EV Isolation

1. Pick up a single colony from stock cultures and inoculate into an Erlenmeyer flask containing 20 ml of MM (Subheading 2.1). Incubate for 48 h at 30 or 37 °C under shaking (150 rpm).
2. Transfer the culture to 600 ml of liquid media contained in 2 l Erlenmeyer flasks. Cultivate yeast cells up to stationary phase (cell density of approximately 1×10^8 cells/ml) at 30 or 37 °C with shaking (150 rpm).
3. Remove fungal cells and debris by sequential centrifugations at 4000, 10,000, and 15,000 × *g* for 15 min, at 4 °C. Discard pellets after each centrifugation step. Vacuum-filter cell-free supernatants through 0.4–0.8 μm polycarbonate membranes to remove possible cellular contaminants.
4. Concentrate supernatants approximately 20-fold using the ultrafiltration system (100 kDa cutoff membrane). Ice baths may be used to prevent microbial contamination.
5. Ultracentrifuge concentrated supernatants at 100,000 × *g* for 1 h at 4 °C; resulting pellets are usually not visible. Discard ultracentrifugation supernatants and gently suspend pellets in PBS. Repeat the ultracentrifugation protocol twice, always discarding supernatants. Suspend pellets in 200 μl PBS (crude EV fractions) or, alternatively, use dry pellets for lipid extraction. For *C. neoformans* EVs, removal of contaminant glucuronoxylomannan (GXM) is required (*see* **Note 4**). For further EV fractionation, iodixanol density gradient may be used (*see* **Note 5**).

3.2 Analysis of Sterol and Protein EV Content

1. Add 300 μl methanol and 600 μl chloroform to a 120 μl suspension of crude EVs. Alternatively, the 900 μl methanol-chloroform mixture can be added to dry ultracentrifugation pellets. Vortex vigorously for 10 s. White precipitates (protein aggregates or polysaccharides) are usually formed.

2. Spin down for 10 s at 2000 × *g* at 4 °C; a two-phase system will be formed. Discard the upper phase and concentrate the lower phase to dryness under a N_2 stream.
3. Fractionate the dry lower phase according to the Folch partition method [20] by dissolving the sample in 1 ml of dissolving buffer. Vortex vigorously and collect the lower phase enriched with neutral lipids. Concentrate to dryness under a N_2 stream.
4. Dissolve the sample in 60 μl of a 2:1 (v/v) chloroform:methanol mixture. Using a microliter syringe, load 20 μl of the lipid mixture into an HPTLC silica gel plate to form band-shaped loading areas 0.5–1 cm wide, 1 cm above the bottom of plate. Sterol standard (ergosterol, 2 μg) is required for retention factor (*Rf*) comparisons between samples.
5. Place the HPTLC plate in a chromatography chamber under the conditions described in Subheading 2.3. Remove plates from the chamber when the distance between the solvent front and the plate's edge is approximately 1 cm. Wait for natural solvent evaporation in a chemical hood (10–15 min, room temperature).
6. Spray the dry plate with the sterol detection reagent (subheading 2.3) and heat it at 100 °C for 5 min (Fig. 1). Sterols develop as purple bands. For densitometry analysis of the spots, we recommend ImageJ software (imagej.nih.gov/ij/).
7. Quantify sterols with the Amplex Red Cholesterol assay kit, following the manufacturer's instructions.
8. Quantify proteins with the BCA protein assay kit [5] (*see* **Note 6**). Protein identification can be performed through LC-MS/MS proteomics, as previously described [4, 14]. Proteomics protocols are out of the scope of this chapter and will not be described here.

