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Progress, challenges, and prospects of small extracellular vesicles isolation and characterization

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ABSTRACT

Exosomes are nanoparticles that can be secreted by almost all cells into the extracellular space and carry active substances such as nucleic acids, lipids, and proteins and can participate in intercellular signaling. Exosomes are consequently used as a natural medicinal ingredient and can also play a role as carriers of biomarkers and drugs. The heterogeneous nature of exosomes suggests that they have considerable potential for diagnosing and treating multiple diseases. However, standardized methods for exosome isolation are still lacking to ensure the yield, purity, and quality of exosomes, which consequently limits their applications. Therefore, isolation methods that produce exosomes with a high yield, purity, and stability and are supported by standardized characterization techniques need to be further developed. In 2018, the International Society for Extracellular Vesicles released guidelines for the isolation and characterization standards of exosomes, and in this review, we have prepared a comprehensive discussion based on these guidelines that describes the biogenesis of exosomes and the principles, advantages, disadvantages, and application prospects of their isolation techniques to provide basic information for the study of exosomes.

1. Introduction

Exosomes are a subtype of membrane-contained extracellular vesicles (EVs) that are 40–200 nm in diameter and that are secreted by cells into their surroundings.[1] Exosomes can be used for treating many diseases as a natural medicine. For example, many studies have found that exosomes are involved in many physiological and pathological processes, such as cancer, inflammation, and atherosclerosis.[2–4] The extensive pharmacological effects of exosomes are due to the cargo of functional molecules, such as proteins, DNA, lipids, and RNA, which have regulatory effects, that they carry and transfer and that play a crucial role in regulating multiple disease process such as tumor growth, metastasis, and angiogenesis and can be used as a prognostic marker, for instance, as a grading basis for patients with cancer.[5] Furthermore, exosomes contain various antiapoptotic, anti-inflammatory, and antioxidant components that hold considerable promise in treating various diseases.[6] As a cell-free therapy, exosomes can be used in advanced drug delivery and therapeutic applications because of their key features such as low immunogenicity, high physicochemical stability, ability to penetrate tissues, and innate capacity to communicate with other cells over long distances. Compared with liposomes and other nano-delivery systems that are synthesized in vitro, the endogeneity and heterogeneity of exosomes provide extensive and unique advantages in disease diagnosis and treatment.[7,8] Thus, therapeutic methods using exosomes are expected to be developed that utilize the advantages of exosomes to overcome the limitations of traditional chemical drugs.

EVs are divided into at least three categories according to their subcellular origin and size: exosomes, microvesicles, which range in size from 100 nm to 1 μm, and apoptotic bodies, which range in size from 50 nm to 5000 nm.[9,10] The concept of "exosomes," which can be produced by virtually any cell in a normal physiological or pathological state, was first proposed in 1981.[11,12] For a long time, exosomes were considered to be a "superfluous substance".[12] However, with the development of exosome isolation technology and analysis of exosome components, the functions of exosomes in disease progression and cellular communication and as drug carriers and diagnostic markers have been elucidated, and

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more and more studies have suggested that exosomes have broad application prospects in immunotherapy and regenerative medicine.

However, several limitations still restrict the expansion of exosomes as therapeutic agents and biomarkers in biomedicine. A relative paucity of studies has described how to ensure the yield, purity, and integrity of exosome isolation. Previous studies of exosome characterization techniques have suffered from notable methodological weaknesses that cannot completely ensure the quality of exosomes. Furthermore, as the heterogeneity among exosomes has not been fully studied, components with similar properties to exosomes are often isolated from biological samples during the isolation process. Therefore, to ensure the quality of exosomes and their clinical application, the choice of exosome isolation techniques is critical, and the isolation method will directly affect the physicochemical properties and content of exosomes.

Notably, because of the limitations of current isolation methods, the ISEV 2018 guidelines recommend that the name "exosomes" be changed to "small extracellular vesicles" (sEVs).[13] Therefore, the name "sEVs" will be used from here onward. This review will compare the different ways that sEVs are isolated by existing methods such as ultracentrifugation (UC), ultrafiltration (UF), size-exclusion chromatography (SEC), polymer precipitation-based methods, immunoaffinity-based methods, emerging microfluidic technologies, and several kit products.[7] Specifically, we focus on the isolation methods of sEVs, analyze their advantages and disadvantages, and then discuss the challenges of the current isolation techniques and prospects for application. In addition, we describe the characterization methods of sEVs to provide a reference for researchers in the isolation and application of sEVs.

2. Biogenesis of sEVs

SEVs originate with the inward budding of the plasma membrane to form early endosomes, whose membranes then partly invaginate and bud into the surrounding lumina with cytoplasmic content to form intraluminal vesicles (ILVs).[14] The late endosomal structures comprising dozens of ILVs are called multivesicular bodies (MVBs), which then fuse with the plasma membrane to release sEVs into the extracellular environment.[15] MVBs are eventually transported to the trans-Golgi network for endosome recycling, delivered to lysosomes for degradation of all carried material, or fused with the plasma membrane and released sEVs into the extracellular space (Fig. 1).[14,16] The endosomal sorting complex required for transport (ESCRT) mechanism plays an important role in processing MVBs and biogenesis of ILVs. The ESCRT mechanism comprises four complexes, termed ESCRT-0, –I, –II, and –III, with associated proteins, including apoptosis-linked gene 2-interacting protein X (ALIX, encoded by PDCD6IP), vesicle trafficking 1 (VTA1), vacuolar protein sorting-associated protein 4 (VPS4), and tumor susceptibility gene 101 protein (TSG101).[5,17] Of these, ESCRT-0, –I, and –II are presumably involved in cargo sorting while ESCRT-III is involved in membrane deformation and fission. Recruitment of the VPS4 complex to ESCRT-III drives vesicle neck scission and the dissociation and recycling of the ESCRT-III complex.[14,18] In addition to this, the biogenesis of sEVs involves non-ESCRT pathways; for example, the pathways mediated by lipids and related proteins, such as the four-transmembrane protein (tetraspanin) on the surface of the cell membrane.[19]

