Comparison of ultracentrifugation and a commercial kit for isolation of exosomes derived from glioblastoma and breast cancer cells

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Abbreviations

ABC, ammonium bicarbonate; AGC, automatic gain control; BC, breast cancer; BCA, bicinchoninic acid; dd, data-dependent; DLS, dynamic light scattering; DMEM/F12, Dulbecco's modified eagle medium with nutrient mixture F-12; ER, endoplasmic reticulum; EVs, extracellular vesicles; FA, formic acid; FBS, fetal bovine serum; FSG, fish serum gelatine; GBM, glioblastoma multiforme; GO, gene ontology; IAM, 2-iodoacetamide; ISEV, The International Society of Extracellular Vesicles; LC-MS/MS, liquid chromatography tandem MS; MP, mobile phase; MVBs, multivesicular bodies; NMR, nuclear magnetic resonance; RPMI, Rosewell Park Memorial Institute; RT, room temperature; S/N, signal to noise ratio; TEM, transmission electron microscopy; UC, ultracentrifugation; WB, Western blot

ABSTRACT

Exosomes are small extracellular vesicles around 30-100 nm in diameter that are secreted from cells and can be found in most body fluids. Exosomes can be a vital source of biomarkers as they contain various substances (e.g. lipids, RNAs, metabolites and proteins) that can reflect the cell of origin (e.g. cancer cells). For isolation of exosomes present in biological matrices, ultracentrifugation (UC)-based procedures are most common. Other approaches exist, including commercial kits developed for easy and low sample volume isolation. In this study, differential UC and an isolation kit from a major vendor (Total Exosome Isolation Reagent from Thermo Fisher Scientific) were compared. Exosomes were isolated from cell culture media of two different cell sources (patient derived cells from glioblastoma multiforme and the breast cancer cell line MDA-MB-231). For both isolation methods, transmission electron microscopy, dynamic light scattering and western blotting indicated the presence of exosomes. The kit- and UC isolates contained similar amounts of protein measured by the bicinchoninic acid (BCA) assay with absorbance at 562 nm. Using western blot, positive exosome markers were identified in all isolates, and additional exosome markers were identified using MS-based proteomics. For the glioblastoma exosome isolates, the number of proteins identified with liquid chromatography tandem MS (LC-MS/MS) was higher for the UC isolates than the kit isolates when injecting equal protein amounts, contrary to that for the breast cancer exosome isolates. However, negative exosome markers were also found in glioblastoma isolates using LC-MS/MS. Thus, we would not use the term “exosome isolation” as impurities may be present with both isolation methods. Notably, potential biomarkers for both diseases were identified in the isolates using LS-MS/MS. In our opinion, the two isolation methods had rather similar performance, although with some minor differences based on cell of origin.

1 Introduction

Exosomes are extracellular vesicles (EVs) with membrane-surrounded bodies of 30-100 nm sizes which are secreted from cells to the extracellular environment as a part of the endocytic pathway (1). Exosomes are formed by invagination of an endosome membrane to create intraluminal vesicles inside the endosome (i.e. multivesicular bodies (MVBs)) and are secreted when the endosomes fuse with the plasma membrane (2). Exosomes commonly contain proteins originating from the cellular cytosol and the plasma membrane, nucleic acids (e.g. DNA, mRNA, microRNA and non-coding RNA), lipids and metabolites (1, 3-8), and are believed to take part in e.g. cell-cell communication, transfer of proteins/nucleic acids, coagulation and antigen presentation (6, 9).

Cancer cells have been found to release more exosomes than stromal cells (10, 11) and exosomes are associated with metastasis and tumor progression (7, 12, 13). Hence, cancer exosomes may be a source of biomarkers for diagnosing cancers such as breast cancer (BC) and glioblastoma multiforme (GBM) when e.g. isolated from body fluids. BC is the predominant type of female cancer (14), with recurrent metastatic disease being responsible for the majority of BC-caused deaths (15). GBM is the most frequently and malignant form of brain cancer (16-18). The diagnosis of both BC and GBM rely on highly invasive patient tissue biopsies at relatively late stages (16, 19, 20). Thus, a non-invasive disease monitoring is desirable for both BC and GBM, and can be achieved by measuring biomarkers in accessible body fluids, such as blood (liquid biopsy), for early diagnosis and prognosis assessment (16, 21-23). Hence, the isolation of exosomes for cancer biomarker discovery has emerged as an alternative to invasive methodologies (23-31).

Isolation of exosomes is predominantly performed from body fluids (e.g. blood, urine, and saliva) or cell culture media by centrifugation-based methods (e.g. sucrose density gradient centrifugation or ultracentrifugation (UC)) (32, 33). In addition, other isolation protocols and principles have been developed to overcome the drawbacks of UC such as the large amounts of starting material needed, low yield, and poor reproducibility (8, 34-41). Moreover, there is a great need for exosome isolation protocols tailored towards smaller starting volumes (< µL) for e.g. miniaturized cell culture models like organoids and "organ on a chip" (42, 43). However, there is a lack of consensus as the methods for rigorous isolation are still largely empirical.

The protein content of exosomes has previously been characterized using western blot (WB) as standard method but also liquid chromatography tandem MS (LC-MS/MS) has been applied (41, 44). Tetraspanins (e.g. CD9, CD63 and CD81) are commonly used as positive exosome protein markers for targeted analysis as they are particularly known to be enriched in exosomes compared to cells (1, 45-48). Positive exosome markers generally take part in exosome biogenesis, and are hence expected to be present in, but are not specific to, exosomes (49). A broad range of positive and negative exosome markers for exosome characterization are provided by The International Society of Extracellular Vesicles (ISEV) (45). In addition, protein databases covering proteins occurring in exosomes and other extracellular vesicles are available (50-52). ISEV also recommends using supplementary characterization methods in addition to WB and LC-MS/MS (e.g. size distribution and imaging), to study the heterogeneity and morphology of vesicles present in the isolated samples.

In the present study, we have compared two exosome isolation methods (UC and a commercial kit for precipitation of exosomes) with a particular emphasis on the characterization methods used for identifying exosomes and evaluating the purity (the presence of positive exosome markers and absence of non-exosome proteins) of the isolated exosomes. The methods were evaluated using characterization techniques recommended by ISEV: WB, transmission electron microscopy (TEM), dynamic light scattering (DLS), quantitative total protein analysis using UV-Vis spectrophotometry and LC-MS/MS for untargeted proteomic analysis. Exosomes were isolated from cell culture media from free floating patient-derived primary cell cultures from GBM biopsies (T1018) and a traditionally serum cultivated, adherently growing BC cell line (MDA-MB-231). Comparison of exosome isolation techniques for these cell culturing conditions has not been performed, and studies on exosome presence and purity are also limited for such cell cultures.

2 Experimental Procedures

Unless otherwise stated, water (commonly type 1 water purified by a Direct-Q® water purification system from Millipore (Billerica, MA, USA)) was used as solvent. For detailed information about chemicals, solutions and experimental methods used, see **Supplemental Methods**.

2.1 MDA MB-231 cell culturing

The BC cell line was purchased from American Type Culture Collection (ATCC, Sesto San Giovanni, Milan, Italy) and is derived from a triple-negative human metastatic breast carcinoma. The cells were maintained in Rosewell Park Memorial Institute (RPMI) 1640 growth medium depleted of phenol red (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % exosome-

depleted fetal bovine serum (FBS) (System Biosciences, Palo Alto, CA, USA) and 1 % penicillin/streptomycin (Sigma- Aldrich). The cells were incubated in a humidifying atmosphere at 5 % CO_2 and at 37 °C. Prior to exosome isolation, 1-2.3 million cells (in T75-T175 culturing flasks) were incubated for 6-7 days (always using a passage lower than 12). The incubated cell culture medium was centrifuged at 906 × *g* (30 minutes at 23 °C).

