Chapter 13

Isolation of Exosomes for Subsequent mRNA, MicroRNA, and Protein Profiling

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Abstract

Exosomes are nano-sized, cell membrane surrounded structures that are released from many cell types. These exosomes are believed to transport a range of molecules, including mRNAs, miRNAs, and proteins; the contents depending on their cell of origin. The physiological and pathological relevance of exosomes has yet to be fully elucidated. Exosomes have been implicated in cell-to-cell communication. For example, in relation to the immune system, such exosomes may enable exchange of antigen or major histocompatibility complex–peptide complexes between antigen-bearing cells and antigen-presenting cells; in cancer, they may contain molecules that not only have relevance as biomarkers, but may also be taken up and cause adverse effects on secondary cells. Furthermore, exosomes have been proposed as autologous delivery systems that could be exploited for personalised delivery of therapeutics. In order to explore the contents and functional relevance of exosomes from medium conditioned by culture cells or from other biological fluids, prior to extensive molecular profiling, they must be isolated and purified. Here, we describe differential centrifugation methods suitable for isolating exosomes from conditioned medium and from other biological fluids, including serum, saliva, tumour ascites, and urine. We also detail Western blotting and transmission electron microscopy methods suitable for basic assessment of their presence, size, and purity, prior to progressing to global mRNA, miRNA, or protein profiling.

Key words: Exosomes, Multivesicular bodies, Extracellular, Cell line, Conditioned medium, Serum, Plasma, Urine, Saliva

1. Introduction

Exosomes are membrane-bound nanoparticles (30–100 nm in diameter) that are released by many cell types. These small, right-side-out structures form intracellularly by inward budding of endosome membranes (1), resulting in vesicles-containing endosomes

Lorraine O'Driscoll (ed.), *Gene Expression Profiling: Methods and Protocols*, Methods in Molecular Biology, vol. 784,
DOI 10.1007/978-1-61779-289-2_13,

called multivesicular bodies (MVBs). When MVBs fuse with the cell membrane, they release their internal vesicles into the extracellular environment. Once released into extracellular space, these microvesicles are termed exosomes (2).

The physiological and pathological role(s) of exosome is of great interest. Exosomes have been implicated in cell-to-cell communication via trans-cellular signalling (3), transfer of membrane receptors, proteins, mRNA, microRNA (miRNA) (4), and organelles (e.g. mitochondria) between cells. Other roles with which exosomes have been associated include the delivery of infectious and toxic agents (e.g. chemotherapeutic drugs) into cells (5). The contents of exosomes depend on their cell of origin (6). Exosomes derived from cells of the immune system enable exchange of antigen or major histocompatibility complex (MHC)-peptide complexes between antigen-bearing cells and antigen-presenting cells. Exosomes have also been described as having both immunostimulatory and anti-tumour affects in vivo (7). Exosomes derived from tumour cells have been associated with accelerating tumour growth (6, 8) and invasiveness (9–11).

Based on the studies of conditioned medium (CM) derived from cell lines and primary cell cultures, as well as from analysis of bodily fluids, including serum/plasma, urine, and saliva, ourselves and others have also reported evidence to suggest that extracellular mRNAs, miRNAs, and proteins may be contained and protected in membrane-bound structures (12, 13). This is supported by the fact that the RNA contained within exosomes remains amplifiable, implicating protection from RNase degradation by the exosome membrane (6, 14, 15). Circulating exosomes have been identified as having potential diagnostic relevance in various cancer types, including ovarian cancer (16), glioblastomas (6), and lung cancer (17). In fact, these structures have been proposed to be involved in horizontal transfer of information between cells, suggesting that mRNAs/miRNAs/proteins could be carried from a cancer cell and be taken up and subsequently cause adverse effects on secondary cells. Intriguingly, emerging data suggest that exosomes may have a role as autologous delivery systems that could be harnessed for personalised delivery of therapeutics into secondary cells.

Following isolation of pure populations of exosomes, these entities are suitable for a range of gene expression profiling approaches in order to determine their contents and so contribute to our understanding of their relevance. Such profiling may include RT-PCR, qPCR, and multiplex PCR (as outlined in Chapters 1 and 2); global mRNA/whole genome microarray analysis (as in Chapter 3); global miRNA analysis (as in Chapter 7); proteomics, including 2D gel electrophoresis, mass spectrometry, and Western blotting (as in Chapters 8 and 9). Thus, detailed protocols for the isolation of

exosomes from CM and bodily fluids using centrifugation and subsequent basic characterisation of exosomes (i.e. their presence and purity) are reported in this chapter.