3. Existing isolation methods for sEVs

Given their multiple functions and regeneration potential, it is critical to obtain sEVs with high yield, purity, and quality. Currently, many isolation techniques for sEVs isolation have been developed based on their biophysical and/or biochemical features, such as size, density, and specific surface markers. However, because of the difference in requirements and the complexity of biological fluids, the choice of isolation techniques must be carefully considered. Furthermore, the interfering presence of non-sEVs must be considered as these can coisolate with sEVs to influence subsequent observations.[20] The most frequently used isolation techniques for sEVs are shown in (Table 1) and discussed hereafter.

3.1. Ultracentrifugation

UC uses a centrifugal force of 100,000×*g* to effectively isolate small particles such as viruses, bacteria, and organelles.[31] UC can be broadly classified into three types based on the key mechanism used: differential centrifugation (DC), iso-density-gradient centrifugation, and rate zone centrifugation (RZC). UC has long been considered the "gold standard" for the isolation of sEVs.[31] However, the complexity, specific composition, and physical properties of each biofluid constitute a technical barrier to obtaining reproducible and pure sEVs preparations.[32] Furthermore, the rotor type, g-force, and centrifugation times significantly influence sEVs yield during centrifugation-based isolation procedures.[7,33] Therefore, the selection of appropriate UC techniques is crucial for the isolation of sEVs.

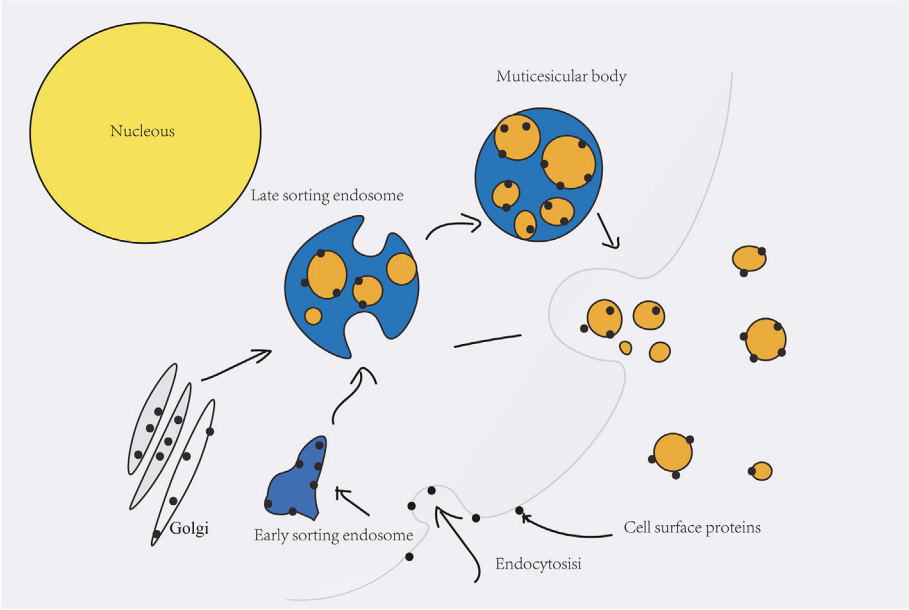

Fig. 1. Biogenesis of sEVs.

Table 1
Principles, advantages, and disadvantages of sEVs isolation techniques.

Isolation techniques	Principles	Advantages	Disadvantages	Reference
Differential centrifugation (DC)	Size Density	Easy to operate Simple equipment Allows both low and large sample sizes	Expensive equipment Labor-intensive Potential destruction of sEVs	21
Size-exclusion chromatography (SEC)	Size	Reduce processing time Increase throughput Does not require special equipment	Cracking Deformation of sEVs	22
Ultrafiltration (UF)	Size	High yield Reproducibility Low time and cost	Low purity Clogging	23–25
Polymer precipitation	Hydrophilicity of polymer	Simplicity Rapidity Nonspecialized handling Unnecessary for expensive equipment	Low purity	26,27
Immunoaffinity-based isolation technique	Antibody-antigen binding	High purity	Expensive antibody Low-sample volume	28
Microfluidics	Viscoelastic Acoustic Electrical	Simplicity Cost-effectiveness Precise Fast processing	Low-sample volume Clogging	29,30

3.1.1. *Differential centrifugation*

This technique relies on the sequential isolation of particles by sedimentation, which is dependent on their size and density, by using a series of centrifugal forces and duration.[34,35] DC is performed through multiple cycles of centrifugation with centrifugal forces between 300 and 100, 000×*g* 4 °C.[36] Cells, cell debris, and apoptotic bodies are sequentially removed by controlling different centrifugal forces and centrifugation times. After the final centrifugation (i.e., 100,000×*g*), sEVs are collected by removing the supernatant (Fig. 2). The DC method is easy to operate, has simple equipment, and allows both low and large sample sizes, and consequently has been widely used for isolating sEVs. However, the DC method has several limitations such as being time-consuming, requiring expensive equipment that is cumbersome to operate, and disrupting the integrity of sEVs at too high a rotational speed.[37] Furthermore, some lipoproteins can coprecipitate with sEVs.[38] Therefore, density-gradient centrifugation was derived to improve the purity of sEVs.

