Scientific Papers of the University of Pardubice,
Series A; Faculty of Chemical Technology
29 (2023) 105–124.

UNIVERSITY OF PARDUBICE
FACULTY OF CHEMICAL TECHNOLOGY

Current trends in exosome isolation methods: A systematic review

Ondřej Morávek and Zuzana Bílková*

Department of Biological and Biochemical Sciences,
The University of Pardubice, CZ−532 10 Pardubice, Czech Republic

Received: September 4, 2023; Accepted: September 14, 2023

Exosomes, extracellular vesicles crafted via a regulated process involving endocytosis, biomolecule packaging, and exocytosis, possess diverse functions. Exosomes participate in many processes crucial for the functionality of cells and tissues, e.g. in intercellular communication, immune response, programmed cell death, inflammation, and morphogen transport. Based on their multifunctional activity, the exosomes also play a significant role in many pathological conditions, such as malignancy or neurodegeneration. Nanosized exosomes carry various biomolecules as nucleic acids, proteins, lipids, metabolites. It has already been revealed that the composition of exosomes often corresponds with the (patho)physiological status of a cell, tissue, organ, and, ultimately, an organism. That is why the exosomes/oncosomes become promising structure suitable for studying the pathogenesis of the relevant disease or for the early and precise diagnosis of many serious diseases. The barrier for their wider use is the difficulty to isolate exosomes in the desired quantity and quality. To date, various isolation strategies have been proposed, spanning physico-chemical and affinity-based methods. Even the accelerated exosome isolation techniques exploiting the benefits of magnetic carriers or microfluidic platforms are still facing some limitations, and it is already clear where new trends can be expected. A concise review of strategies and technologies for exosome isolation is given here.

Keywords: Extracellular vesicles; Exosome; Isolation; Physicochemical methods; Affinity-based methods; Microfluidics

* Corresponding author, ✉ zuzana.bilkova@upce.cz

Introduction

Exosomes (Figure 1) as cell-derived nanoparticles ranging in size from 30 to 150 nm are formed by a strictly regulated process [1]. Firstly, the early endosomes have to be created. Then, the bioactive molecules like enzymes, signaling molecules, DNAs, microRNAs, non-coding RNAs and lipids are packed into intraluminal vesicles. Finally, membrane-enclosed exosomes are produced via exocytosis of those multivesicular bodies into the extracellular environment within the late endosome and plasma membrane fusion [2]. Based on the biogenesis, the membrane-derived vesicles contain a mix of components related to the cells and tissues from which they originate [3]. Even so, the exosomes contain an evolutionarily conserved set of protein molecules, typical molecular pattern of human exosomes consists of tetraspanins like CD9, CD63, CD81, and CD82, membrane transport and fusion proteins like Annexins, GTPases, and flotillin, proteins associated in multivesicular body biogenesis like ALIX (ALG-2-interacting protein X) or TSG101 (tumor susceptibility gene 101 protein), as well as lipid-related proteins and phospholipases [4–6].

Their major functions are being cell-to-cell communication and the exchange of bio-active molecules (such as mRNA and miRNA, DNA, proteins etc.) into recipient cells. They are involved not only in intercellular communication transferring various effectors or signaling molecules between the specific cells [7], but they also engage in processes, such as immune response, programmed cell death, angiogenesis, coagulation, and morphogen transport [8–11].