3.3 Sample Preparation by High-Pressure Freezing and Freeze Substitution for Routine TEM Observation and/or Electron Tomography

1. Prepare yeast cell or EV pellets as detailed in Subheading 3.1. Place pellets between two types of aluminum carriers, protecting the biological material within the 200 μm cavity on one carrier (fill the whole cavity). Alternatively, cells or EVs may be placed in 200 μm wide cellulose capillaries previously cut into 2 mm pieces (for fitness into 3 mm HPF carriers) (*see* **Note 7**).
2. Mount the sandwiched samples in the HPF holder and freeze it using high-pressure freezing equipment (HPM 010 or 100, Bal-Tec, Corp., Liechtenstein). The machine should be previously loaded with liquid nitrogen. After freezing, carriers are removed from the holder and stored in liquid nitrogen.
3. Remove samples carefully from liquid nitrogen and immerse them in the substitution medium (*see* **Note 8**).

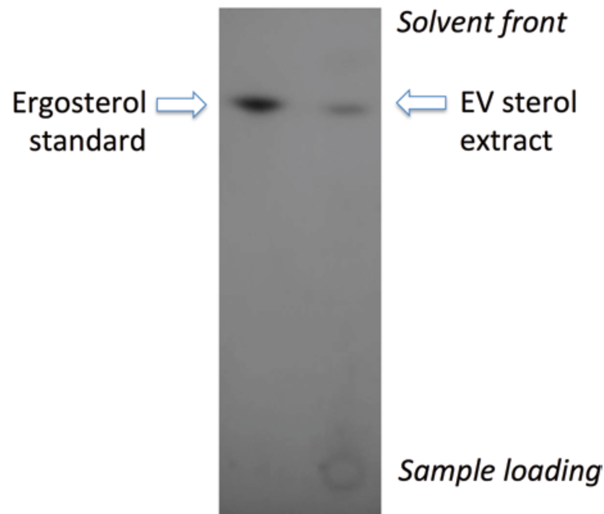

Fig. 1 Illustrative HPTLC analysis of a sterol extract obtained from crude EV fractions

4. Apply a substitution-heating curve consisting of keeping the samples for 72 h at 90 °C, 15 h at −20 °C, and 2 h at 4 °C.
5. After substitution, wash the samples three times with acetone at room temperature. Embed samples in epoxide resins (Epon or Spurr). Polymerize at 60 °C for 72 h.
6. Using a ultramicrotome, obtain 70 nm thick sections for conventional transmission electron microscopy or 200 nm thick sections for electron tomography and collect the sections in 200 mesh copper grids. Alternatively, series of sections may be obtained and collected on formvar-coated slot grids.
7. Post-stain the grids for 20 min in 5% uranyl acetate in water and 5 min in 1% lead citrate (post-staining solutions, 2.7). For electron tomography, incubate both sides of the grids in 10 nm colloidal gold for 5 min and wash with distilled water.
8. Transfer the grids to a transmission electron microscope and acquire images using either a film or a digital acquisition system (CCD camera).
9. For electron tomography, transfer the grids containing the 200 nm thick sections to a transmission electron microscope equipped with electron tomography capabilities, operating at 200 kV.
10. Position the grid to ensure that the grid's bars are parallel to the axis of the tomography holder to minimize obstruction of the electron beam at high tilt angles. Similarly, for slot grids containing serial sections, position the slot (assuming that the ribbon of serial sections is placed along the slot axis) parallel to the holder axis.

11. After defining the region of interest, perform the corrections of eccentric height and focus, and record the tilt series from −70° to +70° with an angular increment of 1° or 2°.
12. At the end of the tomography acquisition, a single file comprising the tilt series will be generated (MRC file). Align the tilt series based on fiducial markers (colloidal gold applied as mentioned above) with appropriate software (for example, IMOD software package—University of Colorado, USA).
13. After alignment of the tilt series, perform the three-dimensional (3D) reconstruction using, for example, weighted back projection or SIRT. We suggest the IMOD software package (University of Colorado, USA). A 3D model can be generated automatically by manual segmentation of the structures of interest in the 3D volume (Fig. 2). Different software is available for this purpose, including IMOD software package (University of Colorado, USA), AMIRA (Visage Imaging, USA), and Cytoseg (National Center for Microscopy and Image Research, USA).