2. Materials

2.1. Cell Culture and Conditioned Medium Collection

1. MCF7 (American Type Tissue Collection) – RPMI-1640, 2-mM L-Glutamine, 5% foetal bovine serum (FBS) from which exosomes have been eliminated (see Notes 1 and 2).
2. Tissue culture grade vented flasks (e.g. 175 cm^2).
3. 30- and 50-mL centrifuge tubes.
4. 0.22-μm filters.
5. 20- and 50-mL syringes.
6. Biosafety cabinet, pipette aids, etc. (as for basic mammalian cell culture).

2.2. Serum Collection

1. Non-heparinised tube(s) for blood procurement (by trained Phlebotomist).
2. Bench-top centrifuge.
3. Cryovial tubes.
4. –80°C freezer.

2.3. Exosome Collection

1. Refrigerated bench-top centrifuge.
2. 30- and 50-mL centrifuge tubes.
3. Ultracentrifuge and fixed-angle or swinging-bucket rotors (for details on Beckman Coulter ultracentrifuge, rotors, and rpm to *g* conversions, see: http://www.beckmancoulter.com/resourcecenter/labresources/centrifuges/rotorcalc.asp).
4. Appropriately sized polyallomer or polycarbonate tubes for the rotor(s) mentioned in Item 3.
5. Phosphate-buffered saline (PBS): sodium chloride 8 g/L, potassium chloride 0.2 g/L, di-sodium hydrogen phosphate 1.15 g/L, and potassium dihydrogen phosphate 0.2 g/L, pH 7.3 at 25°C (Sigma).

   For the additional/option step of exosome purification on sucrose cushion
6. Tris/sucrose/D_2O: 30 g protease-free sucrose, 2.4 g Tris base, and 50 mL D_2O. Adjust pH to 7.4 with 10 N HCl. Bring to 100 mL total volume with D_2O. Pass through a 0.2-μM filter. Store for up to 2 months at 4°C.
7. 5- and 50-mL syringes.

8. 18-G needle.
9. 0.2-μM filter.
10. SW 28 ultracentrifuge rotor and appropriately sized polyallomer tubes.
11. 45 Ti ultracentrifuge rotor and appropriately sized polycarbonate tubes.

2.4. Western Blotting

1. Laemmli sample buffer (2×): 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, and 0.125 M Tris–HCl, pH 6.8. Stored at –20°C.
2. Resolving buffer (4×): 1.5 M Tris–HCl, pH 8.8, 0.4% SDS. Stored at room temperature.
3. Stacking buffer (4×): 0.5 M Tris–HCl, pH 6.8, 0.4% SDS. Stored at room temperature.
4. Acrylamide/Bis-acrylamide, 30% solution (Sigma). Stored at 4°C.
5. *N*,*N*,*N*,*N*′-Tetramethyl-ethylenediamine (TEMED; Sigma). Stored at 4°C.
6. Ammonium persulfate: 10% solution in double-distilled water, freshly prepared.
7. Running buffer (10×): 250 mM Tris, 1.92 M glycine, 1% (w/v) SDS. Stored at room temperature.
8. Prestained Molecular weight markers: PageRuler™ Prestained Protein Ladder (Fermentas, Burlington, Canada).
9. 1-D Electrophoresis system (Bio-Rad Laboratories Inc., Hercules, CA).
10. Blotting buffer: 25 mM Tris–HCl pH 8.3, 192 mM glycine and 20% (v/v) methanol. Stored at 4°C. Methanol to be added immediately before use.
11. Immun-Blot PVDF membrane and extra thick blot paper (Bio-Rad Laboratories Inc.).
12. Ponceau S solution (Sigma).
13. Tris-buffered saline (TBS, 10×): 100 mM Tris–HCl pH 7.5, 1.5 M NaCl. Stored at room temperature.
14. Blocking buffer: Membranes were blocked in 5% low-fat dry milk (Bio-Rad).
15. Washing buffer (TBS-T): 1× TBS solution supplemented with 1% Tween-20. Stored at room temperature.
16. Antibody dilution buffer: 1× TBS supplemented with 3% (w/v) bovine serum albumin (BSA) and 1% Tween-20. Single aliquots frozen at –20°C.

