University of South Bohemia in České Budějovice

Faculty of Science

Comparative analysis of different extracellular vesicle isolation kits and their effect on the structure of their biomolecular corona

Bachelor thesis

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Abstract

Recent studies highlight the potential role of the corona-like surface that surrounds the extracellular vesicles (EVs) of various organisms. From a functional perspective, this structure indicates a particular role in internalization by target cells. However, it is commonly lost during the process of purification. Moreover, different EV isolation techniques affect the composition, structure and enrichment of different EV subpopulations. Therefore, this bachelor thesis focuses on studying of the impact of three different purification techniques: i) a commercial Size exclusion chromatography (SEC) kit; ii) a commercial MagCapture kit; iii) a commercial Precipitation kit, on the structure of EVs and biomolecular corona recovery.

Prohlášení

Prohlašuji, že jsem autorem této kvalifikační práce a že jsem ji vypracovala pouze s použitím pramenů a literatury uvedených v seznamu použitých zdrojů.

V Českých Budějovicích,

Dne ..... Podpis studenta .....

Poděkování

Poděkování patří zejména mému konzultantovi Hynkovi Mazancovi za cenné rady, metodickou a odbornou pomoc při zpracování práce a školiteli Romanu Kuchtovi za odborné vedení, podnětné připomínky a čas, který mi věnoval ke konzultacím. Dále děkuji pracovníkům laboratoře helmintologie Parazitologického ústavu Biologického centra AV ČR, zejména Tomáši Scholzovi, za poskytnutí příjemného zázemí. Děkuji také Nikol Buškové a Oldřišce Kadlecové za pomoc s prací v laboratoři. Dále bych ráda poděkovala Zdenkovi Gardianovi z laboratoře elektronové mikroskopie Parazitologického ústavu Biologického centra AV ČR a CF Nanobiotechnology, Brno, ČR za pomoc s analýzou dat. V neposlední řadě bych ráda vyjádřila díky své rodině a přátelům za podporu a trpělivost, jež mi v průběhu celého studia prokazovali.

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1. INTRODUCTION

A fundamental ability of all cells is to perceive and respond to signals from their surrounding microenvironment. Such interactions between cells can be achieved through many ways (endocrine, paracrine, autocrine) (Camussi et al., 2010; Campanella et al., 2019). In the past decade, there has been an outbreak of studies that emphases the role of extracellular vesicles (EVs) as potential transmitters of biological signals and immune responses between cells (Marcilla et al., 2014). EVs are a heterogeneous group of membrane-delimited nanoparticles released from the plasma membrane of a wide variety of prokaryotic and eukaryotic cells (Yáñez-Mó et al., 2015). Moreover, unlike their cells of origin, EVs are not capable of replication (Théry et al., 2018). EVs released through various pathways persist in the extracellular space or in various biological fluids from where EVs can be internalised by recipient cells (Yáñez-Mó et al., 2015; Zaborowski et al., 2015). Their role in the cell-cell communication is determined by their structure and composition. Moreover, EVs have been found to carry a plethora of different molecules such as lipids, proteins or various nucleic acids (e.g., DNA, miRNA, siRNA) (Marcilla et al., 2014; Yáñez-Mó et al., 2015).

The first report of the EV-like structures comes from the second half of the 1940s when based on electron microscopy images they were described as "a variety of minute breakdown products of blood corpuscles" in the study of human plasma (Chargaff & West, 1946). More than two decades later, electron microscopy images were published and captured particles from ultracentrifuged fresh human plasma were reported as "platelet dust" (Wolf, 1967). After few years further images of these structures from human and other mammalian organisms' blood were reported as "microparticles" (Crawford, 1971). In subsequent studies focusing on the structure of the cell membrane in sheep and rat reticulocytes, it was found that the fusion of multivesicular bodies (MVBs) with the membrane leads to the release of EVs into the surrounding environment (Pan & Johnstone, 1983; Harding et al., 1984; Johnstone et al., 1987).

Raposo et al. (1996) discovered that the externalized vesicles (exosomes) presented antigenic peptides through MHC II and were able to induce specific T-cell responses (Fig. 1). These findings suggested the involvement of EVs in cell signalling and intercellular communication, which was later supported by the presence of bioactive molecules (proteins,

lipids, nucleic acids, etc.) within and on the surface of EVs (Raposo et al., 1996; Ratajczak et al., 2006; Valadi et al., 2007).

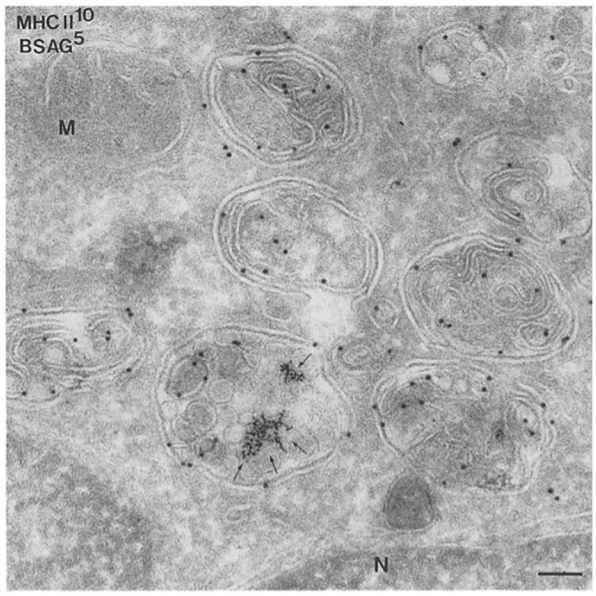

Fig. 1. Ultrathin cryosection of an RN (HLA-DR 15^{+}) B cell immunolabeled for MHC class II with 10-nm gold particles. **N**: nucleus; **M**: mitochondrion. Bar: 0,1 μm (Raposo et al., 1996).

Based on their biogenesis mechanism, EVs can be classified in two major subtypes: i) **exosomes** and ii) **microvesicles** (MVs) (Fig. 2) (Raposo & Stoorvogel, 2013; Marcilla et al., 2014). However, EV subtypes can also be further categorised by physical characteristics, for example density and size, biochemical composition, or cell of origin (Théry et al., 2018).

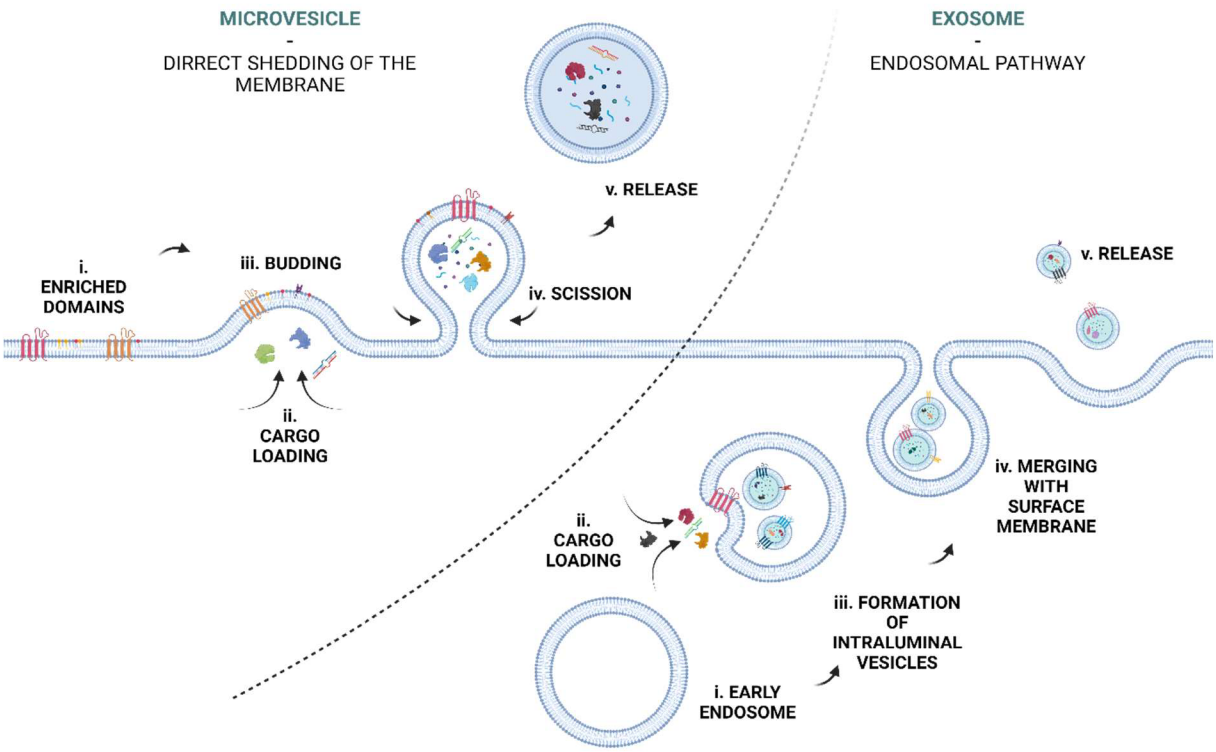

Fig. 2. Scheme of biogenesis of the extracellular vesicles (Bušková, 2021).

