Critical comparison of ultracentrifugation and a commercial kit for isolation of exosomes derived from glioblastoma and breast cancer cell lines

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Abbreviations: ABC, ammonium bicarbonate; ACN, acetonitrile; AGC, automatic gain control; BC, breast cancer; BSA, bovine serum albumin; dd, data-dependent; DLS, dynamic light scattering; DTT, dithiothreitol; ECL, enhanced chemiluminescence; 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 mass spectrometry; MP, mobile phase; MS, mass spectrometry; MVBs, multivesicular bodies; nuclear magnetic resonance, NMR; PBS, phosphate buffered saline; RPMI, Rosewell Park Memorial Institute; RT, room temperature; S/N, signal to noise ratio; TEM, transmission electron microscopy; TFA, trifluoroacetic acid; UC, ultracentrifugation; WB, Western blot

Key words: *Exosomes, Ultracentrifugation, LC-MS, Proteomics, Glioblastoma, Breast cancer*

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 isolating 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, the performance of differential UC and an isolation kit from a major vendor (Total Exosome Isolation Reagent from Thermo Fisher) were compared. Exosomes were isolated from cell culture media of two different cell lines (patient derived cells from glioblastoma multiforme and the breast cancer cell line MDA-MB-231). Transmission electron microscopy (TEM), dynamic light scattering (DLS) and Western blot (WB) indicated the presence of exosomes for both isolation methods. The isolation kit (using <10 % of the sample volume than for UC) and UC contained similar amounts of protein determined by bicinchoninic acid assay (BCA) with absorbance at 562 nm. Using WB, positive exosome markers were identified in all samples. Using mass spectrometry (MS)-based proteomics, additional exosome markers were identified. Regarding glioblastoma exosomes, the number of proteins identified with MS was higher for the UC samples when injecting equal protein amounts, opposite to the breast cancer exosomes. However, negative markers could also be found using MS. Thus, we are cautious to use the term “exosome isolation” as impurities may be present regarding both procedures. Notably, known potential biomarkers for both diseases were detected in the samples using 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 small membrane-bound 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 (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 (for both common proteins and proteins specific to the cell type), 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 are linked to metastasis and tumor progression [7, 12, 13]. Hence, cancer exosomes, when e.g. isolated from body fluids, may be a source of biomarkers for diagnosing cancers such as breast cancer (BC) and glioblastoma multiforme (GBM). BC is the most dominant type of cancer in women [14], with recurrent metastatic disease being responsible for the majority of BC deaths [15]. GBM is the most frequent occurring form of malignant glioma (tumor originating in glial cells in the brain) in adults and is a highly aggressive astrocytoma [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 e.g. for early diagnosis and prognosis assessment using biomarkers in more accessible body fluids such as blood (liquid biopsy) is desirable for both GBM and BC [16, 21, 22]. Hence, the isolation of exosomes for cancer biomarker discovery has emerged [23-30].

The isolation of exosomes is mostly performed from body fluids (e.g. blood, urine, and saliva) or cell culture media by centrifugation-based methods (e.g. sucrose gradient ultracentrifugation and ultracentrifugation, UC) [31, 32]. In addition, other isolation protocols and principles have been developed to overcome the drawbacks of ultracentrifugation such as large amounts of starting volume needed and poor reproducibility [8, 33-39]. Moreover, there is a great need for exosome isolation protocols to tailor towards the isolation of smaller starting volumes (< µL) for e.g. miniaturized cell culture models like organoids and “organ on a chip” [40, 41]. However, there is a lack of consensus between the laboratories as the methods for rigorous isolation are still largely empirical.

The protein content of exosomes has been previously extensively characterized using Western blotting, but also by liquid chromatography tandem mass spectrometry (LC-MS/MS) [42-44]. Tetraspanins (e.g. CD9, CD63 and CD81) have been 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]. These 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 large range of exosome markers (both positive and negative) for exosome characterization are presented by The International Society of Extracellular Vesicles (ISEV) [45]. In addition, larger protein databases covering occurring proteins in exosomes and other extracellular vesicles are available [50-52]. ISEV also recommends using other characterization methods in addition to WB and LC-MS/MS, to study the heterogeneity and morphology of vesicles present in the isolated sample.

In the present study, we have compared two exosome isolation methods (UC and a Total Exosome Isolation kit from Thermo Fisher). 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 patient-derived GBM cells (T1018) and one BC cell line (MDA-MB-231). The impact of different isolation techniques for these cell lines have not yet been studied, and studies of exosome presence and purity are also limited for these cell lines. We also present a critical view of the characterization methods used to detect exosomes and evaluate the purity of the exosomes samples.

2 Material and Methods

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 **Supplementary Materials 1** (**SM-1**).

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 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 $^{\circ}$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 spun down at a RCF of 906 *g* (30 minutes at 23 °C).

2.2 Glioblastoma cell culturing

The glioblastoma (GBM) cell line (T1018) was obtained from the Langmoen Lab at Oslo University Hospital, Norway. The cell line is derived from primary glioblastoma multiforme tumours. The cells were maintained in DMEM/F12 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The medium was supplied with HEPES buffer (10mM) and penicillin/ streptomycin from Lonza (Basel, Switzerland), B27 w/o Vit A from Thermo Fisher, 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). The cells were incubated in a humidifying atmosphere at 5 % CO_2 and 37 °C. Prior to exosome isolation, the incubated cell culture media was centrifuges twice at a RCF of 453 g and 1811 *g* for 5 minutes each.