17. Primary antibody: mouse anti-TSG101 antibody (Abcam): 1:500 dilution in 3% Blotting-Grade Blocker and 0.1% Tween (Sigma).
18. Secondary antibodies: Horseradish peroxidase (HRP) conjugated anti-mouse antibody (Cell Signalling).
19. Enhanced chemiluminescent (ECL) reagents: Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA).
20. Stripping buffer: 62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS. Stored at room temperature. Warm to 70°C and add 100 mM β-mercaptoethanol before use.
21. Wet electroblotting system (Bio-Rad Laboratories Inc.).
22. Visualisation: Proteins were visualised by chemiluminescence (Millipore).
23. Imaging system: Detection was performed with the Chemidoc exposure system (Bio-Rad Laboratories).

2.5. Transmission Electron Microscopy

1. Transmission electron microscopy (TEM).
2. PBS: Sodium chloride 8 g/L, potassium chloride 0.2 g/L, di-sodium hydrogen phosphate 1.15 g/L, and potassium dihydrogen phosphate 0.2 g/L, pH 7.3 at 25°C (Sigma).
3. 4% paraformaldehyde: Dissolve 4 g paraformaldehyde in 90 mL of 0.1 M sodium phosphate buffer. Heat to 65°C while stirring. Carefully add 1N NaOH, dropwise, until the solution clears. Bring to 100 mL with 0.1 M sodium phosphate buffer, allow to cool and then filter through a 0.22-μm filter. This may be used immediately or frozen (once) and stored at –20°C for up to 6 months.
4. 1% glutaraldehyde: Dilute glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, to a final concentration of 1%. This may be used immediately or frozen (once) and stored at –20°C for up to 6 months.
5. Uranyl acetate, pH 4.0: Dissolve 2 g of uranyl acetate in 50 mL distilled H_2O. This may be stored at 4°C in the dark for up to 6 months. Just prior to use, filter required volume through a 0.22-μm filter.
6. Methyl cellulose, 2%: Dissolve 4 g methyl cellulose (Sigma) in 196 mL of distilled H_2O, which has been heated to 90°C. Stirring will be required to dissolve completely. Continuing to stir while rapidly cooling on ice to 10°C, and then more slowly cool to 4°C overnight while stirring. Leave to rest for 3 day, and subsequently bring to a final volume of 200 mL with water. Centrifuge at 100,000 × *g* for 95 min at 4°C. Collect supernatant 2% methyl cellulose. This may be stored at 4°C for up to 3 months.

7. 0.15 M oxalic acid: dissolve 0.945 g oxalic acid in 50 mL distilled H_2O.
8. Uranyl-oxalate, pH 7.0: Mix uranyl acetate (pH 4.0) with 0.15 M oxalic acid (1: 1). Adjust to pH 7.0 by the dropwise addition of 25% (w/v) NH_4OH. This solution may then be stored, in the dark, at 4°C for up to 1 month.
9. Formvar-coated grids.
10. Parafilm.
11. Whatman no. 1 filter paper.
12. Forceps.
13. Grid storage box.

3. Methods

A number of methods exist for the isolation of exosomes from medium conditioned (CM) by cell lines and from bodily fluids (e.g. serum, urine, etc). As the most commonly used method for this purpose is differential centrifugation, we detail this approach here.

3.1. Collecting CM for Subsequent Exosomes Isolation

1. Grow cells of interest (MCF7 included here, as example) until they reach 70% confluency (see Note 3).
2. Remove the medium and replace with fresh FCS-/exosome-free medium, from which cell MCF7-secreted exosomes are subsequently collected. Incubate for 48 h (see Note 4).
3. Collect the CM supernatant and transfer to 30- or 50-mL polypropylene tubes. Centrifuge at 300 × *g* for 10 min at 4°C to remove any free cells. Carefully collect the CM supernatant with a pipette; do not pour (see Note 5).
4. Transfer the CM into a fresh centrifuge tube and spin at 2,000 × *g* for 20 min at 4°C, to remove large cell particles/cell debris. As mentioned above, carefully collect the CM with a pipette and proceed as outlined in Subheading 3.3.