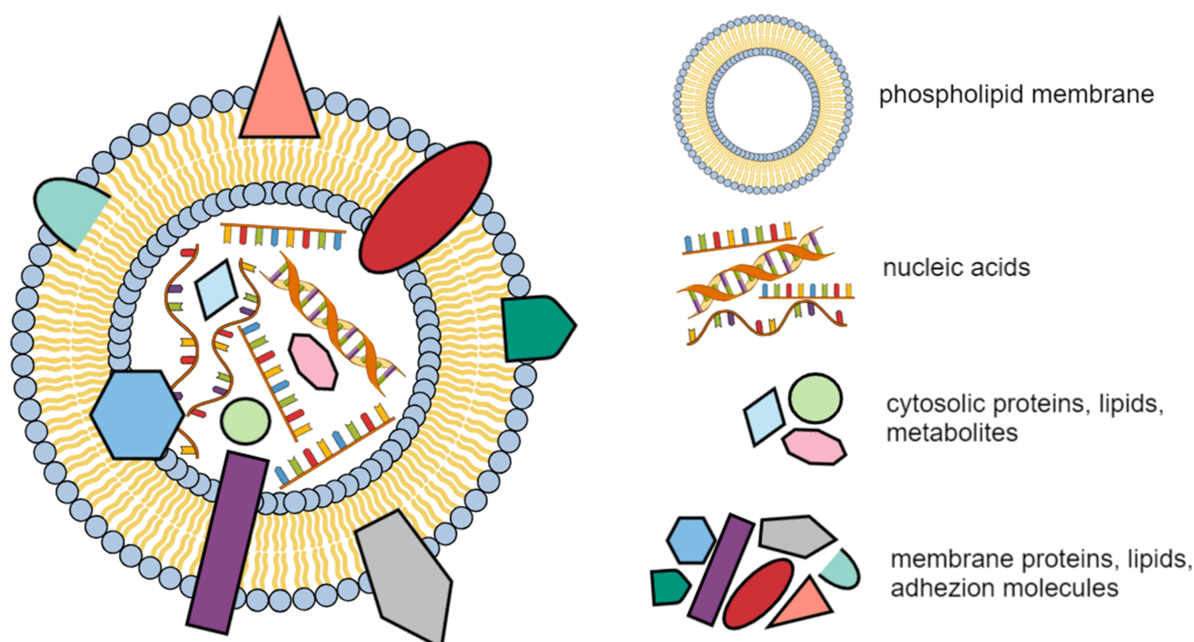

Fig. 1 Exosome structure visualization

The role of exosomes has been studied under the different patho-physiological conditions. Cancer-derived exosomes activate signaling pathways, promote tumor growth, affect immune responses, and deliver tumor antigens to dendritic cells to provoke T-cell-mediated antitumor responses. Exosomes derived from mesenchymal stem cells fulfil a protective role in stroke models. Exosomes are also involved in the release and propagation of misfolded proteins, contributing to neurodegenerative diseases like Huntington's, Alzheimer's, and Parkinson's disease [12–14].

Methodological strategy applicable for exosome isolation

In exosome-related research or in clinical practice, there is a great demand for a simple, fast, and highly effective method which provide the exosomes in sufficient purity and in maximal yield. Despite the various approaches available today [15], there is no consensus with respect to the best methodology for isolating exosomes with substantial yields and reliable quality.

Because exosomes are secreted into the extracellular space, they are found in body fluids, such as serum, plasma, urine, saliva, breast milk, amniotic fluid or cerebrospinal fluid [16]. The liquid nature of biological material facilitates the process of exosome isolation. The principles of the methods used today rely on traditional separation techniques applied routinely to isolate bioactive molecules, organelles, or whole cells. A different strategy is chosen if one wants to perform isolation and structural analysis of exosomes, or when there is a need for sufficient amount of intact exosomes for the subsequent functional tests.

Methods can be divided according to the separation mechanism into two groups – physicochemical and affinity-based approaches. It should be also stressed that different methods provide products of different quality (purity, intactness) and quantity. The higher yield or purity of exosomes can be increased by combination of two or three separation steps of different separation principles. Other parameters, such as the amount of biological material, the cost per one analysis, the proper equipment of the laboratory and the experience of a research team, all that must be taken into the account.

Methods based on physicochemical principles

Separation techniques exploiting the differences in physicochemical properties of various components to be separated. Parameters as solubility, charge, molecular size, shape, and polarity of compounds are the most useful in this respect. Usually, more complex separation procedures are required for multicomponent samples. This section is intended to give an overview on the methods already proven for the isolation of exosomes from the body fluids mentioned above.

Ultracentrifugation

Ultracentrifugation (UC) is a common method for pelleting various substances, including lipoproteins, protein complexes, and aggregates, with a g-force. However, UC in traditional arrangement is time-consuming and labor-intensive [17], in practice, differential UC and UC in a density gradient have proven themselves for exosome isolation.

Differential ultracentrifugation (dU; Figure 2), also known as the pelleting method, involves sequential centrifugation cycles with varying forces to separate extracellular components based on their density [18]. The first step includes centrifugation at 300–400 × g for 10 minutes, which leads to cell pelleting. The next step is centrifugation at 2,000 × g for 10 minutes to remove cell debris and then at 10 000 × g to exclude structures with higher density than exosomes, like apoptotic bodies and biopolymer aggregates. The final centrifugal step runs at 100,000 to 200,000 × g for 1 to 2 h [16,18]. In case of high sample heterogeneity, fractions with exosomes result in lower purity [18].