2.3 Exosome isolation by ultracentrifugation

For the BC and GM cells, 9-12 mL and 60 mL cull culture media were used for centrifugation. Cell culture media was thawed on ice and centrifuged at a RCF of 1811 *g* (5 minutes). The supernatant was centrifuged at a RCF of 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 supernatant was transferred to polycarbonate ultracentrifugation tubes (Beckman Coulter) and diluted with phosphate buffered saline (PBS) until the tubes were full. The tubes were centrifuged twice at a RCF of 100 000 *g* (90 minutes at 4 °C) with an L-80 ultracentrifuge (45 Ti rotor) from Beckman Coulter. The supernatant was removed (leaving suspension 1 cm above the pellet) and the pellet was suspended with PBS between the centrifugations. Upon centrifugation, the supernatant was discarded and the pellet was suspended in either PBS (3 mL for DLS- and 50-100 µL for TEM analysis) or the preferred lysis buffer (**Section 0 and 0**).

2.4 Exosome isolation by isolation kit

The isolation of exosomes with the use of the Total Exosome Isolation Reagent (from cell culture media) was from Thermo Fisher (catalog nr. 4478359). The isolation was performed according to the protocol of the supplier [53]. Starting volumes were ranging from 0.5-9 mL cell culture media for the BC cells and 5-6 mL for the GBM cells. The samples were centrifuged with the Allegra 25R centrifuge, and the pellet was suspended as with ultracentrifugation (**Section 0**).

2.5 Protein extraction and measurement with UV-Vis spectrophotometry

Cell and exosome protein extracts were made by adding RIPA buffer (Thermo Fisher) containing protease inhibitors (Protease Inhibitor Coctail Tablets, Roche, Basel, Switzerland) and phosphatase inhibitors (PhosStop Tablets, Sigma-Aldrich). The BC exosomes were extracted in 50 µL of the RIPA solution, and the BC cells in the 100 µL RIPA solution. The GBM exosomes were extracted in 300 µL of the RIPA solution, and the cells in 1 mL RIPA solution. The extracts were incubated while rotating for 30 minutes (4 ℃), and then snap frozen (at -80 ℃). Extracted samples were thawed and centrifuged at a RCF of 20 570 *g* (30 minutes at 2℃) using a Heraeus Fresco 21 centrifuge or Eppendorf Centrifuge 5424R. The pellet was discarded. The protein amount was determined using Pierce™ BCA protein Assay Kit (Thermo Fisher), by measuring the absorbance at 562 nm. 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). The GBM protein measurements were performed on a Nanodrop 2000 Spectrophotometer (Thermo Fisher).

2.6 Western blotting

The samples containing 3-15 µg protein were diluted giving equal volume of water before adding 5x loading buffer (see **SM-1** for solution preparation) to yield 1x. Prior to electrophoresis, the samples were boiled for 3 minutes at 92 ℃. The samples with added PageRuler™ Prestained protein ladder (Thermo Fisher) were loaded on to 4-12 % Bis-Tris gels (Thermo Fisher) 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. MOPS or MES was used as SDS running buffers (Thermo Fisher). Proteins were transferred to a 45 µm nitrocellulose membrane (Thermo Fisher) 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 (5:95, *w/v*) non-fat dry milk (PanReac AppliChem ITW reagents, Darmstadt, Germany) in tween-20/ TBS (0.05:99.95, *v/v*) (Medicago, Uppsala, Sweden) for 1 hour on a mixing plate. The proteins were then stained with primary antibodies (at 4 °C overnight) in blocking solution and with secondary antibodies for 2 hours at room temperature with rocking in blocking solution. The antibodies CD9 (10626D), CD63 (10628D) and CD81 (MA5-13548 and 10630D) were purchased from Thermo Fisher. 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 tween-20 in TBS (0.05:99.95, *v/v*) 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 ChemidocTM touch imaging system (Bio-Rad). The antibody actin (A2066, from Sigma) was used as a positive control (results not shown).

2.7 Immunogold labelling and transmission electron microscopy

One drop of 5-50 µL of the exosome samples 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 adsorbed 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 (diluted 1+ 9 with fish serum gelatine (FSG) in PBS (1:99, *v/v*)) for 20 minutes. 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 on one drop of the diluted protein A-gold solution (1:50 in the FSG in PBS solution) (10 nm particle size) for 20 minutes, 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 room temperature (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.

2.8 Dynamic light scattering

The dynamic light scattering (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 polymer solutions 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 **SM-1** (**SM-1.6 DLS**).

2.9 Protein digestion

2.9.1 In-gel digestion

Prior to in-gel digestion, the samples were prepared and run using the same procedures as with gel electrophoresis (**Section 0**). Bovine serum albumin (BSA) was prepared in parallel. 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). The lane containing BSA was cut in the mass range of 55-70 kDa. Each fraction was transferred to Protein LoBind tubes. The samples were further reduced, alkylated and digested using the protocol of Shevchenko et al. [54], with trypsin from Promega Biotech AB (Nacka, Sweden). The digested samples were concentrated to dryness and kept at -20 °C until further use (not more than 7 days).

2.9.2 In-solution digestion with peptide desalting

The samples were concentrated, and the pellet was dissolved with 25 µL 6 M urea in 100 mM ammonium bicarbonate (ABC). Then, the samples were reduced with 9.5 mM dithiothreitol

(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 samples, with 28 mM DTT (30 minutes at 30 °C). The proteins in the BC exosomes were initially digested by adding 0.1 µg Lys-C (120 minutes at 37 °C), before the samples were diluted to a final concentration of 19 mM ABC. The trypsin digestion of the samples was performed with 1 µg trypsin (16 hours at 37 °C) and the protease activity was terminated with formic acid, FA/water (0.9:99.1, *v/v*). The desalting and concentrating of the samples were performed using ZipTip® (silica particles with C_{18} resin) from Millipore. The ZipTip was wetted with neat acetonitrile (ACN) and equilibrated with trifluoroacetic acid, TFA/water (0.1:99.9, *v/v*). The peptide samples were then pipetted 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*). The filtrate was concentrated to dryness at 30°C and frozen at -20°C until further use (not more than 30 days).