3.4 Cryoultramicrotomy and Immunogold Electron Microscopy

1. For immunogold labeling, fungal cells in the fixation medium (Subheading 2.8) for 1 h at 4 °C.
2. Wash the cells twice in 0.1 M sodium cacodylate buffer and spin down to obtain strongly tight pellets. If firm pellets are not formed, alternatively embed sediments in 10% gelatin. With a toothpick, transfer the pellet and the surrounding buffer to a parafilm-covered Petri dish. Section the sediment in 2 mm wide cubes using a razor blade. Transfer pellet pieces to the polyvinylpyrrolidone/sucrose cryoprotectant solution (Subheading 2.8) and incubate for 2 h. Mount the pellet in cryoultramicrotomy holders and plunge it into liquid nitrogen, making a fast circular movement to avoid freezing by nitrogen gases formed around the sample (Leidenfrost effect).
3. Transfer the sample to a previously cooled cryoultramicrotome loaded with a glass or cryodiamond knife. Sample and chamber temperatures should be adjusted to −90 °C; knife temperature must be −70 °C. Obtain ultrathin cryosections, arrange them with an eyelash, and collect them in 2.3 M sucrose or 3% methylcellulose (Subheading 2.8), using a wire loop.
4. Thaw the sections over formvar-coated 300 mesh nickel grids and transfer them to the grid washing solution, cells facing down. Incubate the grids for 30 min in quenching solution (Subheading 2.8).
5. Wash the grids three times for 5 min with the grid washing solution.
6. Incubate the grids with the primary antibody for 1–3 h.

