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Exosome Isolation: Is There an Optimal Method with Regard to Diagnosis or Treatment?

Mustafa Kotmakçı and Gülşah Erel Akbaba

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Abstract

Extracellular vesicles (EV) gained considerable interest in recent years as both diagnostic tools and templates for therapeutic applications. EVs carry a number of cell-specific markers which gave researchers the opportunity of employing them as liquid biopsies causing no discomfort to patients. On the other hand, they are very exciting candidates for drug delivery due to their eobiotic origin, physicochemical and size characteristics. Isolation of EVs is performed by several strategies, having advantages and disadvantages over each other. As such, the method of EV isolation and in particular exosome isolation determines the quality and purity of obtained vesicles. In this chapter, extracellular vesicle isolation methods are evaluated with regard to their further use. Methods such as ultracentrifugation with different modifications, size exclusion chromatography, ultrafiltration, affinity and precipitation are compared with respect to the yield efficacy and purity of isolates. Furthermore, the advantages and disadvantages of different methods according to the purpose of use are revealed. Recent progress and remaining challenges in the isolation of EVs with regard to diagnosis and treatment is reviewed and discussed. In order to select the most suitable method researchers should clearly define purity, yield, quantity and quality requirements for exosomes, and consider disadvantages of distinct isolation methods.

Keywords: exosomes, extracellular vesicles, ultracentrifugation, size exclusion chromatography, precipitation, ultrafiltration, affinity isolation

1. Introduction

Extracellular vesicles (EVs) are nano-sized membrane vesicles, released by almost every cell types. EVs are shown to play crucial roles in many physiological events, as well as many pathological processes. In the past decade, extensive research has been done using exosomes as

vehicles for diagnostic and therapeutic application. Previous studies showed that exosomes are promising systems for drug and nucleic acid delivery. Also, they might be promising tools for diagnosis. Different types of EVs, including exosomes, microvesicles and apoptotic bodies are released by cells. The vesicles are diverse and their quantity and quality depend on the type and origin of the cells. Among these, exosomes are the smallest vesicle type, with sizes ranging from 30 to 120 nm. They originate from multivesicular bodies (MVBs), the form of endosomes at a later stage of maturation. Exosomes are formed by inward budding of the endosomal membrane and accumulate in these MVBs. Later, these small vesicles in MVBs are released from the cells upon fusion of MVBs with the plasma membrane [1, 2]. In contrast, microvesicles (MVs) are larger (50 nm–1 μ m) and more heterogeneous in size. MVs are formed through direct outward budding of the plasma membrane. A heterogeneous population of vesicles (50 nm–5 μ m) which are named apoptotic bodies are released during late stages of the programmed cell death. Each EV subtype contains different amounts of cargo molecules the identity of which is more or less similar among EV subtypes [3].

In different applications, it is important to make a clear distinction between MVs and exosomes because of the fact that they are different both in protein and genetic material content, and in size characteristics. This is important when post-purification processes such as protein characterization/isolation, RNA sequencing, targeted or conventional application in therapeutics delivery are to be investigated. This issue is still scarcely addressed and more research is needed in order to develop specific isolation methods for different EV subtypes. Also, there is still not enough knowledge about how the isolation methods affect physicochemical properties of exosomes. As can be seen from their size distribution and similarities between their cargo molecules, there is no strict border separating different EV subtypes. As the result, it is difficult to obtain highly purified exosome isolates that are completely devoid of other EV types. Given the fact that more and more research focuses on the potential of EVs for diagnostic and therapeutic application, the need of a reproducible method for their purification becomes more prominent.

Due to the complex nature of both intracellular matrix and extracellular environment from which EVs are isolated, not only the desired structures are attained. From biological fluids, contaminants such as proteins, lipoproteins and nucleic acids are also isolated together with the EVs [4–6]. Isolates from cell culture media are contaminated by supplements such as antibiotics and extraneous proteins and EVs coming from fetal bovine serum (FBS) [5, 7]. Prokaryotic contamination is also reported for body fluid EV preparations [8]. All of these contaminants affect the downstream applications of EVs.

In diagnostic applications, these contaminants could lead to false-positive results and subsequently erroneous interpretations. For example, free proteins can lead to over-estimation of the protein cargo of EVs, and, if the protein concentration is considered for normalization of samples, this could lead to significant inconsistencies between results of different research groups.

In order to circumvent any potential interference with the therapeutic efficacy of the active compound, and to minimize the risk of unpredicted side effects, the composition simplicity of drug delivery systems is of utmost importance. Even though there are small number of ingredients in conventional nanoparticle-based drug delivery systems (liposomes, solid

lipid nanoparticles, polymeric nanoparticles, etc.), there are still many debated aspects related to their safety. Considering their nature, EVs are obviously much more complicated than conventional nanoparticulate therapeutics. Therefore, in the therapeutic application field, impurities in EV isolates can be much more confusing. When EVs are isolated from cells designed to express a particular RNA molecule, other changes could also occur in the cellular machinery, and as a consequence, these could contribute to loading of unknown impurities into the EV lumen, leading to false-positive results or even toxicity [9]. This problem can be accomplished, at least in part, by optimizing the isolation protocols, applying extra purification steps and by investigation of sensitive detection techniques for biomolecules [10–14]. However, currently, there is still not single method which ensures EV isolation fully devoid of impurities. In this respect, different isolation methods will be discussed for their applicability in isolation of exosomes intended either for diagnostic or therapeutic purposes.

2. Isolation methods and their convenience in different applications

EVs can be isolated from different types of bodily fluids such as blood, urine and saliva. Depending on the source cell, EVs took part in different roles in the body ecosystem and they are able to overcome natural barriers as cellular membrane, blood-brain barrier and escape the immune system, etc. [1]. Moreover, their immunologic and cytotoxic activities are very low. Despite EVs' promise for diagnostic and therapeutic applications, effective and pure isolation is still a problem which should be overcome. Existing isolation methods cause difficulty in terms of purity and reproducibility [15]. Besides, in some applications, it is important to make a clear distinction between MVs and exosomes because of the fact that they are different both in protein and genetic material content, and in size characteristics. This is important when post-purification processes such as protein characterization/isolation, RNA sequencing, targeted or conventional application in therapeutics delivery are to be investigated, as each EV subtype contains different amounts of cargo molecules [3]. This issue is still scarcely addressed and more research is needed in order to develop specific isolation methods for different EV subtypes.

