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Review

# Advances in exosomes technology

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A B S T R A C T

Exosomes, also called extracellular vesicles (EVs), are membranous structures measuring between 40 and 100 nm. Exosomes, secreted by various cells of the human body into body fluids, contain protein, mRNA, miRNA, and signaling molecules. Physiologically, exosomes assist in the intercellular transport of protein and RNA. Immunologically, exosomes exhibit antigen-presenting capability. In recent studies, exosomes were found to be associated with the pathophysiology of cardiovascular, renal, neurological, and ocular diseases. In addition, exosomes may play a major role in cancer metastasis. Due to the extremely small size and scarcity of exosomes in living samples, many early studies utilized sucrose density gradient ultracentrifugation for exosome collection. However, sucrose density gradient ultracentrifugation is rather time consuming and requires large biological sample quantities. Newer exosome studies combined immunoaffinity and microfluidic system approaches for more efficient exosome collection. Our review summarizes existing methods for EV isolation and notes their advantages and disadvantages. These promising approaches are all characterized by isolation efficiency, and savings in cost, labor, and time. Optimization of current methods is a necessary step toward clinically-relevant diagnostic product production, but the fact that EVs are already widely used in disease diagnosis and treatment encourages continued efforts.

## 1. Introduction

The traditional technique for exosomes collection, sucrose density gradient ultracentrifugation, requires considerable processing time, relatively large starting sample quantities, and expensive ultracentrifuges. Commercial kits for exosomes collection are available, but come with a high price. In recent years, microfluidic systems have been used to improve the efficiency, speed, and cost of exosomes purification. These approaches included the use of immunoaffinity beads, microfluidic filtration systems, and microfluidic laminar flow systems. The latest study used both microfluidic and immunoaffinity systems in combination.

## 2. Review of different systems to isolate exosomes

Sucrose density gradient ultracentrifugation and non-centrifugation-based methods, including filtration, chromatography, precipitation, and the use of microfluidic systems, are reviewed below.

### 2.1. Sucrose density gradient ultracentrifugation

As the name suggests, this method uses both ultracentrifugation and a sucrose density gradient for exosomes isolation. Sucrose density gradient ultracentrifugation is currently the most used and most studied method for organelle, protein, and other biomolecule isolation. Methodology is based on EV size and density. This gold standard method has been used to isolate EVs from conditioned culture media, bodily fluids, and tissues [1,2]. Ultracentrifugation itself carries the risk of contamination with other non-vesicles including aggregates and lipoproteins also pelleted by a high-speed spin. Accordingly, using a sucrose density gradient as a continuation of ultracentrifugation allows for better separation of particles when extra purity is needed [3]. Fig. 1 depicts a conventional scheme for employing this combined methodology [4–8]. Serial ultracentrifugation is performed at 300 $\times g$ to remove living cells. This is followed by subsequent ultracentrifugations at 2000 $\times g$ and 10,000 $\times g$ to eliminate dead cells and cell debris, respectively. Additional ultracentrifugations at 100,000 $\times g$ reduce the amount of contaminating

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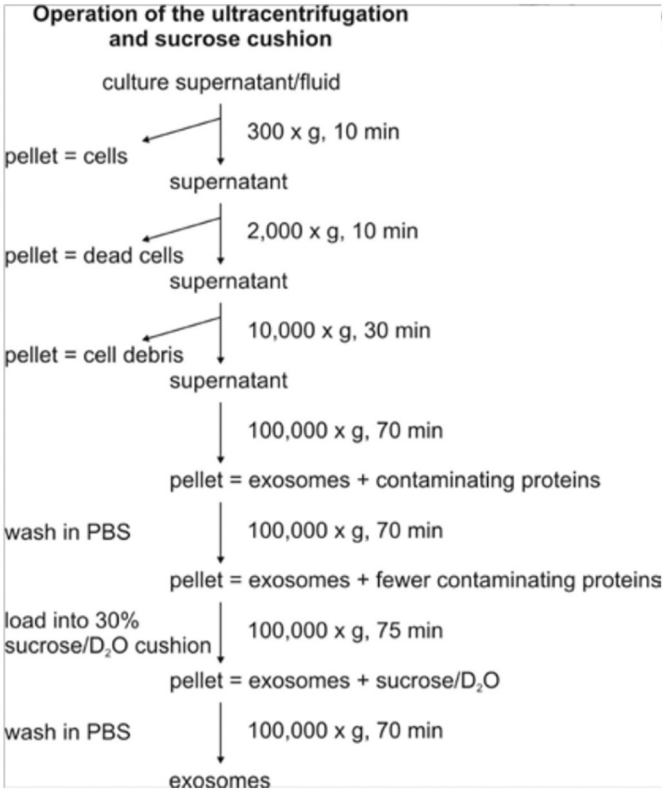