2.2 Glioblastoma cell culturing

The GBM cells (T1018) were derived from biopsies from a primary GBM tumour, obtained after informed consent through a biobank approved by the Regional Ethical Authorities operated at Oslo University Hospital (2016/1791). The cells were maintained in Dulbecco's modified eagle medium with nutrient mixture F-12 (DMEM/F12, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with HEPES buffer (10 mM) and penicillin/streptomycin (100 U/mL) from Lonza (Basel, Switzerland), B27 without vitamin A (1/50) from Thermo Fisher Scientific, epidermal growth factor (20 ng/mL) and basic fibroblast growth factor (10 ng/ mL) from R&D Systems (Minneapolis, MN, USA) and heparin (2.5 µg/mL) obtained from LEO Pharma AS (Ballerup, Denmark). Under these culturing conditions cells express stem cell markers *in vitro*, differentiate upon removal of growth factors and give rise to diffusely infiltrative tumors upon xenografting (53). The cells were incubated in a humidifying atmosphere at 5 % CO_2 and 37 °C in T25 flasks (Thermo Fisher Scientific). Prior to exosome isolation, the incubated cell culture medium was centrifuged twice at 453 × *g* and 1811 × *g* for 5 minutes each. The cell pellets were harvested for WB analysis.

2.3 Exosome isolation by ultracentrifugation

For the BC and GBM cells, 9-12 mL and 60 mL cell culture media were used for centrifugation, respectively. Cell culture media were first centrifuged at 1811 × *g* (5 minutes at 20 °C). The supernatants were then centrifuged at 20 000 × *g* (20 minutes at 20 °C) with an Allegra 25R centrifuge (with TA-14-50 rotor) from Beckman Coulter (Brea, CA, USA) and the supernatants were transferred to polycarbonate ultracentrifugation tubes (Beckman Coulter) and diluted with PBS until the tubes were full (~60 mL in each). The tubes were centrifuged twice at 100 000 × *g* (90 minutes at 4 °C) with an L-80 ultracentrifuge (45 Ti rotor) from Beckman Coulter. The supernatants were removed (leaving suspension 1 cm above the pellets) and the pellets were suspended with PBS between the centrifugations. Upon centrifugation, the supernatants were discarded and the exosome pellets (UC isolates) were suspended in either PBS (3 mL for DLS- and 50-100 µL for TEM analysis) or the preferred lysis buffer (**Section 2.5**).

2.4 Exosome isolation by isolation kit

The isolation of exosomes with kit was performed with the Total Exosome Isolation Reagent (from cell culture media) from Thermo Fisher Scientific (catalog nr. 4478359). The isolation was performed according to the protocol of the supplier (54). Starting volumes ranged from 0.5 mL to 9 mL cell culture medium for the BC cells and 5 mL to 6 mL for the GBM cells. The samples were centrifuged with the Allegra 25R centrifuge, and the exosome pellets (kit isolates) were suspended as with UC (**Section 2.3**).

2.5 Protein extraction

Cell and exosome protein extracts were made by lysis with RIPA- or Nonidet™ P40 (NP40) buffer (both from Thermo Fisher Scientific) containing protease inhibitors (Protease Inhibitor Coctail Tablets, Roche, Basel, Switzerland) and phosphatase inhibitors (PhosStop Tablets,

Sigma-Aldrich). For BC isolates, the NP40 buffer was prepared from NP40 detergent solution as described in **Supplemental Methods** (**S-2.1**), while "ready to use" NP40 buffer from the vendor was used for GBM isolates. For WB analysis, the BC isolates were lysed in 50 µL of the RIPA solution and the BC cells in 100 µL RIPA solution. The GBM isolates were lysed in 300 µL of the RIPA solution, and the GBM cells in 1 mL RIPA solution. For LC-MS/MS analysis, BC- and GBM isolates were lysed in 50 µL NP40 buffer. All extracts were incubated while rotating for 30 minutes (4 ℃), and then snap frozen (at -80 ℃). The extracted samples were thawed and centrifuged at 20 570 × g (30 minutes at 2℃) using a Heraeus Fresco 21 centrifuge or an Eppendorf 5424R centrifuge. The pellets were discarded.

2.6 UV-Vis spectrophotometry

The protein amount was measured using Pierce™ BCA protein Assay Kit (Thermo Fisher Scientific), by measuring the absorbance at 562 nm. The GBM protein measurements were performed on a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). The BC protein measurements were performed using a Wallac Victor2 1420 multilabel counter and Wallac 1420 Workstation software (version 3.00), both from Perkin Elmer (Waltham, MA, USA).

2.7 Western blotting

The protein extracts containing 3-15 µg protein were diluted with water giving equal protein concentrations before adding 5x loading buffer (see **Supplemental Methods** for solution preparation) to yield 1x. Prior to electrophoresis, the protein extracts were boiled for 3 minutes at 92 ℃. The protein extracts together with PageRuler™ Prestained protein ladder (Thermo Fisher Scientific) were loaded on to 4-12 % Bis-Tris gels (Thermo Fisher Scientific) and run at 70-75 V for 1 hour. The voltage was increased to 110 V after the first hour if the bromophenol blue lane

was horizontally distributed. The SDS running buffers used were MOPS or MES, both purchased from Thermo Fisher Scientific. Proteins were transferred to a 45 µm nitrocellulose membrane (Thermo Fisher Scientific) by semi-dry electroblotting (1 W, at 4 ℃ overnight) with a transfer chamber from Bio-Rad (Hercules, CA, USA). The transferred proteins were blocked with a blocking solution consisting of non-fat dry milk (5/95, w/v) (PanReac AppliChem ITW reagents, Darmstadt, Germany) in 0.05 % Tween-20/TBS (0.05/99.95, v/v) (Medicago, Uppsala, Sweden) for 1 hour on a mixing plate. The proteins were subsequently stained with primary antibodies (at 4 °C overnight) in blocking solution and with secondary antibodies in blocking solution for 2 hours at room temperature (RT) with rocking. The antibodies CD9 (10626D), CD63 (two 10628D batches) and CD81 (MA5-13548 and 10630D) were purchased from Thermo Fisher Scientific. The antibody TSG101 (T5701) was purchased from Sigma-Aldrich, and anti-flotillin-1 (610821) and anti-calnexin (610523) antibodies were purchased from BD Biosciences (San Jose, CA, USA). The secondary antibodies (sc-2954 and sc-2955) were purchased from Santa Cruz (Dallas, TX, USA). Next, the membranes were further washed with 0.05 % Tween-20-TBS for 30 minutes on a mixing plate. Protein bands were visualized using an ECL-prime from GE Healthcare (Buckinghamshire, UK) and Transparency films from Nobo (integrated part of ACCO Brands Corporation, Lake Zurich, IL, USA). The bands were developed in a Chemidoc™ touch imaging system (Bio-Rad). The antibodies actin (A2066, from Sigma) and GAPDH (sc-32233, from Santa Cruz) were used as a positive control (results not shown).

2.8 Immunogold labelling and transmission electron microscopy

One drop of 5-50 µL of the isolates was placed on clean Parafilm, and the formvar coated copper grid (100 square mesh) was carefully placed to float on the drop with the coated side facing the suspension. The material was allowed to adsorb for 5-20 minutes, before rinsing on two large

drops of PBS for 5 minutes followed by incubation on a drop (8 µL) of the primary anti-CD9 (PA5-11559) from Thermo Fisher Scientific (diluted 1+ 9 with fish serum gelatine (FSG)/PBS (1/99, v/v)). Next the grids were again washed on two large drops of PBS for 5 minutes and incubated on a drop (5 µL) of rabbit anti-mouse antibody (Z0259, Dako Glostrup, Denmark) (diluted 1:200 with the FSG in PBS solution) for 25 minutes, before repeating the washing on two drops of PBS for 5 minutes. Prior to gold labelling, the grid was incubated for 20 minutes on one drop of the diluted protein A-gold solution (1:50 in the FSG in PBS solution) (10 nm gold particle size), from Cell Microscopy Core (CMC, University Medical Center Utrecht, Utrecht, The Netherlands). A final wash on 5 drops of PBS (2 minutes) followed by 5 drops of water (3 minutes) was performed before negative staining with uranyl acetate/water (4/96, w/v) for 2 minutes. Excess fluid was removed, and the grids were stored in a storage box at RT until use. The samples were visualized with a JEM-1400Plus transmission electron microscope from JEOL (Tokyo, Japan) and images were recorded at 80 kV.