Based on the main principle employed in the isolation process, there are basically five EV isolation methods. These are (1) Centrifugation-based methods, (2) Chromatography-based isolation, (3) Precipitation-based isolation, (4) Filtration-based isolation and (5) Affinity-based isolation. Each of these methods can be applied either individually or in combination with others in order to achieve higher yield or purity. **Table 1** summarizes the methods for EV isolation.

2.1. Centrifugation-based EV isolation

Owing to their colloidal size, EVs tend to sediment only under high centrifugal forces. The classical method for EV isolation is differential ultracentrifugation, as used in early exosome studies [16]. While this isolation method is still the most widely used approach, often with modifications of the duration, conditions and the speed of centrifugation steps, several sub-techniques have since been developed. Two commonly used techniques based on centrifugation are differential ultracentrifugation and density gradient ultracentrifugation.

Isolation method	Principle	Required instrumentation and consumables
• Differential centrifugation	• Sedimentation under high centrifugal forces based on density and particle size	General purpose centrifuge, ultracentrifuge, conical centrifuge tubes, ultracentrifuge tubes
• Density gradient ultracentrifugation	• Separation of EVs and other non-vesicular components based on their buoyant density in a density gradient under high centrifugal forces	
• Chromatography-based isolation	• Size exclusion chromatography or gel filtration chromatography. Separation is achieved due to penetration of smaller particles into the pores of a matrix material during elution through a column	Prefilled or custom made chromatography columns, matrix material
• Ultrafiltration	• Separation of EV subtypes and proteins using membrane filters. Separation is based on the size of different particles	Ultrafiltration device equipped with peristaltic pump, ultrafiltration cartridges, centrifugal filtration cartridges
• Polymer-based precipitation	• Reduction of EVs' aqueous solubility in the presence of PEG	General purpose centrifuge, conical centrifuge tubes
• Salt precipitation	• Charge neutralization by adding salt solution followed by reduction of EV solubility in low pH	
• Charge-based precipitation	• Enhanced precipitation of EVs by addition of protamine sulphate based on their negative surface charge	
• Affinity purification (specific antibodies, lectins and heparin)	• Capturing different EVs owing to specific molecules present on their membranes	Antibody coated chromatography matrices, microfluidic devices

Table 1. Commonly used EV isolation methods.

3. Differential ultracentrifugation

This method generally employs at least one step of low-speed (2000 g) centrifugation. In this step, whole cells and cell debris such as apoptotic bodies are removed. A second step of centrifugation at higher g-force (5000–10,000 g) ensures removal of large EV aggregates and protein aggregates. The supernatant is then subjected to 1–3 h ultracentrifugation ($\geq 100,000$ g) at 4°C. EVs and high-density proteins are enriched at the bottom of the ultracentrifuge tube in form of a tiny, barely visible sediment. The supernatant containing small proteins, cell culture supplements, buffer ions etc. is carefully discarded and the pellet is washed by adding cold phosphate buffered saline (PBS) and dispersing by vigorous vortexing or pipetting. A second step of ultracentrifugation is

performed. The supernatant is discarded, and finally, washed EVs are collected in little amount of cold PBS. The general steps of differential ultracentrifugation method for EV isolation are presented in **Figure 1**.

Materials required for this isolation method are sterile conical centrifuge tubes, clean and sterile ultracentrifuge tubes, pipettes for handling the liquid material, PBS or other suitable buffer according to the downstream applications. Equipment for the procedure includes conventional benchtop centrifuge with cooling mode; ultracentrifuge capable of performing centrifugation at g-forces higher than 100,000 g; a laminar flow biosafety hood in order to provide aseptic working conditions, especially when EVs are going to be used as therapeutic delivery systems.