1.1. EXOSOMES

This type are small vesicles of endosomal origin with a 30–150 nm diameter ranging (Théry et al., 2009). The process of their biogenesis begins with the formation of a stage called early endosome. These structures are formed by invagination of the plasma membrane (Fig. 2). Early endosomes must then undergo several maturation processes to transform into late endosomes (Akers et al., 2013). Through inward budding of their membrane, **intraluminal vesicles** (ILVs) are formed with simultaneous sequestering of some proteins, lipids and cytosolic components (Guay & Regazzi, 2017). This vesicle-containing organelle is then referred to as a **multivesicular body** (MVB) (Guay & Regazzi, 2017). MVBs then undergo two possible pathways: i) fusion with lysosomes for degradation; or ii) transport towards the plasma membrane (Fig. 2). ILVs released into the surroundings after fusion of MVBs with plasma membrane are called exosomes (Pelchen-Matthews et al., 2004).

The formation of ILVs and MVBs is controlled by different types of machinery, e.g., endosomal sorting complex required for transport (ESCRT) dependent, lipid-raft formation or tetraspanin enriched domains (De Gassart et al., 2003; Van Niel et al., 2006).

1.2. MICROVESICLES (MVS)

The second type, microvesicles (MVs), also known as microparticles or ectosomes, are particles originating directly from the plasma membrane reaching a size of 100–1000 nm in diameter (Veerman et al., 2021). These vesicular bodies are formed as a result of changes in the distribution of proteins and phospholipids in the plasma membrane (Fig. 2). The resulted asymmetry is the leading trigger behind the initial curvature of the membrane. The distribution of phospholipid is usually controlled by aminophospholipid translocases (flippases and floppases), which are proteins whose function is to transfer phospholipids from one side of the plasma membrane to the other. Redistribution of phospholipids can be caused by other external factors, such as calcium ion influx, which leads to increased release of MVs (Pasquet et al., 1996; Akers et al., 2013).

1.3. IMPORTANCE AND APPLICATIONS OF EVs

As for the functions that EVs possess, it was originally suggested that EVs were simply a waste disposal mechanism (Johnstone et al., 1991). After the potential immunomodulatory activity of B cell-derived EVs was reported, interest in evaluating their biological relevance increased (Raposo et al., 1996, Stremersch et al., 2016). Over the past decades, EVs have been established as a very important tool in cell-cell communication, signalling and other biological processes (de la Torre-Escudero et al., 2016; Stremersch et al., 2016; Usman et al., 2018). EVs have been observed to be involved in many different (patho)physiological processes such as blood clotting, tissue repair, immune surveillance etc. (Zhang et al., 2015; Kuipers et al., 2018; Silachev et al., 2019; Zifkos et al., 2021; Buzas, 2023). Due to these observations, EVs are starting to be exploited as potential diagnostic biomarkers of various diseases (cancer, neurodegenerative, infectious and autoimmune diseases) and therapeutic tools (Pant et al., 2012; Corrado et al., 2013; Tominaga et al., 2015; Stremersch et al., 2016; Ciferri et al., 2021). Moreover, the involvement of EVs is observed in many processes across all kingdoms of life and their numbers are steadily increasing, highlighting the evolutionary importance of EVs (Woith et al., 2019).

1.4. BIOMOLECULAR CORONA (BC)

It has recently been discovered that EVs can be naturally surrounded by a "corona". This term refers to a complex of biomolecules that forms a sort of an outer coating on the surface of nanoparticles (Palviainen et al., 2020).

There are two different ways of biomolecular corona (BC) formation on the surface of EVs (Fig. 3). One of them is the forming already during the EV biogenesis. The corona formed in this way is called **innate corona**. The other involves packing of different material after the release of EVs into the extracellular milieu, where they are exposed to a diverse spectrum of biomolecules. In this case, it is referred to as **acquired corona** (Yerneni et al., 2022). Due to the high surface-to-volume ratio and high free surface energy of nanoparticles, various components (proteins, ions, lipids, etc.) may easily be adsorbed in the solution phase. Several different types of binding forces are involved in these interactions, such as Van der Waals interactions, hydrogen bonding, hydrophobic interactions, electrostatic interactions, and π-π stacking (Lee et al., 2020; Witwer & Wolfram, 2021; Buzas, 2022; Radeghieri & Bergese, 2023). Various factors can affect the formation of BC on the surface of EVs, such as physico-chemical attributes (size, diameter, surface curvature, entropy, net charge etc.) (Akhter et al., 2021; Heidarzadeh et al., 2023).

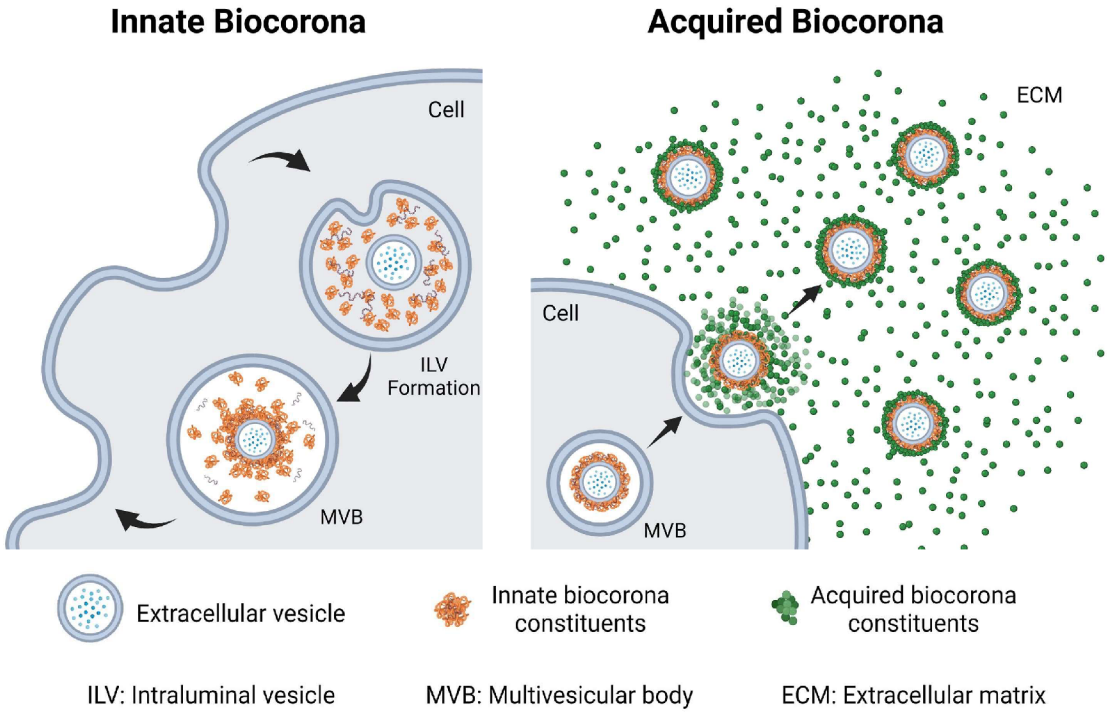

Fig. 3. Scheme of the biomolecular corona formation (Yerneni et al., 2022).

Since biological environments are usually abundant in proteins, these components are the most represented in BC (therefore often referred to as the "protein corona"). However, they are by far not the only types of biomolecules present. Lately, there is growing evidence for the presence of DNA, RNA, and lipids in BC (Shelke et al., 2016; Németh et al., 2017; Lee et al., 2020; Buzas, 2022; Yerneni et al., 2022).