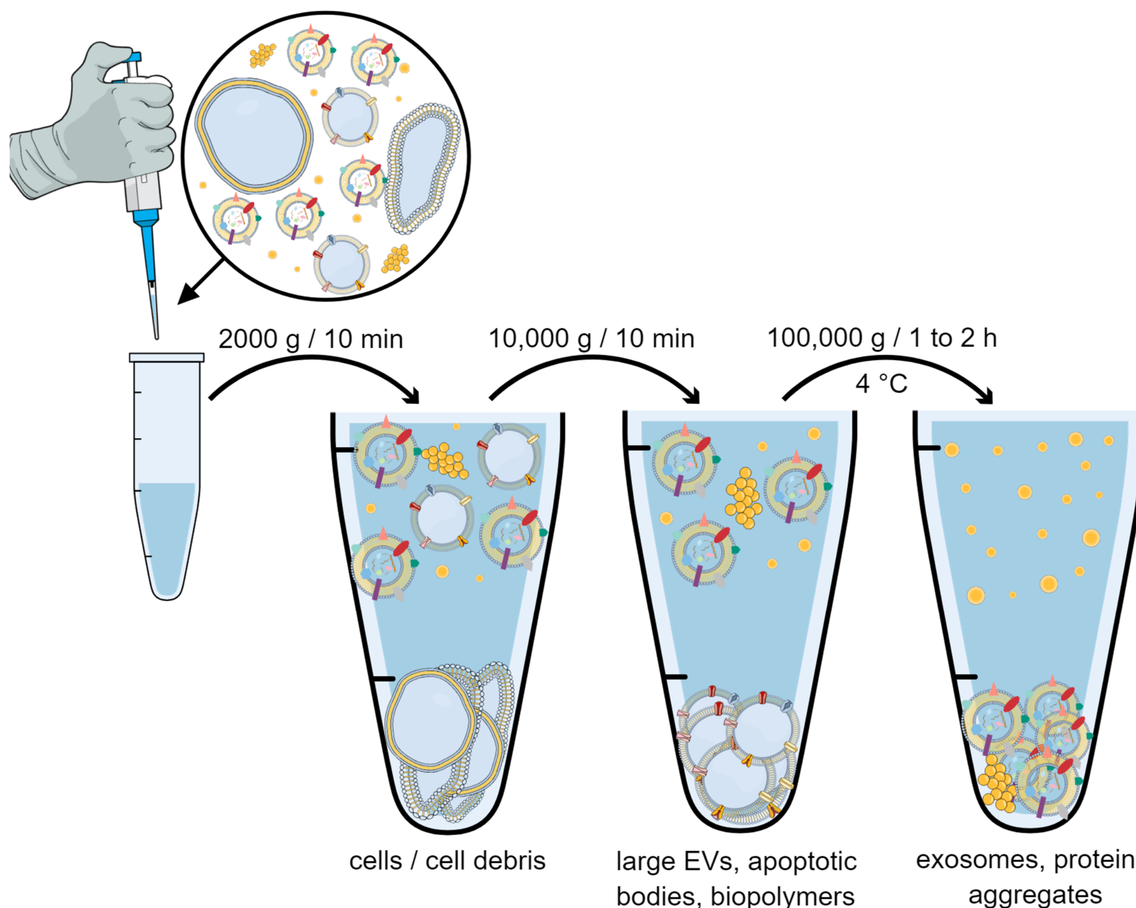

Fig. 2 Differential centrifugation for exosome isolation

Density-gradient ultracentrifugation (dgUC) is a technique when sample with exosomes is applied on the top of the density-gradient medium and then centrifuged at 100,000 to 200,000 × g. This centrifugal force causes the exosomes to move through the gradient at different rates, resulting in distinct zones of separated particles [19]. A continuous gradient is preferred for analysis, while discontinuous gradients are better for exosomes to be harvested. The limitation of this method is smaller loading capacity compared to other centrifugation methods [20].

Ultrafiltration

Ultrafiltration (UF) (Figure 3) is an approach in which extracellular vesicles are effectively differentiated by size using membranes with pores at different molecular weight cut-offs (MWCO). At first, a sample is filtered through a 200 nm filter to remove larger particles than exosomes. A 500 MWCO filter is used for elimination of soluble proteins, and finally, fractions with enriched exosomes are concentrated with a 20 nm filter. Despite its popularity for efficiency and simplicity, this technique has some limitations, including vesicle clogging and potential deformation or fragmentation due to incorrect transmembrane pressure [19,21]. Visan et al. (2022) presented special kind of ultrafiltration called tangential flow filtration. They found this method highly reproducible, time efficient, decreasing clogging concerns and reducing structural damage of exosomes in comparison with common (dead-end) filtration [22].

