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REVIEW

Advances in exosome isolation methods and their applications in proteomic analysis of biological samples

Rui Hou[1] · Yilan Li[1,2] · Zhigang Sui[1] · Huiming Yuan[1] · Kaiguang Yang[1] · Zhen Liang[1] · Lihua Zhang[1] · Yukui Zhang[1]

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Abstract

Exosomes are membrane-bound vesicles secreted by cells, and contain various important biological molecules, such as lipids, proteins, messenger RNAs, microRNAs, and noncoding RNAs. Emerging evidence demonstrates that proteomic analysis of exosomes is of great significance in studying metabolic diseases, tumor metastasis, immune regulation, and so forth. However, exosome proteomic analysis has high requirements with regard to the purity of collected exosomes. Here recent advances in the methods for isolating exosomes and their applications in proteomic analysis are summarized.

Keywords Exosomes · Extracellular vesicles · Isolation · Proteomic analysis

Introduction

Exosomes are membranous vesicles secreted by cells, and contain various important biological molecules, such as lipids, proteins, messenger RNAs, microRNAs, and noncoding RNAs (Fig. 1) [1]. Exosomes can be formed by extracellular stimulation, microbial attack, and other stress conditions [2], and are released through either outward budding of the plasma membrane (microvesicle pathway) or inward budding of the endosomal membrane (exosome pathway). Observed under an electron microscope, exosomes exhibit characteristic cup-shaped morphology, appearing as flattened spheres with diameters ranging from 30 to 150 nm.

Emerging evidence demonstrates that proteomic analysis of exosomes is of great significance in studying metabolic diseases, tumor metastasis, immune regulation, and so forth. The proteins localized on the surface or in the core of exosomes have different properties and functions. The characterized proteins in exosomes include platelet-derived growth factor receptor, lactadherin, transmembrane proteins, lysosome-associated membrane protein 2B [3, 4], membrane-associated proteins (annexins, flotillins), GTPases, heat shock proteins, tetraspanins [5, 6], proteins involved in multivesicular body biogenesis, and lipid-related proteins and phospholipases [7, 8]. In particular, some proteins, such as tumor susceptibility gene 101 and programmed cell death 6 interacting protein [9], which are enriched in exosomes, can be used as specific biomarkers for the isolation and quantification of exosomes [10]. However, the formation and secretion mechanisms of exosomes are not well understood, which might be attributed to the difficulty in the isolation of such low-abundance extracellular vesicles (EVs).

Therefore, highly efficient methods for exosome isolation are prerequisites to obtain substantial breakthroughs. Some excellent reviews have described the isolation and detection of EVs and their applications in therapy and drug delivery [11–13]. However, here we summarize the recent advances in exosome isolation techniques together with their applications in clinical proteomic studies over the past 5 years.

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Rui Hou and Yilan Li contributed equally to this work.

✉ Huiming Yuan
huimingyuan@dicp.ac.cn

✉ Lihua Zhang
lihuazhang@dicp.ac.cn

[1] CAS Key Laboratory of Separation Science for Analytical Chemistry, National Chromatographic R&A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, Liaoning, China

[2] University of Chinese Academy of Sciences, Beijing 100049, China

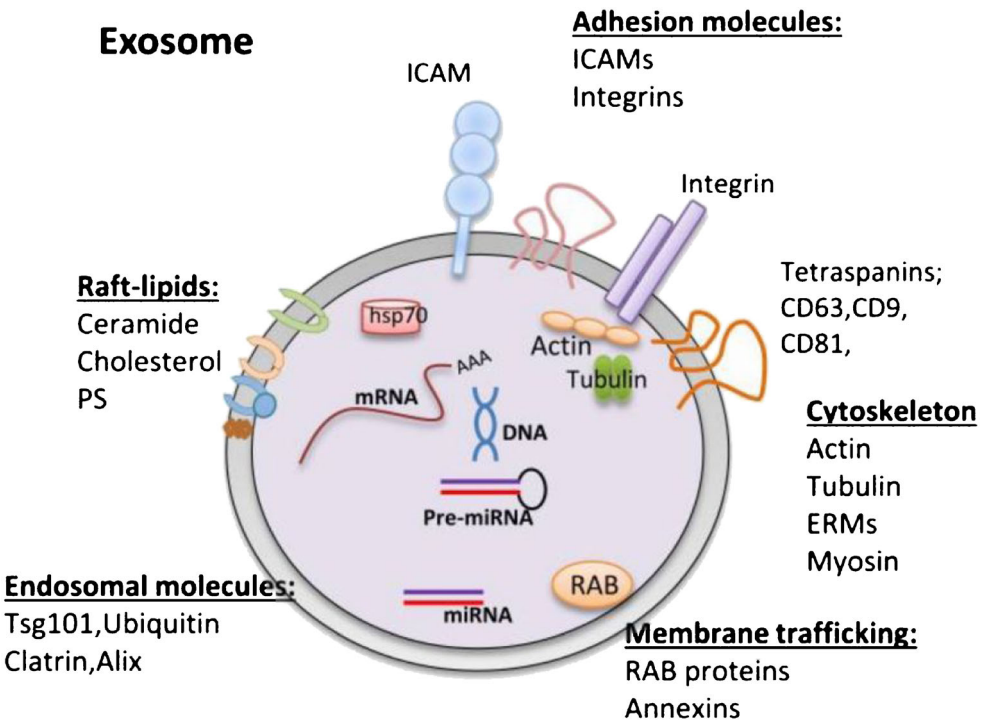

Fig. 1 Components of an exosome. Alix programmed cell death 6 interacting protein, ERM ezrin–radixin–moesin, hsp70 heat shock protein 70, ICAM intercellular adhesion molecule, mRNA messenger RNA, miRNA microRNA, sphingolipids (PS), Tsg101, tumor susceptibility gene 101 protein. (Reproduced with permission from [1])

