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i. Characterization of Extracellular Vesicles by Transmission Electron Microscopy and Immunolabelling Electron Microscopy

LARA CORONA Maribel, HURBAIN Ilse, RAPOSO Graça and VAN NIEL Guillaume

ii. Abstract

Transmission Electron Microscopy (TEM) is currently the only method that enables the observation of extracellular vesicles (EVs) at a nanometer scale. Direct visualization of the whole content of EV preparation provides not only crucial insights on the morphology of EVs but also an objective evaluation of the content and purity of the preparation. Coupled to immunogold labelling, TEM allows the detection and association of proteins at the surface of EVs. In these techniques, EVs are deposited on grids and are chemically immobilized and contrasted to withstand a high voltage electron beam. Under high vacuum, the electron beam hits the sample and the electrons that scatter forward are collected to form an image. Here, we describe the steps needed to observe EVs by classical TEM and the extra steps required to label proteins through IEM.

iii. Key words

Transmission Electron Microscopy (TEM), Immunolabelling Electron Microscopy (IEM), Immunogold labelling, Extracellular vesicles (EVs), Antibodies (Ab).

1. Introduction

Transmission electron microscopy (TEM) witnessed the birth of the Extracellular Vesicles (EVs) field providing the first observation of these entities (Wolf, 1967; Harding & Stahl, 1983; Pan & Johnstone, 1983) and seminal knowledge about their heterogeneity, composition and biogenesis mechanisms (Colombo et al., 2013) (Jeppesen et al., 2019). While complementary methods of characterization of these vesicles are constantly completing the tool set, TEM remains an essential technique to analyze these vesicles and extend our knowledge on their features.

The working principle of TEM and IEM relies on an electron beam in high vacuum that hits the sample, which scatters forward some electrons to form an image (Knoll & Ruska, 1932). Considering that increasing the velocity of an electron will decrease its wavelength, the TEM microscope accelerates electrons in order to decrease their wavelength to the nano or even atomic scale (Winey, et al., 2014). As a result, the resolution is greatly increased and the observation of even small EVs can be achieved with fine detail (Chuo, Chien & Lai, 2018). Low atomic number atoms such as C, H, O, N, P, S, which are abundant in biological samples, scatter few electrons. For this reason, the sample is coated with heavy metals in order to produce electron dense areas that will increase the contrast (Harris, 2014), this is often referred to as negative staining.

Oftentimes, the classification of EVs seems difficult due to the fact that EV composition heavily depends on the cell type and the physiological state of the producing cell (Yáñez et al., 2015). In spite of their heterogenicity, common EV features comprise their ability to reach target cells (Raposo et al., 1996) and their capacity to modify recipient cell functions through various mechanisms (Van Niel, D'Angelo & Raposo, 2018). In the last decades, EVs have gained wide recognition since they have been described for most biofluids (Bano, Ahmad & Mohsin, 2021), rendering them quite promising as non-invasive biomarkers (Lane, et al., 2018; Urabe, et al.,

2020). In addition, the extensive work done on EVs regarding chemical and genetic modifications highlight their potential as nano-vehicles for therapeutical molecules (Mentkowski, 2018).

Vesicle size ranges from 30 nm to several µm of diameter (Doyle & Wang, 2019), and often, EV isolation protocols will result in solutions containing EVs of different sizes. TEM and IEM are techniques used to distinguish between various vesicle populations. In addition, these techniques are helpful to discriminate between EVs, particles of similar size or contaminants that might have been co-isolated with classical EV isolation protocols such as Differential Ultracentrifugation (UC), Size Exclusion chromatography (SEC) or Density Gradient (Stam, et al., 2021; Wills, et al., 2018). In this regard, TEM and IEM can be used to determine the purity of the EV preparation for downstream applications, notably in vivo (Rikkert, et al., 2019).

To date, there is no gold standard for the complete characterization of EVs. However, it is undeniable that Electron Microscopy is still the one of the most robust and reliable techniques to visualize and label EVs. While various protocols have been employed to visualize and label EVs by TEM, we present here two standard protocols that allow the user to observe and label EVs in TEM and IEM, respectively.

2. Materials

Prepare all solutions with ultra pure autoclaved water (prepared by purifying water to reach a resistivity of 18.2 M Ω x cm at 25°C).

Follow all security and waste disposal rules displayed in the product information sheet and institutional procedures.

All materials are prepared with analytical grade reagents.