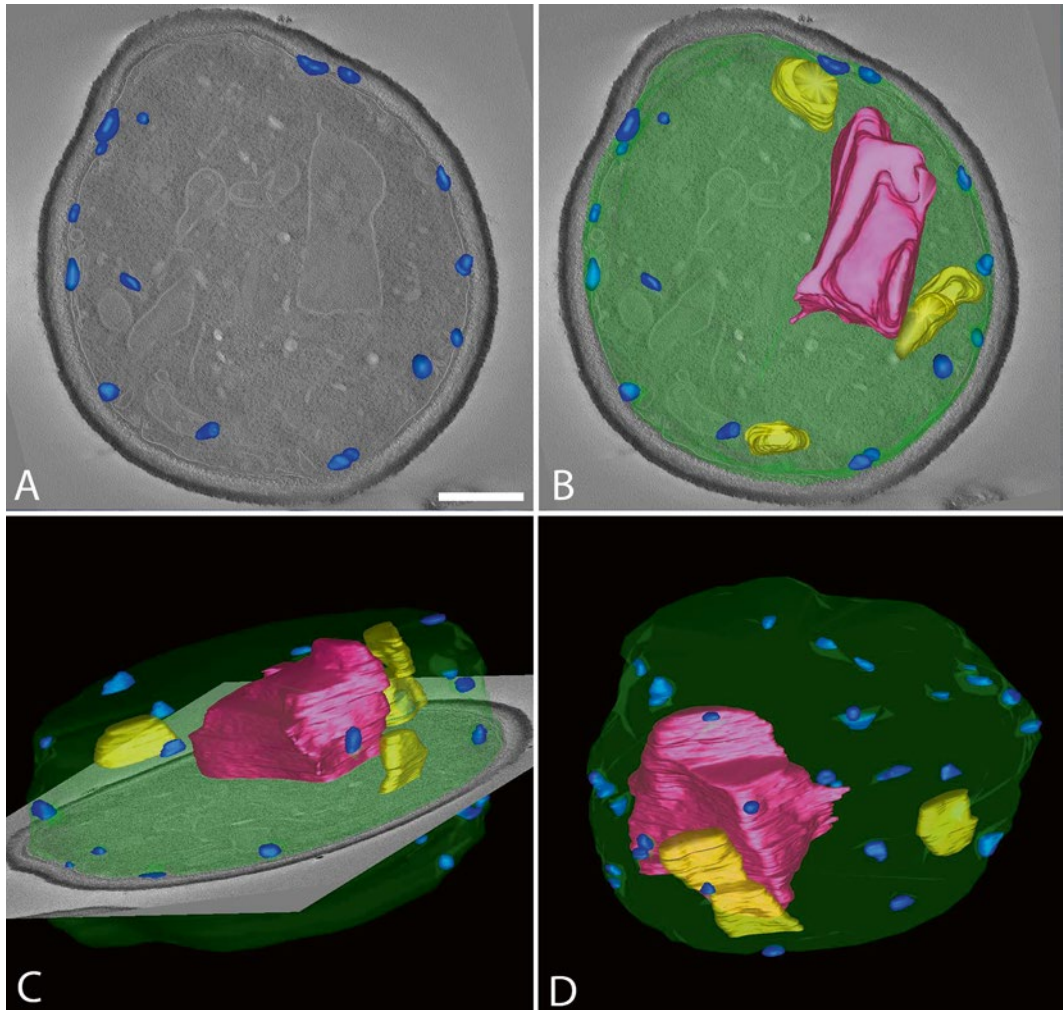

Fig. 2 Three-dimensional reconstruction of a whole *S. cerevisiae* cell by serial electron tomography. Cells were prepared using the high-pressure freezing-freeze substitution protocol described here. A ribbon of 200 nm sections was obtained by ultramicrotomy and collected in formvar-coated slot grids. Tilt series of ±70° at a 1° interval was obtained from each profile of the cell in each section and reconstructed. Segmentation was performed in IMOD, as described before. (**a**) Virtual section of a tomogram showing the general structure of the cell. Budding extracellular vesicles are shown in *blue*. (**b**, **c**) Projection of virtual sections and reconstructed models of intracellular structures at different angles. B—Orthogonal projection, C—tilted view. (**d**) 3D model of the reconstructed volume (whole cell) showing the segmentation of a few surface and intracellular structures. *Blue*: Extracellular vesicles. *Red*: Nucleus. *Yellow*: mitochondria (partial segmentation). *Green*: Plasma membrane-intracellular space. Bar: 1 μm

7. Wash the grids three times for 10 min with the grid washing solution (Subheading 2.8). Incubate the grids with the secondary antibody conjugated with gold particles of varying sizes, depending on the structure that should be labeled (usually between 5 and 15 nm).