2.9 Dynamic light scattering

The DLS experiments were conducted with the aid of an ALV/CGS-8F multi-detector version compact goniometer system, with 8 fiber-optical detection units, from ALV-GmbH, Langen, Germany. The beam from a Uniphase cylindrical 22 mW HeNe-laser, operating at a wavelength of 632.8 nm with vertically polarized light, was focused on the sample cell (10-mm NMR tubes, Wilmad Glass Co., of highest quality) through a temperature-controlled cylindrical quartz container (with 2 plane-parallel windows), vat (the temperature constancy being controlled to within ± 0.01 ^{o}C with a heating/cooling circulator), which is filled with a refractive index matching liquid (*cis*-decalin). The isolates were filtered in an atmosphere of filtered air through a

5 µm filter (Millipore) directly into precleaned NMR tubes. The measurements were carried out at 25 ^{o}C. The measurements revealed two relaxation modes, one fast and one slow mode. This suggests that there is a coexistence between single entities and aggregates in the solution. The analyses of the correlation function data are presented in **Supplemental Methods** (**S-6 DLS**).

2.10 Protein digestion

2.10.1 In-gel digestion

Prior to in-gel digestion, the isolates (from **Section 2.5** and **Section 2.6**) were prepared and run using the same procedures as with the gel electrophoresis described in **Section 2.7**. The gel was covered by a fixation buffer (water/methanol/acetic acid, 40/50/10, v/v/v) overnight (18 hours at 4 °C), stained with Coomassie brilliant blue for 4 hours at RT and destained overnight with water. The gel was cut to yield four fractions from each gel lane; approximately 0-25 kDa, 25-70 kDa, 70-130 kDa (70-250 for BC samples) and 130-up kDa (250-up kDa for BC samples). Each fraction was transferred to Protein LoBind tubes. The fractions were further reduced, alkylated and digested using the protocol of Shevchenko et al. (55), with trypsin from Promega Biotech AB (Nacka, Sweden). The digested fractions were evaporated to dryness and dissolved in 15 µL 0.1 % formic acid (FA) (water/FA, 99.9/0.1, v/v).

2.10.2 In-solution digestion with peptide desalting

The isolates (from **Section 2.5** and **Section 2.6**) were evaporated to dryness and dissolved in 25 µL 6 M urea in 100 mM ammonium bicarbonate (ABC). Subsequently, the isolates were reduced with 9.5 mM DTT (30 minutes at 30 °C) and alkylated with 25 mM 2-iodoacetamide (IAM, 60 minutes, at RT and in the dark). The reduction with DTT was repeated for the BC isolates, with

28 mM DTT (30 minutes at 30 °C). The BC isolates were pre-digested by adding 0.1 µg Lys-C (120 minutes at 37 °C), before the digests (both BC- and GBM isolates) were diluted to a final concentration of 19 mM ABC. The trypsin digestion was performed with 1 µg trypsin (16 hours at 37 °C) and the protease activity was terminated with water/FA (99/1, v/v). Desalting and enrichment of the digests were performed using ZipTip® (silica particles with C_{18}) from Millipore. For the BC digests, ZipTip was wetted with neat ACN and equilibrated with TFA/water (0.1/99.9, v/v). The digests were desalted by pipetting through the ZipTip. Washing the ZipTip was performed with water/methanol/TFA (94.9/5/0.1, v/v/v) and the peptides were eluted with 5 µL water/ACN/TFA (29.9/70/0.1, v/v/v). For the GBM digests, the same procedure was performed with FA replacing TFA. All desalted digests were evaporated to dryness at 30 °C and dissolved in 10 µL 0.1 % FA.

2.11 LC-MS/MS analysis

Unless otherwise stated, the dilutions during sample preparations were performed using water (HiPerSolv Chromanorm®) from VWR. Proteins digested in-gel were analysed in laboratory 1 and proteins digested in-solution were analysed by laboratory 2.

2.11.1 LC-MS/MS analysis at laboratory 1

The precolumn (50 µm ID x 20-50 mm) and analytical column (50 µm ID x 150 mm) were packed with C_{18}-Accucore particles (2.6 µm beads, 80 Å pore size) from Thermo Fisher Scientific, using the developed method as described in our previous study (56). An EASY-nLC 1000 pump (with autosampler) connected to a Q-Exactive™ Orbitrap MS equipped with a nanoFlex nanospray ion source (Thermo Fisher Scientific) were applied throughout the

experiments. Mobile phase A (MP A) was made of 0.1 % FA, while mobile phase B (MP B) was made of ACN/FA (99.9/0.1, v/v) (mobile phase B, MP B). Trapping of the analytes in the pre-column was performed with 100 % MP A at a maximum flow rate restricted not to reach above 500 bar (12 µL). A 120-minute linear gradient elution from 3-15 % MP B with a flow rate of 130 nL/min was set, starting at 3 % MP B for 3 minutes before the percentage of MP B was increased to 15 % in 120 minutes. The MP B was increased to 50 % for 5 minutes before the percentage of MP B increased to 80 % for 2 minutes (flow rate was also increased to 173 nL/min) and kept at 80 % MP B for another 15 minutes. The injection volume was 10 µL for each fraction.

The eluting peptides were ionized at 1.8 kV at 250 °C for the BC isolates and at 275 °C for the GBM isolates. The MS was operated in data-dependent (dd) positive mode to automatically switch between MS and MS/MS acquisition. Survey full scan MS spectra (with a mass filter of *m/z* 350 to 1850) were acquired with a resolution of 70 000, automatic gain control (AGC) of $1x10^6$ and a maximum injection time of 120 ms. For dd/MS/MS, the resolving power was set to 17 500, the AGC to $1 x10^5$ and the maximum injection time to 60 ms. Charges of 1, 7 or ≥ 8 were excluded and dynamic exclusion was set to 70.0 seconds. The method allowed sequential isolation of up to the ten most intense ions depending on signal intensity (intensity threshold $2.0 x10^4$), with isolation window of *m/z* 1.8.

2.11.2 LC-MS/MS analysis at laboratory 2

The analytical column applied was an Acclaim PepMap 100 column (C_{18}, 3 µm beads, 100 Å, 75 µm ID x 500 mm), and an Ultimate 3000 nano ultra-HPLC system from Dionex (Sunnyvale, CA, USA) was used in combination with a Q-Exactive™ Orbitrap MS equipped with a nanoFlex

nanospray ion source from Thermo Fisher Scientific. The MP A was 0.1 % FA and the MP B was ACN/FA/water (90/0.1/9.9, v/v/v). A 207 minute linear gradient elution from 4-35 % MP B with a flow rate of 300 nL/min was set. The percentage of MP B was increased to 50 % in 20 minutes and 80 % MP B in 2 minutes. The injection volume was 5 µL.

The MS was operated in data-dependent (dd) positive mode. Survey full scan MS spectra (with a mass filter of *m/z* 400 to 1700) were acquired with a resolution of 70 000, AGC of $3x10^6$ and maximum injection time of 100 ms. In dd/MS/MS, the resolving power was set to 35 000 and the maximum injection time to 120 ms. The dynamic exclusion was set to 60 seconds. The method allowed sequential isolation of up to the ten most intense ions depending on signal intensity (intensity threshold $1.7x10^4$) and the isolation window was *m/z* 2 without offset.

2.11.3 Data processing and protein identification

Chromatograms and mass spectra were obtained by Xcalibur™ Software (version 2.1, Thermo Fisher Scientific), and the search engine Proteome Discoverer™ Software (version 1.4.0.228, Thermo Fisher Scientific) was used to identify the peptides and proteins. The proteins were identified using both SEQUEST and MASCOT algorithms searching the Swiss-Prot database (human taxonomy, April 2017 (20 198 entries) and June 2017 (20 205 entries) for BC- and GBM isolates, respectively). All searches were performed setting the digestion enzyme to trypsin with maximum two missed cleavages, fragment ion mass tolerance of 0.1 Da and a precursor mass tolerance of 10 ppm. Signal to noise (S/N) threshold was set to 1.5 and the minimum ion count to 1. An automatic decoy search was performed with a false discovery rate (FDR) threshold of 0.01 (strict) and 0.05 (relaxed). Carbamidomethylation of cysteine was specified as static

modification. Oxidation of methionine, acetylation of the protein N-terminus and deamidation of glutamine and asparagine were specified as dynamic modifications. The proteins were identified with high peptide confidence filter, and ≥1 signature peptide was required for all protein identifications. Proteins identified as keratin or trypsin were removed from the list, in addition to proteins found in the blank samples.