2.10 LC-MS/MS analysis

Unless otherwise stated, the dilutions during sample preparations were performed using water (HiPerSolv Chromanorm®) from VWR. A Q-Exactive™ equipped with a nanoFlex nanospray ion source from Thermo Fisher was used for the LC-MS/MS analysis at the two laboratories.

2.10.1 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, using the developed method as described in our previous study [55]. An EASY-nLC 1000 pump (with autosampler) from Thermo Fisher was applied throughout the experiments. The solvents used for peptide separation was of FA/ water (0.1:99.9, *v/v*) (mobile phase A, MP A) and of ACN/ FA (0.1:99.9, *v/v*) (mobile phase B, MP B). Prior to LC-MS analyses, the samples were thawed and dissolved in 10-15 µL FA/ water (0.1:99.9, *v/v*). 10 µL of the sample was injected with a flowrate of 20 µL/min. 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 for the exosome samples, 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 eluting

peptides were ionized at 1.8 kV at 250 °C for the BC exosome samples and at 275 °C for the GBM exosome samples. 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. When 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.

2.10.2 LC-MS/MS analysis at laboratory 2

The analytical column applied was an Acclaim PepMap 100 column (C18, 3 µm beads, 100 Å, 75 µm ID) of 500 mm bed length and an Ultimate 3000 nano ultra-HPLC system from Dionex (Sunnyvale, CA, USA) was connected to the LC-MS system. The MP A was set to FA/water (0.1:99.9, *v/v*) and the MP B was set to be 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 for the exosome samples. The percentage of MP B was increased to 50 % in 20 minutes and 80 % MP B in 2 minutes. 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, automatic gain control (AGC) of $3x10^6$ and maximum injection time of 100 milliseconds. When in dd/MS/MS the resolving power was set to 35 000 and the maximum injection time to 120 milliseconds. The dynamic exclusion was set to 60.0 seconds. The method allowed sequential isolation of up to the ten most intense ions depending on signal intensity (intensity threshold $1.7x10^4$) isolation window was *m/z* 2 without offset.

2.10.3 Data processing and protein identification

Proteome Discoverer (version 1.4.0.228) was used to identify the peptides and proteins. The proteins were identified using both SEQUEST and MASCOT algorithms. All searches were performed setting the digestion enzyme to trypsin with maximum one missed cleavage, fragment ion mass tolerance of 0.10 Da and a precursor mass tolerance of 10.0 ppm. Signal to noise (S/N) threshold was set to 1.5 and the minimum ion count to 1. Carbamidomethylation of cysteine was specified as static modification. Oxidation of methionine, acetylation of the N-terminus and deamidation of glutamine and asparagine were specified as dynamic

modifications. The proteins were identified with high peptide confidence filter. Proteins identified as keratin or trypsin were removed from the list, in addition to proteins found in the blank samples.

3 Results and Discussion

For comparison of the two exosome isolation methods (UC and Total Exosome Isolation kit) using the cell lines T1018 (GBM) and MDA-MB-231 (BC), common characterization methods were investigated 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 performed in addition to total protein determination, DLS and LC-MS/MS. Comprehensive proteome analysis using LC-MS/MS was applied to complement WB for detecting exosome protein markers (hereby referred to as exosome markers) and inclusion of additional positive and negative markers for a more complete investigation.

3.1 Similar protein yields in kit and UC samples

The protein amount in the BC (**Figure 1A**) and GBM (**Figure 1B**) exosomes per million cells was measured using UV-Vis spectrophotometry (i.e. absorbance at 562 nm). The total protein amount measured for exosomes isolated by the kit was 15-28 times higher than for exosomes isolated by UC. Elevated protein amounts in exosomes isolated by kit compared to UC was also observed in a previous study by Van Deun et al. (UC compared to an identical isolation kit for MCF7 derived exosomes) [56]. However, increased absorbance (indicating possible protein contaminations) was measured in the kit blanks (i.e. isolation of cell culture medium grown w/o cells with isolation kit), in comparison to UC blank (i.e. cell culture medium grown w/o cells isolated by UC) where the absorbance was below the limit of quantification. When correcting for the blank (subtracting the protein amount measured in blank samples from the protein amount in exosome samples), the measured protein amount for exosomes isolated by the kit and UC was similar.