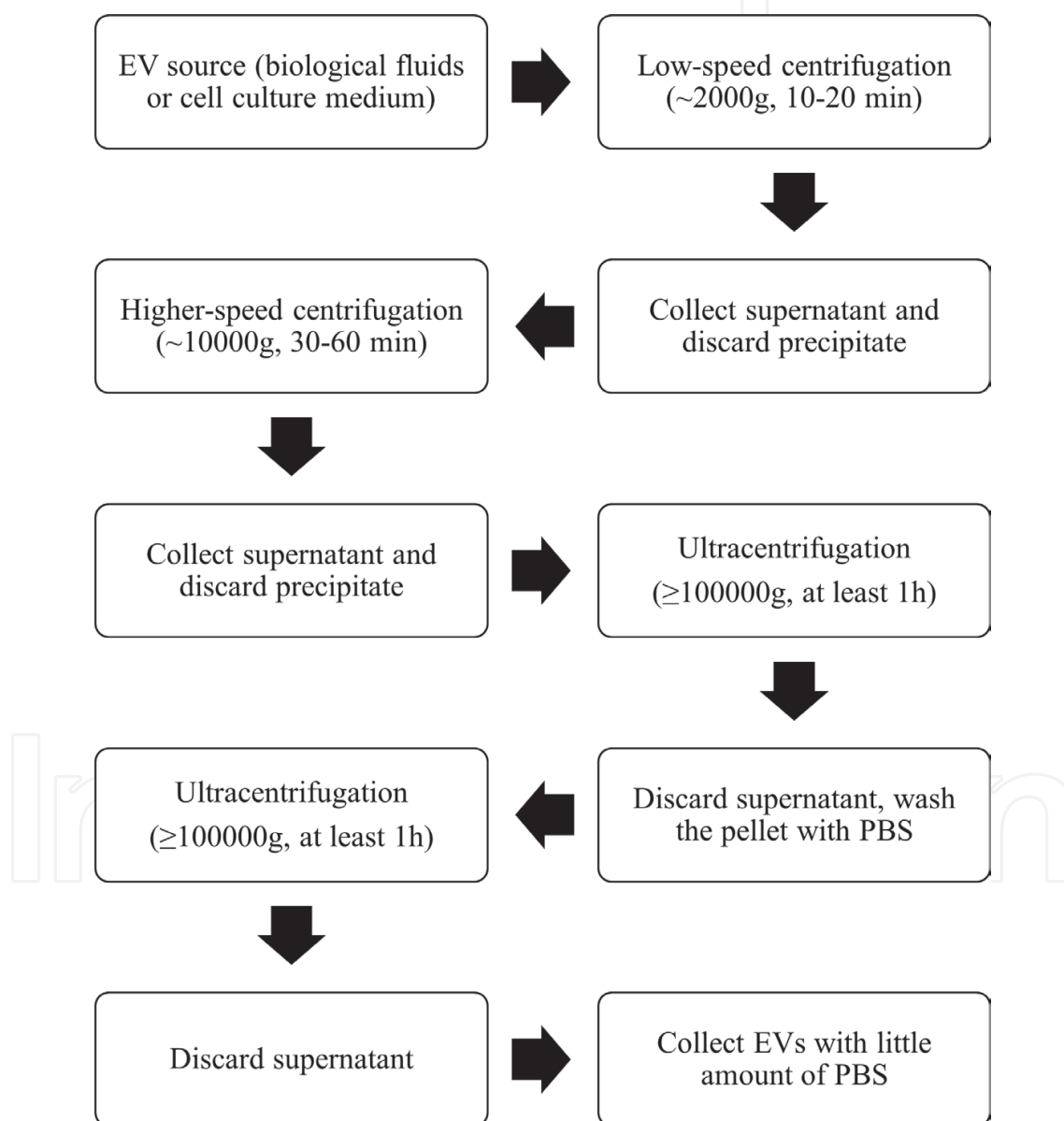


Figure 1. General steps followed during EV isolation by differential ultracentrifugation. In this method, the source of EVs is first cleared from cells and cell debris by performing two steps of centrifugation in a conventional benchtop centrifuge. Afterwards, the cleared supernatant is ultracentrifuged at $>100,000\times$ g for at least 1 h, washed with PBS, ultracentrifuged again, and finally, the EVs are collected from the ultracentrifuge tubes.

The limitations of this method are its time-consuming protocol and possibility of aggregations under high centrifugal forces [15]. Lamparski et al. performed a comprehensive study on the development of clinical grade, good manufacturing practice (GMP)-compliant method for exosome purification [17]. They compared differential ultracentrifugation, ultrafiltration-cushion ultracentrifugation methods. The method of differential centrifugation produced highly variable results for exosome yield. Ultrafiltration followed by cushion ultracentrifugation has given more stable exosome yield with higher recovery, and regulatory compliance [17].

Tauro et al. investigated ultracentrifugation, density gradient separation and immunoaffinity capture methods [18]. Exosomes were isolated from 500 μ l cell culture supernatant and the yield of applied methods were analysed. In this set of experiments, ultracentrifugation has given the best yield (375 μ g), followed by density gradient separation (150 μ g) and immunoaffinity capture (195 μ g). The size uniformity of exosomes has been shown to alter according to the isolation method. In this study, the immunoprecipitation method has been considered the best for exosome capture, as it yielded exosomes that have greater homogeneity and higher exosome-associated protein content [18].

4. Density gradient ultracentrifugation

In density gradient ultracentrifugation, almost the same steps are followed as in differential ultracentrifugation. Materials required for this isolation method are sterile conical centrifuge tubes, clean and sterile ultracentrifuge tubes, pipettes for handling the liquid material, sucrose or iodixanol for preparation of discontinuous gradient, PBS or other suitable buffer according to the downstream applications [19]. Equipments required for performing this procedure are the same as those described in differential ultracentrifugation method.

The EVs are first isolated by applying the differential centrifugation steps and the first ultracentrifugation step, as described under the differential ultracentrifugation, or alternatively, the isolation medium is concentrated using centrifugal filters. Next, a discontinuous sucrose or iodixanol gradient is prepared in ultracentrifugation tubes. For this purpose, sucrose solutions of gradually decreasing concentration are overlaid atop of each other. Crude EV pellet or concentrated isolation medium is then resuspended in little amount of PBS or buffer of choice, loaded on the gradient liquid and ultracentrifuged for extended period of time in order to separate EVs based on their buoyant density in the discontinuous viscous fluid [14, 18, 20]. The general steps of density gradient ultracentrifugation method for EV isolation are presented in **Figure 2**.

The ultracentrifugation method remains the most widely used approach for EV isolation. Lack of technical information about the type and the diameter of the rotor used, the volume and viscosity of the sample all represent challenges for establishing a standardized ultracentrifugation-based method [21].

The limitation of density gradient ultracentrifugation is its even long-lasting ultracentrifugation step as compared to differential ultracentrifugation. Moreover, this method requires an