Overview of exosome isolation

Exosome isolation is usually based on physicochemical properties, such as density, size, and mass, as well as affinity interaction with specific proteins [14, 15]. Characterization is mainly by transmission electron microscopy [16], nanoparticle tracing analysis (NTA) [17], Western blotting, and flow cytometry [18]. The purity and the recovery of exosomes are two key parameters for the evaluation of the performance of exosome isolation. The former is defined by the ratio of the number of exosome particles and the amount of proteins (particles per microgram) [19, 20], which can typically be obtained by NTA and the bicinchoninic acid assay. Moreover, the purity of exosomes can be characterized by the intensity of exosome markers, which are identified by Western blotting [21, 22]. The latter is defined by the ratio of the treated exosome particles and the original exosome particles in samples [23], both of which can be determined by NTA. A typical overview of exosome isolation for proteomic analysis is illustrated in Fig. 2.

Density-based isolation

Ultracentrifugation (UC) is the most widely used method for exosome isolation, and is typically regarded as the gold standard [24]. Johnstone et al. [25] first applied UC for the isolation of exosomes from reticulocyte tissue culture medium. To achieve higher purity of exosomes, the UC protocol was further optimized, by which cells, dead cells, and cell debris are removed by centrifugation at 300*g*, 2000*g*, and 10,000*g*, respectively, and exosomes are further purified by UC (more than 100,000*g*), as shown in Fig. 3.

Kim et al. [26] compared the effects of different UC cycle numbers on the purity of the exosomes. One-dimensional gel images demonstrated that at least five cycles of UC should be performed for the successful removal of nonexosome proteins from isolated exosomes, but the exosome yields were low, ranging from 0.001% to 0.01%.

Sucrose density gradient centrifugation is another density-based method to isolate exosomes [24, 27], which float with density ranging from 1.15 to 1.19 g/mL. Gupta et al. [28] compared differential UC with one-step sucrose cushion UC (SUC) for exosome isolation. In their study, both adipose tissue mesenchymal stem cells and bone marrow mesenchymal stem cells were used as the models and the exosomes were purified by differential UC and SUC, respectively. The concentration of the exosomes obtained by SUC was greatly increased by about two to three times. Furthermore, newer isosmotic gradients (e.g., iodixanol gradient) have been used to maintain the integrity of exosomes of vesicles [29]. Xu et al. [30] modified the traditional density gradient centrifugation through a 17% Optiprep™ cushion (cushion method), followed by a 4-h centrifugation wash, which yielded significantly more exosomes. The total amount of proteins of exosomes from overnight centrifugation through a 17% Optiprep™ cushion was twice that from traditional UC (about 700 μg versus about 300 μg, 1 mL plasma). Calculation of the ratio of exosome particles and the total amount of protein showed the purity achieved by the cushion method was about 1.23 times higher than that achieved by traditional UC. In the study