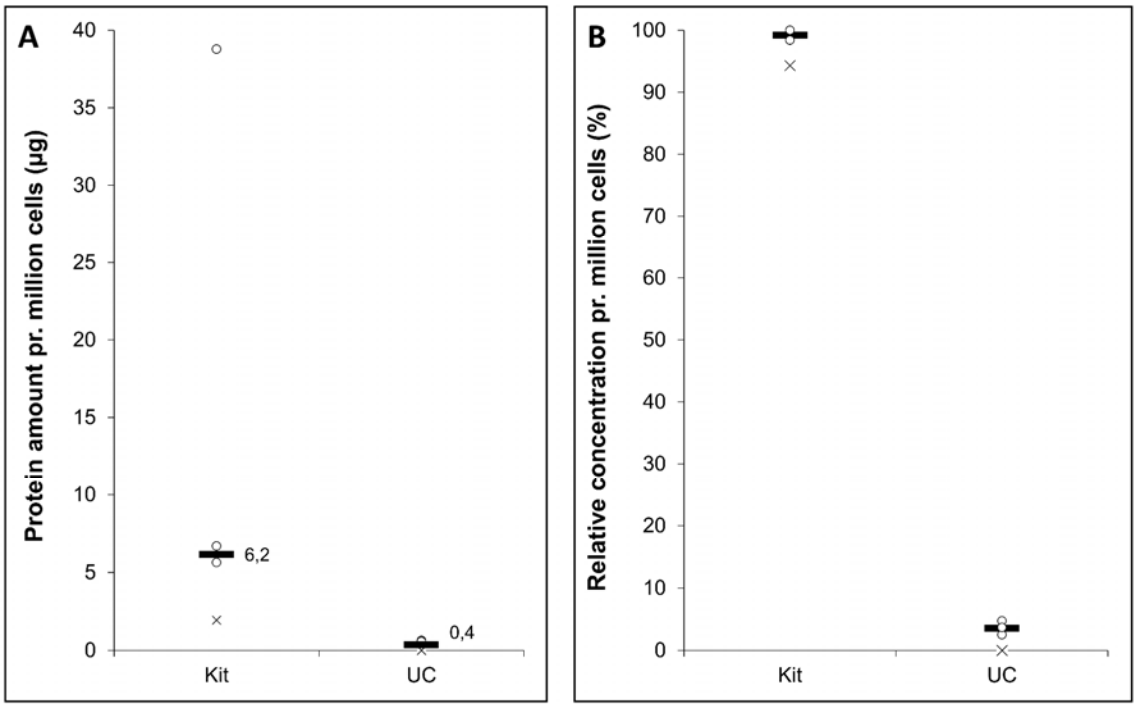


Figure 1: Measured protein amount pr. million cells in exosome samples from GBM- and BC cells isolated by kit and UC (n ≥ 2). A) Measured absolute protein amount (µg) for the BC exosome sample. **B**) The measured relative protein amount (due to low regression coefficients, %) for the GBM exosome samples. Each replicate is depicted as circles, and the median depicted as a line. The X-mark shows the measured protein amount in the blank sample (isolated cell culture medium). The protein amounts were measured by UV-Vis spectroscopy (absorption at λ= 562 nm) after reaction with BCA kit reagents.

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 in the samples isolated with both kit and UC were observed in the micrographs (**Figure 2**). Vesicle structures similar to that described in literature were observed [6, 57, 58]. 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 exosomes (**Figure 2A-I** and **2A-III**) and could not confirm the presence of a membrane enclosing the vesicles. The UC sample presented more distinct double membranes in the expected size range for exosomes compared to the kit sample. The blank samples for both isolation methods did not display membrane structures and seemed pure (**Figure 2A-II** and **2A-IV**). The high

purity was further confirmed by DLS analysis of the UC blank (**Figure 2B**). The DLS-analysis of the exosome samples exhibited particles of similar sizes of 51 and 73 nm (mean) with both isolation methods (**Figure 2B**). Thus, both isolation methods isolated comparable exosome populations.

3.2.2 BC exosomes

Several of the BC vesicle structures were CD9-labelled (**Figure 2B-I** and **B-III**). CD9-labelled vesicles have also been observed in a previous study of the same cell line [59]. Notably, the blank samples displayed contamination (**Figure 2C-II and C-IV**), 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 exosome samples from kit (28 and 95 nm, mean values) while only one was presented using UC (137 nm, mean value), indicating differences in the particle sizes isolated with the two isolation methods.

The sizes observed with DLS correlates well with other studies (30– 250 nm), also displaying a heterogeneity of particle sizes in exosome samples [13, 56, 60-64]. In conclusion, the isolated samples did show structures resembling to those of EVs, but some blank samples were not entirely pure from vesicles or particles. Observations made with TEM are not necessarily detectable with DLS because TEM measure on dry samples, whereas DLS probes on solution or suspension of particles. In addition, the micrographs taken with TEM display a narrow section of the grid, which again represent a small part of the isolated sample.