**Fig. 1.** Protocol of ultracentrifugation for the exosomes purification. After each of the first three centrifugations, pellets including cells, dead cells as well as cell debris were discarded, and the supernatant was kept for the next step. Pellets containing exosomes and contaminant proteins were kept, while supernatants were discarded. (Reproduced with permission from reference [4]).

proteins. During final ultracentrifugation, a 30% sucrose cushion is added for further purification of exosomes. Despite being widely used, sucrose density gradient ultracentrifugation has several drawbacks. It takes a great deal of time to complete multiple centrifugations. It also requires relatively high-volume sampling, rendering it unusable for low-volume clinical sample situations (e.g., aqueous humor). Finally, high velocity ultracentrifugation increases the risk of vesicle rupture and loss [9]. As a result, fewer EVs can be successfully isolated.

### 2.2. Ultrafiltration

Ultrafiltration (UF) uses membranes with different pore sizes to fractionate and isolate exosomes. Permeate carrying material smaller than the selected membrane pore size passes through the membrane while larger particles are left behind. UF procedures can be used to produce highly-purified EMVs in comparison to other methods [10]. However, there are drawbacks to UF including the effects of material shape and electrical charge on separation [11]. Additionally, it is difficult to remove remaining proteins that adhere to the nanomembrane and hamper elution of exosomes [12]. Further, adherent particles block pores, leading to declining flow and low elution efficiency.

### 2.3. Size-exclusion liquid chromatography

Variations of chromatography (e.g., ion-exchange chromatography and affinity chromatography) have been used to isolate numerous biomolecules and chemical compounds, including proteins, enzymes, and antibodies [13–15]. As with ultrafiltration, size-exclusion liquid chromatography (SEC) is effective in isolating exosomes by size. At a 2013 discussion paneI during ISEV, Gardiner proposed combining ultrafiltration and size-exclusion liquid chromatography to isolate exosomes. In 2017, the above two methods were used in combination to isolate EVs with exosomes properties from cell culture media [16]. To accomplish this, samples underwent crude centrifugation and then ultrafiltration. The recovered smaller molecules were then subjected to size-exclusion liquid chromatography to further segregate exosomes by size. In contrast to density-gradient ultracentrifugation, and to prevent biological function loss, this process used buffers with physiological osmolarity and viscosity [17]. Disadvantages of this approach include the amount of labor required, possible sample contamination with lipoproteins, and possible protein aggregation [18].

### 2.4. Precipitation

#### 2.4.1. Immunoaffinity beads

Immunoaffinity beads separate EV based on the expression of surface markers. Magnetic beads coated with an antibody against a target marker attach to EVs expressing said marker [19]. In a 2012 issue of Analytical Biochemistry, Yoo et al. described a method to extract miRNA from exosomes using immunoaffinity beads. They coated immunoaffinity beads with anti-EpCAM antibodies to capture exosomes and then immediately performed exosomal miRNA extraction, as shown in Fig. 2. This method resulted in relatively rapid miRNA extraction, but it was difficult to prove whether these RNAs originated from within the target exosomes. It remains to be seen whether the exosomes captured by these beads can be collected and used for further processing [20]. Immunoaffinity bead methods have demonstrated extensive diagnostic and therapeutic potential. Tauro et al. performed a comprehensive evaluation of ultracentrifugation, density-based separation, and immunoaffinity capture using anti-EpCAM-coated magnetic beads to isolate colorectal cancer-related exosomes. They concluded that immunoaffinity capture was the best method for capturing exosomes as demonstrated by two-fold greater recovery compared to the other two approaches [21]. Mizutani et al. developed an immunoaffinity-based method to isolate prostate cancer-related exosomes from blood. Their study indicated that patients with aggressive prostate cancer exhibited higher levels of prostate cancer-related exosomes in blood [22]. Although immunoaffinity beads are widely used, they are only effective when a high proportion of exosomes present the target protein [23].