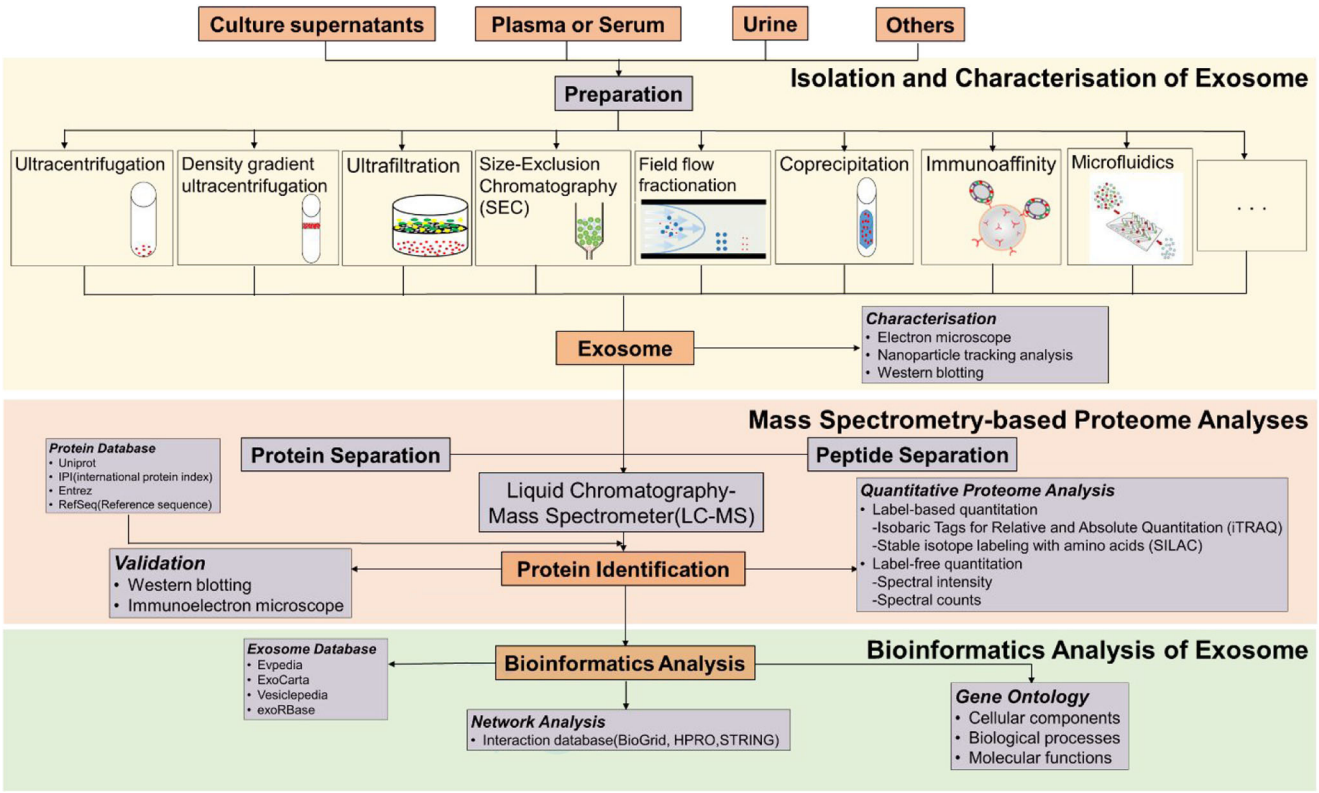

Fig. 2 Overview of methods for exosome isolation and proteomic analysis. (Modified with permission from [14])

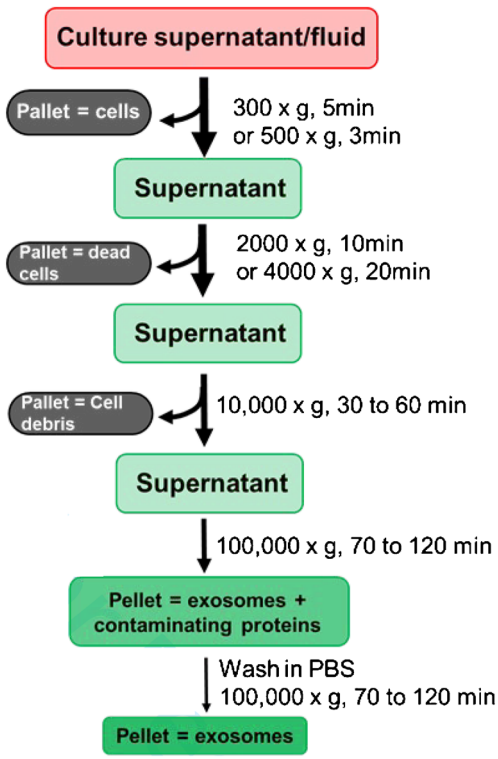

Fig. 3 Flow chart for exosome purification based on differential velocity centrifugation. PBS phosphate-buffered saline. (Modified with permission from [24])

of Yamashita et al. [31], exosomes collected from murine melanoma B16-BL6 cells by several methods were compared with respect to dispersibility, recovery rate after filtering, and clearance from the blood circulation in mice, including simple UC/pelleting (pelleting method), UC with an iodixanol cushion (cushion method), and UC on an iodixanol density gradient (gradient method), among which the recovery with the gradient method was the highest (82%).

Although density-based exosome isolation methods are relatively easy to perform and can achieve exosome purity as high as 10^8–10^9 particles per microgram, they are usually time-consuming and have low recovery (10–80%) since the vesicle of exosomes might be broken during UC. Moreover, the coexistence of protein aggregates and other large biomolecules or particles with similar density is unavoidable.

Size-based isolation

The specific size of exosomes, ranging from 30 to 150 nm, has been well utilized for purification. On the basis of this mechanism, various methods, such as ultrafiltration [polycarbonate track-etched nanoporous membrane and poly(ether sulfone) membrane with a pore size of 30–200 nm were often used] [32–37] and size-exclusion chromatography [36–38], have been applied for exosome isolation. Compared with UC, both of these methods are much

faster and do not require special equipment [39]. However, because of low resolution, it is difficult to separate exosomes of different sizes.

To increase resolution, asymmetric flow field-flow fractionation (AF4) was recently developed and applied to purify exosomes [40–42], by which large exosome vesicles (90–120 nm) could be separated from small exosome vesicles (60–80 nm) and nonmembranous nanoparticles, termed "exomeres" (approximately 35 nm). To further characterize and quantify exosomes, Sitar et al. [41] optimized the operation conditions of AF4 and coupled it with a multidetection system (UV and multiangle light scattering). Compared with NTA, AF4 with UV and multiangle light scattering detection revealed the presence of two particle subpopulations, the larger exosomes with an average size of approximately 113 nm and the smaller vesicle-like particles with an average size of approximately 23 nm, indicating higher resolution of this method.