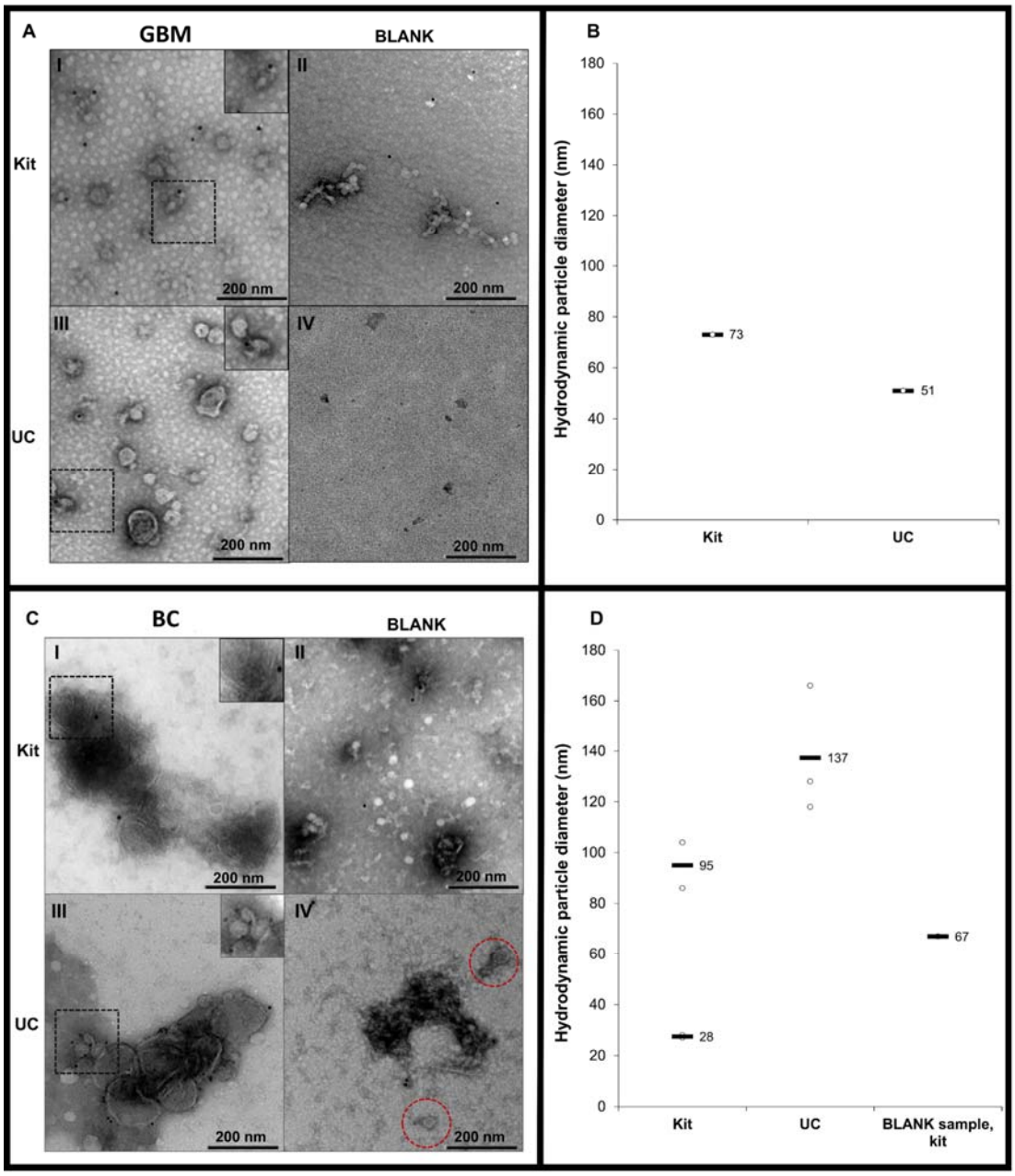


Figure 2: Transmission electron micrographs and hydrodynamic particle size (nm) distribution by DLS analysis of exosome samples from GBM- and BC cells isolated by kit and UC. The TEM-samples were immunogold labelled with anti-CD9, with gold particles depicted as 10 nm black dots. Images were taken with a magnification of 400 000, and the dashed areas were additionally zoomed. **A**) Micrographs of CD9-labelled GBM exosome samples. **I** depict the micrograph from a kit sample, **II** the kit blank, **III** the UC sample, and **IV** the UC blank. **B**) DLS analysis of GBM exosome samples isolated by kit and UC (n = 1). No particles were detected in the blank sample isolated with UC (n = 1). DLS analysis of the kit blank was not performed. **C**) Micrographs of CD9-labelled BC exosome samples. **I** depicts the micrograph from the kit sample, **II** the kit blank, **III** a UC sample, and **IV** the UC blank. **D**) DLS analysis of BC exosome samples isolated by kit (n = 2) and UC (n = 3), including the kit blank (n = 1). No particles were detected in the blank sample isolated with UC.

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

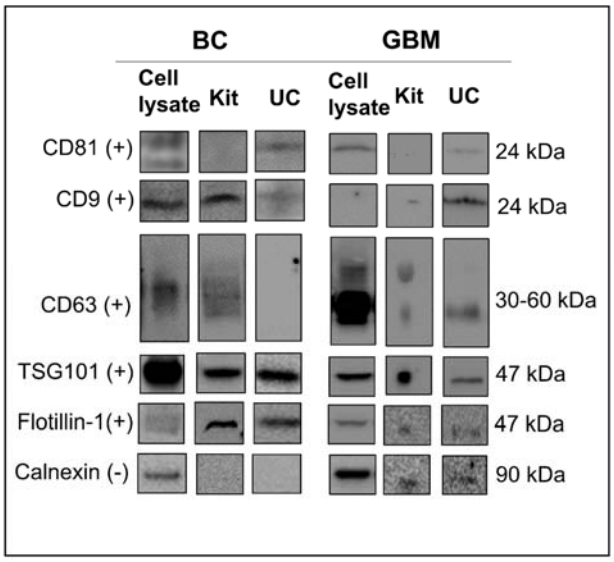
According to ISEV, at least three markers should be included for characterization of exosomes, originating from both transmembrane proteins (e.g. tetraspanins), cytosolic proteins (e.g. TSG101 or annexins) and negative markers (e.g. calnexin) [45]. In the present study, WB was performed using antibodies 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 have been absent in exosome samples in some studies [45, 60]. Hence, the presence of calnexin is assumed to signalize ER-contamination (which enables possibilities of contaminations from other cellular organelles).

3.3.1 GBM exosomes

For the GBM cells and exosomes, all exosome markers (both positive and negative) were found in samples from both kit- and UC isolation except for CD81, which was only found in UC samples (**Figure 3**). The WB-bands were more apparent for most positive markers for exosomes isolated by UC (even with higher protein amount loaded for the kit samples), which is also in accordance with Van Deun's study [56].