Various studies have found that the corona could be divided into two parts: i) the **inner "hard" corona** and ii) the **outer "soft" corona**. These parts differ in the strength of binding affinity and the residence time on the surface (Gunawan et al., 2014; Saie et al., 2015). The hard corona is composed of macromolecules firmly adsorbed on the EV surface, such as plasma proteins immunoglobulins and apolipoproteins. These layers are characterized by a longer turnover time of their composition and therefore appear to be more stable. However, it has been found that the composition of the hard corona can also alter with changes in the biological environment (Gunawan et al., 2014; Wang et al., 2021; Musicò et al., 2023). In contrast, the soft corona exhibits a looser structure and a faster exchange of components. It consists mainly of weakly bound proteins that bind primarily to other corona proteins (rather than to the surface of the EVs themselves), such as the plasma protein albumin (Gunawan et al., 2014; Heidarzadeh et al., 2023; Radeghieri & Bergese, 2023).

More and more studies suggest that these outer protein layers have an essential impact on the EV functions, EV-cell interactions, and EV cellular uptake (Tóth et al., 2021; Ramos et al., 2022). Proteins interacting with the surface of EVs can either enhance (LDL, IgG and C3b) or decrease (albumin, ApoA4, ApoC3 and clusterin) cellular uptake (Buzas, 2022; Wolf et al., 2022). The EV corona could also possibly provide a role in the recognition of foreign materials or various biomarkers, which could potentially be applied in diagnostics (Walker et al., 2020).

1.5. METHODS OF EXTRACELLULAR VESICLES ISOLATION

The information on the presence of BC significantly differs between various studies. It has recently been discovered that in the process of EVs isolation, the unintended disruption of the structure or even complete shaving of the BC may occur (Wolf et al., 2022). This is mostly caused by methodological differences in the isolation of EVs. It is due to the fact

that BC biomolecules are not covalently bound to the EV membrane and are therefore prone to dissociation from the membrane (Eisenstein, 2022). Here is an overview of the six most common methods used to isolate EVs, their strengths and weaknesses.

1.5.1. ULTRACENTRIFUGATION (UC)

1.5.1.1. DIFFERENTIAL ULTRACENTRIFUGATION (DUC)

Probably the most commonly used method for EVs isolation to date is differential ultracentrifugation (DUC) (Witwer et al., 2013). This method is based on a series of successive centrifugation steps (Li et al., 2017). The first several steps are used to remove unwanted components (e.g., intact cells, dead cells or cell debris) from the sample (Szatanek et al., 2015). Between centrifugations, the supernatant is always transferred to a new test tube and the generated pellets are thrown away. After the final ultracentrifugation (100,000 x *g*) the pellet with EVs is obtained and subsequently used for further studies (Théry et al., 2006). This technique is rather time-consuming and requires specialized laboratory equipment (Monguió-Tortajada et al., 2019). Exposing the sample to such high speeds might disrupt the structure of the EVs or even cause their rupture (Lang et al., 2022). EVs can also fuse with other co-precipitated compounds such as proteins, which can affect the physical properties of EVs (e.g., unnatural formation of BC) and following analysis (van der Pol et al., 2012; Baranyai et al., 2015; Linares et al., 2015; Böhmert et al., 2020).

1.5.1.2. DENSITY GRADIENT ULTRACENTRIFUGATION (DGUC)

This is another ultracentrifugation method applied to isolate EVs. The centrifuge tubes are filled with a series of solutions of different densities forming a density gradient (Brakke, 1953). Widely used density media for this purpose are sucrose and iodixanol (e.g., OptiPrep) (Cantin et al., 2008; Zhang et al., 2018). Separation of the solution components is then achieved within the tube based on the migration of substances to regions of corresponding density (Brakke, 1953; Cantin et al., 2008). Like DUC, this method is relatively time-consuming and requires expensive specialized equipment (Zeringer et al., 2015). Other drawbacks of this technique are the possibility of contamination with co-precipitated substances and disruption of the EVs structure (Konoshenko et al., 2018; Tiwari et al., 2021). Unlike the previous method, DGUC allows to obtain EV fractions of higher purity (Van Deun et al., 2014; Konoshenko et al., 2018). Moreover, BC may be affected by its shaving (Wolf et al., 2022).

1.5.2. SIZE EXCLUSION CHROMATOGRAPHY (SEC)

Sample solution is introduced to a filtration column, which is filled with porous packing (e.g., Sepharose 2B or CL-4B) with a particular pore size (Sabapatha et al., 2006). Due to the different sizes of the compounds in solution, particles smaller than the pore size are free to penetrate the stationary phase (porous matrix) and are retained, thus eluting later, while larger particles are washed out earlier. This results in differently sized EV fractions (Böing et al., 2014). Compared with other methods, it is one of the cheaper and faster alternatives (Monguió-Tortajada et al., 2019). SEC is also characterized by the purity of the isolated EVs and higher recovery (Böing et al., 2014). Nevertheless, a disadvantage of this method may be shaving of BC while the sample passes through the column (Witwer et al., 2013; Böhmert et al., 2020). SEC is commonly coupled with a low-speed centrifugation step (removal of larger cells, cellular debris, organelles, etc.) and with a filtration step as pre-concentration of EVs (Witwer et al., 2013; Szatanek et al., 2015).

1.5.3. POLYMER PRECIPITATION (PP)

The principle of this method is the introduction of a water-excluding polymer solution, such as polyethylene glycol, into the sample (Doyle & Wang, 2019; Tiwari et al., 2021). Its purpose is to tie up the water molecules, causing a decrease in solubility and subsequent precipitation of other components of the sample (Zeringer et al., 2015). Precipitation-based isolation is a very simple and rapid method that does not require expensive or special equipment (Coumans et al., 2017; Tiwari et al., 2021). However, it is mainly a concentration method, so along with EVs, non-EV particles and other contaminants are precipitated (Peterson et al., 2015; Zarovni et al., 2015). One of the drawbacks may be the unnatural formation of BC resulting from high contamination in the sample and the application of a centrifugation step. In order to reduce the level of contamination, so-called sample pretreatment, such as filtration and/or ultracentrifugation steps, is introduced (Li et al., 2017).

1.5.4. AFFINITY-BASED ISOLATION (ABI)

A variety of biomolecules (lipids, proteins, polysaccharides) and other materials are exposed on the outer surface of EVs and molecules from the external environment can

specifically interact with them. This can be utilized for the isolation of EVs (Konoshenko et al., 2018).

1.5.4.1. IMMUNO-AFFINITY (IA)

The principle is the application of a specific antibody (usually monoclonal) that binds a specific marker found on the surface of EVs. Antibodies for a specific antigen of interest can be attached to plates (e.g., ELISA), microbeads, resins, or microfluidic devices (Zhang et al., 2018). These methods can be used to isolate EVs from biological fluids, cell culture, or tissues (Gurunathan et al., 2019). The main disadvantage here is that the protein or antigen must be expressed on the surface of EVs. If it is enclosed inside, the antibody will be not recognized (Li et al., 2017; Doyle & Wang, 2019). One of the advantages of these techniques is their selectivity and specificity. It is possible to isolate very pure and specific subpopulations of EVs. On the other hand, this is compensated by very low yield (Batrakova & Kim, 2015; Szatanek et al., 2015). Another very important advantage is the combinability of these methods with other methods, e.g., flow cytometry, western blotting or "real-time" PCR. They are also often used after exosomal enrichment by ultracentrifugation or ultrafiltration (Szatanek et al., 2015; Doyle & Wang, 2019).

1.5.4.2. PHOSPHATIDYLSERINE-BINDING PROTEINS (PSBP)

A variant of affinity-based isolation of EVs, it utilizes agents that bind to the molecule phosphatidylserine (PS), which is naturally incorporated into the membrane of EVs (Théry et al., 2002; Miyanishi et al., 2007). Several different proteins (e.g., Annexin 5, Tim4) are exploited for this purpose. These molecules are then adhered to magnetic beads which are used for separation of EVs from cultivation media (Konoshenko et al., 2018). The disadvantages here are the higher cost of the material and low yield. On the other hand, high purity of isolated EVs is achieved (Nakai et al., 2016; Konoshenko et al., 2018; Yoshida & Hanayama, 2022).