However, the aforementioned size-based methods, including ultrafiltration, size-exclusion chromatography, and AF4, lack specificity. By these methods, the purity of collected exosomes is about 10^8 particles per microgram, and exosome recovery can reach more than 80%, both of which should be further improved, especially in the application of proteomic analysis.

Polymer precipitation

Because an aqueous polymer reduces the hydration of EVs and causes precipitation, the precipitated EV products can be easily and reproducibly isolated with low centrifugal forces. On the basis of this mechanism, various commercial kits, such as ExoQuick, Exo-Spin, and Pure-Exo, were developed. By these methods, the purity of exosomes achieved is about 10^7–10^9 particles per microgram. In our laboratory, Weng et al. [43] developed a polyethylene glycol (PEG)-based precipitation approach by which exosomes can be collected from cell culture supernatant with high enrichment efficiency and low cost. Exosomes were isolated with PEG from cell culture supernatant for in-depth proteome profiling by tandem mass spectrometry analysis. In their study, 6299 protein groups encoded by 5120 genes were characterized from HeLa cell culture supernatant, including numerous exosome proteins that overlapped with 97% of the top 100 exosome marker proteins recorded in the ExoCarta database. Although polymer-precipitation-based exosome isolation methods can provide high yields of EVs with a well-kept structure, interference by co-precipitated proteins is inevitable because of the nonspecific interaction between polymer and proteins, which might be solved by the integration of other separation methods.

Immunoaffinity

The surface of exosomes carries a number of specific membrane proteins, such as CD9, CD63, CD81, CD82, programmed cell death 6 interacting protein, annexin, epithelial cellular adhesion molecule (EPCAM), and RAB5, which can be used as specific markers for the isolation of exosomes. Various immunoaffinity capture-based techniques have been developed [15, 44–46]. Cai et al. [19] constructed immunoaffinity superparamagnetic nanoparticles by combining antibodies with superparamagnetic nanoparticles through host–guest interactions between β-cyclodextrin and 4-aminoazobenzene, by which $(8.8 \pm 1.3) \times 10^9$ particles per microgram could be obtained with high recovery of 80%. Furthermore, exosome cellular uptake experiments were used to confirm the structural and functional integrity. Exosomes were labeled with PKH67 and incubated with MCF-7 cells for different times. Intracellular localization of exosomes was tracked by confocal laser scanning microscopy. All the results showed the immunoaffinity superparamagnetic nanoparticle method was superior in retaining the structural and functional integrities of exosomes compared with conventional UC, PEG-based precipitation, and a polymer-based commercial kit. Furthermore, Tauro et al. [47] compared different methods for isolation of exosomes from LIM1863 human colon cancer cell concentrated culture medium, including UC at 100,000*g*, OptiPrep™ density gradient centrifugation, and EPCAM immunoaffinity capture. They used a proteomic approach to profile the protein composition of exosomes, and label-free spectral counting to evaluate the effectiveness of each method. EPCAM immunoaffinity capture was found to be the most effective method to isolate exosomes (highest purity and moderate recovery).

Although immunoaffinity-capture-based techniques can be used to obtain exosomes with higher purity than exosomes obtained by the other methods, commercially available antibodies are limited and expensive [19]. Furthermore, some antibodies cannot be expressed on the surface of exosomes, and thereby the numbers of recycled exosomes are underestimated, which hinders the wide application of immunoaffinity-capture-based techniques in exosome purification.

Other methods

The emerging microfluidic-based technology shows great promise for exosome isolation [46, 48–50]. A size-based EV isolation tool, ExoTIC (exosome total isolation chip), has been developed that not only is simple, easy to use, and modular, but also facilitates high-yield and high-purity exosome isolation compared with UC and PEG-based precipitation. Liu et al. [49] first investigated the ability of the ExoTIC device to process low volumes (10–500 μL) of plasma. They found that the yield of exosomes purified from 500 μL of healthy human

plasma by the ExoTIC device was approximately 1000 times higher than that obtained by UC. Compared with commercial PEG precipitation kits, the ExoTIC device achieved threefold to fourfold higher exosome yields. Besides, a viscoelasticity-based microfluidic system was designed to isolate exosomes from cell culture supernatant or serum in a size-dependent and label-free manner [51]. With a small amount of biocompatible polymer as the additive in the medium to control the viscoelastic forces exerted on exosomes, high separation purity (more than 90%) and recovery (more than 80%) of exosomes were achieved. In addition, Woo et al. [20] presented a rapid, label-free, and highly sensitive method for EV isolation and quantification using a lab-on-a-disk integrated with two nanofilters (Exodisc) with a size of 600 and 20 nm. Starting from crude biological samples, such as cell culture supernatant or cancer patient urine, fully automated enrichment of EVs in the size range from 20 to 600 nm was achieved within 30 min with a tabletop-sized centrifugal microfluidic system. Quantitative tests using NTA confirmed that the Exodisc allowed greater than 95% recovery of exosomes from cell culture supernatant.