3.1.2. *Density-gradient centrifugation*

Two types of density-gradient ultracentrifugation are used: isopycnic ultracentrifugation and RZC.[39] DC often suffers from contamination and sEVs losses because of the heterogeneity of sEVs and considerable overlap in the size of extracellular vesicles. UC is often coupled to isopycnic or RZC techniques to allow the sEVs of relatively low densities to float for further purification. This technique can also improve the quantity of sEVs isolated. A typical density-gradient UC includes the following steps: first, layers of biocompatible medium with varying densities (e.g., iodixanol or sucrose) covering the range of particle densities in the sample are placed into a tube, with gradually decreasing densities from the bottom to the top. The sample of interest is then added onto the top of the density-gradient medium, followed by extended centrifugation for a prolonged period (e.g., 100,000×*g* for 16 h) (Fig. 3).[38,40] Compared with ordinary UC, density-gradient centrifugation effectively improves the purity of sEVs.[41] For example, bone marrow cells were isolated from

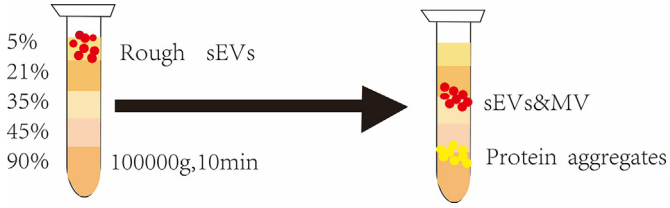

Fig. 3. Isodensity gradient centrifugation.

porcine bone marrow by density-gradient centrifugation.[42] However, the sucrose-density-gradient UC isolation process is time-consuming and requires a large amount of biological sample. The EVs subpopulations cannot be distinguished, and large vesicle populations may be lost by DC.[43] Kuipers et al.[44] found that for centrifugation processes in small-density gradients, the use of iodixanol is preferred over sucrose as the sEVs can reach equilibrium in an iodixanol gradient at a relatively faster rate. Consequently, the optimization of sEVs isolation can be improved by selecting a suitable inert medium and thus improving the isolation results.

3.1.3. *Rate zone centrifugation*

RZC is an isolation method based on the size and density of sEVs. With RZC, the gradient has a lower density throughout the entire gradient compared to density-gradient centrifugation to ensure that the distance a particle travels through the gradient is solely dependent on the particle diameter.[45] RZC comprises two gradient media with densities less than the sEVs, and upon addition of the sample, the sEVs will settle to the bottom of the tube under centrifugal force to achieve isolation. However, because of the low densities of the two media, after a sufficiently long centrifugation time, all components with other densities will settle to the bottom. Therefore, for RZC, time control of the isolation is crucial.[40,44] In

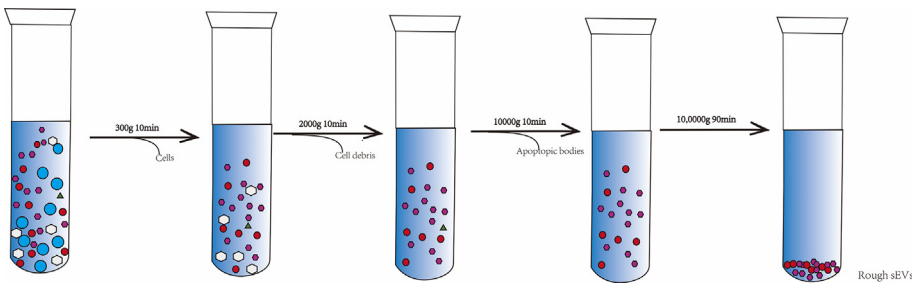

Fig. 2. Differential centrifugation.

addition, the medium used affects the isolation efficiency of sEVs. This also increases the operation time and makes the operation procedure more cumbersome, and because of the addition of inert media, the viscosity of the sample solution is increased, which consequently prolongs the time required for the settling of sEVs. In addition to this, the hypertonic medium may damage the integrity of the sEVs.

3.2. Size-based isolation methods

3.2.1. Ultrafiltration

UF, which isolates sEVs by using membranes with different molecular weight cutoffs (MWCO), is an isolation method based on the size of the substance and is generally used in combination with other methods for further purification (Fig. 4A).[46,47] Compared to UC, UF increases vesicle isolation to significantly reduce processing time and increase throughput but does not require special equipment.[23–25] However, UF is prone to cracking as well as deforming sEVs because of the shear force, causing a loss of sEVs. Based on the principle of UF, tangential flow filtration (TFF) and sequential filtration methods were derived. In the TFF mode, the sample fluid enters in a direction parallel to the membrane, which regulates the transmembrane pressure, reduces the loss of sEVs and the clogging of the membrane and prolongs the service life of the membrane (Fig. 4B). TFF surpasses UC in terms of throughput, reproducibility, time, cost, and scalability.[48] Sequential filtration is another commonly used method where the sample is first passed through a 1000-nm filter to remove cellular debris as well as apoptotic vesicles and then through a second filter with a 500-kD MWCO for TFF to remove free proteins. Finally, 50–200-nm diameter material can be passed through a 200-nm filter for isolation (Fig. 4C). This method has the advantages of being gentle, automated, and suitable for large particles and can be used to produce uniformly sized sEVs, which largely ensures their integrity and purity.[49]

To overcome the limitations of conventional filtration, Chernyshev et al.[50] developed asymmetric depth filtration (DF) as an easily accessible method with high yield and low contamination of sEVs. Conventional filtration is generally categorized into surface filtration and DF. During surface filtration, large particles are retained because of their size, and a "cake" of these particles will eventually form on the surface of the filtration medium, whereas with DF media, large voids allow particles to enter the pores of the medium. Consequently, the asymmetric DF method is based on the principle that small particles can be eluted as the sEVs are immobilized on the surface and within the depth of the porous media. This method is suitable for isolating therapeutic sEVs from large volumes of growth medium used to culture EV-secreting producer cells.