3.2. Serum Collection for Subsequent Exosomes Isolation

1. Allow blood in non-heparinised tube(s) to clot for 30 min minimum to 1 h maximum after procurement.
2. Centrifuge at 400 × *g* for 15 min.
3. Gently remove the serum and dispense (as approx. 0.5 mL volumes) into labelled cryovial tubes.
4. Cryovial tube label should include: (a) anonymised identifier; (b) date; (c) duration of time from procurement to placing at −80°C. Keep in mind that the time from procurement to placing at −80°C should be <3 h.

3.3. Isolating Exosomes from CM

1. Progressing from Step 4 of Subheading 3.1, transfer the CM into a polyallomer or polycarbonate tube(s) appropriate to the ultracentrifuge rotor to be used (see Note 6).
2. Using a waterproof marker, mark one side of each ultracentrifuge tube and carefully place the tubes into the rotor in such a way that the mark is facing out/up, as a reference to where the pellet will be following centrifugation. For fixed-angle rotors, the pellet will be found near the bottom of the tube and on the side of the tube facing up; for swinging-bucket rotors, the pellet will be at the bottom of the tube.
3. Centrifuge at 10,000 × *g* for 30 min at 4°C.
4. Again, gently removing the CM supernatant, repeat Steps 1 and **2** of Subheading 3.3. Even if a pellet is too small to be visible, assume its position to be as mentioned in Step 2 and leave approximately 0.5 cm of medium behind, to avoid disturbing the pellet and thus contaminating the CM supernatant.
5. *Importantly*, for CM from many cell types, Steps 3 and 4 inclusively can be avoided by passing the CM through a 0.22-μm filter to remove any remaining large cell particles/cell debris (see Note 7).
6. After placing the CM supernatant into fresh, marked ultracentrifuge tubes, spin at 110,000 × *g* for 70 min at 4°C; this time to pellet the exosomes.
7. Carefully remove and discard the supernatant CM, saving the exosome pellet.
8. To wash any protein contamination off the exosome pellet, gently re-suspend the pellet in 1 mL PBS (see Note 8). Pool exosome suspensions from all tubes containing exosomes from the same CM and place into a fresh ultracentrifuge tube. Fill the tube with PBS.
9. Centrifuge at 110,000 × *g* for 70 min at 4°C.
10. Re-suspend the pellet in a small volume (typically 50–100 μL) of PBS.

3.4. Isolating Exosomes from Bodily Fluids

In principle, the method for isolating exosomes from bodily fluids, such as serum, plasma, saliva, broncho-alveolar lavage, tumour asites, etc., by differential centrifugation is similar to that for CM. However, as some of these fluids are more viscous than CM, diluting in PBS as well as increasing the duration and – in some cases – the speed of centrifugation is recommended. As mentioned above, the method outlined here is used successfully for isolating exosomes from serum. However, a similar approach is suitable for procuring exosomes from other bodily fluids.

1. Progressing from Step 4 of Subheading 3.2, dilute the serum with an equal volume of PBS and subsequently bring to a 10 mL total volume with PBS.

2. Pass this suspension through a 0.22-μm filter to remove any large cell particles or cell debris.
3. Transfer the serum supernatant into a 30- or 50-mL centrifuge tube and centrifuge at 2,000 × *g* for 30 min at 4°C.
4. As mentioned above, without disturbing the pellet and so risk avoiding contamination, carefully collect the serum supernatant with a pipette and transfer into a polyallomer or polycarbonate tube appropriate to the ultracentrifuge rotor to be used (see Note 6). As outlined in Step 2 of Subheading 3.3, mark the ultracentrifuge tubes and subsequently centrifuge at 12,000 × *g* for 45 min at 4°C. *Importantly*, as an alternative to this spin at 12,000 × *g*, at this stage the serum supernatant can be passed through a 0.22-μm filter.
5. Place the resulting serum supernatant into a fresh tube, and centrifuge at 110,000 × *g* for 2 h at 4°C to pellet the exosomes.
6. Carefully remove the supernatant, this time saving the exosome pellet.
7. To wash the exosome pellet, gently re-suspend in 1 mL PBS (see Note 8). Where relevant, pool exosome suspensions from all tubes containing exosomes from the same serum specimen and place into a fresh centrifuge tube. Fill the tube with PBS.
8. Centrifuge at 110,000 × *g* for 70 min at 4°C.
9. Re-suspend the washed exosomes pellet in a small volume (typically 50–100 μL) of PBS.