1.6. MODEL ORGANISM

The studies on EVs are predominantly done on traditional model organisms (e.g., mice, human). However, this approach ultimately limits our understanding of EVs as an evolutionary conserved mechanism. The rat tapeworm *Hymenolepis diminuta* (Rudolphi, 1819) (Cestoda: Cyclophyllidea) has been used for more than a century as a suitable model

for ecological, biochemical and physiological studies including host-parasite interrelationship (Arai, 1980; Smyth & McManus, 1989; Řežábková et al., 2019). This species has a relatively simple indirect life cycle, which involves an insect intermediate host that are various beetle species (e.g., *Tenebrio molitor*, or *Tribolium confusum*) and definitive host that are rats (accidentally human) (Fig. 4). It consists of three distinct developmental stages: i) the egg with hexacanth larva, ii) the cysticercoid larva in the body cavity of intermediate host and iii) the sexual adult stage in the intestine of definitive host (Arai, 1980; Řežábková et al., 2019).

This tapeworm species has been intensively studied even as a potential candidate for helminth therapy of human autoimmune diseases (Řežábková et al., 2019). Moreover, data on its genome and proteome are already available for this species (Bień et al., 2016; Młocicki et al., 2018). Analysis of excretory-secretory products (ESPs) and surface-related proteins was performed by Bień et al. (2016) and later updated by Młocicki et al. (2018). Mazanec et al. (2021) then conducted the first study of EVs obtained from *H. diminuta* adults using qualitative and quantitative characterisation of EVs content as well as their visualisation by several ultrastructural methods. Two distinct subtypes of EVs were observed on the surface of the adult tapeworms and proteomic analysis was used to detect their protein cargo including potential drug targets and vaccine candidates. Furthermore, proteins involved in the ESCRT-dependent biogenesis pathway were also documented (Mazanec et al., 2021).

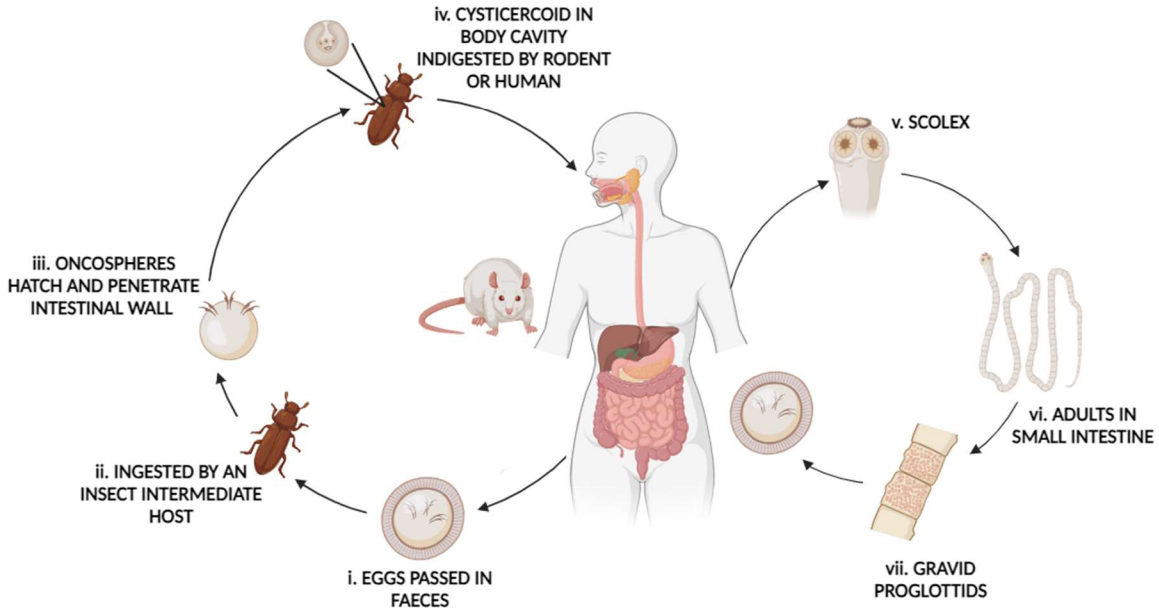

Fig. 4. *Hymenolepis diminuta* life cycle scheme. **i, ii**: embryonated eggs with hexacanth larva passed in the faeces are ingested by an intermediate host (various beetle species); **iii**: oncosphere larvae penetrate the mid-gut wall and enter haemocoel of the beetle; **iv**: cysticercoid larvae develop in body cavity of the beetle in approximately 10 days; **v, vi, vii**: development of the adult tapeworms in the intestine of definitive host (rats, accidentally human), after 2–3 weeks the tapeworms start to produce eggs (created with Biorender.com).

2. AIMS OF STUDY

1. Isolation of extracellular vesicles from collected cultivation medium with *Hymenolepis diminuta* by three different isolation techniques.
2. Compare differences in yields through a particle size distribution analysis and protein concentration across the applied isolation methods.
3. Compare the differences in the morphology of biomolecular corona via transmission electron microscopy.

3. MATERIALS AND METHODS

3.1. HOST-PARASITE MODEL

The tapeworms used for this study (*Hymenolepis diminuta*) were obtained from experimentally infected Wistar rats *Rattus norvegicus* (Berkenhout) kindly provided by the Laboratory of Parasitic Therapy (Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic). A total of 4,51 g of adult tapeworms were obtained by parasitological dissection from the intestine of 3 experimentally infected rats.

3.2. CULTIVATION OF TAPEWORMS

Freshly obtained adult tapeworms were cleaned from intestinal contents by washing in 1× PBS and RPMI-1640 medium following protocol of Mazanec et al. (2021). The tapeworms were then weighed, divided into two replicates and transferred into sterile filtered RPMI-1640 medium supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin and cultivated in incubator for 48 hours at 37 °C and 5 % CO_2 (ICO Memmert, Germany). The medium was collected and replaced after 24- and 48-hours post-dissection. The recovered medium without tapeworms was then concentrated using a stirred cell (Amicon®, USA) with 10 kDa ultrafiltration discs (Millipore, USA) and 30 kDa centrifugal filters (Millipore, USA) to a final volume of 4,5 ml (Fig. 5). The final concentrate (excretory-secretory products, ESPs) was then either used immediately for downstream isolation of extracellular vesicles or stored at -80°C until further use.

3.3. EVs PURIFICATION

The obtained ESPs were centrifuged at 300 *g* for 10 min, 1500 *g* for 30 min and 5000 *g* for 30 min using centrifuge XPN-90 Beckman Coulter (USA) to remove large particles and tissue and cell debris (Fig. 5). At each step, the supernatant was transferred to a new tube while the pellet was discarded. The final supernatant of a protein concentration of approximately 500 µg/ml was then used for EVs isolation. Three different techniques were applied:

i) commercial **polymer precipitation kit (PP)**

ii) commercial **size exclusion chromatography columns (SEC)**

iii) commercial **magnetic beads-based isolation kit (MBB)**

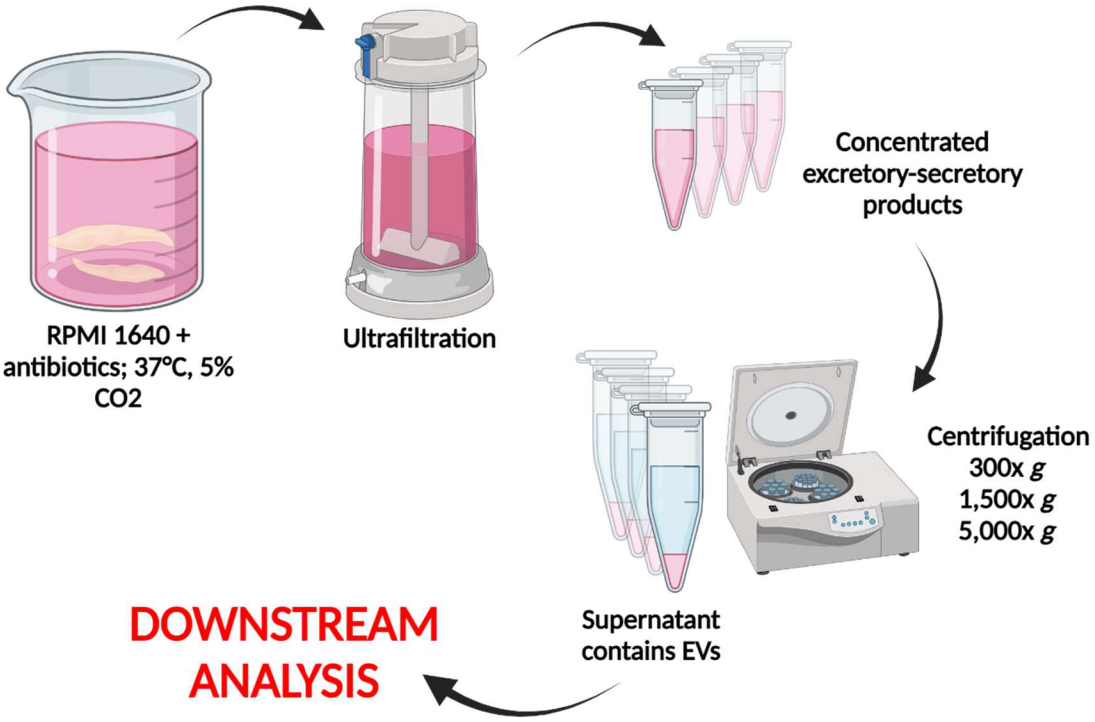

Fig. 5. Isolation and purification of EVs (created with Biorender.com).