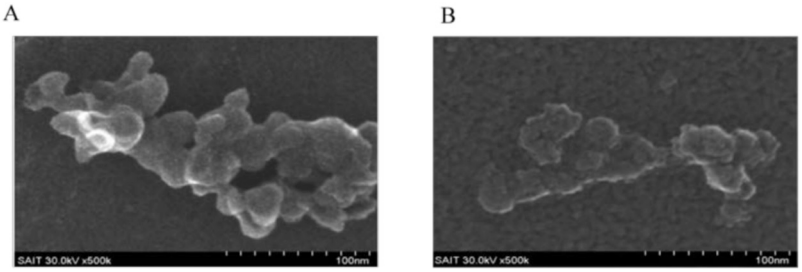


**Fig. 2.** SEM showed debris after being mixed with extra-bound beads at 30 ˚C (A), and 70 ˚C (B). (Reproduced with permission from reference [20]).

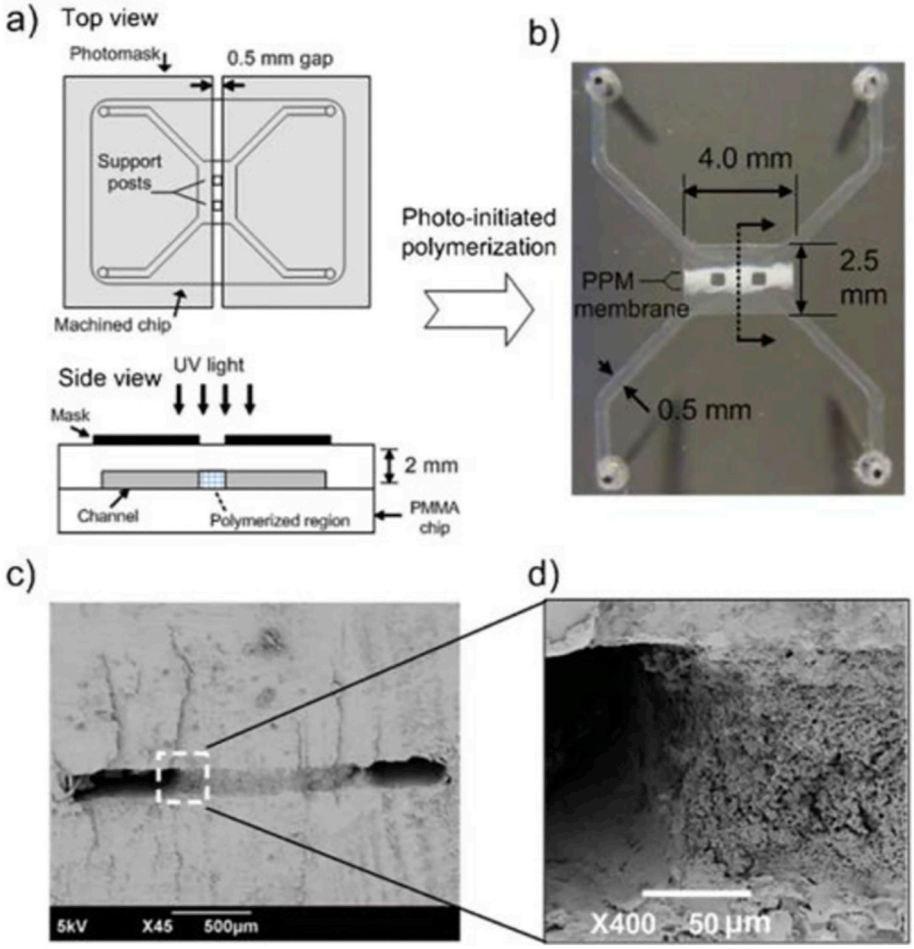


**Fig. 3.** The proposed microfluidic device. (a) Schematic diaphragm of microfluidic system. (b) The porous polymer monoliths (PPM) as a semi-permeable filter; (c) and (d) different magnifications porous polymer monoliths showing SEM strongly integrated into the channel walls. (Reproduced with permission from reference [30]).