3.5. Further Purification of Isolating Exosomes (Optional)

The procedures outlined above are generally adequate for exosome isolation. However, prior to progressing to extensive characterisation, it is recommended that the purity of the initial exosome population isolated from a given source be assessed, e.g. by using electron microscopy. If the population is found not to be of an acceptable purity (e.g. if it includes large protein aggregates), an additional step involving further purification of the exosomes on a sucrose gradient is recommended. To do so:

1. Gently re-suspend the exosome pellet (for CM, from Step 9 of Subheading 3.3; for serum, from Step 8 of Subheading 3.4 in 25 mL PBS).
2. Place 4 mL of Tris/sucrose/D_2O solution into a polyallomer tube (appropriate for SW 28 ultracentrifuge rotor).
3. Carefully layer the diluted exosomes onto the cushion of sucrose without mixing.
4. Centrifuge at 110,000 × *g* for 70 min at 4°C.
5. Using the 18-G needle on the 5-mL syringe, pierce the lower part of the tube and gently draw off ~3.5 mL of the Tris/sucrose/D_2O solution that now contains the exosomes.

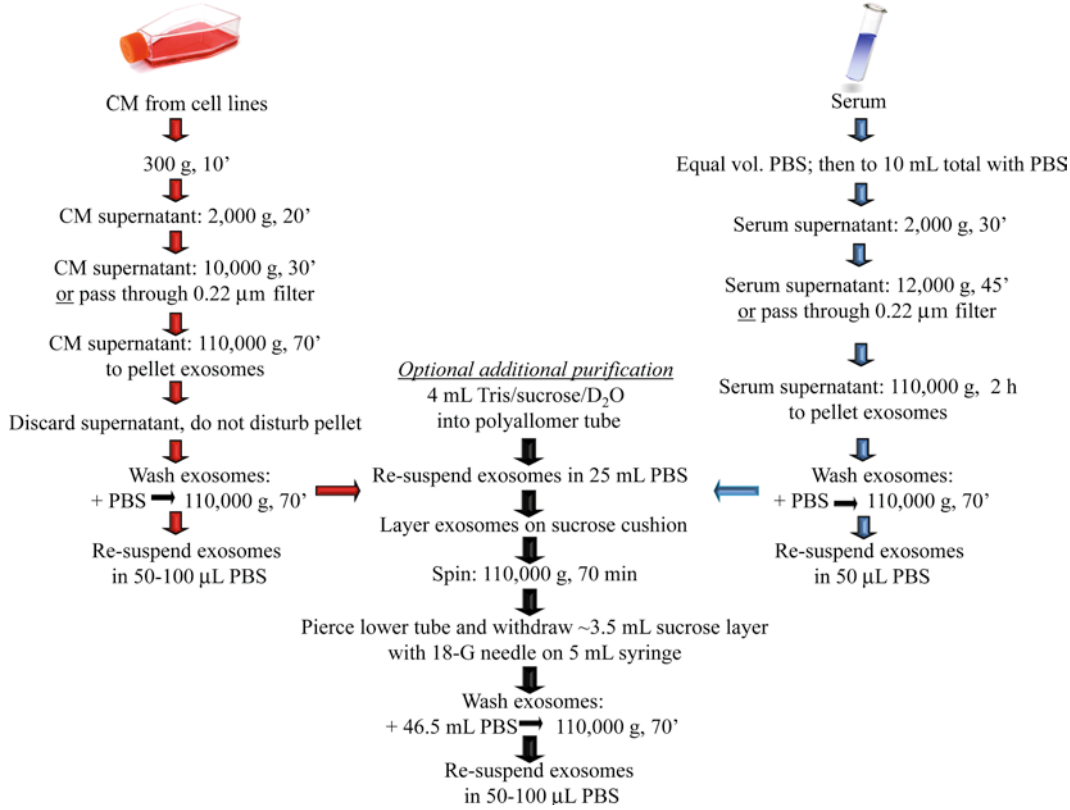

Fig. 1. Schematic representation of exosomes isolation by differential centrifugation. Exosomes can be isolated from medium conditioned by cell lines or primary cultures and from serum, using the procedures illustrated here. If sample analysis of the exosome isolates (e.g. by transmission microscopy) indicates an impure preparation, an additional step of purifying the exosomes on a sucrose cushion is recommended. All centrifugation steps should be performed at 4°C.