3.3.2 BC exosomes

For the BC exosomes, inconsistency for several positive exosome markers were observed between the kit and UC samples (**Figure 3**). The positive marker CD63 was only detected in kit samples, while CD81 was only detected in samples from UC (similar to GBM exosomes). The proteins TSG101, flotillin-1 and CD9 (barely visible in the UC samples) were detected for both isolation methods. Although, a study by 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 on UC samples from the same cell line [23, 65, 66].



The reason for the absence of some tetraspanins in kit and others in UC samples could be due to protein concentrations below detection limits or poor antibody quality. Several antibodies for CD63 and CD81 (from different companies) were tested on the BC exosomes before a signal was obtained, which 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 samples 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 methods indicate that the samples do not contain contamination from the ER. However, only one negative marker is insufficient to exclude cell organelle impurities. Further investigation by implementing more comprehensive methods like LC-MS/MS was considered to be beneficial.

Figure 3: Western blot of common protein exosome markers. The protein markers CD81, CD9, CD63, TSG101, flotillin-1 (positive markers, +) and calnexin (negative marker, -) were targeted in cell lysates and exosome samples isolated with kit and by UC ($n \geq 2$). For the BC exosomes, 15 µg protein was loaded for kit samples and 3 µg for UC samples. For the GBM exosomes, ~14 µg was loaded for kit samples and ~8 µg for UC samples. Uncropped Western blots are presented in **Supplementary Materials 2** (**SM-2**).

3.4 LC-MS/MS studies confirmed and complemented the WB study

LC-MS/MS was performed to confirm the WB observations, using in-house packed nano separation columns [55]. One positive marker (annexin A2) and one negative marker (serine/threonine-protein kinase 26) was added to complement the WB study. The detection of the selected exosome markers using LC-MS/MS is presented in **Table 1**, with chromatograms and MS/MS spectra of a CD9 signature peptide (BC, **Figure 4A**) and calnexin signature peptide (GBM, **Figure 4B**). Similar to the WB analysis, the same tetraspanins including calnexin observed in kit and UC samples were detected for the GBM exosomes using LC-MS/MS. However, the tetraspanins detected from kit samples were only found in one replicate, indicating low concentrations and high detection uncertainty. Flotillin-1 was also detected, but only for UC samples with LC-MS/MS. TSG101 was identified in neither kit nor UC (GBM exosomes) using LC-MS/MS in contradiction to WB.

Table 1: A selection of common protein markers (from LC-MS analyses) with a specific exosome related function. The exosome markers were identified in exosome samples from

MDA-MB-231 and glioblastoma isolated with kit and by UC. In addition, proteins identified in blank samples 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

For the BC exosomes, the LC-MS/MS analysis is contradictory to the trend observed in the WB analysis where kit samples provided more pronounced signals for positive markers than the UC samples. Using LC-MS/MS, several positive markers were absent in the kit samples when injecting similar amounts of protein as with UC samples. The reason for the difference to WB could hence be partially due to higher protein amount loaded onto the gel for kit samples. Calnexin was, similar to the WB, not detected in the BC samples. Annexin A2 (positive marker) was found in all samples and serine/threonine-protein kinase 26 (second negative marker) was not detected in any samples.

Other negative markers from peroxisomes (PMP70), mitochondria (prohibitin-1, hexokinase-2 and mitochondrial phosphoenolpyruvate carboxykinase [GTP]), Golgi apparatus (GM130, translocation protein SEC62, translocation protein SEC63 and protein disulfide-isomerase TMX3), nucleus (Bcl-2-associated transcription factor 1 and c) and ER (calreticulin), as used in other studies [56, 67], were not detected in any BC samples in our study (only translocation

protein SEC63 and protein disulfide-isomerase TMX3 detected in GBM samples). The absence of several negative markers in the BC exosomes supports the low cell organelle contamination observed with WB and LC-MS/MS.

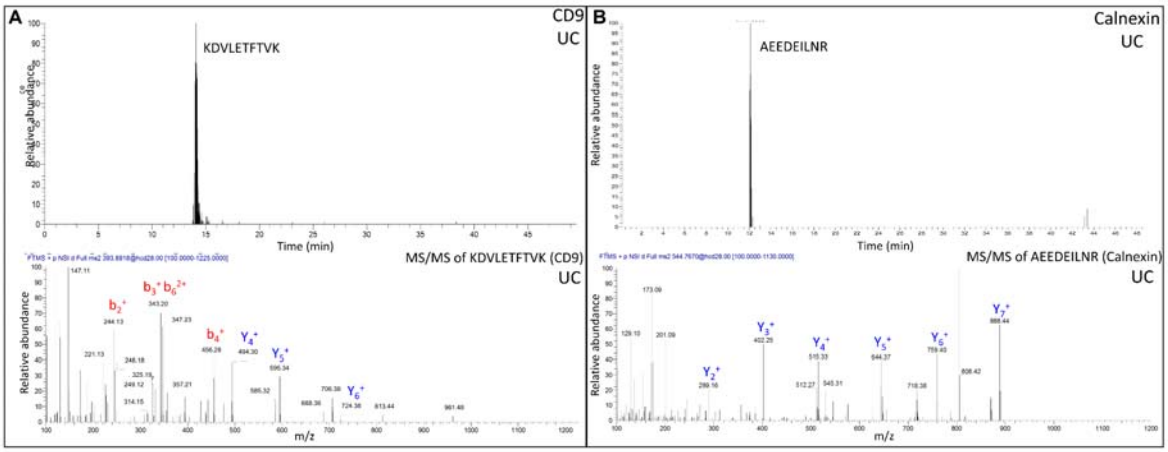


Figure 4: Chromatograms and MS/MS spectrums from LC-MS/MS analysis of GBM- and BC exosome peptides. A) Chromatogram with corresponding MS/MS spectrum for the CD9 signature peptide KDVLETFTVK (*m/z*=393.89, z=3) in BC exosomes isolated by UC. **C)** Chromatogram with corresponding MS/MS spectrum for the calnexin signature peptide AEEDEILNR (*m/z*=544.77, z=2) from GBM exosomes isolated by UC.