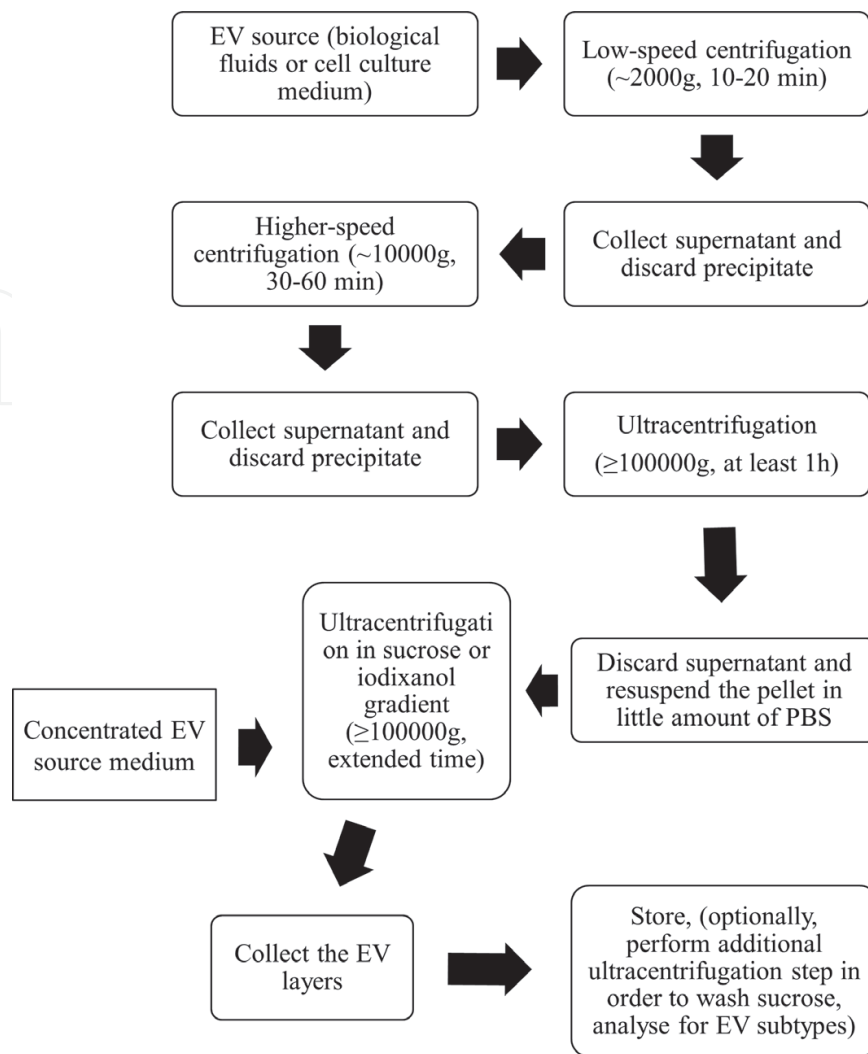


Figure 2. General steps followed during EV isolation by density gradient ultracentrifugation. This method is similar to differential ultracentrifugation to the first ultracentrifuge step. The collected EVs are then transferred on top of a gradient-forming agent and ultracentrifuged for extended period of time. EVs are separated as individual layers owing to the differences in their buoyant density and particle size. Finally, EVs are collected and analysed.

additional wash step in order to remove the density gradient-forming agent. On the other hand, its primary advantage is that several layers can be drawn after density gradient ultracentrifugation and each of these layers can be characterized in order to distinguish between different EV subtypes separated owing to their buoyant density.

4.1. Chromatography-based EV isolation

Size exclusion chromatography (SEC), also known as gel filtration chromatography, employs size difference of exosomes, microvesicles, apoptotic bodies, proteins and other components present in biological materials. The source material for EV isolation is loaded on a column prefilled with a stationary phase such as Sepharose® and Sephacryl®. A mobile phase, usually phosphate buffered saline, is then allowed to pass through the column. While the mobile phase

passes through the column, it draws EVs into the stationary phase. During this process, smaller molecules such as proteins and small vesicles—the exosomes interact with the pores of the stationary phase, leading to relative deceleration of their movement speed as compared with that of larger structures. As the result, EV subtypes are separated from each other as individual populations. Generally, these particle populations are collected in small fractions and each fraction is then analysed in terms of particle size and specific markers. Suitable fractions are then pooled and used for further downstream applications. General steps followed during SEC purification of EVs are schematized in **Figure 3**. Equipments required for isolation of exosomes by SEC are a prefilled column and a mobile phase to perform the elution (usually PBS). Fractions are collected in microcentrifuge tubes for later analysis and downstream applications. Alternatively, the SEC columns can be custom-designed for investigational purposes—a syringe with removed plunger, or a small-volume burette could perform well as an empty column to be filled with a suitable chromatography resin of researchers’ choice.

An efficient single step EV isolation based on chromatography is described by Böing et al. [12]. They used Sepharose CL-2B to create a separate column for size exclusion chromatography. As compared to the ultracentrifugation method from the literature, having highly varying EV yields (2–80 %), this method was superior with 43% stable recovery of EVs, and almost complete removal of contaminating proteins. Furthermore, the method takes as little time as less than 20 minutes to complete [12]. Disadvantages of this method are (1) the accessibility of the chromatography column to contamination, therefore aseptic working conditions should be ensured especially if the isolated EVs are intended for therapeutic use; (2) a large number of fractions should be collected and analysed in order to make sure complete separation of EV subtypes and contaminating proteins and (3) contrarily to the simplicity and time effectiveness of the separation protocol, post-isolation analysis of each fraction may be quite time consuming.

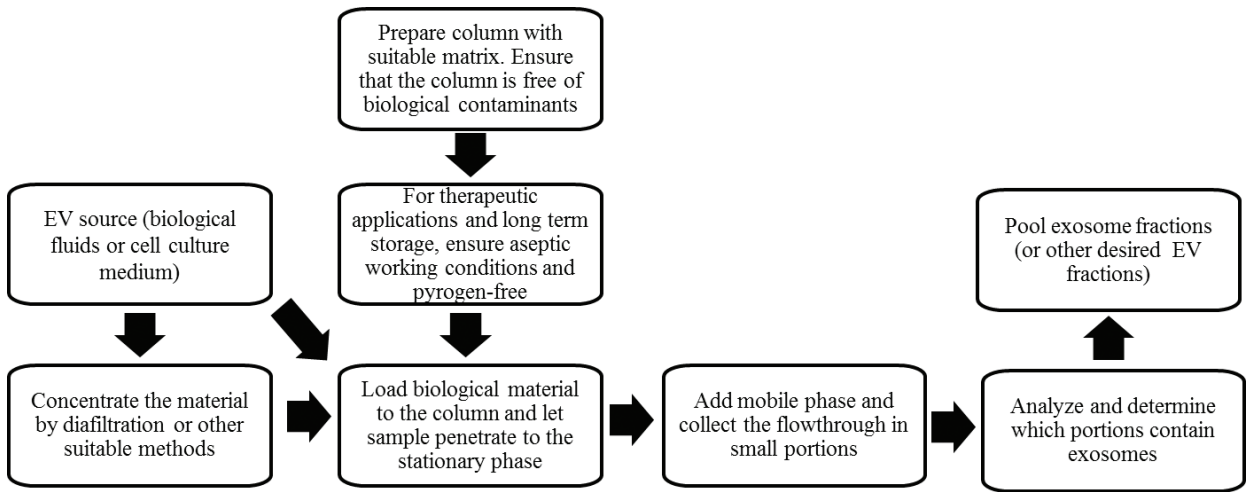


Figure 3. General steps followed during EV isolation by size exclusion chromatography. EV source is either directly loaded to the column, or first concentrated by a suitable method in order to increase the yield and then loaded to the column. Subsequently, the mobile phase is added and gravity-driven elution is performed.