2.12 Experimental Design and Statistical Rationale

GBM procedures

For GBM samples, 3 kit- and UC isolation replicates were used for the UV-Vis spectrophotometry (one replicate excluded in **Section 3.1** due to uncertainty in the isolation volume) and LC-MS/MS analysis in both laboratories (all isolates divided in two, no injection replicates due to low protein amounts). For TEM analysis, one isolation replicate from both isolation methods are presented. From the isolation replicate, 2 technical replicates were analysed with TEM. Three additional UC isolation replicates from a second batch were performed for verification, and SW480 isolates were used as a positive control (result not shown). For the DLS analysis, only one isolation replicate was performed since the particle sizes obtained in the DLS analysis were in correspondence with that seen by TEM analysis. For the WB analyses of exosome isolates, 1-2 replicates for each antibody are presented (**Figure WB.7** in **Supplemental Western Blots**), and 3 replicates from a second batch of UC isolates were performed for confirmation and investigation of the repeatability (**Figure WB.9** in **Supplemental Western Blots**). For WB analysis of GBM cells, 6 replicates were used (three replicates from two cell batches) with the antibodies for actin or GAPDH used as a loading control (result not shown). A

kit blank and UC blank were used as negative controls for all exosome analyses (except of no kit blank for the DLS analysis).

BC procedures

For BC samples, 3 kit- and 2 UC isolation replicates were used for the protein measurement by UV-Vis spectrophotometry. For the TEM, one isolation replicate and 2 technical replicates were analysed, in addition to a positive control (SW480 isolate, results not shown). DLS analyses were performed with the use of 2 isolation replicates (kit), 3 isolation replicates (UC) and one blank isolation replicate (isolated with both isolation methods). For the WB analyses, 3 kit- and 2 UC isolation replicates were used. WB analyses of BC cells were also performed, using 3 cell replicates (results not shown). The antibodies for actin or GAPDH were used as loading controls for the cells (results not shown) and anti-TSG101 was used as a loading control for the isolates. Prior to LC-MS/MS, 2 isolation replicates were analysed in laboratory 1 (digested in-gel) and one isolation replicate was analysed in laboratory 2 (in-solution digested) (no injection replicates due to low protein amount). One blank isolation replicate (isolated with both isolation methods) was used for analysis with LC-MS/MS in each of the two laboratories.

3 Results and Discussion

For comparison of the two exosome isolation methods (UC and Total Exosome Isolation Reagent (kit)) using GBM (T1018) and BC (MDA-MB-231) cell culture media, common characterization methods were used and evaluated for their ability to prove the presence of exosomes and/or determine the purity. The standard techniques TEM, for morphological analysis, and WB, for

exosome marker analysis, were used in addition to the measurement of total protein amount, DLS and LC-MS/MS. Comprehensive proteome analysis using LC-MS/MS was applied to complement WB for detecting exosome protein markers (hereafter referred to as exosome markers), and additional positive and negative markers were included.

3.1 Similar content of protein measured in kit- and UC isolates

The protein amount per million cells (hereafter referred to as protein amount) in the BC- (**Figure 1A**) and GBM (**Figure 1B**) isolates was measured using UV-Vis spectrophotometry (with absorbance at 562 nm). The total protein amount measured for kit isolates was 15-28 times higher than for UC isolates. A higher protein amount in exosomes isolated by the kit compared to that by UC were also observed in a study by Van Deun et al., who compared UC to the same isolation kit used in the present study for MCF7 derived exosomes (57). However, the measured absorbance in the kit blanks (i.e. cell culture medium grown without cells and isolated by kit) was high in comparison to UC blanks (i.e. cell culture medium grown without cells and isolated by UC), where the absorbance was below the limit of quantification. The high absorbance in the kit blanks could indicate protein contaminations. When correcting for the blank (subtracting the protein amount measured in blank samples from the protein amount in exosome isolates), the measured protein content for exosomes isolated by the kit and UC was similar.

3.2 TEM and DLS detected vesicles in the expected size range for exosomes

Morphological analysis of the exosome samples was performed using TEM and immunogold labelling of CD9. In addition, the hydrodynamic particle size distribution was measured using DLS analysis. Clusters of vesicles were observed in the micrographs of the samples isolated with both kit and UC (**Figure 2**). Vesicle structures similar to that described in literature were

observed (6, 58, 59). The DLS experiments disclosed the coexistence of two populations of moieties, single entities and clusters, both with a narrow size distribution.

3.2.1 GBM exosomes

No CD9-labelling was observed for the vesicle structures observed in the GBM isolates (**Figure 2AI** and **2AIII**) and the presence of a membrane enclosing the vesicles could not be confirmed. Compared to the kit isolates, the UC isolates presented more distinct double membranes in the expected size range for exosomes. The blank samples for both isolation methods did not display membrane structures (**Figure 2AII** and **2AIV**). The absence of vesicles was further confirmed by DLS analysis of the UC blank (**Figure 2B**). The DLS-analysis of the GBM isolates exhibited particles of similar sizes of 51 and 73 nm (mean) with both isolation methods (**Figure 2B**). Thus, both isolation methods gave rise to comparable exosome populations.

3.2.2 BC exosomes

Several of the BC vesicle structures were CD9-labelled (**Figure 2CI** and **2CIII**). CD9-labelled vesicles have also been observed in a previous study of the same cell line (60). Notably, the blank isolates displayed contamination (**Figure 2CII and 2CIV**), e.g. exosome-resembling vesicles were found in the UC blank (red dashed circles). However, no contaminations were found in the UC blank using DLS, while the kit blank displayed 67 nm (mean) contaminations (**Figure 2D**). The DLS analysis also presented two distinct particle diameters in kit isolates (28 and 95 nm, mean values) while only one particle diameter was present in UC isolates (137 nm, mean value), indicating differences in the particle sizes isolated with the two isolation methods.

The sizes observed with DLS correlates well with that found in other studies (30–250 nm) (13, 57, 61-65). In conclusion, the isolates showed structures resembling those of EVs, but some blank were not entirely devoid of vesicles or particles. Observations made with TEM are not necessarily detectable with DLS because TEM analyses dry material, whereas DLS measures on solutions or suspensions of particles. In addition, the micrographs taken with TEM display a narrow section of the grid, which again represents only a small part of the isolate.

3.3 Western blot analyses indicated the presence of exosomes, but detected impurities exclusively in the GBM exosome isolates

According to ISEV, for characterization of exosomes at least three exosome markers should be included; transmembrane proteins (e.g. tetraspanins), cytosolic proteins (e.g. TSG101 or annexins) and negative markers (e.g. calnexin) (45). In the present study, WB was performed using antibodies for a selection of positive exosome markers (the tetraspanins CD81, CD9 and CD63, TSG101 and flotillin-1). Calnexin was selected as a negative marker for purity evaluation as recommended by ISEV. This protein is located at the endoplasmic reticulum (ER) and has been absent in exosome samples in some studies (45, 61). Hence, the presence of calnexin is assumed to signalize ER-contamination. Thus, contaminations from other cellular organelles cannot be excluded.

3.3.1 GBM exosomes

For the GBM cells and exosomes, positive and negative exosome markers were detected in isolates from both kit- and UC. The positive marker CD81 was only found in the UC isolate from the first batch (**Figure 3**). The WB-bands were also more apparent for most positive markers for

exosomes isolated by UC (lower protein amount loaded than for the kit isolates), and thus is in accordance with the study of Van Deun et al. (57). The kit isolate bands were also circular, which implies higher detection uncertainty.

3.3.2 BC exosomes

For the BC cells and exosomes, inconsistency on the presence of several positive exosome markers were observed between the kit and UC isolates (**Figure 3**). The positive marker CD63 was only detected in kit isolates, while CD81 was only detected in UC isolates (similar to GBM exosomes). The proteins TSG101, flotillin-1 and CD9 (barely visible in the UC isolates) were detected using both isolation methods. However, Harris et al. did not detect TSG101 using WB on BC exosomes isolated by UC (13). The proteins CD9, CD81 and flotillin-1 were detected in other WB-studies of UC isolates from the same cell line (24, 66, 67).