3.3.1. SIZE EXCLUSION CHROMATOGRAPHY (SEC)

Before applying SEC qEV original/35 nm isolation columns (IZON, New Zealand), the sample was centrifuged at 15, 000 *g* for 1 h at 4 °C in centrifuge Z 326 K Hermle Labortechnik (Germany). The supernatant containing a fraction of smaller and less dense EVs (SEC fraction), was then transferred to a new tube. The pellet was resuspended in 250 µl of 1× PBS, washed twice and centrifuged at 15,000 *g* for 1 h and stored at -80 °C. SEC columns were then used according to the manufacturer´s protocol. Briefly, column was equilibrated to the room temperature (18–24 °C) and loaded with 500 µl of the SEC fraction previously thoroughly vortexed. After the sample is completely soaked into the filter, we immediately start continuously adding 1× PBS, which serves as a driving force for the sample to pass through the column. Different fractions were then collected continuously immediately after the sample loading: 2× 1,250 µl of void fraction, 4× 500 µl of small-EV fraction and 2× 1,500 µl of soluble protein fraction (Fig. 6). The individual fractions were stored at -80 °C until further use.

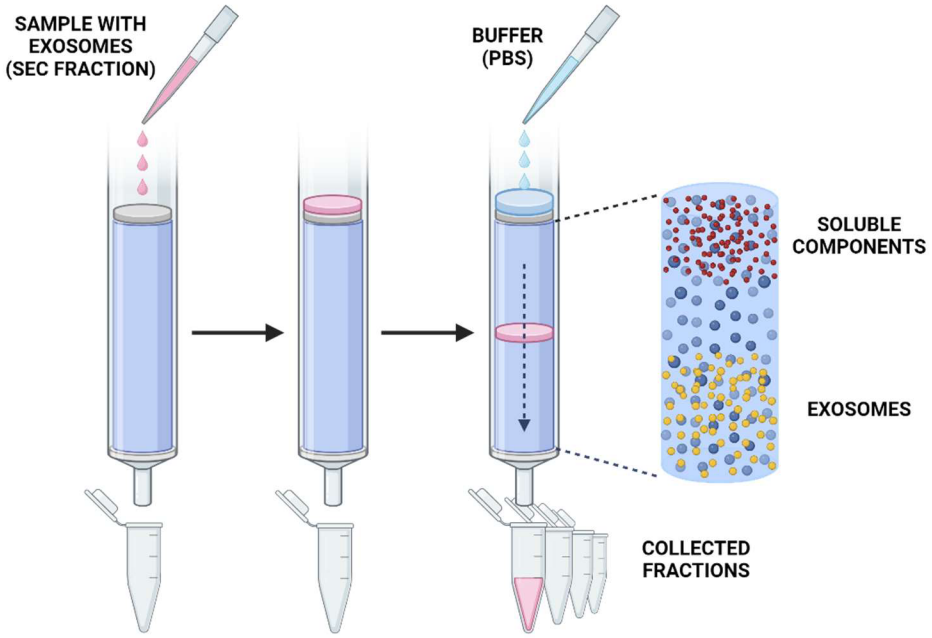

Fig. 6. Principle of size exclusion chromatography (created with Biorender.com).

3.3.2. POLYMER PRECIPITATION (PP)

Total Exosome Isolation Reagent (from cell culture media) kit (Invitrogen, USA) was used according to the manufacturer´s protocol. Briefly, 375 µl of total EVs isolation reagent was added to 750 µl of the sample, mixed by vortexing and incubated in the refrigerator overnight (Fig. 7). After incubation, the mixture was centrifuged at 10,000 *g* for 1 h at 4 °C. The supernatant was discarded, the pellet was resuspended in 100 µl of 1× PBS and stored at -80 °C until further use.

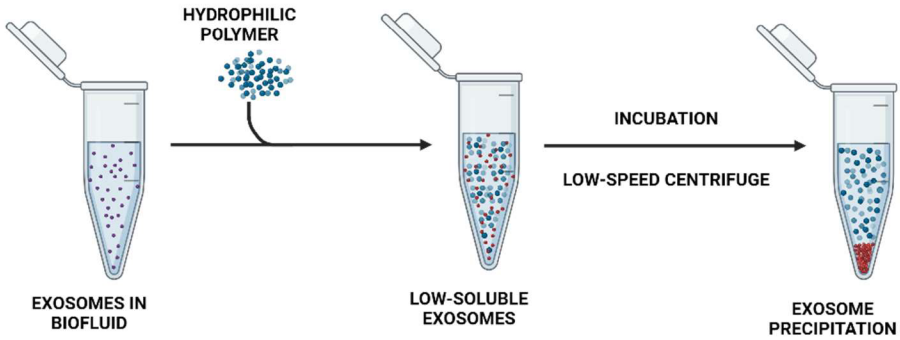

Fig. 7. Principle of polymer precipitation (created with Biorender.com).

3.3.3. MAGNETIC BEADS-BASED ISOLATION (MBB)

MagCapture Exosome Isolation Kit PS (FUJIFILM, USA) was used according to the manufacturer´s protocol. In brief, the sample was centrifuged at 10,000 *g* for 30 min at 4 °C to remove large EVs. The supernatant was then transferred to a new tube (Mag EVs). 800 µl of the Mag EVs fraction was added to the magnetic beads. The mixture was incubated for 3 h on a rotating mixer at 4 °C. Subsequently, the beads were washed three times. EVs were eluted by adding 50 µl of elution buffer twice (Fig. 8). The supernatant containing isolated EVs (100 µl in total) was transferred to a new tube and stored at -80 °C until further use.

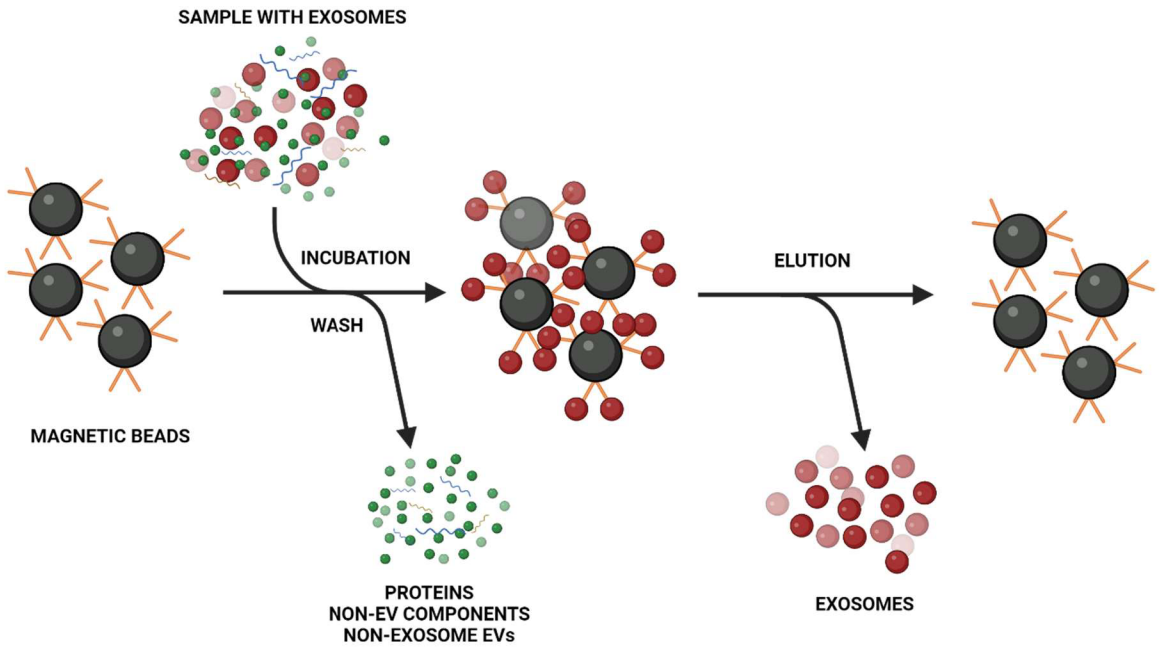

Fig. 8. Principle of magnetic beads-based isolation (created with Biorender.com).