4.2. Filtration-based EV isolation

Filtration-based isolation of EVs relies on separation of different EV subtypes from each other and from contaminating proteins due to their size. A series of filtrations is performed and cell debris, microvesicles, exosomes and free proteins are efficiently separated by this method. General steps of the filtration procedure can be seen in **Figure 4**. Required equipments for this method are: a peristaltic pump to circulate the EV suspension during the process, filtration cartridges in order to perform the separation, a sample chamber and a filtrate collection chamber. A proof-of-concept study describing the use of tangential flow filtration method for exosome isolation is performed by Heinemann et al. [22]. Authors made clear distinction between exosome and other EVs vesicles and aimed to efficiently separate them. Vesicles with much greater poly-dispersity are obtained as compared with differential filtration method. It was concluded that this method produces exosomal preparations with very high purity [22]. This method may be considered superior to ultracentrifugation method especially in cases where specific features of only exosomal fraction of extracellular vesicles are to be investigated, or if only exosomal fraction is desired for use in therapeutic delivery studies.

4.3. Precipitation-based EV isolation

A common and easy to handle way of isolating EVs is precipitation. In precipitation protocols, polymers such as polyethylene glycol (PEG) [7, 23, 24] or salt solutions such as sodium acetate [25] are used for isolation. In this method, the sample is first incubated with the precipitating agent. During incubation, the polymers reduce EVs' solubility and lead to their precipitation. After precipitation is completed, the pellet is simply collected by low-speed centrifugation. Later, it was observed that EV yield increases when the precipitation with polymer is performed in acidic pH [26]. Another approach may be precipitation of the solubilized proteins, leaving a supernatant enriched with extracellular vesicles. This method is called 'Protein Organic Solvent Precipitation (abbreviated as PROSPR)'. Acetone chloroform and trichloroacetic acid are used to precipitate proteins. After proteins are removed, EVs are concentrated by filtration or vacuum-dried for proteomic analysis [27]. More recently, charge-based precipitation of EVs has been reported. Researchers hypothesized that negatively charged EVs could interact with positively charged protamine sulphate. It was shown that charge-based precipitation in conjunction with polymer gives higher yield as compared with PEG-precipitation and

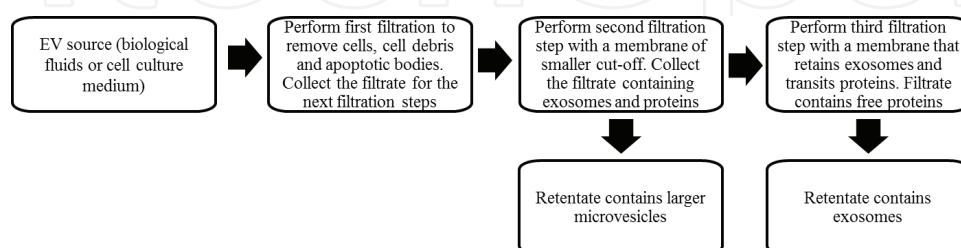


Figure 4. General steps followed during EV isolation by filtration. In this method, the source of EVs is sequentially passed through filtration cartridges with narrowing pore size. In the first step, cells and cell debris are removed. In the second filtration cartridge, the membrane passes exosomes and proteins into the filtrate while retaining microvesicles. Finally, the filtrate is passed through a cartridge with smallest pore size which passes free proteins and retains exosomes.

ultracentrifugation [23]. General steps followed during EV isolation by precipitation methods are represented in **Figure 5**. A salting-out procedure for exosome precipitation is proposed by Brownlee et al. [25]. This method employed addition of acetate ions to EV source followed by immediate precipitation of EVs due to charge neutralization. They compared the exosomes obtained with this method to those obtained by ultracentrifugation and showed that exosomes isolated by these methods are indistinguishable in respect to their size and shape characteristics. In precipitation methods, the necessary equipments are: suitable tubes for performing the precipitation, a precipitating agent of choice (polymers, electrolytes or organic solvents), buffers for performing the washes and a benchtop centrifuge to collect the formed precipitate.

4.4. Affinity-based EV isolation

Perhaps the most promising method for specific exosome isolation is the affinity precipitation in which specific antibodies are used. The most commonly employed antibodies in this method are monoclonal antibodies against specific exosomal membrane proteins (CD63, CD81, CD82, CD9, EpCam and Rab5). These antibodies are used alone or in combination [2]. Practically, the antibodies could be fixed on different types of materials such as magnetic beads [19, 28] or microfluidic devices [29–31]. The isolation is based on the binding efficiency of specific antibody to the specific antigen protein present on the exosome membrane (e.g. CD63). Magnet-based kits are commercially available for specific isolation of CD81, CD63, CD9 or EpCam-containing exosomes.

Using saccharide residues on the exosomal surface is also another approach in affinity methods [32]. This approach is easy to apply, however, due to the huge number of cells that contain