The reason for the variation in tetraspanin appearance in the BC kit- and UC isolates could be due to protein concentrations below detection limits or poor antibody quality (see **Figure 3**). Several antibodies for CD63 and CD81 (different batch number/catalog number) were tested for the BC isolates before a signal was obtained (signal obtained for CD81 using catalog number 10630D), and this could indicate poor antibody quality. On the other hand, the WB was performed under reducing conditions. When the epitope binds to cysteine-conserved protein domains (i.e. tetraspanins), performing WB under non-reducing conditions is more commonly selected. The stronger signals for the kit isolates from BC could be due to the higher loaded protein amount. Nevertheless, the presence of positive markers indicates the presence of exosomes in the isolates obtained using both methods. The absence of calnexin in BC exosomes from both isolation

488 methods indicates that the isolates are not contaminated with the ER. However, only one negative
489 marker is insufficient to exclude cell organelle impurities. Further investigation by implementing
490 more comprehensive methods like LC-MS/MS was therefore considered to be beneficial.
491
492 **3.4 LC-MS/MS studies confirmed and complemented the WB study**
493 LC-MS/MS was performed to confirm the WB observations, using in-house packed nano
494 separation columns (laboratory 1, (56)) and a commercial column (laboratory 2). One positive
495 marker (annexin A2) and one negative marker (serine/threonine-protein kinase 26) were added to
496 complement the WB study. The identification of the selected exosome markers using LC-MS/MS
497 is presented in **Table 1**, with chromatograms and MS/MS spectra of a CD9 signature peptide
498 (BC, **Figure 4A**) and calnexin signature peptide (GBM, **Figure 4B**). Similar to the WB analysis,
499 the same tetraspanins including calnexin observed in kit and UC isolates were identified in the
500 GBM exosomes using LC-MS/MS. However, the tetraspanins identified from kit isolates were
501 only found in one replicate, indicating low concentrations and high detection uncertainty.
502 Flotillin-1 was also identified with LC-MS/MS, but only for UC isolates. TSG101 was not
503 identified in neither kit nor in UC isolates with GBM exosomes using LC-MS/MS, in
504 contradiction to that found by WB.
505
506 For the BC exosomes, the findings by LC-MS/MS analysis was contradictory to the trend
507 observed in the WB analysis, where kit isolates provided higher intensity bands for positive
508 markers than the UC isolates. Using LC-MS/MS, several positive markers were not found in the
509 kit isolates when injecting similar amounts of protein as for UC isolates (**Table 1**). The reason for
510 the difference in the identified proteins between LC-MS/MS and WB could hence be partially

511 due to higher protein amount loaded onto the gel for kit isolates (see **Figure 3**). Calnexin was not
512 found in the BC isolates using LC-MS/MS, similar to that observed by WB. Annexin A2
513 (positive marker) was found in all isolates and serine/threonine-protein kinase 26 (second
514 negative marker) was not detected in any isolates.
515
516 Other negative markers from peroxisomes (PMP70), mitochondria (prohibitin-1, hexokinase-2
517 and mitochondrial phosphoenolpyruvate carboxykinase [GTP]), Golgi apparatus (GM130,
518 translocation protein SEC62, translocation protein SEC63 and protein disulfide-isomerase
519 TMX3), nucleus (Bcl-2-associated transcription factor 1 and c) and ER (calreticulin), as used in
520 other studies (57, 68), were not detected in any BC isolates in our study (only translocation
521 protein SEC63 and protein disulfide-isomerase TMX3 detected in the GBM isolates). In the BC
522 isolates, the absence of the selected negative markers can imply low cell organelle contamination.
523

524 **3.5 GO annotations revealed proteins annotated to cell organelles in BC exosomes**

525 However, the possibility of protein contaminants being present in BC isolates cannot be
526 excluded. Other general proteins related to e.g. the nucleus, Golgi apparatus, mitochondrion and
527 ER were indeed identified in the BC exosomes using LC-MS/MS and gene ontology (GO)
528 annotations (**Figure 5**). The proteins identified in BC isolates were classified based on their GO
529 annotations to different cellular localizations, where one protein can be annotated to several
530 cellular localizations. Out of the 668 proteins identified in the UC isolates and 814 in the kit
531 isolates, 615 and 749 DAVID ID`s were annotated to selected cellular localizations. Both
532 isolation methods generated isolates enriched in exosome related proteins. Several proteins were
533 annotated to the cellular organelles mitochondria (11-13 %), ER (6-8 %) and Golgi apparatus (7

534 %). Interestingly, 35-42 % of the proteins were also annotated to the nucleus (e.g. histones),
535 which seems to imply impure exosome isolates (45). On the other hand, a high percentage (20-40
536 %) of other proteins related to the nucleus has also been found in isolates from other studies (69,
537 70). The presence of cell organelle annotated proteins could point toward cellular impurities in
538 the isolates, which would not have been discovered by targeted protein characterization methods
539 (e.g. WB). However, there is not sufficient knowledge on whether cell organelle proteins derive
540 exclusively from cell impurities, or if they occur naturally in EVs. To summarize, from our point
541 of view, complete information about exosome purity cannot be obtained by any of the common
542 characterization techniques used today, and one can argue that the term "exosome isolation" can
543 be misleading.
544

545 **3.6 The number of cancer related proteins identified was dependent on the isolation**
546 **method and cell source**

547 The total number of proteins identified in the GBM and BC isolates using LC-MS/MS is
548 presented in the Venn diagrams in **Figure 6** (see **Supplemental Proteins** for a list of all
549 identified proteins). For the GBM isolates, the number of identified proteins reflects the findings
550 in both WB and LC-MS/MS exosome marker investigations. UC isolates provided more unique
551 proteins than the kit isolates (75 % higher number of identified proteins). An increased number of
552 potential biomarkers for GBM (e.g. heat shock proteins 70 kDa and 90 kDa (71-73), chondroitin
553 sulfate proteoglycan 4 (71, 74), CD44 (71, 74, 75) and CD276 (76)) were also identified in the
554 UC isolates compared to the kit isolates using LC-MS/MS. The identification of relevant
555 biomarkers is of great interest for further studies on exosomes. However, the identified

556 biomarkers cannot exclusively be related to exosomes due to the presence of negative exosomes
557 markers indicating cellular contaminations with both isolation methods.
558
559 For the BC exosomes, the opposite was observed; kit isolates provided 12 % higher number of
560 identified proteins than UC isolates. However, there was no correlation between the injected
561 protein amount or the starting volume used for isolation, and the number of identified proteins
562 with kit or UC for the BC exosomes (result not shown). Thus, the reason for the variation in the
563 number of identified proteins between the two cell sources and isolation methods is unknown.
564 The identification of biomarkers related to triple negative breast cancer (e.g. histone H4 (77), heat
565 shock 90 kDa α and β protein (78), calmodulin and epithermal growth factor receptor (79)) was
566 similar for both isolation methods (see **Supplemental Proteins**).
567
568 When comparing cell sources, the number of identified proteins was lower in GBM isolates than
569 BC isolates, but the number of identified proteins for GBM isolates is comparable to another LC-
570 MS/MS study on GBM exosomes (80).
571
572 **3.7 Choosing the proper exosome isolation method is not straight forward**
573 A complete comparison of the characteristics of the two exosome isolation methods is given in
574 **Table 2**. For all isolates, the kit and UC isolates displayed similarities and differences.
575
576 *3.7.1 GBM exosomes*
577 For the GBM exosomes, one of the positive markers detected in the UC isolates (CD81) was not
578 found in kit isolates by WB. In TEM, double membrane structures were more defined in the UC

isolates, but the existence of double membranes cannot be excluded by looking at the micrographs from the kit isolates. The largest differences between the two isolation methods for the GBM exosomes were found by the LC-MS/MS studies (positive markers and number of identified proteins). All tetraspanins investigated were identified in the UC isolates in several replicates. In the kit isolates, CD81 was not found, and the detected tetraspanins (CD63 and CD9) were only found in one replicate each. A larger number of proteins and biomarker candidates were also identified in the UC isolates compared to kit isolates. However, the negative marker calnexin was detected in more replicates for the UC than the kit using LC-MS/MS. In total, from **Table 2**, UC appear to be the method of choice for isolation of GBM cell culture exosomes.