To determine the protein concentration of isolated EVs by all three techniques, Qubit 4 fluorometer (Invitrogen™, USA) was used according to standard protocol.

Isolated EVs were then aliquoted for separate downstream analyses to avoid multiple freeze-thaw cycles and stored at -80°C unless used immediately.

3.4. NANOPARTICLE TRACKING ANALYSIS (NTA)

Fractions of isolated EVs were compared based on their protein yield and then subjected to Nanoparticle Tracking Analysis (NTA) at CF Nanobiotechnology, Brno, Czech Republic. The size and frequency distribution of individual vesicles were determined using a NanoSight NS300 (Malvern, UK).

3.5. TRANSMISSION ELECTRON MICROSCOPY (TEM)

For the room temperature TEM analysis of EVs, the negative staining method was used. Purified EVs were visualised using a JEOL JEM-2100F transmission electron microscope (Jeol, Japan) operated at an accelerating voltage of 200 kV. Samples were placed on glow-discharged carbon-coated copper grids and stained with 1,5% uranyl acetate. TEM images were taken using a bottom-mounted Gatan CCD Orius SC1000 camera (Gatan, USA).

For Cryo-TEM of the purified EVs, samples were loaded onto perforated carbon grids and flash frozen using an immersion freezer LEICA EM GP2 (Leica Microsystems, Germany). A 200 kV JEOL JEM-2100F microscope equipped with a Gatan ELSA cryo transfer holder was used to visualise the samples. Images were captured using a Gatan K2 Summit direct electron detector.

All the micrographs from TEM were taken by Zdenko Gardian at the Laboratory of Electron Microscopy, BC, CAS, Czech Republic.

Obtained micrographs from TEM were analysed using ImageJ software (NIH, USA).

4. RESULTS

In order to compare the methods of EVs isolation collected culture medium from *Hymenolepis diminuta* was used for all three different techniques. Total of 2 replicates were obtained by each method (**SEC, PP** and **MBB**).

4.1. BASIC COMPARISON

The individual methods were compared with each other based on the following parameters: i) cost; ii) time requirement; iii) maximum applied amount of excretory-secretory products; iv) need for specialized equipment; and v) ease of implementation (Table 1).

Table 1. Comparison of applied isolation kits (**SEC**: Size exclusion chromatography by qEV original/35 nm; **PP**: Polymer precipitation by Total Exosome Isolation Reagent; **MBB**: Magnetic beads-based isolation by MagCapture Exosome Isolation Kit PS) based on their price, time and complexity of performance. Time and price are based on one isolation using the maximum isolation volume. Difficulty of implementation: +++ = easy, ++ = medium.

Isolation kit	Cost	Total time	Maximum load of sample	Specialized equipment	Difficulty of implementation
SEC	≤10 €	2 h	500 µl	No	+ +
PP	<5 €	17 h	N/A	Centrifuge	+ + +
MBB	~50 €	5 h	1 ml	Centrifuge, Magnetic stand	+ +

4.2. NANOPARTICLE TRACKING ANALYSIS

To evaluate the size distribution and abundance of the isolated particles by different isolation methods, NTA was performed. All methods showed a fairly wide particle size distribution.

The **SEC** method displayed a narrower distribution in terms of particle size, with most particles in the range of 50–300 nm. One major peak revealed at 136 nm shows the highest particle concentration (Fig. 9A). The **PP** method shows a very broad size range of 50–750 nm with a tendency to isolate slightly larger particles than **SEC**. The highest particle concentrations were detected by two main peaks at 133 and 171 nm (Fig. 9B). In addition,

this method seems to isolate a considerably larger number of particles than the other two methods. The **MBB** resulted in the lowest particle count /ml compared to the other methods and we also observed here that most particles were smaller than 200 nm. The particle concentration shows significant peak at 103 nm (Fig. 9C). One small peak was visible at 700 nm.

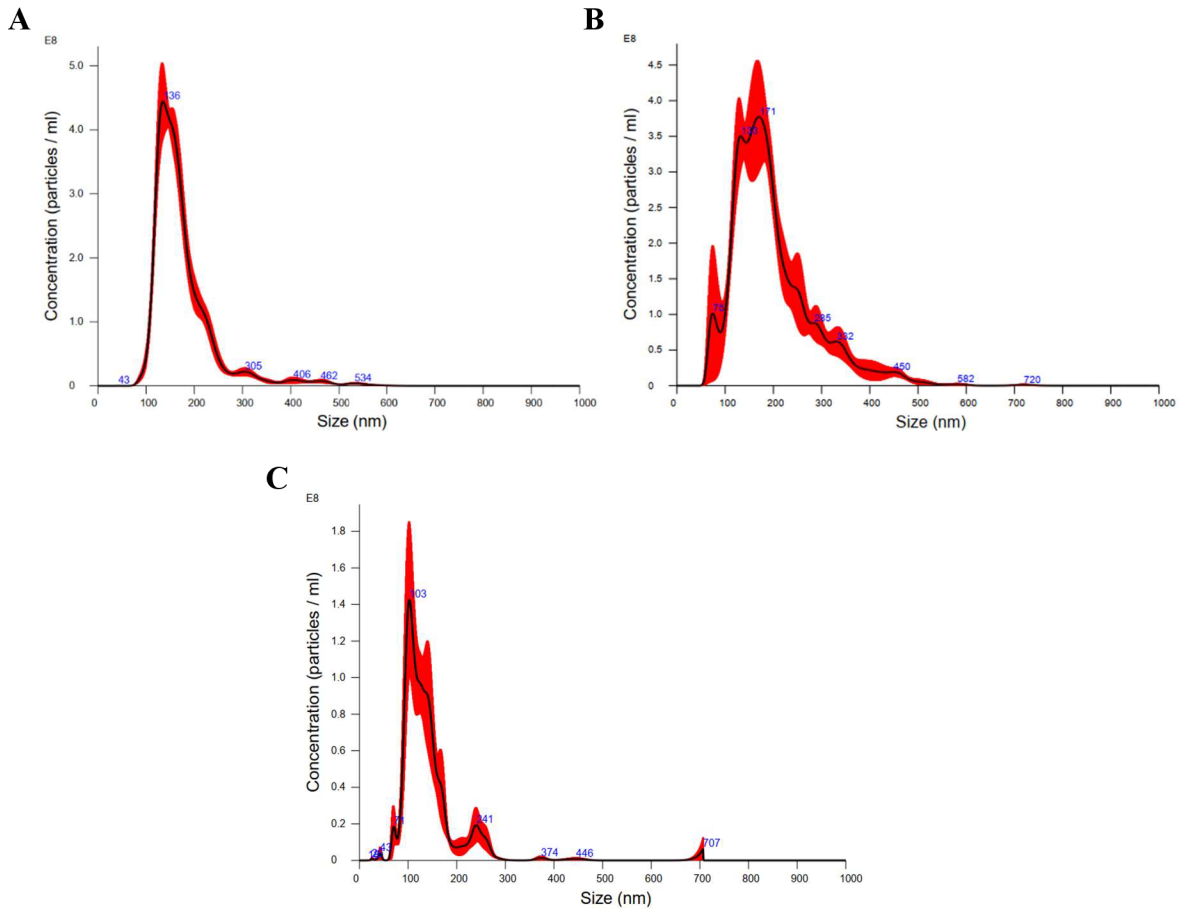

Fig. 9. The size distribution and abundance of the isolated EVs of *Hymenolepis diminuta* by Nanoparticle tracking analysis (NTA) using **SEC**: size exclusion chromatography (A), **PP**: precipitation (B) and **MBB**: MagCapture (C).

4.3. PROTEIN YIELD

In addition to particle number, the total protein concentration in the fractions of isolated EVs was assessed (Table 2, Fig. 10). The **PP** method resulted in many-fold higher levels of isolated proteins than other two methods. On the other hand, the lowest yield was observed for the **MBB** method.