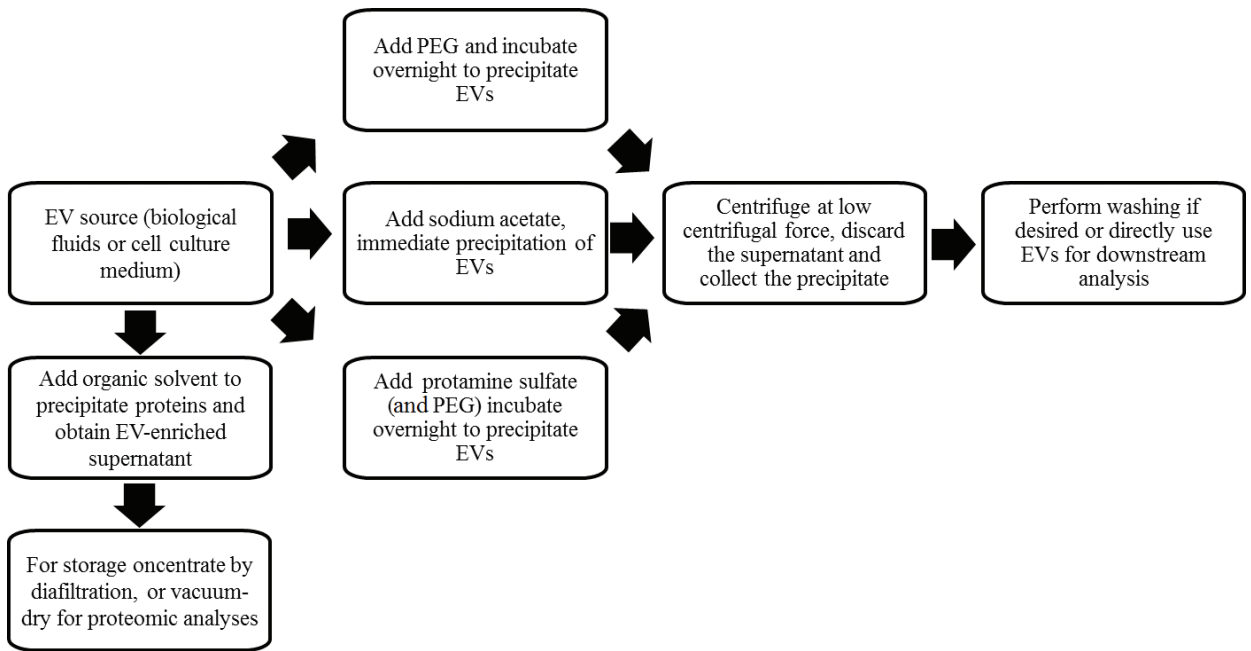


Figure 5. General steps followed during EV isolation by precipitation. One of the following methods of precipitation can be chosen by the researcher: protein organic solvent precipitation, polymer-driven precipitation, salting out with electrolyte solution or ionic precipitation by using cationic protamine. After an incubation period, the precipitate is collected by low-speed centrifugation.

mannose on their surface, the specificity of this affinity method is weak. Another affinity-based method relies on heparin affinity of EV. Based on previous observations that heparin blocks entry of EVs to recipient cells, a group of researchers hypothesized that heparin can bind directly to heparin sulphate proteoglycans on EVs' surface and can be used for their isolation [33]. Like in the case with saccharide affinity, the fact that many cell types contain heparin sulphate proteoglycans the specificity of this capturing method is weak too. Different steps needed in affinity-based approaches for EV isolation can be seen in **Figure 6**.

4.5. Commercial isolation kits

The increased research on exosomes for diagnostic and therapeutic applications has led to the development of commercially available isolation kits. Commercial kits for EV isolation involve precipitation of the proteins on the outer membrane of EVs along with contaminating proteins of non-EV origin. So, it is very important to determine the contaminating proteins before using their quantity for normalization of further experiments. Commercial kits for EV isolation include column-based isolation kits, immunocapture-based isolation kits and precipitation-based kits. **Table 2** summarizes the commercially available exosome isolation kits. Each of these kits is designed for isolating extracellular vesicles for various post-isolation applications, and can be found on the manufacturers' product lists.

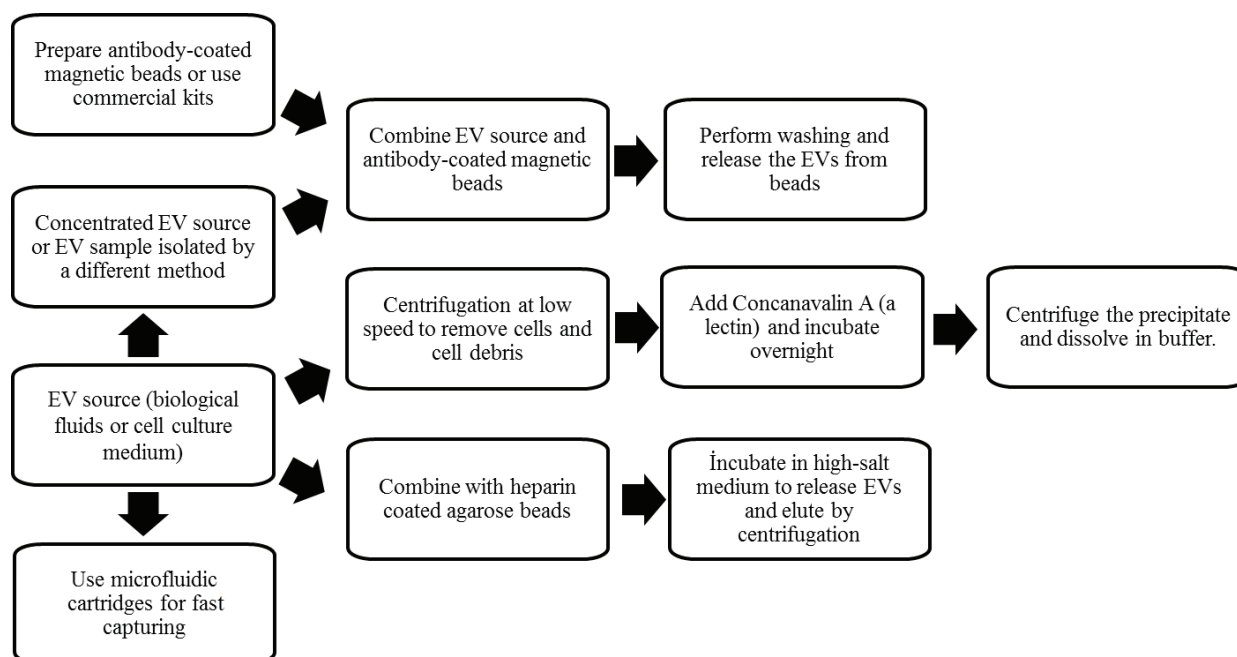


Figure 6. Main steps followed during different affinity-based EV isolation methods. Antibody affinity, lectin affinity or heparin affinity can be employed depending on specific properties of expected EVs. For antibody affinity, a concentrated source medium or EV isolate previously obtained by another method is combined and incubated with antibody coated beads or plates. Afterwards, the exosomes are washed and eluted. For lectin affinity isolation, a low-speed centrifugation is performed in order to eliminate intact cells and cell debris, and the medium is incubated in the presence of lectins. Then EVs, selectively bound to the lectins, are collected by centrifugation. For heparin affinity isolation, the EV source is combined with heparin-coated agarose beads and incubated. Later, EVs are released by adding concentrated salt solution and eluted by centrifugation. Very promising alternative is the use of microfluidic devices pre-coated with antibodies. This method ensures very fast capturing of specific EV subtypes.