6. To wash the exosomes of sucrose, transfer this solution to a fresh ultracentrifuge tube; dilute to 50 mL with PBS; and centrifuge at 100,000×*g* for 70 min at 4°C in a 45 Ti rotor.
7. Re-suspend the pellet in a small volume (typically 50–100 µL) of PBS (see Fig. 1 *for summary of exosome isolation procedures*).

3.6. Basic Characterising of Exosomes

Prior to progressing with extensive mRNA/miRNA/protein profiling, it is recommended that samples of exosome isolates be assessed for presence, size, and purity. While there are a range of possible options, typically Western blotting is used to show the presence of common exosomal proteins (e.g. Alix, 96 kDa; Tsg101, 44 kDa; CD9, 25 kDa) and TEM can be used to show the presence, size – and to some extent, purity – of exosomes isolates.

3.6.1. Western Blotting

For protein isolation and quantification prior to Western blotting analysis, techniques as outlined in Chapter 8 may be used.

1. The protocol here reported refer to the use of mini gels 1-D Electrophoresis system (Bio-Rad Laboratories), but can be adapted to other formats.
2. Carefully clean glass plates with 95% (v/v) ethanol and assembly the front and back glasses in the opposite clamps. It is advisable to pour dH_2O inside the plates to check that there is no leakage from the bottom of the set-up. Then pour off the dH_2O.
3. Prepare a 7.5% gel solution by mixing 2 mL of 4× resolving buffer and 2 mL of 30% acrylamide/bis-acrylamide solution with 4 mL of water. Subsequently, add 100 μL of ammonium persulfate solution and 40 μL of TEMED; mix and immediately pour the gel, leaving enough space for the stacking gel. Overlay with ethanol and allow to polymerise.
4. Pour off the ethanol and rinse with water. Then pour the stacking gel solution prepared by mixing 1 mL of 4× stacking buffer, 0.5 mL of 30% acrylamide/bis-acrylamide solution, and 2.5 mL of water. Immediately insert the combs and allow to polymerise.
5. Carefully remove the glass plates containing the gel from the holder and assemble the gasket with the electrodes. Remove the comb, fill the gasket and the outer chamber with running buffer (1×), and then wash the wells with a syringe fitted with a thin gauge needle.
6. Load the samples and the molecular markers in the wells.
7. Assemble the unit and connect to the power supply. Apply a constant voltage up to 130 V to carry samples through the stacking gel, and then increased to 150 V to run through the running gel. Turn off the power supply immediately after the bromophenol blue dye has run off the gel and disconnect the power supply.
8. Cut a sheet of PVDF paper to a size that is slightly larger than the gel size and place in a tray containing methanol to activate the membrane. After 1 min, transfer the PVDF in another tray filled with blotting buffer. Wet two sheets of extra thick paper and two sponges in blotting buffer.
9. Disassemble the gel unit, cut and remove the stacking gel with a blade, then transfer the resolving gel into a tray containing blotting buffer. If desired, a corner of the gel can be cut to allow the tracking of the gel orientation.
10. Assemble the transfer cassette by lying a sheet of paper onto a sponge and the PVDF membrane on the top; the gel is then carefully laid on top of the membrane; another sheet of thick paper and a sponge are then positioned on top of the gel. Make sure that no air-bubbles exist between the gel and the membrane. The transfer cassette is then locked.