2.1 EV deposition

1. Grids : TEM Grids 200 Mesh Cu/Pd. Formvar/carbon supported grids. (see Note 1)

2.2 Fixation

1. Phosphate buffer (PB): 200 mM, pH 7.4. Weigh 27.59 g of NaH₂PO₄.H₂O₋ Add water to a volume of 1L. Weigh 35.60 g of Na₂HPO₄.2H₂O₋ Add water to a volume of 1 L. Mix 1 volume of 0.2M NaH₂PO₄ and 4 volumes of 0,2M Na₂HPO₄. Adjust pH to 7.4 if necessary. Store at room temperature. (see Note 2)

2. Fixative A: 2% paraformaldehyde (PFA) (see Note 3), 1 % glutaraldehyde in 100 mM PB, pH 7.4. To prepare 10 mL of fixative A, mix 5 mL of 200 mM phosphate buffer pH 7.4 with 0.4 mL of 25% glutaraldehyde, then add 1.25 mL of 16% formaldehyde. Make up to 10 mL with 3.35 mL of water. Use the fixative fresh.

3. Fixative B: 2% PFA in 100 mM PB, pH 7.4. To prepare 10 mL of fixative B, mix 5 mL 200 mM PB pH 7.4 with 1.25 mL 16% of PFA/H2O. Make up to 10 mL with 3.75 mL of water. Use the fixative fresh.

4. Fixative C: 1 % glutaraldehyde in 100 mM phosphate buffer pH 7.4. To prepare 10 mL of fixative C, mix 5 mL of 200 mM PB pH 7.4 with 0.4 mL of 25% glutaraldehyde/H2O. Make up to 10 mL with 4.6 mL of water. Use the fixative fresh.

2.3 Quenching/Blocking

1. Phosphate buffered saline (PBS): 0.017 M KH₂PO₄, 0.05 M Na₂HPO₄, 1.5 M NaCl, pH 7.4. (see Note 4).

2. PBS/BSA 1%: Weigh 100 mg of Bovine Serum Albumin (BSA) and. Add PBS to a final volume of 10 mL.

3. PBS/BSA 0,1%: Mix 1 volume of PBS/BSA 1% and 9 volumes of PBS.

4. PBS/glycine: 50mM. Weigh 375 mg of glycine. Add PBS to a final volume of 100 ml. Store at 4°C.

2.4 Contrasting

1. 2% Methylcellulose: Weigh 4 g of methylcellulose (25 centipoises) and transfer it to a cylinder. Add water at 90°C to a volume of 200 mL and stir the solution. While stirring, cool down the methyl cellulose solution on ice rapidly, then seal it and leave it overnight at 4° still mixing. The next morning, stop mixing and leave it for 2 days more at 4°C for polymer maturation. Add water to a final volume of 200 mL and centrifuge at 100 000 x g during 1.5 h at 4°C. Divide the solution in aliquots of 15 mL and store at 4°C.

2. 4% Uranyl acetate: Weigh 4 g of Uranyl acetate and transfer it to a cylinder (see Note 5). Add water to a volume of 100 mL. Let it mix on a rotor (120 rpm) until it dissolves. Store it protected from light at 4°C

3. Contrasting solution: Mix gently with a micropipette 900 μ L of 2% methylcellulose and 100 μ L of 4% uranyl acetate. Keep the solution in the dark at 4°C. Prepare fresh before use.

2.5 Immunolabelling

1. Primary antibody: Primary Antibody of choice, diluted according to the manufacturer's recommendation (approximatively 1-20 µg/ml)

2. Protein A Gold (PAG): EM-Grade 10 nm, diluted according to the manufacturer's recommendations.

3. Bridging antibody: if the primary Antibody of choice has low or no affinity for Protein A (mouse IgG1), use a rabbit anti mouse IgG as a bridge and diluted according to the manufacturer's recommendation (approximatively 1-20 μ g/ml)

3 Method:

1. Conventional Transmission Electron Microscopy (TEM)

The following steps are performed at room temperature, unless indicated otherwise.

- 1. Unroll parafilm on the bench for EV deposition, fixation, washing and contrasting steps (see note 6).
- 2. Deposit 4 µl of EVs at a concentration of 10⁹ to 10¹¹ particles/mL on formvar coated, carbonated grids for 20 min (see note 7). Cover the grids with a Petri dish lid to protect them from air impurities and dehydration during all the incubation steps. (Fig. 1a).