3.5 GO annotations reveal proteins annotated to cell organelles in BC exosomes

However, the possibility of contaminants being present in BC samples cannot be excluded. Other general proteins related to e.g. the nucleus, Golgi apparatus, mitochondrion and ER was indeed identified in the BC exosomes using LC-MS/MS (**Figure 5**). The proteins identified in BC exosomes were classified based on their gene ontology (GO) annotations to different cellular localizations, where one protein can be annotated to several cellular localizations. Out of the 668 proteins identified from the UC and 814 from the kit, 615 and 749 DAVID ID`s were annotated to selected cellular localizations. From them, both isolation methods generated samples enriched in exosome related proteins. Several proteins were annotated to the cellular organelles mitochondria (11-13 %), ER (6-8 %) and Golgi apparatus (7 %). Interestingly, 35-42 % of the proteins were also annotated to the nucleus (e.g. histones), which seems to imply impure exosome samples [45]. On the other hand, a high percentage (20-40 %) of other proteins related to the nucleus has also been found in exosome samples from other studies [68, 69]. The presence of cell organelle annotated proteins could point toward cellular impurities in the samples, which would not have been discovered by targeted protein characterization methods (e.g. WB). However, there is not sufficient knowledge on

whether cell organelle proteins derive exclusively from cell impurities, or if they occur naturally in EVs. To summarize, from our point of view, complete information about exosome purity cannot be obtained by any of the common characterization techniques used today.

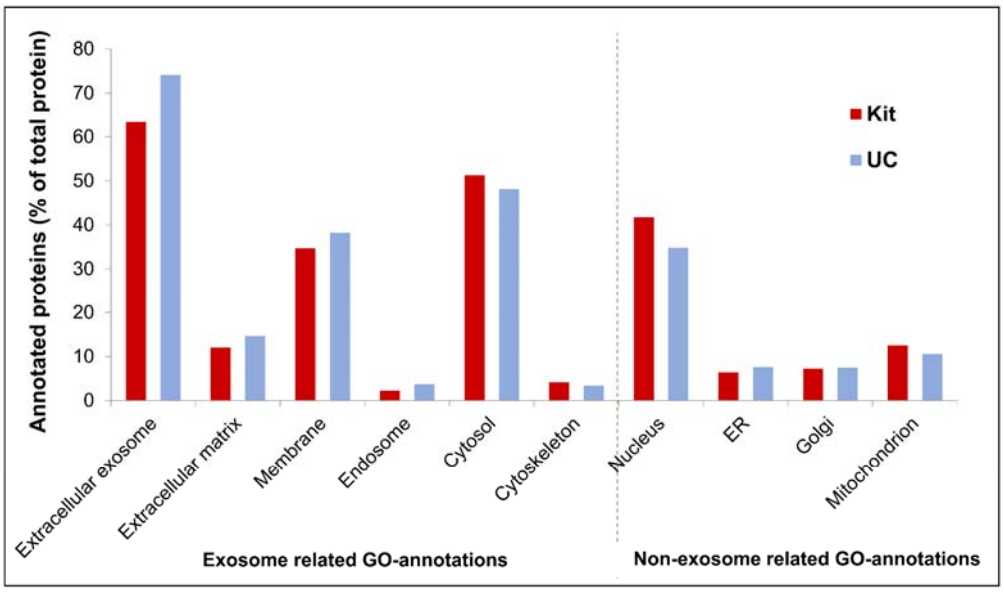


Figure 5*: GO annotation of proteins in BC exosomes to different cellular locations.* The identified proteins classified by their cellular location (GO annotations) grouped based on their positive/ negative relevance towards exosomes. The annotated proteins (% of total proteins) and their cellular location, with proteins annotated from the kit samples are shown in red (from 749 DAVID ID's), while proteins annotated from the UC samples are shown in blue (from 615 DAVID ID's).

3.6 The isolation method and cell line influence the number of cancer related proteins identified

The total number of proteins identified in the GBM and BC exosome samples using LC-MS/MS (performed in two different laboratories) is presented in the Venn diagrams in **Figure 6** (see **SM-3** for a list of all identified proteins). For the GBM exosomes, the number of identified proteins reflects the findings in both WB and LC-MS/MS exosome marker investigations. UC-isolated exosomes provided more unique proteins than the kit-isolated exosomes (75 % higher number of identified proteins than the kit). An increased number of potential biomarkers for GBM (e.g. heat shock proteins 70 kDa and 90 kDa [70-72], chondroitin sulfate proteoglycan 4 [70, 73], CD44 [70, 73, 74] and CD276 [75]) were also identified in the UC samples compared to the kit samples using LC-MS/MS. The

identification of relevant biomarkers is of great interest for further studies on exosomes. However, the identified biomarkers cannot exclusively be related to exosomes due to the presence of negative exosomes markers indicating cellular contaminations.