11. Insert the cassette in the transfer tank, carefully checking the orientation, the membrane must be oriented towards the anode, while the gel towards the cathode. Put an iced-freezer pack in the tank, add enough blotting buffer to cover the cassette and activate a magnetic stir-bar in the tank to avoid heating of the buffer.
12. Close the lid, connect the unit to the power supply and begin transfer, with a constant current of 200 mA for 1.5 h.
13. Following this, disconnect the power supply and then disassemble the transfer cassette. Remove the sponge, the paper, and the gel. If using prestained markers, check that their corresponding bands are clearly visible on the membrane.
14. Place the membrane in a small dish and add 10 mL of Ponceau S staining solution and incubate the membrane for 2 min. The Ponceau S solution can be reused several times.
15. Wash the membrane briefly with TBS to remove excess staining. Continue washing the membrane until the staining is gone. If stains persist, wash the membrane with TBS containing 0.02% NaAzide for 1–3 min and then rinse once with TBS.
16. The membrane is then incubated in 15 ml blocking buffer for 1 h at room temperature on a rocker with gentle shaking.
17. After blocking, the membrane is rinsed twice with TBS and then incubated for 3 h with primary antibody solution at room temperature with gentle shaking. Alternatively, the incubation can be performed overnight at 4°C to enhance the signal.
18. The primary antibody is then removed and the membrane is washed three times for 10 min each with 15 ml of TBS-T with vigorous shaking.
19. Freshly prepared secondary antibody solution is then added to the membrane for 1 h at room temperature with gentle shaking.
20. The secondary antibody solution is discarded and three washes for 10 min each with TBS-T are performed with vigorous shaking.
21. The ECL reagents are mixed together at a ratio of 1:1 immediately before use and evenly added to the blot for 3 min.
22. The excess ECL is removed and the membrane is put in a tray. Proceed with image acquisition as detailed in the next section.
23. Once a satisfactory signal has been obtained, wash the membrane and then proceed with the stripping procedure to clear the membrane before re-probing for a housekeeping gene.
24. For the stripping procedure, warm 30 ml stripping buffer at 70°C and then add. Incubate the membrane in this solution

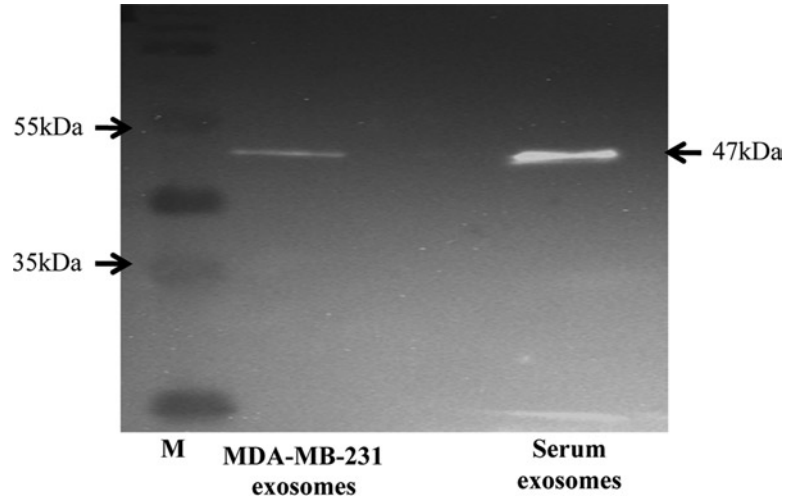

Fig. 2. Western blot analysis of samples of exosome isolates. Probing for tumour susceptibility gene 101 (TSG101), a protein typically assessed as an exosome marker, showed successful isolation of exosomes from cell line conditioned medium (CM: example shown is MDA-MB-231) and from human serum.

for 30 min and then perform extensive washes with TBS. Repeat the blocking step again before re-probing with the primary antibody solution.

25. Detection and imaging can be performed using a Chemidoc exposure system, as outlined in Chapter 8, Subheading 3.4 (see Fig. 2 e.g. *TSG101 Western blot*).

3.6.2. Transmission Electron Microscopy

The procedure outlined here is an adaptation of a technique previously reported, which can be successfully used for analysis of exosomes (18).

1. Using 4% paraformaldehyde, re-suspend exosomes that have been pelleted at 110,000 × *g* at 4°C. Final volume should be ≤100 μL (see Note 9).
2. Place a drop (~10 μL) of this suspension onto a small sheet of parafilm and invert a formvar-coated EM grid onto this, allowing the grid to float on the suspension for 20 min (see Note 10).
3. Gently wash the exosomes-containing grid in the same way, i.e. transfer onto a drop (50 μL) of PBS and leave for 1 min. Repeat this step by transferring onto fresh drops of PBS; 2× more times.
4. Fix the exosomes-containing grid by transferring onto 50 μL of 1% glutaraldehyde for 5 min.
5. Transfer the exosomes-containing grid onto 100 μL distilled H_2O for 2 min. Repeat this step by transferring onto fresh drops of PBS; 4× more times.