3.2.2. Size-exclusion chromatography

SEC involves the use of a stationary phase held in a column that allows a liquid mobile phase containing the analyte in an aqueous buffer solution to pass through and leave the column at a rate proportional to its size, or more accurately the hydrodynamic volume.[51,52] SEC is another method of isolation based on size, where large molecules cannot enter the gel pores and elute along the gaps between the porous gels with the moving phase, whereas small molecules are retained in the gel pores and ultimately eluted in the mobile phase.[53] SEC can remove most of the overabundant soluble plasma proteins that are not discarded using UC or precipitating agents while being more user friendly and less time-consuming than gradient-based EV isolation. SEC can also maintain the major characteristics of sEVs, including their vesicular structure and content, which guarantees their use in subsequent applications.[22] Tsutsumi et al.[54] found that a higher recovery and purity of sEVs were achieved by using SEC after polymer precipitation and DC. Thus, higher purity of sEVs can be obtained by coupling SEC with other methods, such as UF and UC.[55–57] Yang et al.[58] found that combining SEC and UC methods could skillfully solve the shortcomings of the SEC isolation, resulting in a higher purity of sEVs obtained from serum. Comparing SEC and UC, Soares et al.[59] found that, although both techniques isolated sEVs, UC yielded a significantly higher number of particles while SEC produced sEVs that were purer, with fewer protein contaminants or aggregates.

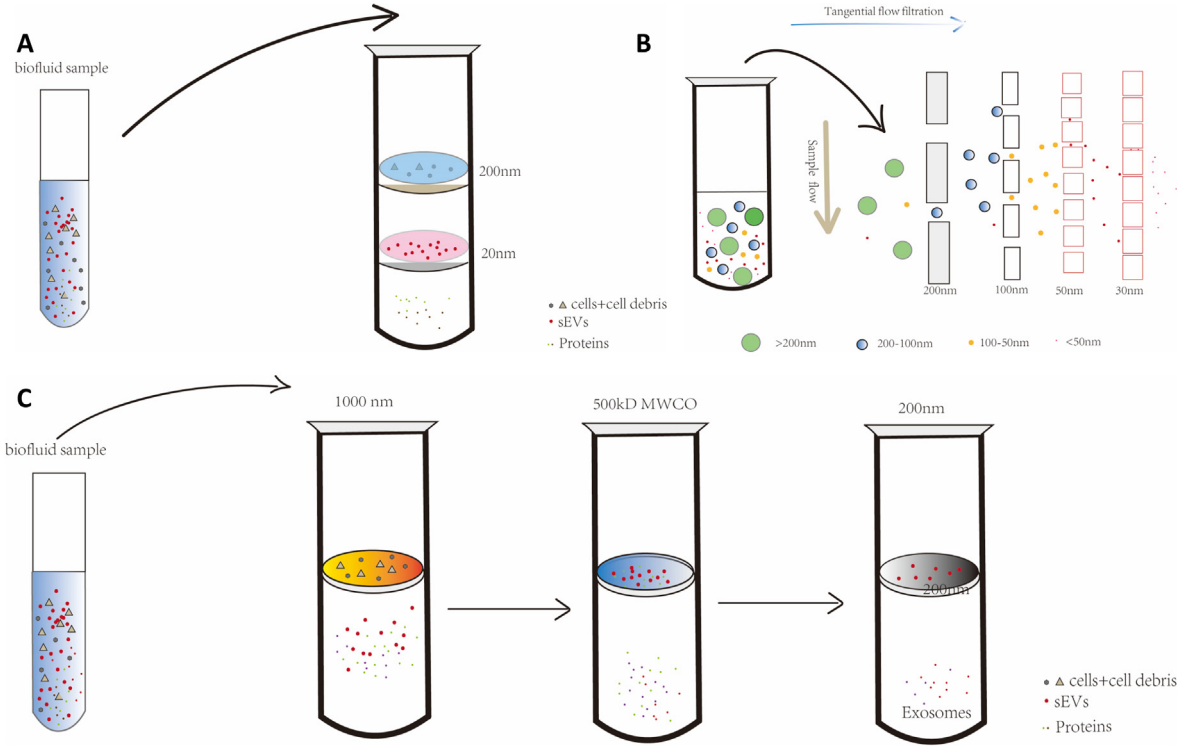

Fig. 4. Isolation methods for sEVs. A: UF; B: TFF; C: sequential filtration.

3.3. Polymer precipitation

The precipitation method is performed by adding polymers to the sample to be isolated and then precipitates by forming a mesh structure with water molecules and sEVs during centrifugation at a low speed (Fig. 5).[60] This method was first used to isolate viruses and was applied for extracting and isolating sEVs because of the similarity in size between viruses and sEVs. Either natural (e.g., chitosan) or synthetic polymers (e.g., polyethylene glycol; PEG) can be used, and PEG is the most commonly used as this has low toxicity and can be altered to be water-soluble. Rider et al.[61] proposed an optimized PEG precipitation method: "ExtraPEG," which allowed for the rapid and large-scale enrichment of sEVs; the isolated sEVs were identified as not being biologically compromised and outperformed those purified using a commercially available kit (ExoQuick). Natural polymers are more biocompatible, nonimmunogenic, and biodegradable, and have lower toxicity than synthetic polymers, and therefore will be more suitable for clinical production. Kumar et al.[62] demonstrated that chitosan can be used to isolate sEVs from a wide range of biological samples by interacting with the sample to form a chitosan-sEV complex, which then undergoes sedimentation via centrifugation. However, natural polymers are more expensive. Overall, polymer precipitation has the advantages of simplicity, rapidity, and nonspecialized handling without the need for expensive equipment, which makes this a suitable isolation technique for large-scale production. Although the yield of sEVs isolated by PEG precipitation is considerably improved, the degree of contamination is also increased and results in a low purity. This consequently affects the downstream analysis of sEVs and further purification via SEC is required. Currently, kits developed based on polymer precipitation include the ExoQuick kit from System Biosciences (Palo Alto, CA, USA) and the Total Exosome Isolation Kit from Thermo Fisher Scientific.[63]