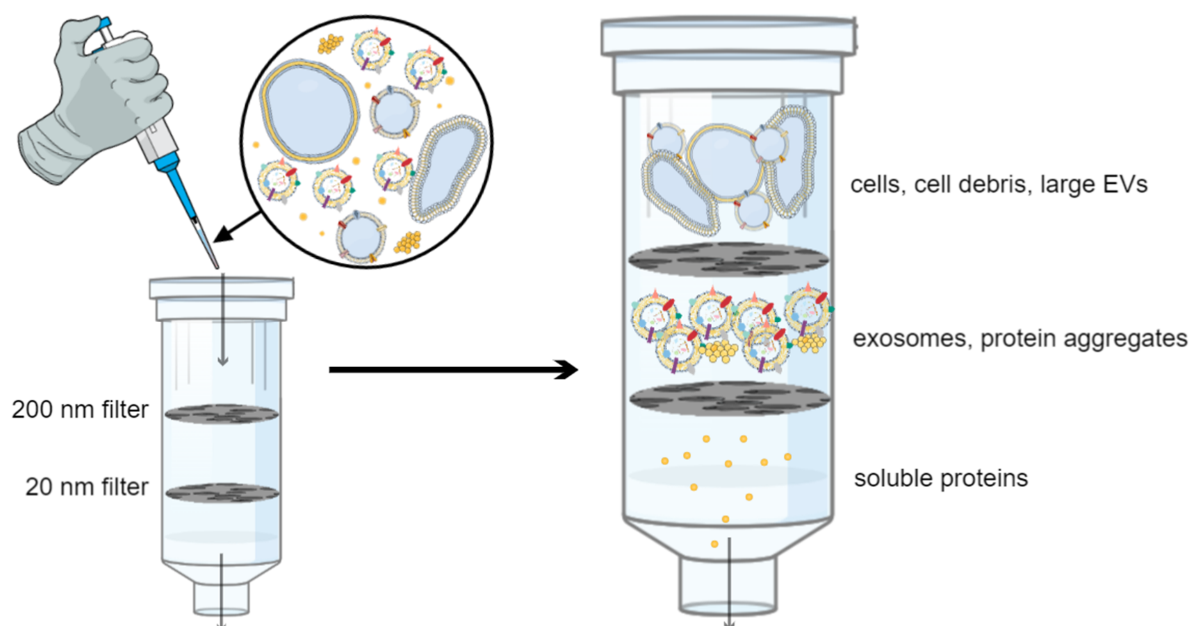

Fig. 3 Ultrafiltration method for exosome isolation

Precipitation

The next separation strategy utilizes the change in extracellular vesicles (EVs) solubility resulting in their precipitation. This technique involves mixing the sample with precipitation-based reagent polyethylene glycol (PEG), incubation, and subsequent low-speed centrifugation to separate the precipitated EVs (Figure 4). Despite its simplicity, this approach often provides fractions with exosomes that are contaminated by proteins, lipoproteins, and nucleoproteins [18,23].

Another precipitation method involves the use of protamine, a positively charged molecule precipitating negatively charged EVs. The combination of protamine with PEG enables a more efficient precipitation and less contaminated EVs fractions. The drawbacks as residual protamine contamination and longer processing time are balanced by obtaining the intact EVs with preserved biological activity [24,25].