- 4. Prepare on a parafilm firmly stretched on the bench, the drops of fixative A and water. One line of drops per EV samples as grids are not distinguishable.
- 5. Recover the grids with tweezers and place them upside down on a drop of fixative. Fix the grids in fixative A for 20 min at room temperature (see note 8).
- 6. Wash the grids with water 6 x 1 min at room temperature (see Note 9).
- 7. Work on ice. Put a parafilm in a Petri dish and deposit 50-100μl drops of the contrasting solution of Methylcellulose/Uranyl Acetate. Incubate the grids upside down on the solution for 10-12 min. Protect the sample from light with aluminium foil (see Note 10).
- 8. Remove the grids from the drop with loops that have a slightly larger diameter than the grid.
- 9. Soak the excess of fluid by gently pushing the loop sideways (with an inclination of 45°) on a Whatman filter paper No 1 placed on a parafilm (to avoid blotting uranyl acetate on the bench) so that a thin film is left covering the section side of the grid (see Note 11) (Fig 1b) Discard the parafilm and the filter paper which were in contact with UA as indicated in institutional procedures.
- 10. Air-dry the grid for at least 10 min up to 1h. After drying, the film should show a dark gold to blue interference colour (Fig. 1c).
- 11. Remove the grids from each loop with fine tweezers. Store the grids in a EM gridbox at room temperature.
- 12. Use an electron microscope to take pictures of the grids (Fig. 2a).

2. Immunolabelling

The following steps are performed at room temperature, unless indicated otherwise.

- 1. Unroll parafilm on the bench for EV deposition, fixation, immunolabelling, washing and contrasting steps (see note 6).
- 2. Deposit 4 μ l of EVs at a concentration of 10⁹ to 10¹¹ particles/mL on formvar coated, carbonated grids for 20 min (see note 7). Cover the grids with a Petri dish lid to protect them from air impurities and dehydration during all the incubation steps. (Fig. 1a).
- 3. Prepare on a parafilm firmly stretched on the bench, the drops of fixative B and water. One line of drops per EV samples as grids are not distinguishable.
- 4. Recover the grids with tweezers and place them upside down on a drop of fixative. Fix the grids in fixative B for 20 min (see note 8).
- 5. Wash the grids with water 6 x 1 min at room temperature (see note 9).
- 6. Incubate the grids in PBS/Glycine 50 mM 4x 2 min. (see note 12)
- 7. Incubate the grids in PBS/BSA 1% for 5 min (see note 13).
- 8. Prepare the antibody solution in PBS/BSA 0.1% and incubate the grids in this solution for 30 min. (a 10-20μl drop for one grid).

For a primary antibody raised in rabbit or if it is a mouse IgG2a or IgG2b, go directly to step 10. If the primary raised in rat or is a mouse IgG1, perform the bridging steps 7 and 8 and continue with step 9. (Note 14)

- 9. Incubate the grids in BSA/PBS 0.1% 5 x 2 min
- 10. Prepare the bridging antibody in BSA/PBS 0.1% and incubate the grids for 20 min (a 10-20 μ l drop for one grid).
- 11. Wash the grids in BSA/PBS 0.1% 5 x 2 min.

- 12. Incubate the grids with Protein-A-Gold (PAG) in BSA/PBS 0.1% for 20 min (see Note 15 and 16). (a 10-20µl drop for one grid).
- 13. Wash with PBS 5 x 2 min.
- 14. Fix in Fixative C for 5 min.

For a single protein labelling go directly to the step 14. For multiple labelling, go back to step 4 and repeat the instructions (see Note 17 and 18).

- 15. Wash with water 6 x 1 min (see Note 9).
- 16. Work on ice. Put a parafilm in a Petri dish and deposit 50-100µl drops of the contrasting solution of Methylcellulose/Uranyl Acetate. Incubate the grids upside down on the solution for 10-12 min. Protect the sample from light with aluminium foil. (see Note 10)
- 17. Remove the grids from the drop with loops that have a slightly larger diameter than the grid.
- 18. Soak the excess of fluid by gently pushing the loop sideways (with an inclination of 45°) on a Whatman filter paper No 1 placed on a parafilm (to avoid blotting uranyl acetate on the bench) so that a thin film is left covering the section side of the grid (see Note 11) (Fig 1b)
- 19. Air-dry the grid for at least 10 min up to 1h. After drying, the film should show a dark gold to blue interference colour (Fig. 1c).
- 20. Remove the grids from each loop with fine tweezers. Store the grids in a gridbox at room temperature.
- 21. Use an electron microscope to take pictures of the grids (Fig. 2b).