*2.4.2. Heparin affinity*

Heparin is a highly sulfated glycosaminoglycan, and it can bind to a variety of proteins as a glycoprotein. Clinically, heparin activates antithrombin III and a cascade of reaction to achieve anticoagulation. Leveraging its capacity to bind to various types of proteins, heparin can be used to bind to the membrane proteins on exosomes and aggregate them [18]. In Leonora's 2015 study, he showed that EVs could be purified from cell culture media and human plasma using ultrafiltration (UF) and heparin-affinity beads. The purified EVs displaying marker Alix displayed lower levels of protein contamination and could bind to and be taken up by cells [24].

*2.4.3. Polyethylene glycol precipitation*

Polyethylene glycol (PEG) can be used to change cell membrane surface structure. Subsequent PEG precipitation avoids degradation of endogenous lipids and phospholipids. Further aggregation for exosomes can be achieved via cell adhesion and surface tension. In this manner, isolated exosome aggregates can be recovered via PEG-based precipitation [25]. Surveys such as the one carried out by Weng (2016) have shown that a PEG-based approach can be used to harvest exosomes from cell culture supernatant. This approach demonstrated several advantages; no specialized equipment was required, cost was minimal, and EV recovery was very pure [26].

*2.5. Microfluidic system*

Microfluidic isolation devices have been developed to minimize technology size and cost. They reduce sample size requirements and reduce reaction time by simultaneously performing multiple steps [27,28].

*2.5.1. Microfluidic filtration system*

Microfluidic system filters have been used to sort molecules using varying pore sizes. Park et al. employed porous polymer monoliths (PPM) as filters in their microfluidic system as shown in Fig. 3. PPM were composed of glycidyl methacrylate, GMA (cross-linker), ethylene glycol dimethacrylate, EGDMA (photo-initiator), 2,2-dimethoxy-2-phenylacetophenone, DMPA, and methanol. In another example, Siwoo Cho et al. used a filtration-based capture system that employed extracellular vesicles on a nanoporous membrane and electrophoretic migration. The resulting EV pellet could be physically peeled off the membrane, leading to minimally irreversible EV destruction [29]. Overall, advantages of microfluidic systems include rapid partition of molecules by size, and smaller sample requirements compared to other methods. However, larger molecules are known to block the filtering pores, which decreases filtering capacity over time [30].

*2.5.2. Microfluidic layer current separation system*

This method uses the varied sedimentation rates of different molecules to isolate molecules by specific size in a double current layer system. As shown in Fig. 4, samples in this approach are loaded from the top current layer and they migrate down through a carrier buffer zone in the lower current layer. Targeted molecules can be isolated by sedimentation rate [31]. This method overcomes the congestion issue found with simple microfluidic systems. However, microfluidic layer