3.4. Immunoaffinity-based isolation

Immunoaffinity-based isolation is a technique based on the specific binding of antigens to antibodies. sEVs have an abundance of surface proteins that can be used as antigens, such as the family of quadruple transmembrane proteins (CD9, CD63, CD81, and CD82). As such, several affinity-based EV capture approaches have been employed, making use of solid surfaces such as chips or beads coated with antibodies,[64,65] aptamers,[66] and even peptides.[67] sEVs can be isolated by immobilizing the antibodies to these specific markers on a carrier, which binds to sEVs on contact with the carrier with isolation achieved via elution (Fig. 6). For example, benefiting from the presence of an adsorption window between sEVs and proteins under the effect of a hydrophilic polymer, sEVs tend to selectively adsorb onto the surface of magnetic beads and can be isolated from biological fluids with high purity by simple magnetic separation.[68] The technique can also be used for the sorting of highly heterogeneous sEVs, where different subpopulations of sEVs can be captured by directly targeting different surface markers.[69] Affinity-based methods for sEVs isolation can produce highly specific and efficient isolation results. However, literature is lacking that summarizes these methods and their effects on the downstream molecular analysis of sEVs.[28] Benecke et al.[70] found that the sEVs obtained by the immunomagnetic bead-based method "EXÖBead" were of a higher purity as compared with those isolated via size-exclusion methods. However, the relatively high price of antibodies, the long binding time of antibodies to the surface antigen of sEVs, and the elution conditions will directly affect the isolation effect, and the choice of eluent will also affect the structure of sEVs. In addition to this, the number of isolated sEVs can be limited by the number of binding molecules available, increasing the difficulty of isolating intact sEVs from antibody beads. Yoshida et al.[71] developed a TIM4-affinity isolation method that targets phosphatidylserine (PS), a component of the sEVs membrane. TIM4 binds to PS via Ca^{2+}-binding to PS, which allows intact sEVs to be eluted from TIM4 beads in the presence of the chelating reagent ethylenediaminetetraacetic acid. The TIM4-affinity isolation method helps to overcome the limitations of the affinity isolation method and allows for the isolation of heterogeneous sEVs at a high purity.

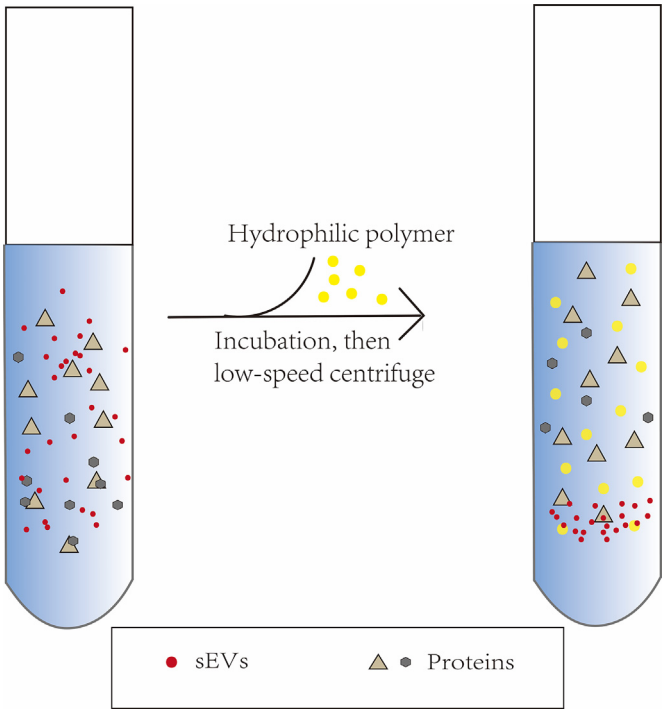

Fig. 5. Polymer precipitation method.

3.5. Microfluidics

Microfluidics is an automated platform that integrates the isolation, detection, and analysis of sEVs. Currently, several commercial sEVs isolation kits have been developed based on different principles such as charge neutralization-based precipitation, gel-filtration, and affinity purification using magnetic beads.[72] The affinity purification technique achieves automated specific isolation by immobilizing magnetic beads and nanowires loaded with specific antibodies and aptamers onto a chip to which the sample is added as mentioned above (2.4). The other techniques can be categorized into passive and active isolation, with passive isolation involving the use of mechanical filtration and hydrodynamic aggregation while active isolation encompasses methods that use magnetic, electric, and acoustic fields.[73–75] For example, Kanwar et al.[76] developed an isolation device called "ExoChip," which is a microfluidic device composed of polydimethylsiloxane and functionalized with CD63 antibody to achieve specific isolation. Active isolation is exemplified by acoustics, where the isolation of different particles is based on the differences in their size and density, which is achieved by injecting the sample into the sample chamber under the action of ultrasound. Microfluidics have advantages in terms of affordability, purity, sensitivity and specificity, simplicity, rapidity, adjustability, gentleness, and compatibility with limited sample volumes when compared with traditional techniques.[77,78] However, microfluidic techniques are often not applicable to large sample volumes and are prone to clogging.

4. Characterization of sEVs

Existing methods of sEVs characterization are shown in Table 2.