Moreover, some innovative sorting methods, including acoustic [52], electrophoretic [51], and electromagnetic [53] methods, have been developed. The group of Heller [54] developed an alternating current electrokinetic (ACE) microarray chip by which glioblastoma exosomes could be rapidly isolated and recovered from undiluted human plasma samples. Such an ACE device required only 30−50 μL plasma, and could concentrate the exosomes into the high-field regions of ACE microelectrodes within 15 min. By this method, the concentration of exosomes obtained reached about 5×10^{10} particles per milliliter. Furthermore, they further applied this method in the rapid detection of pancreatic cancer in patient blood [55]. They found that glypican 1 and CD63 could be used as biomarkers of pancreatic ductal adenocarcinoma in serum, and could distinguish pancreatic ductal adenocarcinoma patient samples and healthy individual samples with high sensitivity and specificity. Because of fast speed and simplicity, such an ACE method could achieve seamless "sample-to-answer" liquid biopsy screening, which is beneficial to improve early-stage cancer diagnosis.

Ultrasonic standing waves were used to separate exosomes according to size and density by an acoustic system [52]. With this automated acoustic-based technique, termed "acoustic trapping," Ku et al. [56] enriched exosomes from cell culture conditioned medium, urine, and blood plasma, with the required sample volume decreased below 300 μL and the enrichment time decreased to 30 min. Acoustic trapping was comparable to UC with regard to enrichment from plasma (2.4×10^8/mL compared with 3.0×10^8/mL), urine (4.4×10^7/mL compared with 2.4×10^8/mL) or conditioned medium (5.0×10^8/mL compared with 1.4×10^9/mL). UC-enriched samples had consistently larger particle size distributions than the input sample, which is consistent with previous findings [24, 57].

Wu et al. [58] reported an exosome isolation method by acoustofluidics (Fig. 4) that consists of a microscale cell-removal module that can first remove larger blood components, followed by EV subgroup separation in the exosome-isolation module. In the first module, the isolation of 110-nm particles from a mixture of micro-sized and nano-sized particles was achieved with yield greater than 99%. In the second module, exosomes were isolated from the EV mixture with purity of 98.4%. With the integration of such two modules onto a single chip, they isolated exosomes from whole blood, with a blood cell removal rate greater than 99.999%.

In summary, although exosome purification techniques have been developed, it is still hard for a single method to solve all problems. Therefore, the combination of different methods would be a better choice since they have their own advantages, as shown in Table 1.

Exosome proteomic analysis of biological samples

Proteomic analysis of exosomes has great prospects to study and evaluate the development, diagnosis, treatment, and prognosis of diseases. Compared with traditional circulating markers, such as cytokines and hormones, exosomes can remain stable in body fluids for several months at -80 °C without repeated freezing and thawing. Compared with needle biopsy and histopathology examination, the analysis of exosomes in body fluid specimens is more acceptable because of the advantages of easy sampling and less trauma. Therefore, there are many potential applications for exosomes to be used as biomarkers in clinical studies, as shown in Fig. 5.

Proteomic analysis of exosomes in culture supernatants

During cell culture, exosomes are secreted into the cell culture medium. They contain a lot of information and play important roles in cell-to-cell communication, immune responses, and so forth. Therefore, research on the exosome proteome in cell culture is of great significance to understand the mechanisms of diseases.

Palazzolo et al. [59] analyzed the proteome of exosomes collected from serum-starved MDA-MB-231 subconfluent cell cultures derived from breast cancer cells by UC. Two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry were used to identify proteins. It was found that vesicular components of breast cancer cells involved in tumor survival and expansion account for differing abilities in