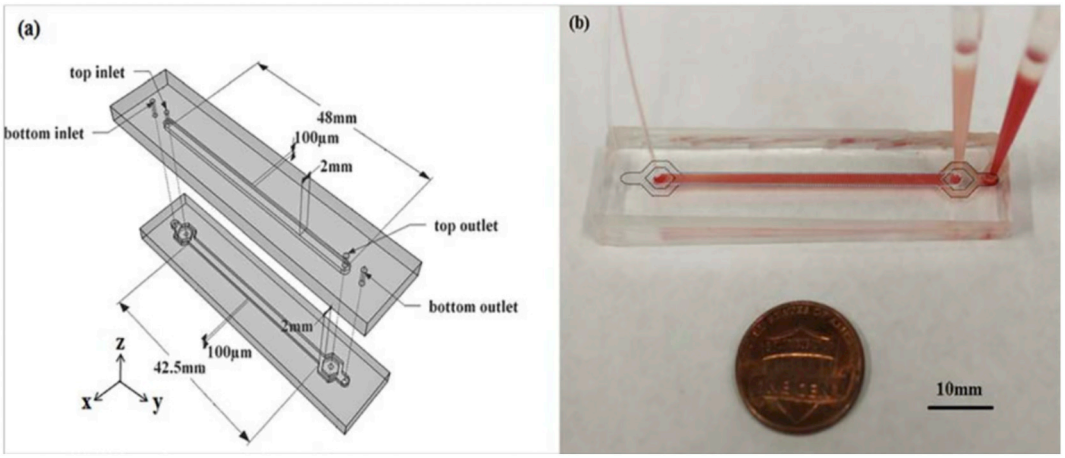


**Fig. 4.** Schematic diagram of the device structure containing two layers of microchannels: top layer for the biological sample to be injected into; bottom layer injected into with a carrier buffer to separate the cells from viral particles (a). A photograph showing the working device: the dark dash line shows the bottom layer channel and the light dash line shows the top layer channel (b). [31]. (Reproduced with the permission of AIP Publishing, https://doi.org/10.1063/1.3609262).

current separation systems can only isolate molecules with similar size and electrical charge. They cannot determine whether these molecules are exosomes measuring 40–100 nm.

### 2.5.3. Microfluidic immunoaffinity system

Similar to immunoaffinity beads, this method dots the fluidic system with antibodies in order to capture exosomes. Since the antibodies are immunospecific, this system allows for efficient and high-concentration exosomes collection. In contrast to the immediate direct extraction of miRNA carried out using the immunoaffinity beads method, the microfluidic immunoafinity method requires traditional RNA extraction. This method allows for structural preservation of the exosomes and subsequent additional laboratory research on exosomes structures and membrane proteins (Fig. 5) [32,33]. This method has been used in studies focused on disease diagnosis. Zheng et al. developed a microfluidic approach using immunomagnetic beads and an enriched preparation of blood plasma exosomes. This method, which was used for blood-based diagnosis of ovarian cancer by multiplexed measurement of exosomal tumor markers, showed significant diagnostic power [34].

### 2.6. Paper-based immunoaffinity system

A paper-based immunoaffinity platform was developed by modifying the paper surface using a chemical conjugation approach. An antibody with high affinity to specific EV was selected as the capture molecule [35]. Both size and number of captured EVs can be evaluated using either transcriptome analysis, paper-based enzyme-linked immunosorbent assays (P-ELISA), or scanning electron microscopy (SEM), respectively [36]. This isolation method is efficient, time-saving, and only requires small sample volumes. Furthermore, a combination of cellulose-based devices and microfluidic chip techniques was developed for the isolation of exosomes from aqueous humor, given that exosomes play an emerging roll in the pathogenesis of major blindness-threatening diseases [37].