Name of the commercial kit	Manufacturer	Principle
ExoCap™ exosome isolation kit	JRS Life Sciences GmbH Co.	Immunocapture and magnetic separation
Exosome-Human CD81/CD63/CD9/EpCAM isolation kits	Life Technologies Inc.	Immunocapture and magnetic separation
Exo-spin™ exosome purification kit	Cell Guidance Systems Ltd.	Sedimentation and column filtration
qEV Size Exclusion Column	iZON Ltd.	Size exclusion chromatography
Invitrogen total exosome isolation kit	Life Technologies Inc.	Sedimentation
ExoQuick™ and ExoQuick-TC™ exosome isolation kits	System Biosciences	Sedimentation
ME™ exosome isolation kit	New England Peptide	Sedimentation
miRCURY™ exosome isolation kit	Exiquon Inc.	Sedimentation

Table 2. Commercially available kits for extracellular vesicle isolation.

When, the commercial ExoQuick exosome isolation kit was compared with classical ultracentrifugation, ExoQuick revealed ca. 19 times higher protein quantity in the isolated exosomal dispersion and is proven simpler and faster [34]. Yet no further experiments are performed to clarify if all the protein yields are truly of exosomal origin. Optiprep density gradient was successfully used for separation of exosomes, microvesicles, free proteins, non-exosome small vesicles and proteasome from the same cells [19].

5. Comparison of exosome isolation methods in diagnosis and treatment of cancer

Table 3 gives examples of studies on EV isolation methods and summarizes the major findings.

As previously mentioned, in order to be applicable for therapeutic purpose, EV product should have a clearly defined origin and well-characterized particles with homogeneous size distribution. Because different EV subtypes are generated by different biogenesis pathways and originate from distinct cellular parts, their molecular cargo differs significantly. Therefore, when EVs will be used for delivery of specific small RNA molecules generated in the donor cells, it is crucial to distinguish whether obtained EVs are microvesicles or exosomes. While for particular applications such as RNA delivery or drug delivery exosomes may be preferable, for other applications such as vaccination and surface antigen display microvesicles might be more relevant [40]. For diagnostic application, especially in proteomics analyses, free protein contaminants may lead to false-positive results. In so far as diagnosis is performed on body fluid samples, attention should be paid to numerous contamination factors like viruses, serum/plasma components, bacteria etc. Suitable method or combination of methods should be selected in order to eliminate all these factors that would affect final results.

Researchers should first consider the downstream application and then decide which method to use according to their advantages and disadvantages. These features of the methods mentioned in this chapter are summarized below.

Source of EVs	Origin	Isolation method(s) used	Findings and outcomes
Cell culture media	CD14+ monocyte-derived dendritic cells from healthy donors	<ul style="list-style-type: none"> • Ultrafiltration → cushion ultracentrifugation • Filtration → centrifugation → cushion ultracentrifugation • Differential Centrifugation 	Ultrafiltration followed by cushion ultracentrifugation has given a stable exosome yield with higher recovery, and regulatory compliance. The method of differential centrifugation produced highly variable results with less effective exosome yield [17]
Whole blood	Healthy donors	<ul style="list-style-type: none"> • Differential centrifugation • ExoQuick (Precipitation) 	Two methods are compared with regard to how they affect the miRNA profile of the isolated exosomes. Comparable results were reported. ExoQuick is not recommended for obtaining exosomes intended for further biochemical and immunological studies [35]
Cell culture media/ biological fluids	NS	Differential centrifugation	Step-by-step description of the ultracentrifugation method is provided [36]
Cell culture media	LIM1863 colon cancer cell line	<ul style="list-style-type: none"> • Ultracentrifugation • Density gradient separation (OptiPrep) • Immunoaffinity capture 	The least yield was achieved by ultracentrifugation, followed by OptiPrep, and the best was achieved by immunoaffinity capture. Ultracentrifugation was the faster method with only 2 hours required to complete. The immunoaffinity has considered the best method [18]
Cell culture media	U87 and 293T cancer cells and normal HUVE cells	<ul style="list-style-type: none"> • Heparin affinity separation • Ultracentrifugation • ExoQuick 	Using the affinity of EVs for heparin, researchers succeeded to demonstrate a simple and effective method to isolate highly pure populations of EVs [33]
Cell culture media	K1735P melanoma cell line	<ul style="list-style-type: none"> • Ultracentrifugation • Salting-out procedure 	Simple and cost-effective ion neutralization in acetate buffer media is described. Increased protein yield is observed in comparison to ultracentrifugation [25]
Cell culture media	Huh-7 liver cancer cell line	<ul style="list-style-type: none"> • Ultracentrifugation • ExoQuick (Precipitation) 	ExoQuick revealed higher protein quantity in the isolated exosomal dispersion, and has been proven simpler and faster [34]. Yet, it still remains to be clarified if the proteins are not of extraneous origin
Cell culture media	MDA231 breast cancer cell line	<ul style="list-style-type: none"> • Sequential filtration • Differential ultracentrifugation 	Vesicles with much greater poly-dispersity are obtained with the sequential filtration method. Exosomal preparations with very high purity are obtained [22]
Body fluids	Platelet concentrate	<ul style="list-style-type: none"> • Size exclusion chromatography 	Efficient, single step, rapid and cost-effective EV isolation method is described [12]
Cell culture media	D3 murine embryonic stem cell line	<ul style="list-style-type: none"> • Differential ultracentrifugation; • Centrifugal extrusion of whole cells to produce exosome-mimetic vesicles 	Based on the protein and RNA amount in obtained EVs whole cell-extrusion method has given nearly 250 times higher vesicle yield than simple exosome isolation [37]. Also, similar results were obtained with in vitro delivery studies