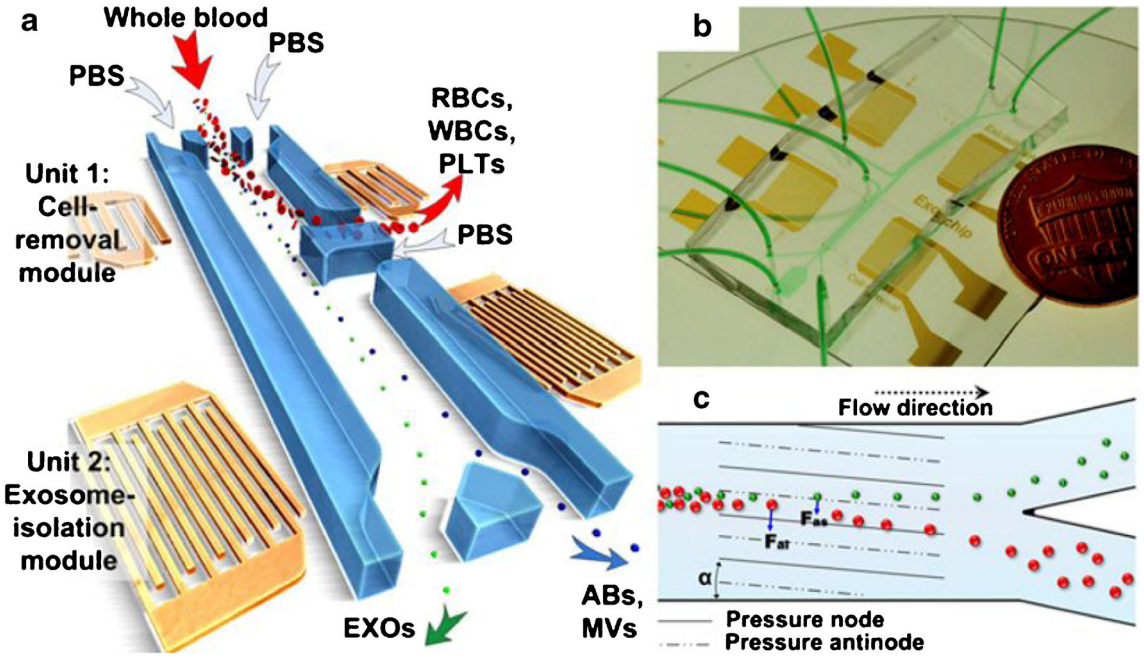

Fig. 4 Schematic illustration of and mechanisms underlying the integrated acoustofluidic device for isolating exosomes [58]. **a** Red blood cells (RBCs), white blood cells (WBCs), and platelets (PLTs) are filtered by the cell-removal module, and then subgroups of exosomes [apoptotic bodies (ABs), exosomes (EXOs), and microvesicles (MVs)] are separated by the exosome-isolation module. **b** Optical image of the integrated acoustofluidic device. Two modules are integrated on a single chip. **c** Size-based separation occurs in each module because of the lateral deflection induced by a tilted-angle standing surface acoustic wave field. PBS phosphate-buffered saline. (Reproduced with permission from [58])

metastasis. Klein-Scory et al. [60] presented a proteomic description of affinity-purified EVs from pancreatic tumor cells incubated in a serum-free medium based on EPCAM-coated magnetic beads. Western blotting and mass spectrometry were used to analyze the exosome proteins. The data showed the composition of exosome proteins from pancreatic cancer cells

Table 1 Summary of current exosome isolation methods

Isolation method	Mechanism	Evaluation parameters			Advantages	Disadvantages
		Purity	Recovery	Time		
Ultracentrifugation [24–27]	Density	++	+	+	High purity, gold standard	Long duration (>4 h); large sample volume; requires ultracentrifuge; low recovery and purity
Ultrafiltration [23, 24,26–37]	Size and molecular weight	++	++	++	Simple and fast operation, high recovery, RNA can be extracted directly	Lacks specificity; difficulty in scaling
Size-exclusion chromatography [36–38]	Size and molecular weight	++	++	++	Simple and fast operation, high recovery, RNA can be extracted directly	Lacks specificity; difficulty in scaling
Field-flow fractionation [40–42]	Size and molecular weight	++	++	++	Broad separation range, wide variety of eluents	Long duration; requires fractionation equipment
Polymer coprecipitation [43]	Surface charge	+	+++	++	Easy and user-friendly processing	Lacks specificity; difficulty in scaling
Immunoaffinity [44–47]	Affinity purification	+++	++	++	High specificity and purity	High cost, low yield, limited use
Microfluidics [46, 48–50]	Density and size	++	++	+++	Fast, low cost, convenient, and easy to automate	Low throughput, complex device
Acoustic nanofilter [52, 56]	Density and size	++	+	+++	Small processing volume, label-free isolation	Seriously affected by environmental factors.