4.1. Electron microscopy

Electron microscopy (EM) is a key technique used for characterizing

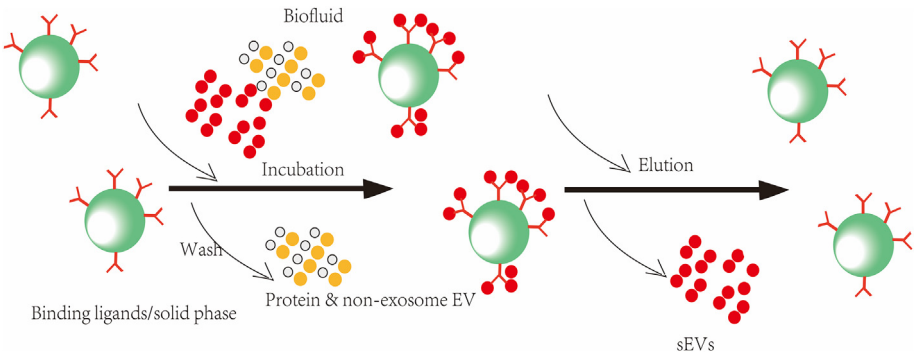

Fig. 6. Immunoaffinity capture.

Table 2
Characterization techniques and their advantages and disadvantages.

Characterization techniques	Advantages	Disadvantages	Applications	Reference
Electron microscope (EM)	Detect and characterize individual sEVs Small sample volume	Time-cost Intensive-cost Requires a high level of skill in operating	Characterize both the size and morphology of individual vesicles	79
Dynamic light scattering (DLS)	Rapid (minutes)	Not suitable for polydisperse solutions	Size typically in the submicron scale Size distribution	80
Nanoparticle tracking analysis (NTA)	Minimal sample preparation	High sample purity required	Distributions Concentration	81
Tunable resistive pulse sensing (TRPS)	Fast sampling	Clogging Reduced sensitivity	Size Distribution Concentration Surface charge	82–84
Western blot (WB)	Qualitative and quantitative analysis of characteristic proteins	Expensive antibody	Protein profile	85
Flow cytometry	Accurate count	Not suitable for particles ≤200 nm	Surface proteins	86
Proteomic	Distinguish between different EVs	Need to consider sample preparation method and the potential contaminants	Biomarker discovery Quantitative and qualitative research	87
RT-qPCR	High sensitivity and accuracy	Limited multiplex capability	Detect the expression levels of mRNA	88,89
ELISA	High sensitivity Specificity Low-sample volume	Limited by antibody availability	Protein profile	83,90
Biosensors	Sensitive Simple Rapid High-throughput	Only detect a single type of sEVs biomarker	Detect RNAs of sEVs	91

sEVs. In studies of biological samples, two types of EM are widely used: transmission electron microscopy (TEM) and cryo-electron microscopy (cryo-EM).[92] TEM can detect and characterize individual sEVs and is therefore considered a powerful tool for studying sEVs, revealing sample purity and molecular composition. Conventional TEM can capture images with a resolution of 1 nm, but does cause damage to the sample because of the dehydration, freezing, or adsorption techniques used to prepare biological samples.[93,94] Furthermore, the process of EM characterization of sEVs requires expensive equipment and specific training and expertise to manage the equipment and the accompanying software.[79] Significant shortcomings in TEM are eluded using cryo-TEM. Cryo-TEM can visualize surface morphology, making this the method of choice for distinguishing between vesicles based on their respective surface structures, thereby providing a path to differentiating vesicle subpopulations and identifying their size distributions.[95] Cryo-SEM also preserves the water content of these cells but can affect the structure of delicate projecting features and cause them to collapse.[95]

Cryo-electron tomography (cryo-ET) avoids ultrastructural changes and redistribution of elements. Additionally, specimens are protected from damage caused by the electron beam radiation through the application of very low temperatures. Inelastic scattering during low-dosing techniques generates high background noise on images, which can be removed by increasing the signal-to-noise ratio to allow computer-based higher resolution for single-particle analysis.[96,97] The standard method for cell preparation (routine EM; fixation, dehydration, embedding, and sectioning) has been applied to reduce the sEVs-drying effect. Routine EM using plastic embedding can avoid or reduce artifacts (changes in volume and shape) caused by denaturation. For chemical fixation, glutaraldehyde can be used for crosslinking (covalent interactions between amino groups). Usually, osmium tetroxide is used for fixing lipids as well as for improving contrast.[98] In addition to the above, cryo-ET can identify the ultrastructural detail of sEVs with high resolution.[99] However, the application of cryo-ET is typically restricted to regions of specimens that are thinner than 500 nm. The inelastic mean-free path of 300 keV electrons in biological specimens embedded in vitreous ice is > 300 nm.[100]

4.2. *Dynamic light scattering*

Dynamic light scattering (DLS), also known as photon correlation spectroscopy is a powerful tool for studying the diffusion behavior of macromolecules in solution. The diffusion coefficient, and hence the hydrodynamic radii calculated from this, depends on the size and shape of macromolecules.[101] DLS is noninvasive and highly sensitive, uses minimal sample volumes, and can be used to investigate a large number of vesicles; consequently, DLS has been used to analyze the size distributions of nanoparticles, sEVs, and liposomes.[74,102] For example, Jiang et al.[103] used DLS to analyze the size and diameter of sEVs and found that their particle-size distribution was around 120 nm. Compared with single-particle–imaging techniques, DLS can obtain information on many particles in a short period. This allows the study of a large number of samples in a batch. In addition, the amount of sample required is small and reusable. Sample preparation is simple and does not involve any invasive steps. However, DLS provides an average value of relatively uniformly sized particles and therefore is not the best technique for evaluating a heterogeneous solution of sEVs. DLS can measure the

diameter range of analyzed sEVs (1 nm–6 μm) but does not provide biochemical data or information concerning the cell from which the sEVs originated.[104] Notably, DLS is much less accurate for heterogenous mixtures of sEVs and provides less precise data.[105,106] The signal from DLS depends on the size and concentration of the macromolecules. Therefore, optimization of the concentration range of sEVs may be necessary to obtain reliable measurements.