3.7.2 BC exosomes

For the BC exosomes, there was a slight difference in favor of the kit method regarding the number of positive markers found by WB and the number of identified proteins (LC-MS/MS). However, using LC-MS/MS, more positive protein markers were found in the UC isolates in contrary to what was found by WB. For the UC isolates, TEM presented double membrane structures with more CD9-labelling. However, the micrograph displays an extremely small part of the whole sample. The isolation methods also performed similarly regarding biomarker identifications. Thus, for BC exosome isolation there is no obvious reason for choosing one method over the other, even though there were some differences in the characteristics (i.e. the identified protein content- and amount, CD9-labelled vesicles, particle sizes) of the isolated exosomes by kit and UC.

The sample volume (e.g. of cell culture medium) and number of samples should also be taken into consideration when choosing the proper isolation method. For the UC isolation, higher starting volumes can be used compared to isolation with kit, while the kit are more compatible with lower starting volumes (81). The high cost of ultracentrifuges has larger impact when a smaller number of samples are to be isolated with UC. On the other hand, larger sample numbers increase the cost for kit isolations due to reagent consumption.

4 Conclusions

The observations made in our study (summarized in **Table 2**) support the view that exosome isolation depends on the isolation protocol used, differences in the behavior of exosomes between cell sources, characterization methods and the conditions applied (82). Hence, we suggest that the application area (e.g. determine exosome purity or for biomarker discovery) and sample volumes available for the exosome isolation should be strong determining factors when selecting the proper isolation method. The characterization methods used in this study are not able to distinguish exosomes from cellular contaminations and other vesicles, but the untargeted proteome analyses using LC-MS/MS provided more extensive and versatile information on the protein content of the samples than targeted WB of a few proteins. Consequently, we suggest that LC-MS/MS should be implemented to a higher extent regarding exosome characterization. Considering our findings, it is important to state that the term “exosome enrichment” is more appropriate than “exosome isolation”.

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Figure Legends

Figure 1: Measured relative protein amount pr. million cells in exosome samples from GBM- and BC cells isolated by kit and UC ($n \geq 2$). *[page 18]*

A) The measured relative protein amount (%) for the BC exosome isolates. **B**) The measured relative protein amount (%) for the GBM exosome isolates. Each replicate is depicted as circles, and the median depicted as a line. The X-mark shows the measured relative protein amount in the

902 blank sample (isolated cell culture medium). The protein amounts were measured by UV-Vis
903 spectrophotometry (absorption at λ= 562 nm) after reaction with BCA kit reagents.
904
905 **Figure 2: Transmission electron micrographs and hydrodynamic particle size (nm)**
906 **distribution by DLS analysis of exosomes isolated by kit and UC from GBM- and BC cells.**
907 *[page 18]*
908 The TEM-samples were immunogold labelled with anti-CD9, with gold particles depicted as 10
909 nm black dots. Images were taken with a magnification of 400 000, and the dashed areas were
910 additionally zoomed. **A**) Micrographs of CD9-labelled GBM exosome isolates. **I** depict the
911 micrograph from a kit isolate, **II** the kit blank, **III** a UC isolate, and **IV** the UC blank. **B**) DLS
912 analysis of GBM exosomes isolated by kit and UC (n = 1). No particles were detected in the UC
913 blank (n = 1). DLS analysis of the kit blank was not performed. **C**) Micrographs of CD9-labelled
914 BC exosome isolates. **I** depict the micrograph from a kit isolate, **II** the kit blank, **III** a UC isolate,
915 and **IV** the UC blank. **D**) DLS analysis of BC exosomes isolated by kit (n = 2) and UC (n = 3),
916 including the kit blank (n = 1). No particles were detected in the UC blank.
917
918
919 **Figure 3: Western blot of common protein exosome markers.** *[page 20]*
920 The protein markers CD81, CD9, CD63, TSG101, flotillin-1 (positive markers, +) and calnexin
921 (negative marker, -) were targeted in cell lysates and exosomes isolated by kit and UC ($n \geq 2$).
922 Monoclonal mouse antibodies were used for CD81, CD9, CD63, flotillin-1 and calnexin, while a
923 polyclonal rabbit antibody was used for TSG101. For the BC exosomes, 15 µg protein was
924 loaded for kit isolates and 3 µg for UC isolates. For the GBM exosomes, ~14 µg was loaded for

kit isolates and ~8 µg for UC isolates. Uncropped western blots are presented in **Supplemental Western Blots**.

Figure 4: Chromatograms and MS/MS spectrums from LC-MS/MS analysis of GBM- and BC exosome peptides. *[page 22]*

A) Chromatogram with corresponding MS/MS spectrum for the CD9 signature peptide KDVLETFTVK (*m/z*=393.89, z=3) in BC exosomes isolated by UC. **C)** Chromatogram with corresponding MS/MS spectrum for the calnexin signature peptide AEEDEILNR (*m/z*=544.77, z=2) from GBM exosomes isolated by UC. An in-house packed 50 µm x 150 mm column with 80 Å Accucore particles with C_{18} stationary phase was used for separation. A 50 µm x ~3 mm in-house packed pre-column with the same column material was used for trapping. The elution was performed with a linear gradient of 3-15 % MP B in 120 minutes. See **Section 2.11.1** for more LC-MS/MS parameters.

Figure 5: GO annotation of proteins in BC exosomes to different cellular locations. *[page 23]*

The identified proteins classified by their cellular location (GO annotations) grouped based on their positive/ negative relevance towards exosomes. The annotated proteins (% of total proteins) and their cellular location, with proteins annotated from the kit isolates are shown in red (from

749 DAVID ID's), while proteins annotated from the UC isolates are shown in blue (from 615 DAVID ID's).

Figure 6: Venn diagram presenting the number of proteins identified by LC-MS/MS in exosomes isolated by kit and UC from GBM- and BC cell culture medium. *[page 24]*

The numbers are the total number of unique proteins identified when trypsin, keratin related proteins and the proteins identified in blank isolates were disregarded. One signature peptide was selected as requirement for positive identifications during database search. Equal amounts of protein were injected for both kit- and UC isolates (~ 1.5 µg protein for GBM isolates (n = 6) and ~2-5 µg protein for BC exosomes (n=3)). A list of all proteins identified is presented in **Supplemental Proteins**.

Tables

Table 1: A selection of common protein markers (from LC-MS/MS analyses) with a specific exosome related function. The exosome markers were identified in BC- and GBM exosome samples isolated by kit and UC. In addition, proteins identified in blank isolates are shown.

Protein marker	Exosome related function	GBM				BC			
		UC	UC blank	Kit	Kit blank	UC	UC blank	Kit	Kit blank
CD81	Membrane protein	x	-	-	-	x	-	-	-
CD9	Membrane protein	x	-	x	-	x	x	x	-
CD63	Membrane protein	x	-	x	-	x	-	-	-
TSG101	Exosome biogenesis	-	-	-	-	x	-	-	-
Annexin A2	Exosome biogenesis	x	-	x	-	x	-	x	-
Flotillin-1	Exosome biogenesis	-	-	-	-	x	-	-	-
Calnexin	Negative	x	-	x	-	-	-	-	-
Serine/ threonine-protein kinase 26	Negative	-	-	-	-	-	-	-	-
Actin	General marker	x	x	x	x	x	-	x	x

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976 **Table 2: Comparison of characteristics of exosome isolated from GBM and BC cell culture**
977 **medium.** In the table, UC and kit were compared for their ability to isolate pure exosomes. The
978 comparison is based on the characterization techniques used in the present study. Increasing

number of + signs indicate positive relation regarding indications of exosomes and/or high purity of exosomes.