6. Place grid on 50 μL of uranyl-oxalate pH 7.0, for 5 min.
7. Transfer onto 50 μL of methyl cellulose-uranyl acetate (9:1 mix of 2% methyl cellulose:4% uranyl acetate); for 10 min on ice.
8. Again and as outlined in Note 10, remove excess methyl cellulose-uranyl acetate solution from the grid by gently touching the side (circumference) of the grid against Whatman no. 1 filter paper.
9. Allow the exosomes-containing grid to air-dry for approximately 10 min (see Note 11).
10. When dry, observe under EM or store in grid box until ready for analysis.

4. Notes

1. MCF7 is included here as example cell line. Of course, this approach can be applied to other cell lines of choice, cultured in their optimal medium.
2. Exosomes are present in FCS. For this reason, the following recommendations should be considered to avoid "contaminating" the exosomes of interest with exosomes arising from the FCS. If the cells of interest can be maintained under serum-free conditions, this is the most straightforward approach. If the cells need some protein to survive, 1% (w/v) BSA may be added instead of FCS. If cells cannot survive without FCS, it is recommended that serum used be depleted of exosomes (as in Steps 3–6 of Subheading 3.4, but keep the serum in this instance, rather than the FCS exosomes), prior to its addition to culture medium. However, as this can be a quite laborious additional step, some researchers choose to use a limited amount of whole FCS (e.g. 1%), but must accept that a small amount of exosomes subsequently isolated may be arising from the FCS.
3. Use as many flasks/"units" of cells as necessary to produce a minimum of 70–100 mL CM. As the yield from the purification procedure increases with the starting volume, it is advisable to purify exosomes from large volumes of CM.
4. Cells must be in a healthy, non-apoptotic stage. For some cell types that grow rapidly, cells may become over-confluent and start to die by 48 h. In this case, 24-h incubation may be more appropriate. For other cell lines that expel exosomes in a limited way, 72-h incubation may be ideal. The timing should be optimised for each cell line of interest.
5. Once CM is collected, it is strongly recommended to proceed immediately with exosomes isolation. However, this is not always

possible (and particular so in the case where human serum specimens are being procedure over time; Subheading 3.2). CM containing exosomes may be stored for a limited number of days at 4°C. Exosomes have been successfully isolated from CM, serum, and other bodily fluids may be stored at −80°C for at least several months. While direct comparisons of immediately analysed versus short-term stored versus long-term stored exosomes have not yet been reported, this storage is likely to lead to some loss of exosomes and the resulting exosomes may be of more limited use (depending on the intended follow-on experiments).

6. To avoid contaminating the exosomes that are to be subsequently isolated, very carefully remove the CM with a pipette, leaving behind approximately a centimetre of medium above the cell pellet. Do not pour off the supernatant as the pellet may become completely or partially dislodged and contamination would then be unavoidable.
7. It is recommended that the initial centrifugation steps and filtration (through 0.22-μm filter) be assessed with CM from any given cell line to determine the yield of exosomes prior to selecting the optimal method for further studies.
8. Exosome pellets are often so small (due to the nano-size of exosomes) that they are not visible to the naked eye. However, from the marking that you have placed on the ultracentrifuge tube, you will know where the small pellet will have formed and so progress accordingly with care.
9. Exosomes fixed in paraformaldehyde may be stored for up to 1 week prior to proceeding to EM analysis.
10. For this and subsequent washing and fixing steps, ensure that the formvar-coated grid containing the exosomes is not allowed to dry out, while keeping the back of the grid dry. Remove excesses of each solution from the grid, prior to progressing to the next step, by gently touching the side (circumference) of the grid against Whatman no. 1 filter paper.

Acknowledgements

Preparation of this chapter was supported by Trinity College Dublin's Start-Up Funds for New Academics 2008/2009; Trinity College Dublin's Postgraduate Studentship; Science Foundation Ireland's funding of Molecular Therapeutics for Cancer, Ireland [08/SRC/B1410] and the Marie Keating Foundation PhD Scholarships at Trinity College Dublin.

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