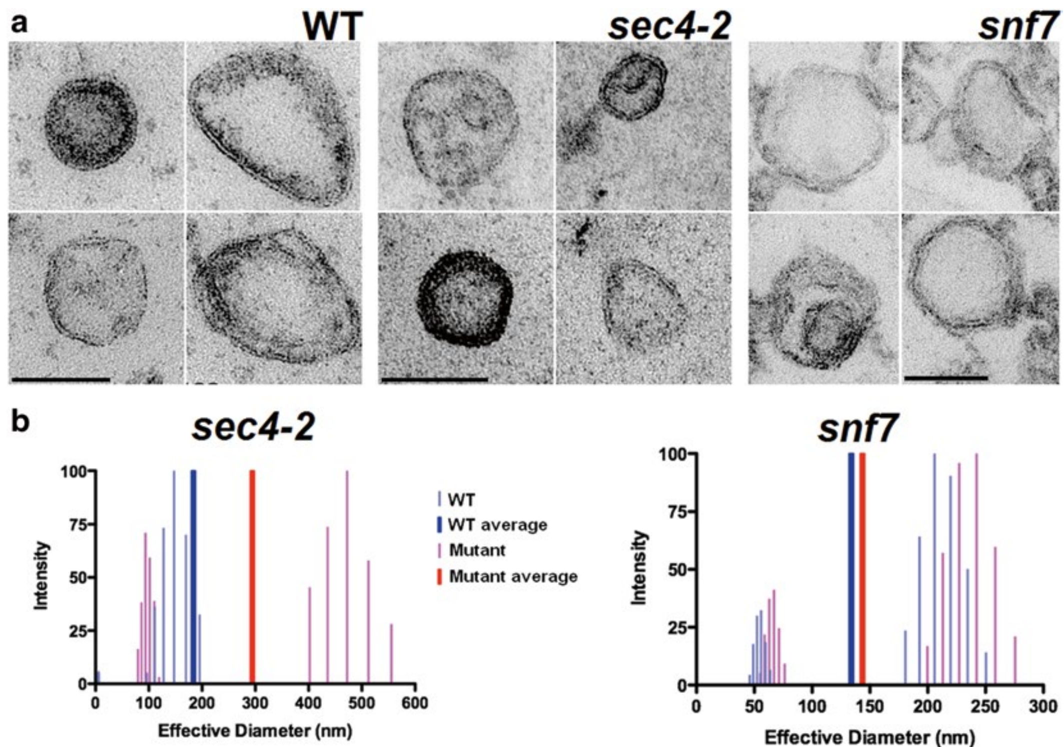

Fig. 3 TEM (**a**) and DLS (**b**) analyses of *S. cerevisiae* EVs. In (**a**), each individual panel exemplifies the typical vesicle morphology for wild-type (WT) and secretion mutants (*sec4.2* and *snf7*). Scale bar, 100 nm. B. Light scattering analysis showing diameter distribution and average values of vesicles obtained from WT or mutant (*sec4-2* and *snf7*) cells. For strain details, *see* ref. 14, from which this figure has been reproduced

8. Wash the grids three times for 10 min with grid washing solution, and then quickly wash in water (30 s each wash). Place the grids in a mixture of PVA-uranyl acetate staining solution for 10 min. Drain off the excess of liquids and let the grids naturally dry at room temperature before observation in a transmission electron microscope (Fig. 3a).

3.5 Physical Chemical Analysis of EVs s by Dynamic Light Scattering (See Note 9)

1. Analyses of fungal EVs by dynamic light scattering (DLS) in our laboratory during the past 5 years have been performed using a quasi-elastic light scattering (QELS) in a 90Plus/BI-MAS Multi Angle Particle Sizing analyzer (Brookhaven Instruments Corp., Holtsville, NY). Suspend purified vesicles in PBS until current count rate is between 10,000 and 90,000 counts per second. DLS analysis should be performed at 22 °C with an equilibrium time of 2 min. A minimum of six replicates with five measurements per replicate should be used.
2. Check the raw correlation data to ensure that the amplitude (*y*-intercept) is stable and the correlograms are smooth (i.e., decay exponentially to a flat baseline) (*see* **Note 10**). Discard

the sample if sediment is visible at the bottom of the cuvette following measurements (*see* **Note 11**).

3. Report the mean *z*-average diameter and mean polydispersity index along with their standard deviations based on the replicates.
4. Obtain the multimodal size distributions of particles (Fig. 3b) by a non-negatively constrained least squares algorithm (NNLS) based on the intensity of light scattered by each particle.

3.6 DiI staining of EVs

1. Prepare 995 μl of an EV suspension with total sterol content adjusted to 2 μg/ml. Add 5 μl of DiI (stock solution, Subheading 2, **item 5**) (*see* **Note 12**). Incubate the suspension for 15 min at 25 °C protecting from light.
2. Ultracentrifuge DiI-stained EVs at 100,000 × *g* for 1 h, 4 °C, and discard the supernatant. Wash stained EVs with PBS three times as described in Subheading 3.2.
3. Suspend DiI-stained EVs to generate samples with sterol concentrations corresponding to 2 μg/ml. For EV suspension, use media suitable for interaction with host cells [4, 8, 17] as mentioned in Subheading 2.4.
4. To prepare negative controls, repeat the procedures above with fractions containing no vesicles.