For the BC exosomes, the tables are turned, and the kit-isolated exosomes provided 12 % higher number of identified proteins than UC-isolated exosomes. However, there was no correlation between the injected protein amount or the starting volume used for isolation, and the number of identified proteins in kit- vs UC samples for BC exosomes (result not shown). Thus, the reason for why the protein number is changed between the two cell lines and isolation methods is unknown. The number of identified biomarkers related to the triple negative breast cancer (e.g. histone H4 [76], heat shock 90 kDa α and β protein [77], calmodulin and epithermal growth factor receptor [78]) in the UC samples compared to kit samples were comparable.

When comparing cell lines, the number of identified proteins was lower in GBM exosomes than BC exosomes, but the number for GBM exosomes is comparable to another LC-MS/MS study on GBM exosomes [79].

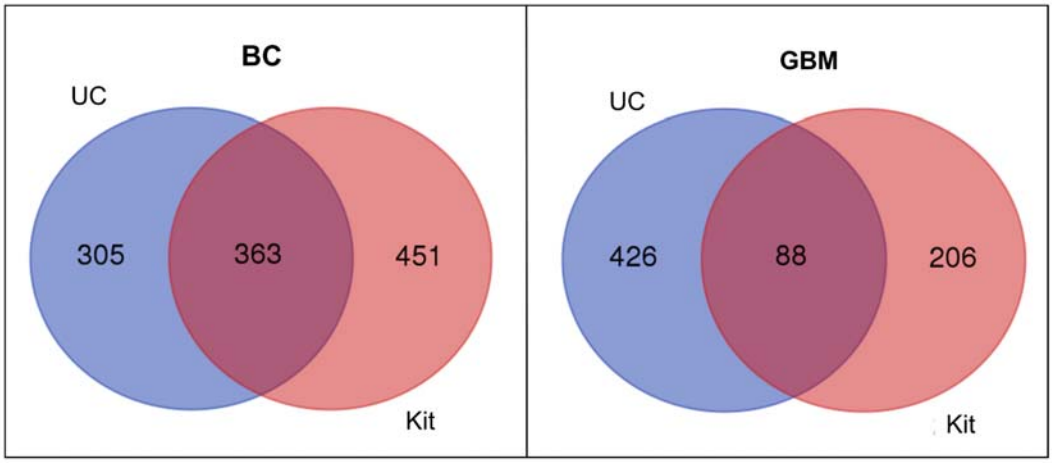


Figure 6: Venn Diagram presenting the number of proteins identified by LC-MS/MS in GBM- and BC exosome samples isolated with kit and by UC. The numbers are the total number of unique proteins identified when trypsin, keratin related proteins and the proteins identified in blank samples were disregarded. One signature peptide was selected as requirement for positive identifications during database search. Equal amounts of protein were injected for both kit and UC (~ 1.5 µg protein for GBM exosomes (n= 6) and ~2- 5 µg protein for BC exosomes (n= 3)). A list of all proteins identified is presented in Supplementary Materials 3 (SM-3).

3.7 Choosing the proper exosome isolation method is not straight forward

A complete comparison of the characteristics of exosome isolation methods is given in **Table 2**. For all exosome samples, the kit and UC samples display similarities and differences.

3.7.1 GBM exosomes

For the GBM exosomes, one of the positive markers detected in the UC samples (CD81) was not found in kit samples by WB. In TEM, double membrane structures were more eminent in the UC samples, but the existence of double membranes cannot be excluded by looking at the micrographs from the kit samples. The largest differences between kit and UC for the GBM exosomes were found in the LC-MS/MS studies recoding positive markers and general protein number. All tetraspanins investigated were detected in the UC samples in several replicates. For the kit, 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 samples compared to kit samples. 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 (T1018) exosomes.

3.7.2 BC exosomes

For the BC exosomes, there was a slight difference in favor of the kit method regarding positive markers in WB and the number of identified proteins (LC-MS/MS). However, using LC-MS/MS, more relevant protein markers were found in the UC samples in contrary to the WB study. In addition, TEM presented slightly more defined double membrane structures with more labeling using UC. However, the micrograph displays an extremely small part of the whole sample. The isolation methods also performed similarly regarding biomarker identifications. Based on the above, there is no obvious reason for choosing one method over the other. However, tendencies towards different characteristics of the isolated exosomes from kit and UC have been observed.

Table 2: Comparison of characteristics of exosome isolated from GBM and BC cell culture medium. In the table, UC and kit was compared for their ability to isolate pure exosomes based on the characterization techniques used. 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 measured (corrected for blank)	+	++	+	++
TEM	++	+	+++	++
DLS	+++	+++	+	++
WB (positive markers)	++	+	+	++
WB (negative marker)	+	+	+++	+++
LC-MS/MS (positive markers)	++	+	+++	+
LC-MS/MS (negative markers)	+	++	+++	+++
LC-MS/MS number of proteins identified	+++	+	+	++
LC-MS/MS relevant biomarkers	++	+	++	++

4 Conclusions

The observations in our study support the perception of exosome isolation being highly dependent on the isolation protocol used, general differences in the behavior of cells/exosomes between cell lines and the characterization methods and conditions applied. The knowledge about potentially natural occurring proteins in exosomes is insufficient and a selection of expected negative markers cannot exclusively be used to determine the purity of exosome samples. Hence, the application area (e.g. proving presence of exosomes, determine exosome purity, biomarker discovery, or lower the dynamic range of proteins in body fluids like blood) and sample volume available for the exosome isolation should be stronger determent factors when selecting the proper isolation method. Thus, the characterization methods used in this study are not able to distinguish exosomes from cellular contaminations and other vesicles. Untargeted proteome analyses using LC-MS/MS did provide more extensive and versatile information on the protein content of the samples than most commonly used targeted WB of few proteins. Consequently, we suggest that LC-MS/MS should be implemented to a higher extend 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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