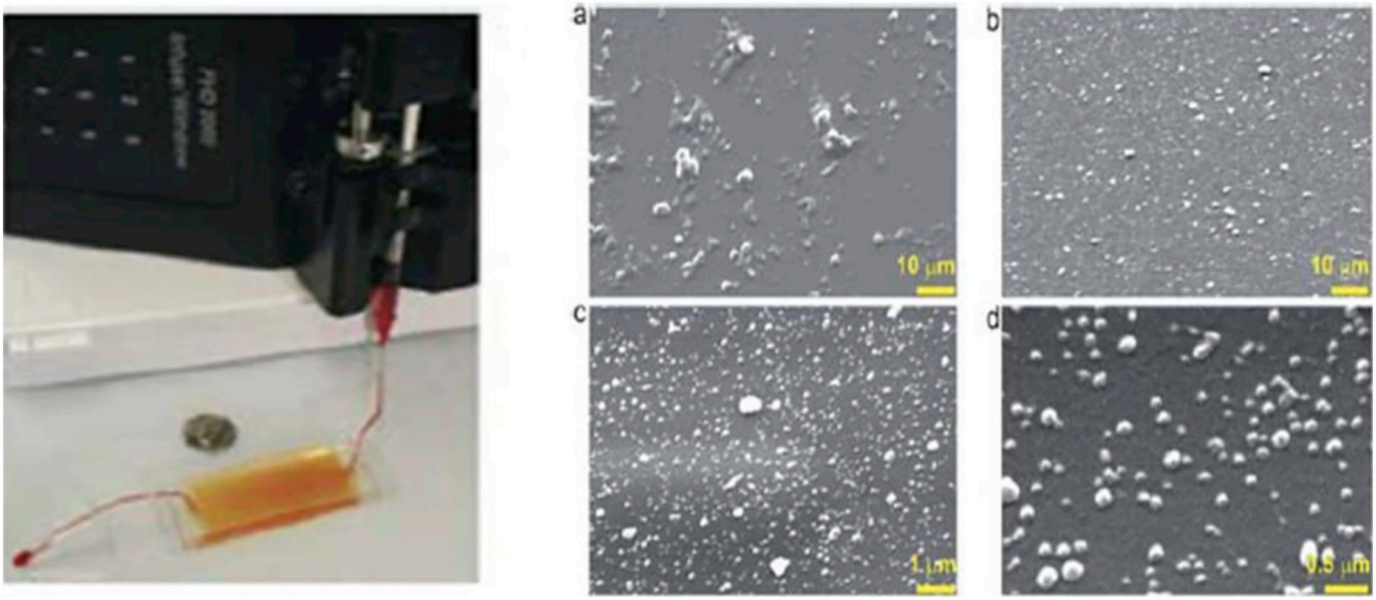


**Fig. 5.** Left column: microfluidic device setting. Right columns: a) SEM view of exosomes from cell culture medium and capsule following ultracentrifugation. b) SEM view of final capsule of exosomes from cerebrospinal fluids of patients with glioblastoma multiforme (GBM) after passage through an immunoaffinity microfluidic device. c and d) SEM views with higher magnification. (Reproduced with permission from reference [32]).

### 2.7. Comparisons between different systems

| Methods | Advantages | Disadvantages | References |
|---|---|---|---|
| **Sucrose density gradient ultracentrifugation** | Most studied and most commonly used, easy to handle with simple principle | Time-consuming, hard to access ultracentrifugation equipment, change in osmotic environment, co-isolating contaminants | [3,18,38,39] |
| **Ultrafiltration** | Faster, no special equipment required, easy to handle compared to ultracentrifugation | Use of force possibly resulting in the deformation and breaking up of large vesicles | [12,40,41] |
| **Size-exclusion liquid chromatography** | Faster, no special equipment required, structurally intact product, high purity, more-efficient in removing contaminants | Need to proceed with other methods, interfering problem between lipoprotein and protein aggregation | [29,42] |
| **Precipitation** | Easy to use, no special equipment required | Easier to precipitate with other non-exosome pollutants within sample | [43,44] |
| **Microfluidic system** | Sample efficiency, reagent consumption, and reduced isolation time | Skilled technique in microfluidic experiments required | [41] |
| **Paper-based Immunoassay system** | Fast, easy to use, no special equipment required, sample efficiency | Separate exosomes with targeted proteins only | [35,36] |

## 3. Conclusion

Improved exosomes isolation, characterization, and content identification have made the potential application of exosomes in clinical practice increasingly possible (e.g., precise diagnosis and treatment of exosome-related diseases). Applications based on exosomes biology can be realized only following optimization of exosomes isolation approaches. Multiple issues such as time-consumption, unsatisfactory reproducibility, and sample volume requirements remain unresolved. In this article, we summarized and compared the advantages and disadvantages of different isolation methods in order to suggest directions for further investigations focused on research design and clinical need. Further efforts will increase understanding of EVs, strengthen methodology for EV quantification and identification, and improve the practical potential of EV-based diagnostic and therapeutic applications.

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