3.7 Interaction of EVs DiI Stained with Host Cells

1. Place RAW 264.7 cells previously suspended in suitable media (Subheading 2.6) into glass cover slip-covered wells of 24-well plates. Initial cell density will correspond to 2×10^5 cells/well. Incubate at 37 °C in a 5 % CO_2 atm until macrophages firmly adhere to the plate (approximately 2 h).
2. Replace culture media with fresh media (500 μl) containing DiI-labeled EV suspensions with sterol concentrations varying from 1 to 0.001 μg/ml. Incubate at 37 °C (5 % CO_2) for 15 min, 1 h, 16 h, and 24 h. After each time point wash monolayers three times with 500 μl PBS and fix the cells with 4 % paraformaldehyde (in PBS) for 1 h at 25 °C or overnight at 4 °C.
3. Transfer glass cover slips to glass slides containing 3 μl of PROLONG GOLD antifade (Life Technologies P36934). Seal with uncolored nail polish and let cover slips dry for 60 min.
4. Observe slides under fluorescence microscopy (excitation, 546 nm; emission, 590 nm). If available, microscopes equipped with deconvolution systems will allow simpler interpretation of the intracellular distribution of EVs, as illustrated in Fig. 4.

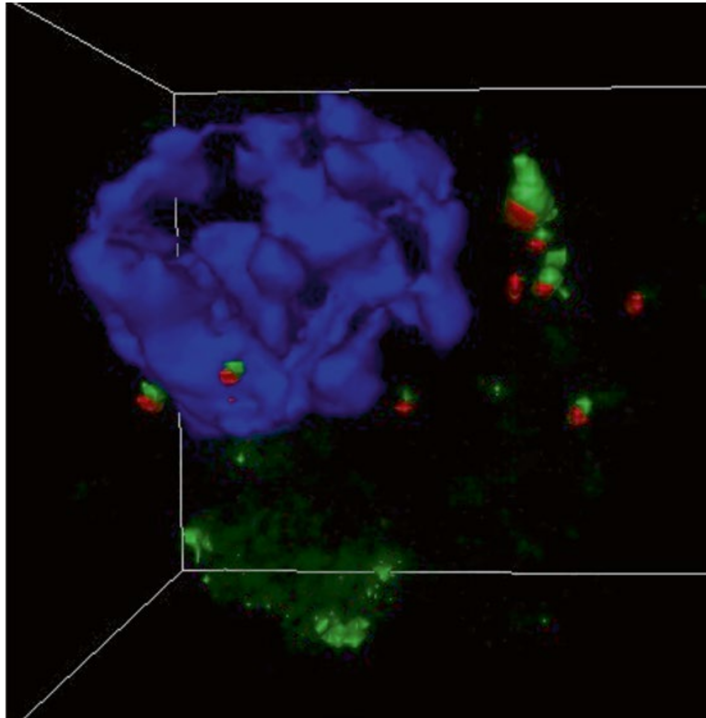

Fig. 4 Tridimensional analysis showing cell surface binding and internalization of DiI-stained *C. albicans* EVs in a dendritic cell model. Z-stacks and deconvolution profiles were obtained on an AxioVision 4.8 (Carl Zeiss International) inverted fluorescence microscope. EVs are shown in *red*; nucleus is stained in *blue* (DAPI) and the cell surface is stained in *green* (β-cholera toxin subunit-FITC; details in [4])