4 Notes

- 1. Put the EV solution on the formvar/carbon coated side. Formvar is a transparent, insulating film that allows the deposition and observation of EVs on the mesh. On the other hand, carbon is highly conductive, but hydrophobic. The glow discharge used to deposit carbon on the grids helps to render the surface grid more hydrophilic. If you carbon-coat the grids yourself, coat the grids with around 2-3 nm of carbon. A very fine coat will quickly damage the sample and a very thick coat will prevent the electrons to be scattered forward.
- 2. Filter the PB solution with a $0.22 \mu m$ pore size filter before use.
- 3. Handle paraformaldehyde with gloves in a fume hood.
- 4. We use a 10X PBS solution diluted in water. Filter the PBS solution with a 0.22 μ m pore size filter before use.
- 5. Uranyl Acetate is a radioactive and carcinogenic substance. Use gloves, glasses and a fume hood when handling this reagent. Follow all institutional rules and the product information sheet to dispose of the materials safely.
- 6. This procedure is performed by placing the grids (EVs facing the buffer) on successive placed on the bench. It is important to keep the grids wet on the section side during all steps, but dry at the opposite side. Each rinsing step is performed on drops of 200-500 μ L while small droplets of 5-15 μ L of immunoreagents are sufficient (this prevents the use of large amounts of antibodies and probes that are generally precious).

- 7. Do not touch the formvar with the tip while depositing the sample and use fine tweezers to handle the grids paying attention to only touch the perimeter of the grid to avoid damaging the area of observation.
- 8. After the EV incubation time, one 200 μ l droplet of fixative can be used to hold 5 grids at the same time, making sure that they do not touch each other or overlap. The combined use of para-formaldehyde and glutaraldehyde allows a quick fixation protocol that maintains EV morphology and structural integrity.
- 9. The washing steps can be simply done by placing the grids on different drops of water. As previously mentioned, several grids of the same condition can be washed on the same water droplet at the same time. This washing step is extremely important to remove traces of phosphate that would precipitate uranyl acetate.
- 10. As uranyl acetate is a radioactive and carcinogenic substance it may be prohibited in some institutions. Alternative contrasting agents are developed such as Uranyless®. The protocol remains the same than with the uranyl acetate/methylcellulose mix except that Uranyless can be used pure.
- 11. During blotting, a quick removal of the uranyl acetate/methylcellulose (UA/MC) will result in a thicker film of UA/MC, whereas a slower removal will result in a thinner film. This step is critical: if the methylcellulose film is too thin, sections can dry out and will not be stable enough under the electron beam. If the methylcellulose film is too thick, structural details on the sections will hardly be visible and will not show any contrast.
- 12. Glycine is used to quench unreacted aldehydes after the fixation step.
- 13. To prevent non-specific binding between the antibody or antibodies and the sample, we use BSA as a blocking agent. Another alternative to reduce the background is the Cold Water Fish Skin Gelatin.
- 14. If you want to perform double labelling, you should do it for antibodies that do not require bridging or for maximum one antibody that requires bridging (using the bridge during the first labelling step).
- 15. For conventional IEM, labelling can only be performed for proteins found at the surface of EVs.
- 16. Protein-A Gold strongly binds to several mammal IgG (for example rabbit) and this interaction facilitates the labelling with antibodies used for other applications like IHC, IF or WB. For multiple labelling, use different sizes of gold particles. It is recommended to prepare the first antibody with the largest gold particle (for example 10 nm) and continue with a smaller one (for example 5 nm). Take in consideration that having several bead sizes in the same sample increases the probability of cross-reactions.
- 17. Before performing any double labelling, perform first a single labelling with each antibody separately to get acquainted with the labelling pattern. It is possible to perform labelling with 2 polyclonal antibodies in combination with protein A-Gold or 1 polyclonal and 1 monoclonal antibody (from different species). Labelling with 2 monoclonal antibodies is not recommended even if they are of different isotypes, because the bridging steps will most likely cause false positives.
- 18. For multiple labelling, it is advised to start with the less abundant antigen, and then repeat the procedure for a more abundant antigen.

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Figures (legends)

Figure 1. Main steps of negative contrast and immunogold labelling. Steps showing the drop of sample deposited on the grid (white arrow) (A), the grid floating on the top of a droplet and the next moves to expect (B).

Figure 2. Blotting step to remove the uranyl acetate and methylcellulose or uranyless. The grid have been recovered on a loop and blotted with an inclination of 45° on whatman paper(A). Blotting should be carefully perform to control the thickness of uranyl and methylcellulose film (B). Air-dried sample on the grid will be removed from the loop to be observed by electron microscopy (C).

Figure. 3. (A) MNT1 derived EVs observed by TEM after negative contrast with uranyl/methylcellulose contrasting. (B) HEK derived EVs observed by TEM after negative contrast with uranyless. (C) MNT1 derived EVs immunogold-labelled for CD63 and revealed with Protein A-gold particle of 10 nm diameter. (C) MNT1 derived EVs double immunogold-labelled for CD63 and revealed with Protein A-gold particle of 5 nm diameter. Scale bars = 200 nm (Credit Roberta Palmulli)

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Figure 2



Figure 3