Characterization method	GBM		BC	
	UC	Kit	UC	Kit
Protein amount (corrected for blank)	+	++	+	++
TEM	++	+	+++	++
DLS	+++	+++	+	++
WB (positive markers)	++	+	+	++
WB (negative markers)	+	+	+++	+++
LC-MS/MS (positive markers)	++	+	+++	+
LC-MS/MS (negative markers)	+	++	+++	+++
LC-MS/MS number of identified proteins	+++	+	+	++
LC-MS/MS biomarkers	++	+	++	++

Figures

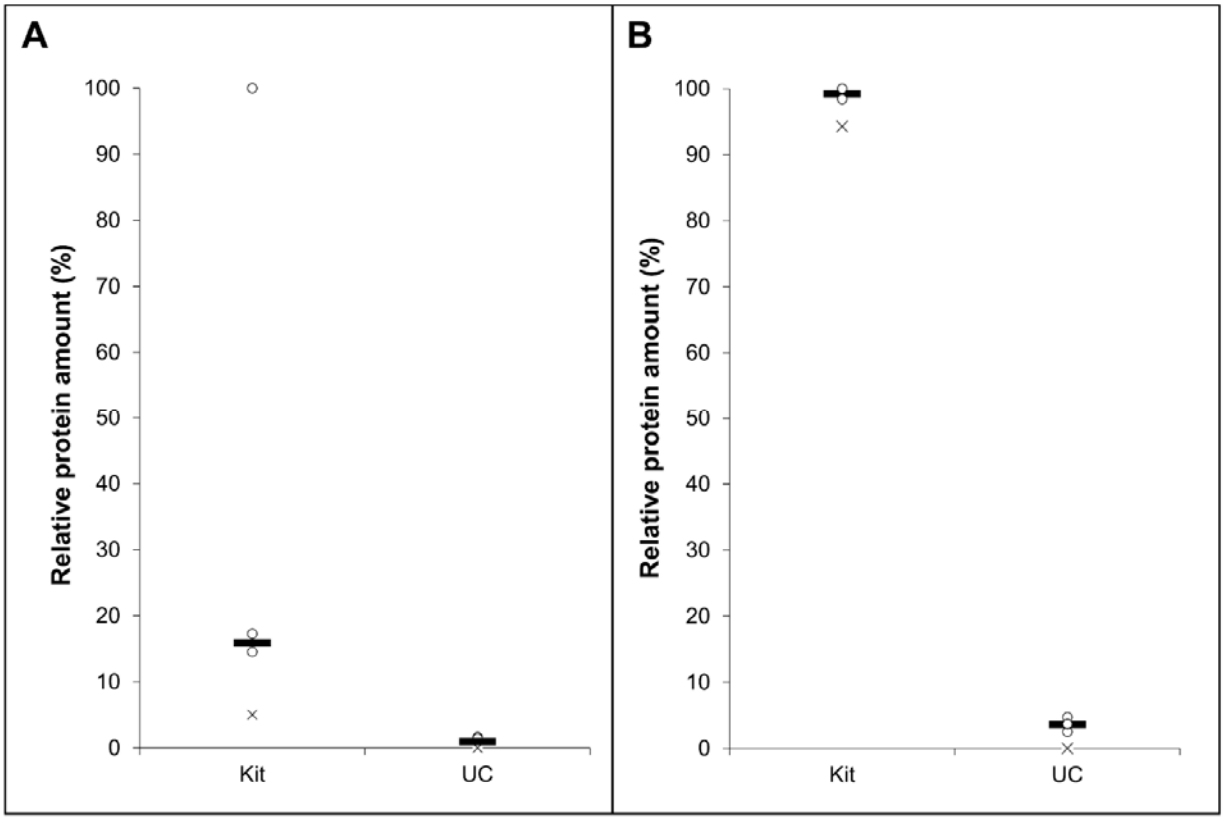
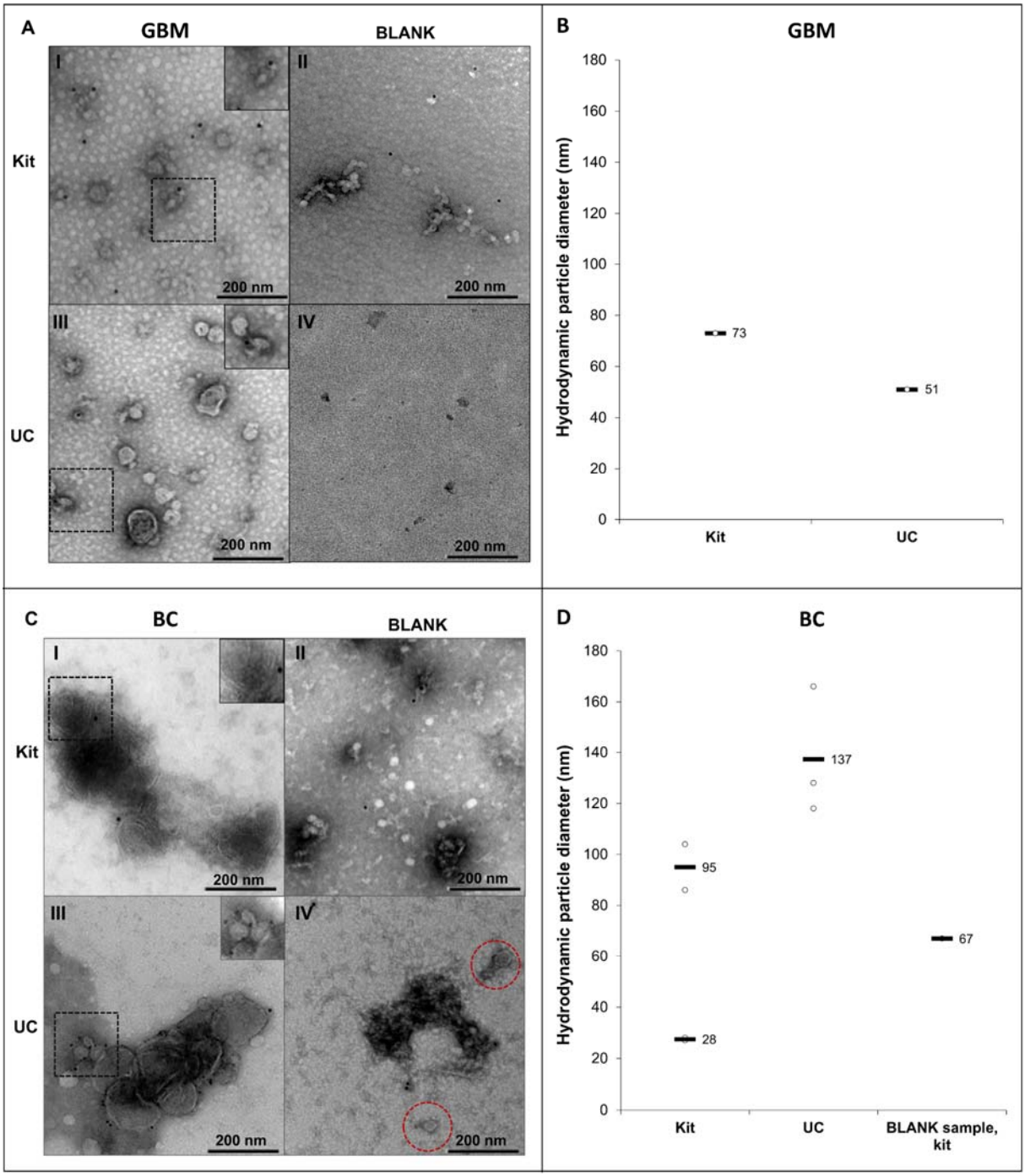