+++, ++ and + represents good, moderate, and bad respectively

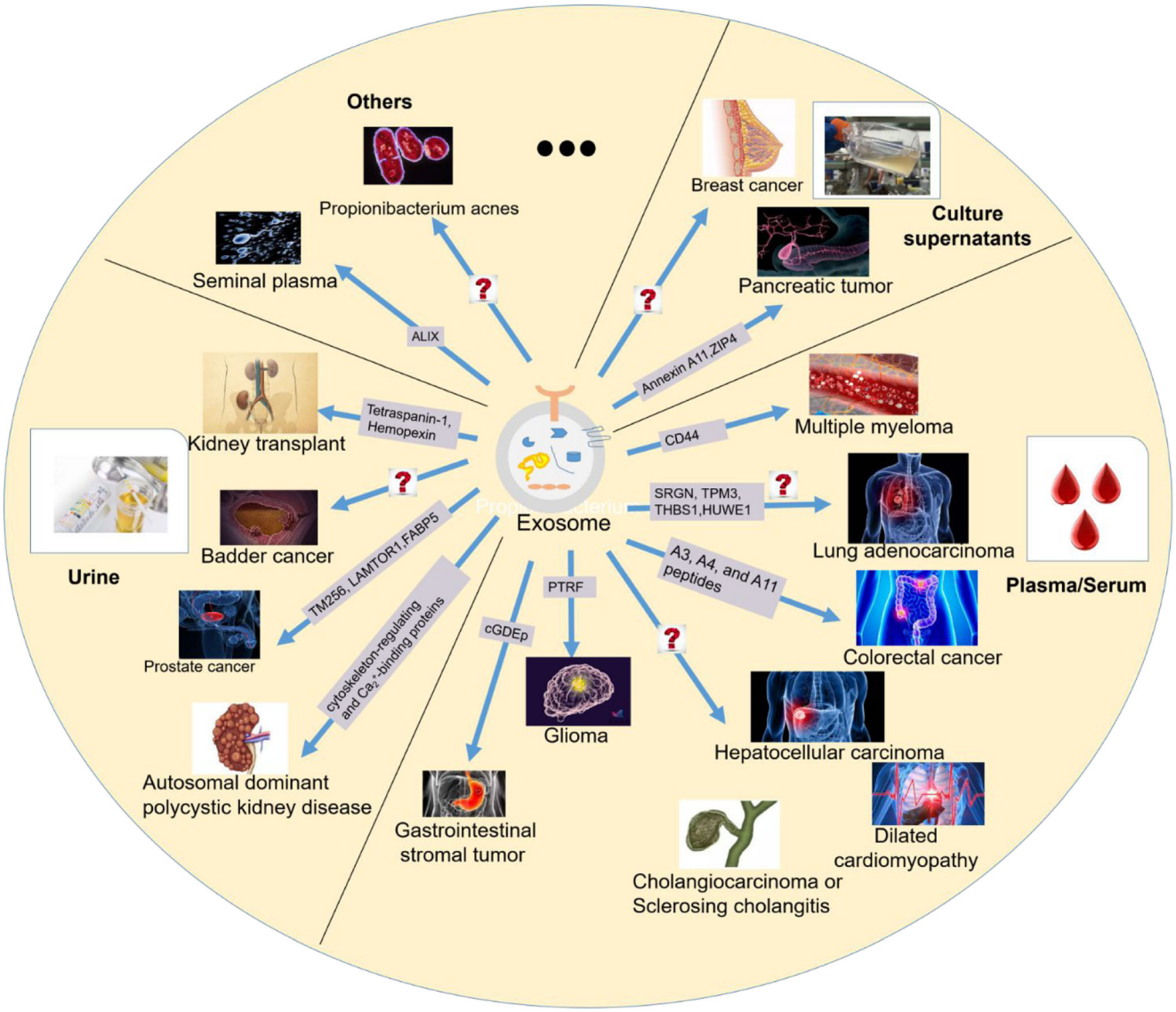

Fig. 5 Exosome proteins as potential biomarkers. The gastrointestinal-stromal-tumor-derived exosome proteome (cGDEp) includes KIT, CD34, anoctamin 1, prominin 1, protein kinase Cθ, endoglin, dipeptidyl peptidase 4, FHL1, cadherin 11, and KCTD12. ALIX programmed cell death 6 interacting protein, FABP5 fatty acid binding protein 5, PTRF polymerase I and transcript release factor, SRGN serglycin, THBS1 thrombospondin 1, TM256 transmembrane protein 256, TPM3 tropomyosin 3

is different from other released proteins from pancreatic cancer cells.

However, cells cultured under starved conditions can hardly reflect the real status of cell secretion. Therefore, it was indispensable to develop exosome isolation methods in conditioned medium with serum added. Braga-Lagache et al. [61] collected the conditioned medium of 60 cell lines from the National Cancer Institute, and exosomes were isolated by PEG precipitation and UC. They provided the largest proteome profiling of exosomes, identifying 6071 proteins, with 213 in common. The differentially expressed proteins between different cell lines might offer potential for cancer diagnosis and prognosis.

Proteomic analysis of plasma exosomes

Exosomes are abundant in plasma, being involved in many physiological and pathological processes, and containing various candidate biomarkers of diseases. Therefore, in recent years, proteomic analysis of plasma exosomes has attracted more and more attention.

Harshman et al. [62] collected exosomes from different multiple myeloma (MM) cell lines, MM patients' serum and bone marrow, and healthy donor serum by UC. After proteome profiling, serum CD44 was screened as a predictive biomarker of overall survival for MM patients. Huang et al. [63] isolated exosomes from the human glioma cell lines LN229,

U87, and U251, the blood of glioma patients, and the serum of glioblastoma multiform (GBM) patients by UC. The proteomic analysis showed a positive correlation between tumor grade and polymerase I and transcript release factor (PTRF) expression in both tumor tissues and exosomes isolated from blood harvested from glioma patients, and after surgery, PTRF expression in exosomes isolated from the sera of GBM patients was decreased, indicating that PTRF might serve as a promising biomarker in the detection of glioma, and potentially as a therapeutic target for GBM. More recently, Gao et al. [23] presented a rapid and efficient method to isolate exosomes from human serum by taking advantage of the specific interaction of TiO_2 with the phosphate groups on the lipid bilayer of exosomes. Serum was centrifuged to remove cells and debris before treatment with TiO_2. Because there are few phosphorylated proteins in serum, high selectivity of exosome isolation was achieved. By comparison of the serum exosomes of pancreatic cancer patients and healthy donors, 59 significantly upregulated proteins were identified, indicating that this method might be a powerful tool for clinical applications