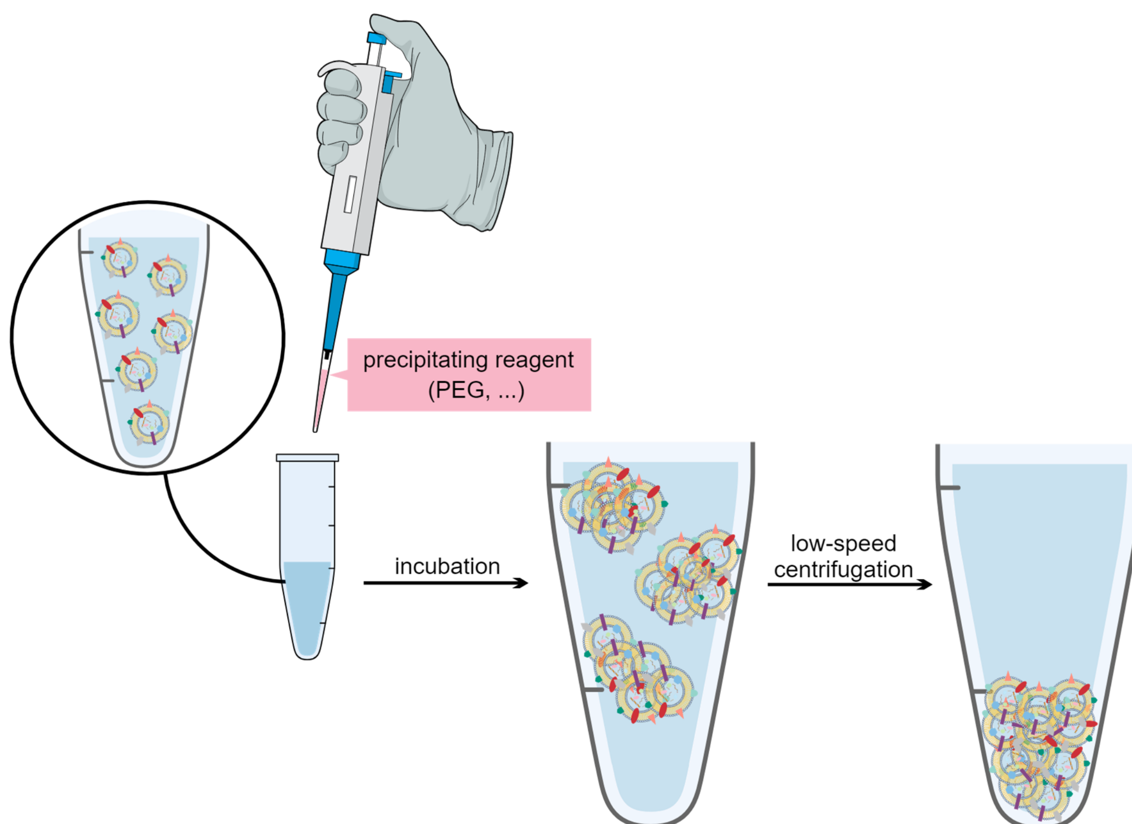

Fig. 4 PEG-based precipitation method for exosome isolation

EV precipitation by sodium acetate is based on neutralizing the surface charge of EVs, causing them to precipitate via hydrophobic interactions [11]. In 2014, Brownlee et al. [26] showed this method working well at specific pH and buffer. Still, it can lead to contamination of the EV fraction with non-EV proteins, especially when isolating EVs from biological fluids like plasma or urine.

A more recent approach involves precipitating by the organic solvent, known as PROSPR (PRotein Organic Solvent PRecipitation). This method was optimized by Gallart-Pallau et al. in 2015 [27]. The isolated EVs were at higher purity, low-protein contamination, and matches well-known exosomal markers. However, there are concerns about EV aggregation into multivesicular structures when using this method, potentially impacting its efficacy [23,27].

Size-exclusion chromatography

Size-exclusion chromatography (SEC) is a size-based separation technique that has been applied to isolate the components differing in molecular weight. SEC involves passing a liquid sample through a column filled with particles of desired porosity. Smaller molecules enter the pores and are slowed down, while larger molecules are eluted earlier as they cannot enter the pores [28] (Figure 5). With the discovery of exosomes, SEC has been adapted for their separation. One of the main advantages of SEC is its ability to preserve the natural biological activities of exosomes. SEC is compatible also with small volumes of high-concentrated samples, requires minimal pre-treatment, and allows one fine-tuning pore size for specific exosome subpopulations [29,30].

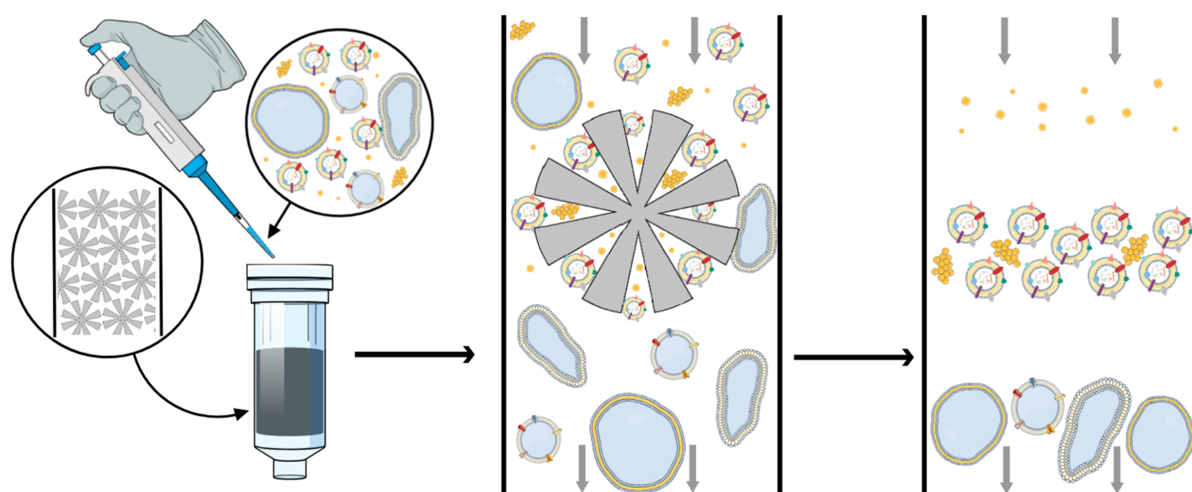

Fig. 5 Size-exclusion chromatography method for exosome isolation

However, SEC faces challenges. Exosomes isolated via SEC may display a wider size distribution, suggesting contamination with similarly sized particles like protein aggregates and lipoproteins [17]. In 2015, Baranyai et al. compared exosome isolation with ultracentrifugation and SEC and they found out higher yield of exosomes with UC with high contamination of albumin. On the other hand, SEC enabled great albumin depletion and gave undamaged exosomes, but with lower yield [31]. Combined strategies involving ultrafiltration and SEC have been

proposed and practiced addressing this, leading to the improved exosome purity and functionality [17]. Despite these challenges, SEC-based exosome isolation is gaining popularity for its versatility and ease of use [32].