4 Notes

1. Autoclave and distribute media into Petri dishes (20 ml per 13 mm plates) before the temperature reaches 50 °C. Keep plates for 5 min at room temperature for agar solidification.
2. Pre-saturate the chromatographic chamber with the solvent mixture by adding the separation solvent to the chamber's bottom part and incubating for 10 min in a gas hood. The mixture must be freshly prepared and used only once to avoid changes in concentration due to evaporation. Volumes will vary depending on chamber dimensions; 0.5 cm solvent height is acceptable.
3. Alternative long-chain lipophilic carbocyanine analogs, such as DiO, can be similarly used for EV staining.
4. To remove contaminant polysaccharide from preparations of *C. neoformans* EVs, suspend pellets from the 100,000 × *g* centrifugation in 50 μl PBS and add to the wells of a 96-well enzyme-linked immunosorbent assay (ELISA) plate previously coated with a monoclonal antibody to the major capsular polysaccharide

of *C. neoformans* (MAb 18B7 to GXM; coating with 10 μg/ml solution, for 1 h at room temperature; antibody provided by Dr. Arturo Casadevall for the studies performed so far). Block with PBS containing 1% BSA (1 h at 37 °C). Incubate the plates for 1 h at room temperature, and collect the unbound fraction, containing vesicles free of soluble polysaccharides. Filter through 0.4 μm membranes to remove any potential aggregate or contaminating cells.

5. Fractionate EVs by loading vesicle samples into an iodixanol gradient (Optiprep). Prepare solutions of iodixanol ranging from 6 to 18% in PBS, with 1.2% concentration increments between each solution. Load iodixanol fractions sequentially from higher (bottom) to lower (top) concentrations into the centrifuge tube. Each fraction will have 300 μl and the final volume in the tube will be 3.3 ml. Gently dispense the crude EV suspension at the top of the 6% gradient fraction and ultracentrifuge at 250,000 × *g* for 75 min. In our experience, a Beckman swing-bucket rotor (50.1Ti) has been successfully used. Keep ultracentrifuge brake off. Collect 11 fractions of 300 μl each from top to bottom. Fractions can be tested for morphological features, biochemical composition, and biological activity [21, 22].
6. The BCA-based method is not efficient for protein detection in *Cryptococcus* EVs, likely because of polysaccharide interference.
7. Samples are taken into the void by capillarity, so it is important to ensure that the space between the capillaries is filled up with 1-hexadecene, allowing pressure and heat transfer to take place.
8. The substitution medium should be precooled to −90 °C using a computer-controlled freeze substitution apparatus.
9. DLS measures the hydrodynamic diameter. In analysis of EV suspensions, light is scattered by vesicles; hence variations in the intensity of the scattered light can be detected. The reason explaining these characteristics relies on differences in the phases of waves scattered by different particles. The DLS experimental time-averaged autocorrelation data contains information on all the diffusional timescales present in the system. The data is usually fitted based on the cumulate approach with a least number of exponential decays sufficient to reproduce the correlation curve. The following equations are used for data interpretation:

$$\Gamma = Dq^2 \tag{1}$$

with D being the diffusion coefficient of the nanoparticles and q the scattering vector given by [23]

$$q = 4\pi n\lambda^{-1}\sin(\theta / 2) \quad (2)$$

where λ is the wavelength of the incident light, n is the refractive index of the suspension medium, and θ is the scattering angle. By using the "Stokes-Einstein" relationship the hydrodynamic diameter d of the particles can then be calculated on the assumption of a log normal size distribution [23]:

$$d = kT / 3\pi\eta D \quad (3)$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the dispersing medium. This technique is extremely precise when there is a simple distribution of decay times, e.g., for scattering from spherical nanoparticles with a narrow distribution of sizes about a single mean, although issues arise in accurately determining the size distribution.

10. Noisy correlograms and/or fluctuating amplitudes for a given sample can be attributed to the presence of dust/foreign particles in the sample, concentration variations from sample precipitation or aggregation, solvent evaporation, or dirty cuvettes.
11. Sediment indicates that the sample either contains a significant portion of large (micrometer) size particles or the target particles are unstable during the time frame of the experiment.
12. DiI and DiO are long-chain lipophilic carbocyanines that diffuse laterally within the plasma membrane. Therefore, staining with DiI can modify EV dimensions, which has been in fact observed in our laboratory.

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