4.3. Nanoparticle tracking analysis

Like DLS, nanoparticle tracking analysis (NTA) is based on the ability to track the Brownian motion of suspended particles. Briefly, during NTA measurements, the sEVs are visualized by the light scattered when irradiated by a laser beam. The scattered light is focused through a microscope onto a camera that records the motion of the particles. The NTA software tracks the Brownian motion of each particle to determine the diffusion coefficient, which is calculated for each particle by using the Stoke-Einstein equation.[107] A typical NTA device comprises a laser module, a microscope connected to a sensitive charge-coupled device or complementary metal-oxide semiconductor camera, a hydraulic pump, and a measurement chamber.[104,108] The use of NTA systems for the detection of different sEVs offers several advantages. The first is the ability to accurately measure small particles with diameters down to 30 nm. Second, the sample collection is performed in the liquid phase, avoiding any variation in the sEVs studied. In addition, sample preparation is simple, with the measurement itself taking only a few minutes. Finally, the sample can be recovered in its natural form after the measurements have been performed, which makes the technique even more attractive.

In addition to the above, the NTA system can also detect fluorescence. This can be used to detect antigens present on sEVs by applying fluorescently labeled antibodies to examine the antigenic composition as well as the size distribution in smaller sEVs, which is not available with other methods, and is expected to be used to monitor phenotypic changes in sEVs in disease.[109] Compared with NTA, DLS slightly favors larger-sized particles when all scattered particles are used for detection, thus swamping the contribution of smaller particles, whereas, in NTA, a more accurate measurement can be obtained by detecting the individual particles.[110,111] However, the NTA technique still has limitations. One is the determination of the dilution of the final sample, and the main obstacle is finding the appropriate dilution required for the NTA camera to record all the sEVs present in the sample and not have the superposition effect of larger sEVs masking the smaller sEVs. As with other methods based on the principle of Brownian motion, the masking of smaller vesicles by larger vesicles would mask their results, generating unreliable data.[112] Another limitation is reflected in the detection of fluorescence signals. Although the NTA system can detect fluorescence, the practical application in sEVs phenotyping is limited as the fluorescence signal must be strong enough to be detected by current NTA systems.

4.4. Tunable resistive pulse sensing

Tunable resistive pulse sensing (TRPS) first appeared in 1976 for detecting and characterizing viruses.[113] TRPS is based on the Coulter principle to detect, measure, and analyze particles at length scales ranging from tens of nanometers to micrometers. With the development of this technique and its associated methods, TRPS is now used as a characterization technique for sEVs.[114] By applying a voltage to a membrane with holes, the sample is moved to one side of the membrane, and as individual particles pass through the holes driven by the differential pressure and voltage, the current through the holes is temporarily reduced because the particles have a higher electrical resistance than the electrolyte, and information about the concentration and size of the sEVs can subsequently be detected. In this case, the concentration is calculated based on the frequency at which the particles pass through the membrane, and the particle size is calculated based on the decrease in current. The membranes used in TRPS are elastic and the pore size can be changed by stretching, allowing optimization of the sensitivity and accuracy of the technique for each sample.[115] TRPS has the advantage of fast sampling compared with optical sensing methods such as NTA. However, TRPS may suffer from pore-clogging and reduced sensitivity when increasing the through-hole transfer rate or decreasing the sampling frequency and bandwidth when quantifying sEVs.[116]

4.5. Western blotting

Western blotting (WB), sometimes referred to as immunoblotting, involves the isolation of natural or denatured proteins by gel electrophoresis, transfer of these separated proteins to a protein-binding membrane, and then detection of the target proteins with target protein-specific antibodies.[85] Typically, sEVs are highly enriched with proteins with various functions such as tetraspanins (CD9, CD63, CD81, CD82), which participate in cell penetration, invasion, and fusion events; heat shock proteins (HSP70, HSP90), which form part of the stress response and are involved in antigen binding and presentation; MVB formation proteins, which are involved in sEVs release (ALIX, TSG101); as well as proteins responsible for membrane transport and fusion (annexins and Rab).[117,118] ALIX, FLOTILLIN, and TSG101 are involved in sEVs biogenesis and are also commonly used as WB-characterized proteins.[119] The 2018 ISEV guidelines highlight three categories of markers that must be analyzed in all bulk EVs preparations to demonstrate the presence of sEVs and assess their purity from common contaminants, but no universal “negative controls” relevant to a particular subtype of sEVs are suggested. The three main categories are transmembrane or GPI-anchored proteins localized at the external membrane of prokaryotic cells and plasma membrane and/or endosomes of eukaryotic cells as representative hallmarks of any type of sEVs, cytosolic proteins (eukaryotic cells and gram-positive bacteria) or periplasmic proteins (gram-negative bacteria), and several proteins that are major constituents of non-EV structures.[13] For example, van de Vlekkert et al.[120] characterized the expression of ALIX, CD9, and CD81 proteins in samples isolated by density-gradient centrifugation to demonstrate the purity of sEVs.