To improve exosome isolation, combination of several techniques is used. For example, Franco et al. used SEC followed by ultrafiltration [33] for extracellular vesicle isolation or Qian et al. combined ultrafiltration with polymer precipitation [34]. In 2022, Visan compared ultracentrifugation with tangential flow filtration, both connecting with size-exclusion chromatography [22].

In general, combinations of two or three physicochemical techniques becomes more and more popular, especially if such combination includes SEC, as showed by Monquió-Tortajada in 2019 [35].

Methods based on affinity interactions

Compounds to be separated are passed by mobile phase through a solid stationary phase inside a column and then separated according to their affinity to mobile/stationary phases. The nature of this interaction allows one a temporary binding and subsequent release of analyte.

TiO_2 microparticles-based exosome isolation

Within exosomal systems, the principal constituents of the lipid bilayer are amphiphilic phospholipids. These lipids contain hydrophilic phosphate head, which is positioned on the outer surface of the membrane. Exosome isolation was carried out by exploiting this feature using electrostatic interactions between positive charge of TiO_2 particles surface and negatively charged phosphate groups on the exosomal membrane surface (Figure 6). The TiO_2-based isolation approach achieved remarkable separation efficiency [36–38]. Table 1 shows the advantages and disadvantages of this method.

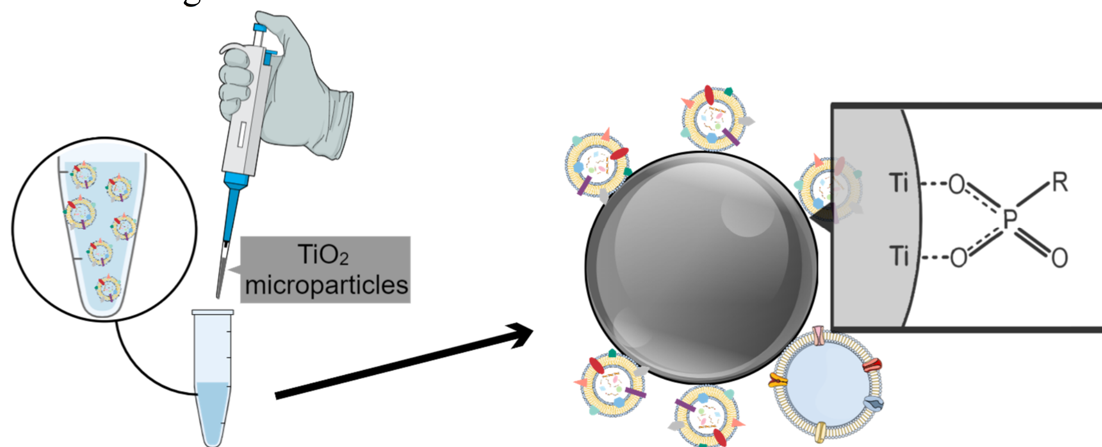

Fig. 6 Exosome isolation with TiO_2 microparticles

Targeting negative charge of exosome surface with poly-L-lysine

Aiming at the negative charge with other molecules than titanium dioxide is also possible. A novel ion-exchange platform called ExoCAS-2 was developed and tested by Kim and Shin in 2021. In this method, magnetic beads functionalized with poly-L-lysine are incubated with filtered plasma. The positively charged poly-L-lysine-coated beads readily attract the negatively charged exosomes through the electrostatic interactions. Subsequently, a magnet is used to catch the exosome-captured beads, followed by the removal of excess liquid [39].

Exosome isolation by immunoaffinity principle

Immunoaffinity-based exosome isolation capitalizes on the presence of common protein markers or receptors on exosomes surface. This technique exploiting the benefits as high specificity and selectivity of molecular recognition principle between specific antibodies, mostly IgG molecules, and antigens, in this case (Figure 7). Some of exosome membrane components unique to exosomes/oncosomes and absent in the extracellular fluid serve as a basis for this isolation method. Over the years, various exosome markers have been identified, including transmembrane proteins [40], heat shock proteins [41], growth factor receptors [42], fusion proteins [43], and lipid-related proteins [19]. Transmembrane proteins like Rab5, CD9, CD63 [44], CD81 [41], CD82, annexin [45], and ALIX [46] have been extensively used for selective exosome isolation, leading to the development of popular exosome isolation products [19].

The use of antibodies against markers overexpressed on tumor-derived exosomes enables to specifically isolate exosomes from both cell culture medium and clinical samples. Commercial systems designed to isolate specific exosome subpopulations then allow one targeted investigations and disease diagnosis [47,48]. Tauro et al. compared already in 2012 immunoaffinity-based approach using anti-EpCAM antibodies combined with ultracentrifugation methods. Immunoaffinity capture outperformed ultracentrifugation yield in exosome isolation by at least twice more effectively [49].