Table 2. Values of measured protein concentrations for total ESPs and fractions of isolated EVs for each method (**SEC**: Size exclusion chromatography by qEV original/35 nm; **PP**: Polymer precipitation by Total Exosome Isolation Reagent; **MBB**: Magnetic beads-based isolation by MagCapture Exosome Isolation Kit PS).

Isolation kit	**Protein concentration [µg/ml]**	
	Total ESPs	**Isolated EVs**
SEC	476	90,8
PP		>520
MBB		26,8

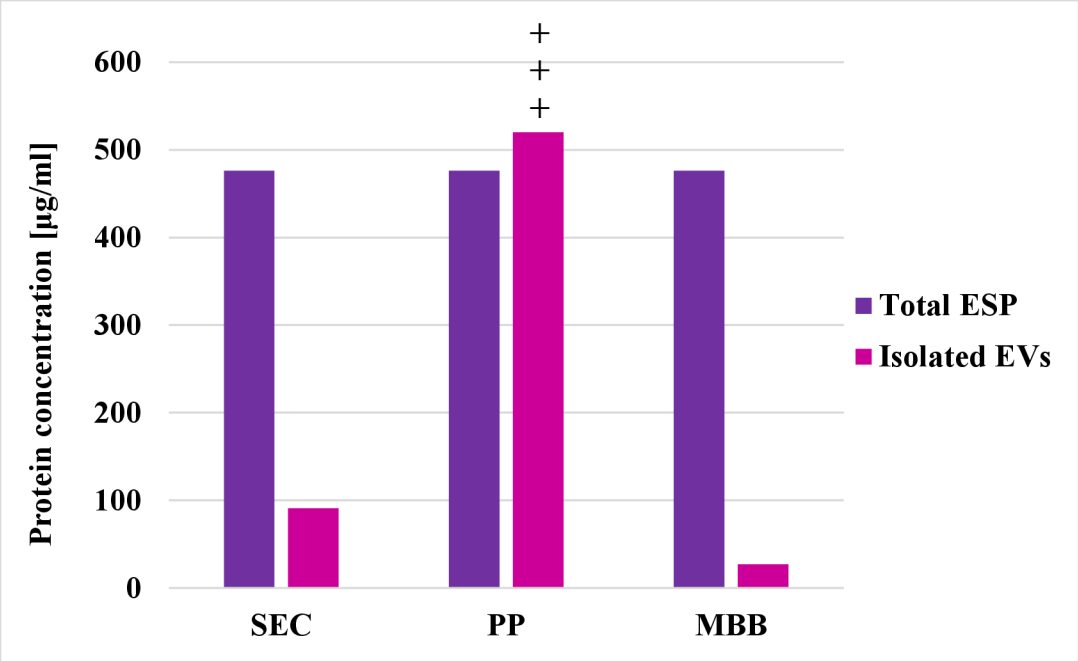

Fig. 10. Comparison of measured protein concentrations in individual fractions of isolated EVs with the initial protein concentration of the total ESPs. + + + indicates that the **PP** method measured a higher concentration than Qubit 4 fluorometer (Invitrogen™, USA) is capable of determining.

4.4. TRANSMISSION ELECTRON MICROSCOPY (TEM)

The morphology and size characterization were conducted by using TEM. The obtained results showed a spherical morphology of the isolated EVs (Fig. 11). For the **PP** method, we

captured a small number of vesicles with an average size of 31,9 nm. The TEM images were rather unclear (Fig. 11B). In contrast, we observed a much larger number of EVs with a wider range of sizes in samples isolated by the **SEC** method. The EVs reached a size of 69,7 nm in diameter on average. At the same time, we obtained much higher sample purity with this method (Fig. 11A). We were unable to get any TEM images from **MBB** method.

The biomolecular corona (BC) was observed on the surface of EVs from **SEC** and **PP** methods (Fig. 11). For both methods we found rather thin layers of BC. However, samples from the **PP** technique showed a slightly thicker corona compared to the **SEC**. We were unable to get any information about BC from **MBB** method.

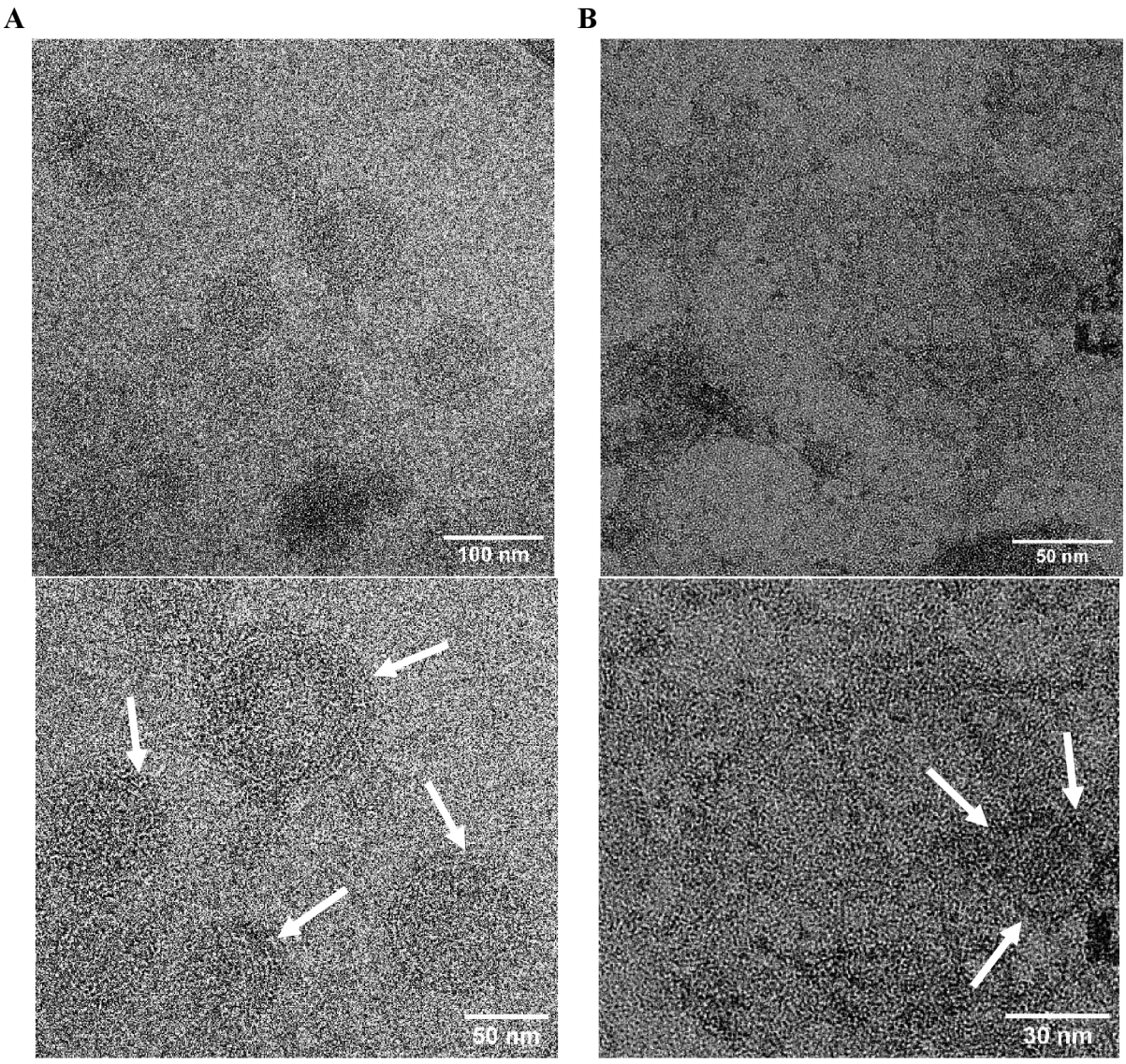

Fig. 11. Transmission electron microscopy images of EVs from *Hymenolepis diminuta* and their biomolecular corona (arrows) isolated by **SEC**: size exclusion chromatography (A) and **PP**: precipitation (B).