Source of EVs	Origin	Isolation method(s) used	Findings and outcomes
Cell culture media	D3 murine embryonic stem cell line	<ul style="list-style-type: none"> Differential centrifugation; Microfluidics-mediated extrusion of whole cells to produce exosome-mimetic vesicles 	Researchers compared the two methods for production of cell-derived vesicles but no comparison of the protein and vesicle yield was made [38]. Similar results were observed with EVs isolated with both methods in <i>in vitro</i> delivery studies
Cell culture media	BT-474 breast cancer cell line	<ul style="list-style-type: none"> Differential ultracentrifugation ExoSpin Exosome Purification Kit Invitrogen Total Exosome Purification Kit PureExo Exosome Isolation Kit 	All four methods are considered non-specific for exosome isolation because of the presence of large particles [39].
Cell culture media	Monocyte-derived dendritic cells, HEK293T, RPE-1, HeLa-CIITA, MDA-MB-231, SHIN, IGROV-1, OV2008	<ul style="list-style-type: none"> Differential ultracentrifugation Optiprep/Iodixanol gradient ultracentrifugation Sucrose gradient ultracentrifugation Immunoaffinity capture 	Subtypes of EVs were isolated by combining different methods such as ultracentrifugation and iodixanol gradient. Subgroups of small non-exosomal vesicles, not carrying the exosomal markers were described [19]

Table 3. Studies dealing with EV isolation and major findings thereof.

5.1. Centrifugation-based methods

Advantages

- these methods are recognized as gold standard for isolation of extracellular vesicles
- as the most commonly used method, ultracentrifugation is acceptable for isolating EVs for many application purposes
- well-established protocols and troubleshooting are available
- ultracentrifugation can be used in combination with other techniques in order to provide better resolution of microvesicles and exosomes
- differential ultracentrifugation is able to discriminate between exosomes, small non-exosome vesicles and microvesicles owing to their different buoyant density

Disadvantages

- the yield of EVs is highly varying in different setups
- requirement for expensive instrumentation and consumables

- disposable consumables like ultracentrifuge tubes meaning
- information about the rotor type and geometry, applied g-force, solution viscosity and salinity should all be considered in order to achieve reproducible results
- the procedure of ultracentrifugation is very time laborious and requires substantial amount of hands-on work. The density gradient ultracentrifugation takes even more time with extra purification steps from start to finish
- in differential ultracentrifugation, not only exosomes are being collected at the end of the isolation. Therefore, for both diagnostic and therapeutic applications, isolates should be purified from contaminating proteins and other EV subtypes in order to avoid

5.2. Size exclusion chromatography

Advantages

- exosomes are isolated in a single step
- short operation time
- efficient elimination of contaminating proteins
- efficient separation of EV subtypes (provided that the sample loading volume is not too large)
- high purity of EV isolates
- no extra compounds are added in order to perform the isolation

Disadvantages

- samples are collected as a large number of fractions
- need to characterize each fraction in order to ensure presence of EVs and proteins
- requirement for aseptic working conditions in order to prevent microbial contamination
- exosomes and small non-exosome vesicles cannot be separated
- large sample loading volume may lead to inefficient separation

5.3. Filtration-based isolation

Advantages

- effective separation of exosomes and microvesicles
- simple and short operation
- efficient elimination of contaminating proteins
- efficient separation of EV subtypes (exosomes and microvesicles)
- high purity of EV isolates

- the method is able to handle large-volume sample
- no extra compounds are added in order to perform the isolation
- suitable for development of microfluidic isolation setups

Disadvantages

- the method cannot be applied on small-volume samples
- exosomes and small non-exosome vesicles cannot be discriminated
- possibility of occlusion of membranes during operation
- membranes should be carefully regenerated or discarded after use

5.4. Precipitation-based isolation

Advantages

- ability to precipitate virtually all EVs in the biological sample
- very high yield
- versatility to perform isolation in different precipitation protocols
- fast and easy application
- protein solvent precipitation method may enhance diagnostic strength of exosomes and ensure contaminant-free isolates for therapeutic applications

Disadvantages

- need to remove the precipitating polymer or salt for downstream applications
- production of highly heterogeneous and protein-contaminated EV mixture
- need for extra purification steps

5.5. Affinity-based methods

Advantages

- provides specific isolation of individual EV subpopulations
- high-purity EV production
- suitable for development of microfluidic isolation setups

Disadvantages

- requirement of specific antibodies and targeting ligands
- need of solid knowledge about EVs' structure
- possibility of functionality loss after detachment from antibodies
- not suitable for high-volume samples

6. Conclusion

Exosomes are gaining continuously increasing interest in biological, medical and pharmaceutical research fields. Treatment and diagnosis of cancer are two particularly promising applications of exosomes. Isolation methods for exosomes are being advanced with the time and new modifications to available methods are being introduced. According to the available literature, there is still no method that is free of shortages. For both applications, scientists should consider carefully the advantages and disadvantages of available methods. It is obvious from published methods that immunoaffinity isolation promises specific capture of exosomes from biological fluids, taking advantage of their membrane structures. Therefore, in cases where exosomes will be subject of investigation for diagnosis of cancer, immunoaffinity may provide isolation of exosomes in the most sensitive and specific manner among all methods. By hyphenation of affinity methods to microfluidics field, even faster isolation, detection and analysis of exosomes can be achieved. The question of 'which method is the best?' currently remains unanswered for therapeutic applications of exosomes. Regulatory requirements for a standardized clinical grade exosome isolation method are yet to be established. Nevertheless, size exclusion chromatography and ultrafiltration methods which do not require incorporation of extra compounds to facilitate the isolation provide exosome isolates with high purity. For cancer therapy, these two methods may be considered optimal.

Author details

Mustafa Kotmakçı^{1*} and Gülşah Erel Akbaba²

*Address all correspondence to: mustafa.kotmakci@ege.edu.tr

1 Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Ege University, Izmir, Turkey

2 Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Izmir Katip Çelebi University, Izmir, Turkey

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