4.6. Flow cytometry

Flow cytometry has also been used to analyze submicron-sized (<1 μm) EVs.[121] Flow cytometry analysis of sEVs follows the same paradigm as lymphocyte analysis, with light scattering used to detect individual sEVs and fluorescence used to detect specific molecular components or physiologically relevant features. Flow cytometry is a powerful single-particle analysis tool, but as its instruments and methods were developed for cell analysis, they are not optimal for the analysis of small, dim sEVs. In addition, the detection limits of commercially available flow cytometers vary considerably. Consequently, flow cytometry for sEVs has not yet gained the same widespread acceptance as flow cytometry for cells. Significant differences are also present in the details of sample preparation and measurement methods, with minimal agreement on standardization, calibration and experimental design principles, and a lack of uniformity in data reporting and archiving.[122]

4.7. Proteomics

With the latest improvements in proteomics techniques, qualitative and quantitative characterization of sEVs proteins has been achieved. Proteomics can be categorized into whole and targeted proteomics depending on the requirements.[123] Proteomics can distinguish between different sEVs by characterizing sEVs proteins and has been used in combination with liquid-mass spectrometry to identify different proteins in sEVs.[124] In addition, proteomics allows for the quantification of proteins in sEVs. For example, Kugeratski et al.[125] used an unbiased quantitative proteomics approach based on Super-stable-isotope labelling by

amino acids in cell culture (SILAC) coupled with high-resolution mass spectrometry to find that Syntenin-1 was consistently the most abundant protein present in sEVs from different cellular origins. Techniques in proteomics or bioinformatics have been applied to understand sEVs heterogeneity, biological function, and molecular mechanism of biogenesis, secretion, and uptake.[126] However, the challenge in protein characterization of sEVs preparations compared with RNA characterization is the lack of amplification programs for the protein. Therefore, a large amount of sEVs must be isolated, which would otherwise affect the analytical sensitivity of sEVs sample testing. In addition, the key issue with mass spectrometry-based proteomics analysis is the sample preparation method and the potential contaminants that can be introduced during this. For example, some commercially available sEVs kits based on the principle of precipitation use substances such as sucrose, which are introduced in density-gradient centrifugation methods for separating sEVs.[43] The proteomic analysis of sEVs surface may hold clues to the mechanism of sEVs formation, secretion, targeting, protein–protein interactions, and host-cell trapping. Increased specificity of sEVs cargos relative to the cell of origin can be realized by examining specific, sorted populations of sEVs. By enabling cell-specific sEVs analysis, a higher specificity in biomarker detection can be achieved. Finally, proteomics techniques are frequently being used to identify new disease biomarkers. As analytical sensitivity increases, new markers will be detected, and new sensitive and specific assays will be developed for early detection and prognosis of diseases and determination of treatment efficacy.

4.8. Other detection methods

In addition to the common characterization methods mentioned above, ELISA and reverse transcription quantitative PCR (RT-qPCR) can also be used to identify sEVs.[127,128] Biosensor correlation methods can be used to detect RNAs (microRNAs and mRNAs) of sEVs. Biosensors are grouped according to their sensing mechanism and can be categorized into fluorescent-,[129] colorimetric-,[130] electrical/electrochemical-,[131] plasma-,[132] and chemiluminescent-based biosensors[133] as well as those that utilize surface-enhanced Raman spectroscopy,[134] inductively-coupled plasma mass spectrometry, and photothermal detection.[135] Methods developed with biosensors can overcome the limitations of traditional RT-qPCR and ELISA in terms of low sensitivity, cumbersome process, large sample size, and high cost. Methods developed with biosensors can achieve sensitive, simple, rapid, high-throughput, low-sample consumption, and cost-effective biomarker detection of sEVs compared with conventional methods.[91] However, because of the unique nature of biomolecules, some methods can only detect a single type of sEVs biomarker, i.e., sEVs proteins or microRNAs. Several researchers have developed an sEVs protein microRNA one-stop (Exo-PROS) biosensor, which not only selectively captures sEVs but also uses a surface plasmon resonance mechanism for in situ simultaneous detection of sEVs protein–microRNA interactions. The Exo-PROS assay achieves rapid, reliable, and low-sample consumption. The Exo-PROS assay has also shown superior diagnostic performance to conventional ELISA and RT-qPCR methods.[136]

5. Conclusion and prospect

Each isolation method has its advantages and disadvantages, and the applicability of the method depends on the type of sample to be isolated, sample volume, and budget.[137] Currently, methods for the isolation, characterization, and analysis of sEVs remain unstandardized, and despite the unavoidable heterogeneity of sEVs, standardized means for isolation methods targeting the same type of sEVs are still required. Immunoaffinity capture is undoubtedly the optimal method for specifically purifying sEVs from biological samples, although the yields still need to be improved and specific isolation markers for sEVs remain lacking. Therefore, the exploration of characteristic markers of sEVs is important for their isolation and even characterization. Currently, the main problems limiting the wide application of sEVs include their purity and yield and that their integrity cannot be guaranteed. Disruption of sEVs structures affects the subsequent analysis, and the optimization of isolation methods is therefore critical to the research of sEVs. The combination of isolation techniques can improve the purity of sEVs, but simultaneously entails more cumbersome operation, which consequently lowers the yield and quality of the purified sEVs.

The emergence of biosensors provides a highly sensitive, simple, rapid, and high-throughput analysis of sEVs, although continuous development and improvement are still needed. First, the detection sensitivity, specificity, and reproducibility of biosensors need to be improved to be able to handle complex biological samples such as blood, urine, ascites, and saliva and to provide accurate measurements of sEVs biomarkers. Second, the diagnostic value of these biosensors must be validated in a large number of patients to assess their reliability and demonstrate their clinical utility. Finally, biosensors should have user-friendly and cost-effective designs to facilitate clinical translation and promote commercialization.

The adaptability of many biosensors to clinical environments is greatly limited by the need for expensive manufacturing processes, costly support equipment, and intensive user training. Therefore, developing accurate, simple, rapid, and inexpensive biosensors for sEVs detection will facilitate the development of sEVs analysis. Furthermore, isolation methods that provide sEVs with high yield, quality, and purity and the development of sensitive detection methods will support the application of sEVs as drug therapy and drug carriers.

CRediT authorship contribution statement

Hongyan Yin: Writing – original draft, Visualization, Software, Conceptualization. **Sihan You:** Visualization, Software. **Xiaomeng Li:** Visualization, Software. **Shuang Li:** Writing – review & editing, Conceptualization. **Chunyan Guo:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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