5. DISCUSSION

EV field is experiencing a rapid development and growth. Based on EVs´ tremendous potential for use in clinical diagnostics and therapeutics, interest in studying the properties and functional applications of EVs has increased significantly in recent years. However, there are many different types of EVs that differ in their functions and composition. Moreover, it has recently been shown that a wide variety of different biomolecules bind to the surface of EVs, which may be crucial for the realization of the functions of these vesicles (Palviainen et al., 2020). Thus, in order to better understand and fully utilize these nanoparticles, there is a need to facilitate their efficient separation from biological fluids while preserving their sufaceome and identifying their co-isolates such as lipoproteins, soluble proteins, etc. Nowadays, novel isolation methods are constantly being developed and an increasing number of new isolation kits are emerging on the market (Gardiner et al., 2016; Welsh et al., 2024). The main focus is to achieve high purity of isolated fractions of EVs in the shortest time and at the minimum cost. However, each method has its advantages and disadvantages. Therefore, in this study, we focused on the characterization and comparison of several selected types of techniques widely used today to isolate EVs.

The different methods were applied on culture medium obtained by cultivating adults of *Hymenolepis diminuta*. We compared the methods with each other based on their protein yield, NTA and TEM.

The isolation columns qEV original from IZON (**SEC**) proved to be the most advantageous of all three kits used (Fig. 9). Based on our results this method showed high particle recovery and satisfactory protein yield. According to Borup et al. (2022), the columns displayed a uniform particle size range in the fraction of isolated EVs, which is in agreement with our results. However, in our case columns tended to isolate more of smaller particles similarly to **MBB** method. On the contrary, subpopulations of EVs of larger sizes were obtained by the **PP** method (Fig. 9). This may have been due to the incorporation of a centrifugation step to separate large EVs (LEVs) from small EVs (SEVs) prior to the actual isolation, which greatly depleted the initial sample of larger particles.

We were also able to detect biomolecular corona (BC) in TEM images using two methods (**SEC** and **PP**). However, compared to the study by Kuipers et al. (2020), who used EVs

obtained from schistosomula of blood fluke *Schistosoma mansoni*, our outer layers of biomolecules are significantly thinner (Fig. 12). This may be due to the isolation methods chosen. Kuipers et al. (2020) used the ultracentrifugation method to isolate EVs, which may result in aggregates formation and unnatural packing of biomolecules on the surface of EVs due to the application of high speeds, and thus secondary BC formation. In contrast, the method we chose may have resulted in shaving of these outer layers as the sample passed through porous packing of the column. It should be also taken into account that the isolation was performed on distinct developmental stages of different organisms, which may differ in their corona, both in composition, structure and thickness, and in the functions that the corona serves. However, there is not yet enough data to compare the different stages with each other. The environment in which the parasite or the cells under study are located also plays an important role.

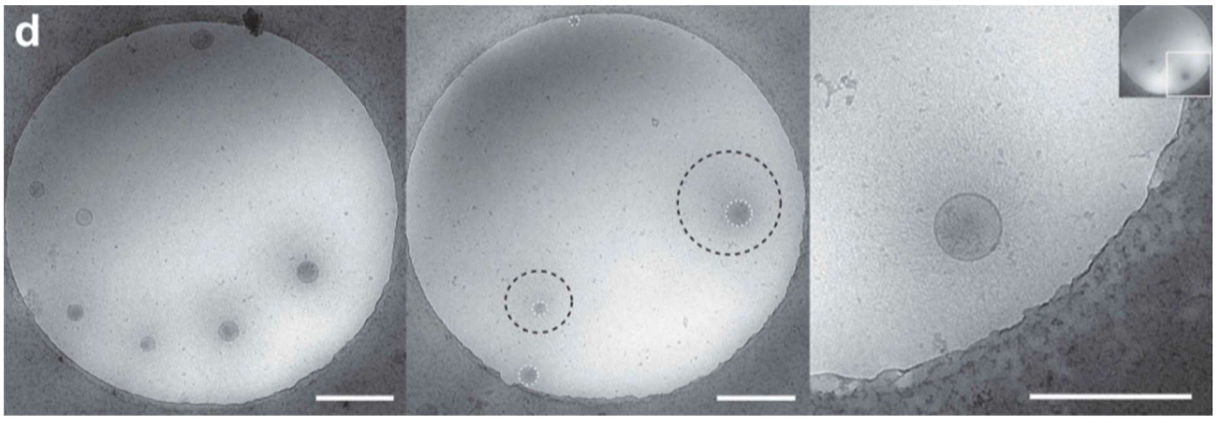

Fig. 12. Cryo-EM images of EVs secreted by schistosomula of *Schistosoma mansoni* isolated by ultracentrifugation (**UC**). The EV membrane and the filamentous structures (corona) are indicated in the middle pane with white and black dashed circles, respectively. Right image shows a close up of one EV. Bar: 0,5 μm (Kuipers et al., 2020).

The polymer precipitation (**PP**) method proved to be the simplest method to perform. The results were in line with our expectations. We achieved the highest protein concentrations and the highest number of particles (Fig. 10, 9B). The protein yield was even higher than that of total ESPs. The reason for such interesting results is the very principle of the **PP** method. Precipitating all components of the input sample (750 μl of ESPs) and then dissolving them in only 100 μl results in a much more concentrated sample than the input ESPs. At the same time, it should be noted that the Qubit 4 fluorometer (Invitrogen™, USA) is only

capable of measuring protein concentrations up to approximately 520 μg/ml. And since our sample reaches much higher values, this fact is therefore indicated in our results (Table 2, Fig.10). According to Van Deun et al. (2014), the overall high values by precipitation techniques are not due to a higher recovery of EVs, but to the presence of non-EV components. Our TEM images are consistent with this statement (Fig. 11B). Also note that, unlike the other two methods, here a centrifugation step to eliminate LEVs was not incorporated, and thus large vesicles are also included in the results, which itself is obviously reflected in the resulting particle and protein concentrations. As with the previous method, we were also able to detect a BC (Fig. 11B), which appears to be slightly thicker compared to the one detected by **SEC** method. However, we are unable to confirm that this surface structure is formed naturally. As with the ultracentrifugation (**UC**) method, centrifugation is applied here, which may result in unnatural BC formation and aggregate formation (Lang et al., 2022).

The last commercial kit tested in this study was the MagCapture Exosome Isolation Kit PS from FUJIFILM (**MBB**). We initially had high expectations for this method because it seemed to promise high purity of isolated EV subpopulations (Yoshida et al., 2017). According to the NTA results from a study performed on human monocytes (line Mono-Mac-6) by Veerman et al. (2021), this method tends to isolate particles of smaller size compared to the other methods studied, and it achieved the lowest particle recovery, which is consistent with our findings. Our results are also supported by the study conducted by Morozumi et al. (2021), where EVs were isolated by different commercially available kits from cow's milk. However, our results achieved much lower protein yields compared to the mentioned studies. Thus, it should be noted here that **MBB** is a very specific method that selects only specific subpopulations of EVs from the sample whose membrane is positive for phosphatidylserine (PS). Affinity-based kits are mostly developed for mammalian EVs and their surface markers (Borup et al. 2020). For helminth EVs are these still unknown. Thus, it is possible that the low efficiency implied by our results is due to the lack of PS in the membrane of helminth EVs. At the same time, we applied a centrifugation step to eliminate LEVs prior to isolation, which likely contributed even more to the reduction in yield. It should also be mentioned that we detected a visible peak at 700 nm in our sample (Fig. 9C). This is most likely due to aggregation or stacking of particles together. Unlike the first two methods mentioned above, we were unable to take images of EVs isolated by **MBB** using TEM. This was most probably due to the very low number of EVs in sample (Fig. 9C).

Unfortunately, the findings of this study did not lead to the elucidation of the impact of different isolation methods on the structure and composition of the BC. In order to better understand and reach more accurate conclusions, it would be necessary to repeat the experiments on additional replicates and also to perform mass spectrometry analysis to compare the total proteome of the extracellular vesicles with their surfaceome.

6. CONCLUSION

1. Three different isolation techniques (size exclusion chromatography, precipitation and affinity-based method) were applied to isolate extracellular vesicles from collected cultivation medium.
2. The individual methods were analysed and compared based on their protein yields and particle size distribution.
3. Biomolecular corona was visualised for the size exclusion chromatography and precipitation method.

We evaluated the **SEC** method as the most useful. This method had a much higher recovery and protein yield compared to the **MBB** technique. At the same time, compared with the **PP** method, the fraction of isolated EVs was highly purified, as we can tell from the TEM images. The method was relatively easy to perform and no specialized equipment is needed, in contrast to the two remaining methods (**MBB** and **PP**). At the same time, the cost is also relatively affordable. The only disadvantage we have noted here is the possibility of unintentional shaving of the BC layers as the sample passes through the porous material of the isolation column. Therefore, if we wanted to focus on studying the BC, then an affinity-based method might be more useful.

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