Exosome isolation by microfluidic platform

Methods described in the two previous chapters provide suitable tools how to obtain exosomes in desired purity and quality. However, it is always necessary to consider time consumption, cost, yield/recovery, laboratory equipment, availability, and quantity of biological material. Recently, the innovations in the form of microfluidic systems, small laboratory system with integrated micro-/nano-structures used for separation of exosomes [50], have been appearing more and more frequently.

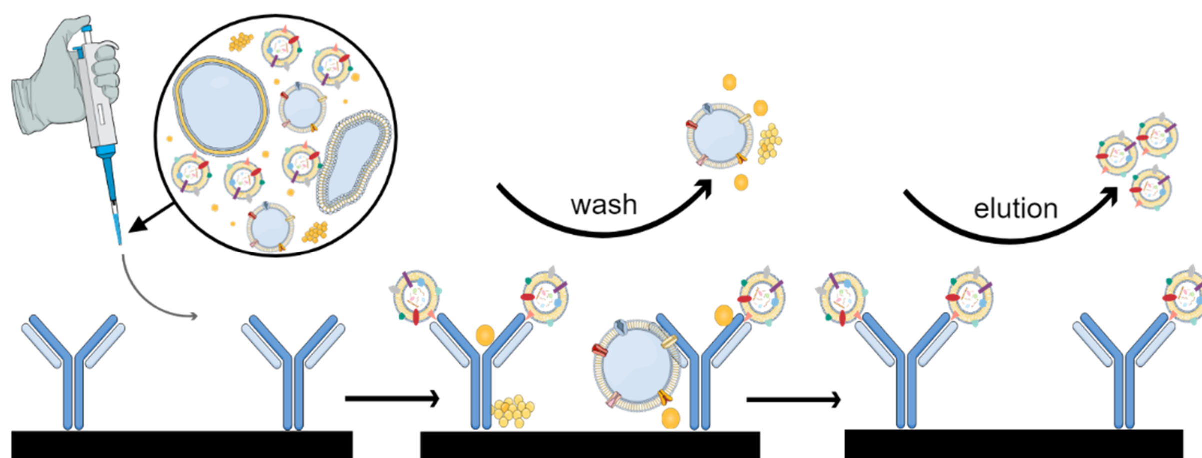

Fig. 7 Exosome isolation with specific antibody

Most microfluidic devices are closed-loop system, in which the flow can be precisely controlled [51]. This bring a considerable number of advantages in terms of reducing of diffusion, more efficient interactions between an analyte and micron-sized structures of the microfluidic chip, reducing the required amount of input material (sample, reagents), as well as duration of the whole process [52–56]. The recovery is significantly higher without the risk to increase the level of contamination. Therefore, conventional methods of isolating exosomes are increasingly being transferred into a microfluidic setup [57].

For example, Davies et al. used nanoporous membranes in microfluidic filtration system for small vesicles isolation [58]. Dehdhani et al. obtained a new microfluidic device (Figure 8) based on tangential flow filtration approach [59]. Chen et al. developed an efficient exosome purification device using negative pressure oscillation and double coupled harmonic oscillator-enabled membrane vibration [60] and many other microfludic platforms have been developed specifically for exosome isolation [61–68].

Tamarin et al. (2021) describe microfluidic technologies figuring in label-free exosome isolation via the mechanism of separation into the following categories: sieving, electrical, viscoelastic, inertial, centrifugal, acoustic, deterministic lateral displacement separations, flow field and pinched flow fractionations [56].

The integration of separation and enrichment of sample, together with its analysis into one complex microfluidic platform, avoids sample loss and cross-contamination. That is welcome and substantial especially in clinical practice. This combination of separation and analysis within one microfluidic pattern is called "lab-on-a-chip", having been highlighted by Surappa et al. in 2023 [57]. Despite the number of advantages, microfluidic techniques also have some limitations, for example, higher acquisition costs associated with the need for specialized equipment compared to other isolation techniques, or, among others, low throughput [69].

For greater clarity, the pros and cons of long-established methods are summarized in Table 1. The microfluidic configuration of these, pointing out its advantages, are also included.

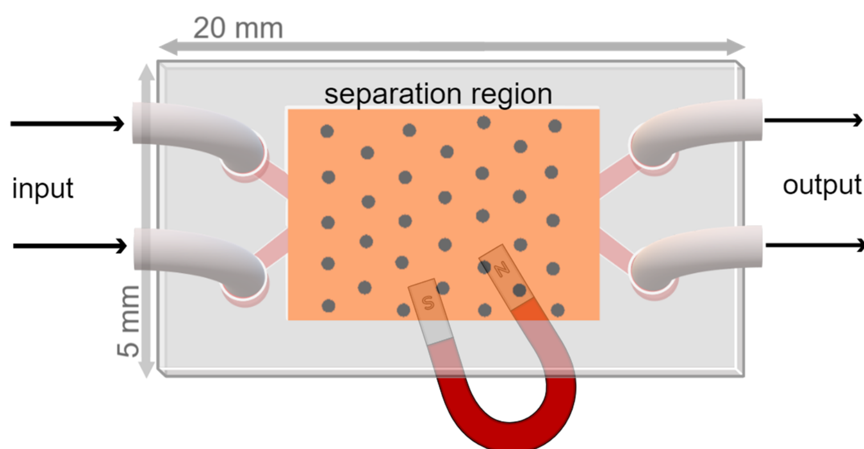

Fig. 8 Schematic representation of microfluidic device – separation region filled with a magnetic carrier biofunctionalized with a specific antibody