The deep proteome profiling of translational modification from plasma exosomes is also important to discover potential biomarkers. Cheow et al. [64] developed an exosome enrichment method for mass spectrometry-based proteome profiling that combined prolonged UC with electrostatic repulsion–hydrophilic interaction chromatography. They identified 127 plasma glycoproteins at a high level of confidence (false discovery rate less than 1%) by mass spectrometry. Fifty-eight glycoproteins were cataloged as exosome proteins in ExoCarta, of which 48 had a concentration ranging from picograms per milliliter to nanograms per milliliter. These results demonstrate that this novel method may facilitate the discovery of more low-abundance proteins from human plasma exosomes. As a consequence, if there are large amounts of contaminated proteins in plasma, they can be identified by Western blot and excluded by mass spectrometry. Plasma exosomes have been successfully isolated by the combination of multiple techniques, and by subsequent proteomic analysis, proteins associated with disease occurrence, development, and recovery have been identified, which is crucial for the study of proteome-driven precision medicine.

Proteomic analysis of urine exosomes

Compared with blood collection, urine collection is noninvasive and urine can be obtained in large quantities. More importantly, An and Gao [65] showed that urine is not subject to homeostatic mechanisms, and the changes in urine are more sensitive than those in plasma. Therefore, it is expected that more candidate biomarkers for the early diagnosis of diseases will be found by proteomic analysis of urine exosomes.

Pocsfalvi et al. [66] used double-cushion UC to isolate exosomes from pooled urine samples of healthy controls and autosomal dominant polycystic kidney disease patients at two different stages. By exosome proteome quantification, 83 differentially expressed exosome proteins were identified by nanoscale high-performance liquid chromatography–electrospray ionization tandem mass spectrometry, among which cytoskeleton-regulating and Ca^{2+}-binding proteins were proven to be closely related to the pathogenic state of tubular epithelial cells in autosomal dominant polycystic kidney disease, and might be used to monitor the status of patients. Fujita et al. [67] aimed to discover a new biomarker for high Gleason score (GS) prostate cancer in urinary exosomes via quantitative proteomic analysis. Exosomes were also isolated from urine by UC from 18 men (negative biopsy result, $n = 6$; GS 6 prostate cancer, $n = 6$; and GS 8–9 prostate cancer, $n = 6$), and 4710 proteins were identified, with 3528 proteins being quantified in the urinary exosomes, among which fatty acid binding protein 5 was screened as a potential biomarker of high-GS prostate cancer. Furthermore, Lee et al. [68] isolated exosomes from patient urine by UC to discover biomolecules related to the pathogenesis of bladder cancer. A total of 1222 proteins were identified by LTQ Orbitrap XL mass spectrometry, and statistical analysis showed that the levels of 56 proteins were significantly increased in bladder cancer urine ($P < 0.05$). Among them, some proteins were selected for further validation of their roles in cancer development and progression.

Proteomic analysis of exosomes in other biological samples

In addition to plasma and urine, saliva, cerebrospinal fluid (CSF), and commensal bacteria have been used in clinical diagnosis. Therefore, the in-depth study of their exosome proteomes is beneficial to understand the mechanisms of diseases and discover more candidate biomarkers.

Human saliva is a unique medium for clinical diagnosis with the merit of noninvasiveness [69]. Salivary exosomes from lung cancer patients and normal controls were isolated by an affinity chromatography column combined with a filter system to efficiently remove the high-abundance proteins and viscous interferents in saliva. Shotgun proteomic analysis identified 113 proteins in the cancer group and 95 proteins in the control group, among which 63 proteins were consistently discovered only in the cancer group. Exosomes from nasal lavage fluid [70] were collected from 14 healthy individuals, 15 individuals with asthma, and 13 individuals with asthma and chronic rhinosinusitis by differential centrifugation. By proteomic analysis, 604 proteins were identified in nasal exosomes, and they showed strong associations with immune-related functions, such as immune cell trafficking. Moreover, exosomes were extracted from human CSF [71]

by UC and ultrafiltration–liquid chromatography to ensure purity. Proteomic analysis indicated that exosome-enriched proteomes could better reflect the intracellular and white matter proteome than whole CSF.

Furthermore, increasing attention is being paid to Gram-positive bacteria as underestimated pathogens in a variety of diseases. Jeon et al. [72] applied UC and density gradient UC in the isolation of exosomes from *Propionibacterium acnes*, and identified 252 vesicular proteins by liquid chromatography–tandem mass spectrometry. Gene Ontology analysis demonstrated these EVs harbor proteins that are involved in many important biological processes, including antibiotic resistance, cell adherence, bacterial competition, immunogenicity, and virulence. These results provide important information for researching the biological role of *P. acnes* and selecting effective targets for *P. acnes* in clinical treatment.

Conclusion and prospects

Exosomes are important mediators of intercellular communication between cells, and can reflect the physiological or pathological conditions of tissues and organs. Although various exosome isolation methods based on physical, chemical, or biological properties, and even the combination of different principles, have been developed, further effort should be made to increase the isolation efficiency and purity of exosomes, especially for proteomic analysis. Moreover, besides their application in clinical diagnosis, exosomes collected from specific cells, such as stem cells, and culture medium might be of great promise for clinical treatment. Therefore, the development of large-scale preparation methods for exosomes should also be given more attention.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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