Figure 1



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998 **Figure 2**

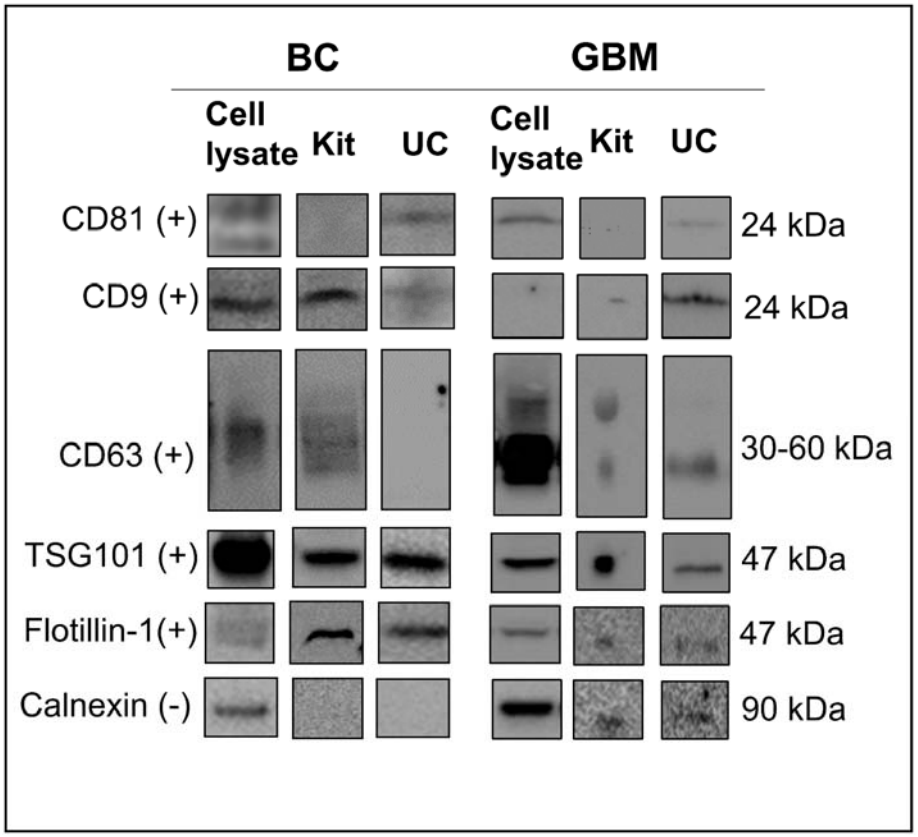

Figure 3

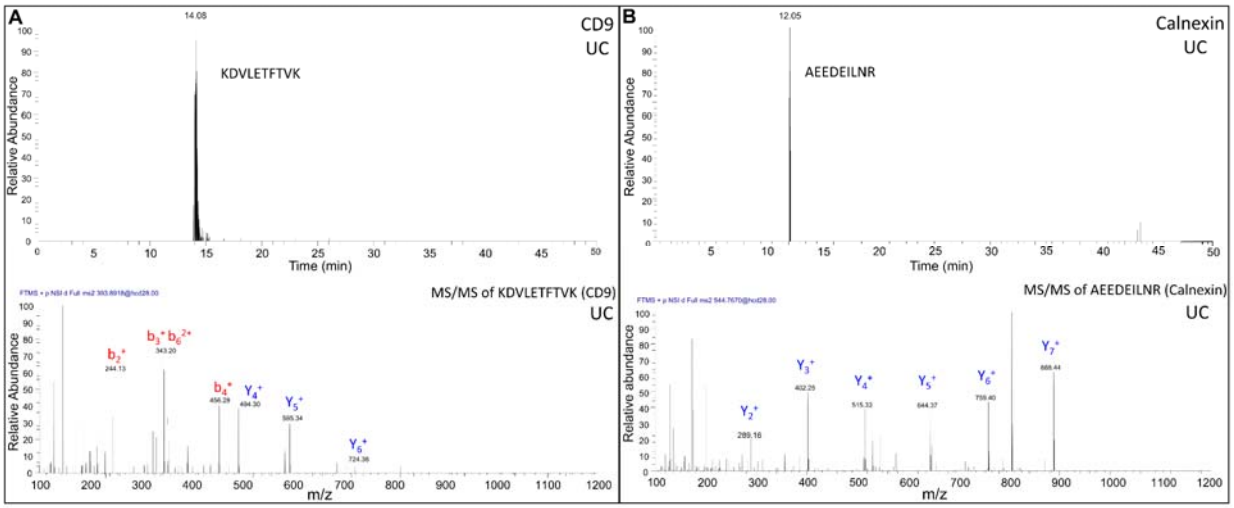

Figure 4

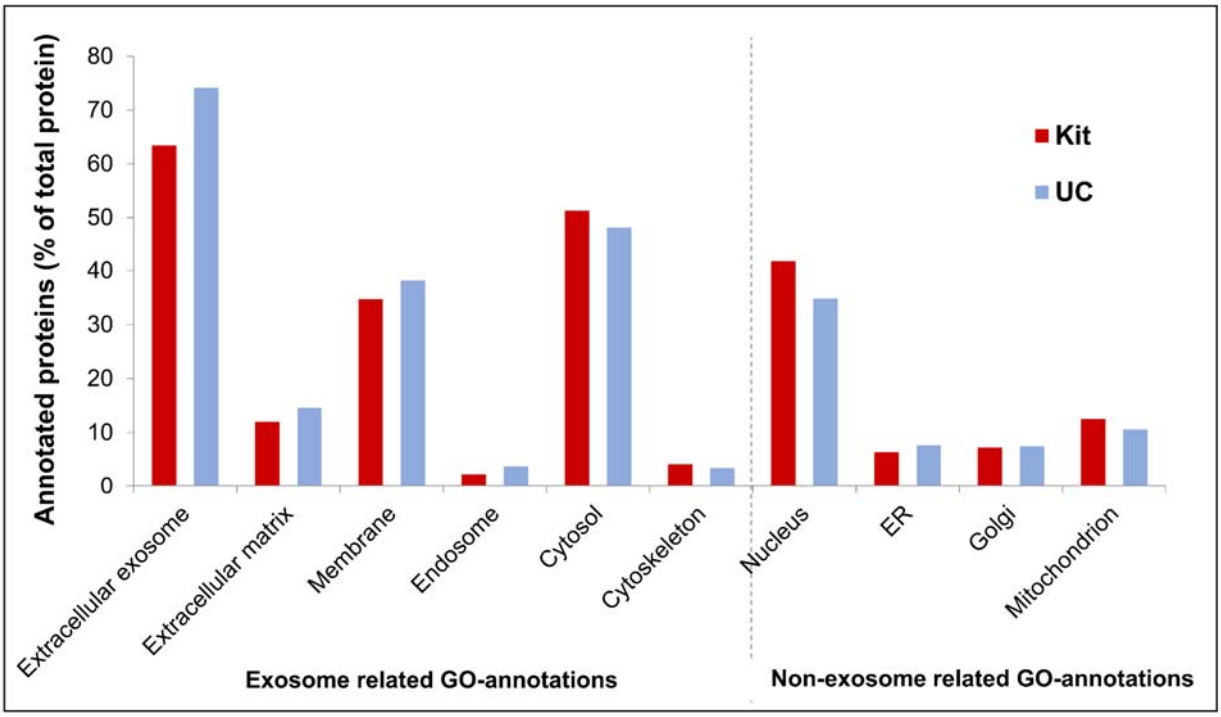

Figure 5

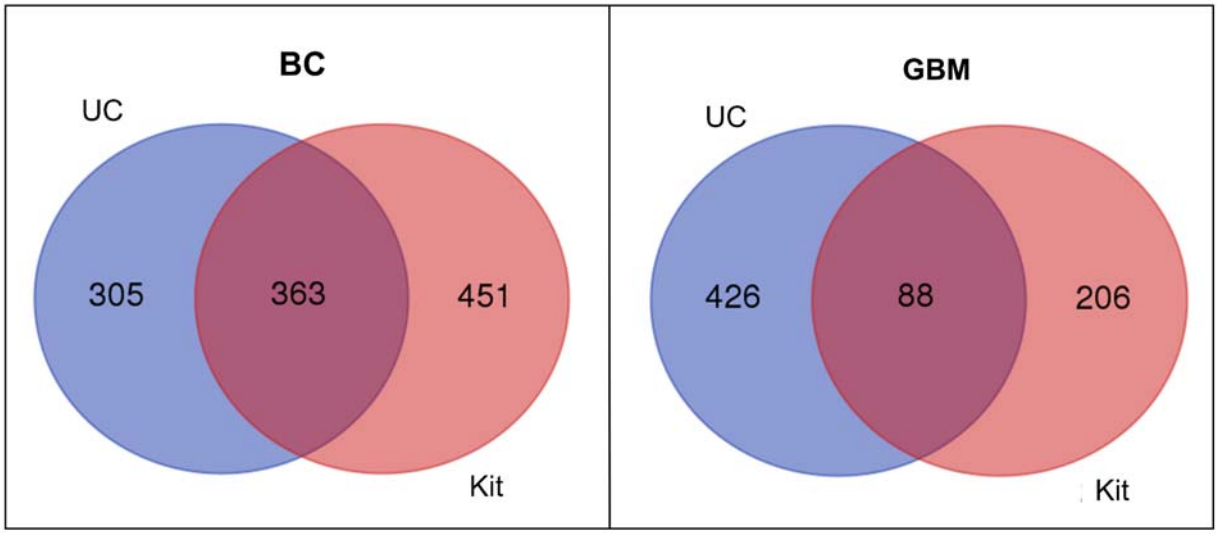

Figure 6