Example of one popular configuration

Conclusions

Nowadays, there are various strategies for isolating exosomes. Some are very effective, but expensive. Others yield large amounts of intact exosomes in one separation step, but contaminants are present. However, it is still true that the isolation and purification strategy has to be optimized for different types of biological materials, body fluids [72,80]. The limitations that each of the methods brings can be minimized by their appropriate combination.

Also, the choice of a suitable procedure is often determined by the purpose, for which the isolation of exosomes is carried out, like observing exosome surface with an electron microscope [29,81], testing the membrane composition [82], testing the cytosolic cargo of exosome [83], or functional analysis [84].

The goal of this article is to provide the basic information about methods, separation strategy, including the respective merits and drawbacks. In overall, rapid progress in exosome isolation techniques with respect to our better understanding of these vital extracellular vesicles, accelerating research in diverse fields, from fundamental biology to diagnostics and therapeutics. As technology continues to evolve, further optimization of isolation strategies will undoubtedly contribute to unearthing of the full potential of exosomes and their applications in modern medicine.

Table 1 List of methods for exosome isolation mostly applied in practice

Separation strategy	Principle	Advantages	Disadvantages	Microfluidic configuration advantages	References
differential ultracentrifugation	different pelleting g-force; based on size and density	for large volume samples, low-cost consumables, no/low contamination with isolation reagents	low yield, exosome deformation/degradation, time consuming, protein aggregates contamination, high equipment requirement, for large volumes only	very fast	[16, 18, 20, 49, 70–73]
density gradient ultracentrifugation	different sedimentation speed in various density gradient; based on size and density	high purity, lower exosomal destruction, time consuming, tolerable yield	time consuming, labor-intensive, high equipment requirement, not practicable for small volume samples	–	[16, 19, 20, 49]
ultrafiltration	different size of filter MWCO cut-off	fast, low-cost consumables, low equipment requirement	possible exosome damage, moderate purity, clogging and membrane trapping	high yield, low exosomal damage, high purity	[16, 19, 21, 22, 74]
polymer precipitation	affecting of exosome solubility causing aggregation	high yield, fast, easy, no special equipment required, low-cost, even for large volume samples	high contamination with proteins/protein aggregates and polymeric materials and other extracellular vesicles, complicated following washing-steps, subsequent purification step needed	–	[12, 16, 18, 20, 23–27, 75]

Table 1 List of methods for exosome isolation mostly applied in practice (continued)

Separation strategy	Principle	Advantages	Disadvantages	Microfluidic configuration advantages	References
size-exclusion chromatography	particle retention in porous stationary phase; based on size	high purity, no/low exosomal damage, simple, moderate cost, good reproducibility, no decrease of exosomal biological functions, combinable with affinity-based methods	lipoprotein contamination, additional method for enrichment is needed	high purity	[16, 17, 28–32, 76, 77]
isolation using TiO_2	electrostatic affinity between TiO_2 and phosphate group on exosomal surface	high exosome catch, quick, low cost, wide possible method arrangement	labor-intensive, other extracellular vesicles contamination, subsequent purification step needed	–	[36–38, 78]
isolation using poly-L-lysine	electrostatic affinity between positively charged poly-L-lysine and negative charge of exosomal surface	quick, low cost, wide possible method arrangement	labor-intensive, other extracellular vesicles contamination, subsequent purification step needed	–	[39]
isolation using specific antibody	immunoaffinity interaction between exosomal antigen and specific antibody	low working volume, high purity (quality), no damage of exosomes, no decrease of exosomal biological activity, wide possible method arrangement	low yield, possibly labor-intensive, quite expensive, pre-purification step convenient	high purity, reproducibility	[19, 47–49, 79]

Abbreviations

ALIX	ALG-2-interacting protein X
dgUC	density gradient ultracentrifugation
dUC	differential centrifugation
EV	extracellular vesicle
MWCO	molecular weight cut-off
PEG	polyethylene glycol
PROSPR	protein organic solvent precipitation
SEC	size-exclusion chromatography
TSG101	tumor susceptibility gene 101 protein
UC	ultracentrifugation
UF	ultrafiltration

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