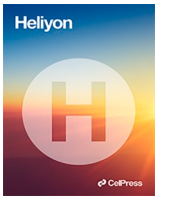
Heliyon 10 (2024) e30328

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Review article

An overview of challenges associated with exosomal miRNA isolation toward liquid biopsy-based ovarian cancer detection

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ARTICLE INFO

Keywords:
microRNA
Exosomes
Isolation
Ovarian cancer
Liquid biopsy

ABSTRACT

As one of the deadliest gynaecological cancers, ovarian cancer has been on the list. With lesser-known symptoms and lack of an accurate detection method, it is still difficult to catch it early. In terms of both the diagnosis and outlook for cancer, liquid biopsy has come a long way with significant advancements. Exosomes, extracellular components commonly shed by cancerous cells, are nucleic acid-rich particles floating in almost all body fluids and hold enormous promise, leading to minimallyinvasive molecular diagnostics. They have been shown as potential biomarkers in liquid biopsy, being implicated in tumour growth and metastasis. In order to address the drawbacks of ovarian cancer tumor heterogeneity, a liquid biopsy-based approach is being investigated by detecting cell-free nucleic acids, particularly non-coding RNAs, having the advantage of being less invasive and more prominent in nature. microRNAs are known to actively contribute to cancer development and their existence inside exosomes has also been made quite apparent which can be leveraged to diagnose and treat the disease. Extraction of miRNAs and exosomes is an arduous execution, and while other approaches have been investigated, none have produced results that are as encouraging due to limits in time commitment, yield, and, most significantly, damage to the exosomal structure resulting discrepancies in miRNA-based expression profiling for disease diagnosis. We have briefly outlined and reviewed the difficulties with exosome isolation techniques and the need for their standardization. The several widely used procedures and their drawbacks in terms of the exosomal purity they may produce have also been outlined.

1. Introduction

Ovarian cancer, having the highest overall gynaecological cancer fatality rate, has been one of the most prevalent cancers among women, with a 5-year survival rate of fewer than 20 % and a very low prognosis when diagnosed at an advanced stage, however, the early-stage diagnosis has shown to have a better survival rate of more than 90 % [1]. Despite the availability of modern advanced diagnostic tools and medical amenities, more than half of cases are identified at an advanced stage as a result, the survival rate is only

Abbreviations: OC, Ovarian Cancer; EOC, Epithelial Ovarian Cancer; miRNA, miR, Micro RNA; qRT-PCR, Quantitative real-time polymerase chain reaction; FIGO, Federation of Gynaecology and Obstetrics; hsa, Homo sapiens; CTC, circulating tumor cell; cfDNA, cell-free DNA; mRNA, messenger RNA; EV, extracellular vesicles; cfmiRNA, cell-free micro-RNA; CD, cluster of differentiation; ADAR, Adenosine deaminases; APOBEC, Apolipoprotein B mRNA Editing Catalytic Polypeptide-like; EMT, Epithelial mesenchymal transition.

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https://doi.org/10.1016/j.heliyon.2024.e30328
Received 30 October 2023; Received in revised form 23 April 2024; Accepted 23 April 2024
Available online 25 April 2024

between 10 % and 30 % [2]. In about 75 % of suspected patients' ovarian cancer is diagnosed at stage III or IV [3]. Based on Cancer Statistics 2022, over 150,000 deaths globally are thought to be caused by OC each year; in the United States itself, 19,710 new cases and 13,270 fatalities are anticipated in 2023 [4]. Nevertheless, with improvements in screening methods and the advancement of tailored treatments for this cancer, almost more than 50 % of ovarian cancer patients continue to pass away because their disease was diagnosed too late and had spread to distant or localized metastases. Although the use of present-day screening tests has assisted in reducing mortality, still they have poor acceptance by the patients due to inadequate sensitivity and specificity of these screenings, their high cost of expenditure, and their intrusive nature.

Although tissue-based biopsy has long been the gold standard for ovarian cancer diagnosis, liquid biopsy has become increasingly popular in diagnosis due to its minimally invasive nature and straightforward sample collection procedure. It has been insightful to develop a fluid-based assay for ovarian cancer detection because, being internally located, its varied origin and molecular traits, tissue biopsy makes the process complex and invasive. Moreover, ovarian cancer has the potential of spreading to other areas while tissues are being collected, and tissue-based biopsies have limits in terms of acquiring the proper section of the sample that contains the bulk of affected cells, as well as inherent potential risks to the patients. Due to this, liquid biopsy is typically used for diagnosis in a bid to avoid these issues.

This study intends to address the challenges in ovarian cancer diagnosis employing exosomal and non-exosomal miRNA-based liquid biopsy approaches. In this review, we have concentrated on the challenges associated with exosome isolation techniques, as well as the complications of the process brought about by the variety of exosome sources and the complexity of biological samples.

Epithelial-mesenchymal transition, tumorigenesis, cancer cell proliferation, and a number of other cancer-related abnormalities in the malignant tissues and adjacent cancer cells have all been linked to miRNAs and their dysregulation. Numerous miRNAs and their roles in various diseases have been thoroughly reviewed in Table 1.

According to the latest patent filed by Mianzhu Peoples Hospital in year 2020 (Patent No. CN112626209A) a Graphene oxide (GO)-based qRT-PCR kit has been developed to detect miRNAs related to ovarian cancer, wherein the expression level of miRNA markers of suspected person is compared, which enabled the early diagnosis of ovarian cancer as well as its prognosis and the assessment of therapeutic effect of anti-tumor drugs. The developed kit can differentiate patients on the basis of different FIGO stages of cancer and will be able to segregate patients with ovarian cancer from women with ovarian benign tumor.

Considering the miRNA significance in cancer diagnosis and response to chemotherapy, another patent by Keller et al., granted in 2016 (Patent No.EP2531611B1) studied the expression level of differentially expressed miRNAs in which miRNAs from the blood of ovarian cancer patients were extracted and screened by microarray analysis which concluded the supporting role of some pre-determined miRNAs including hsa-miR-605, hsa-miR-1248, hsa-miR-342-3p, hsa-miR-133b, hsa-miR-520a-3p, hsa-miR-23b*, hsa-miR-450b-3p,hsa-miR-423-5p, hsa-miR-219-1-3p in ovarian cancer diagnosis. The inventors used blood samples as a non-invasive biomarker source to evaluate the expression of miRNAs at the level of whole-genome in people having ovarian cancer.

Liquid biopsy is the process of collecting blood, plasma, serum, breast milk, or urine samples in order to examine for exosomes, CTCs, or cfDNAs that are released by tumor cells in physiological fluids during necrosis, phagocytosis, or apoptosis [5]. It has numerous pros over tissue-based biopsy, including quick sample collection and dynamic analysis [6]. With a growing body of evidence on the

Table 1
Functional Significance of miRNAs in Ovarian Tumorigenesis.

miRNA	Role in ovarian cancer	Reference
miR-23a-3p	Inhibited the proliferation and invasion of EOC cells	[11]
miR-200b, miR-200a and miR-429	Targeted inhibitor of growth family 5 (ING5)	[12]
miR-1914, miR-203, miR-135a-2, miR-149, and miR-9-1	Targeted genes and proteins involved in EMT and resistance to therapy	[13]
miR-145-5p	Targeted SMAD4 and repressed cell migration via MMP-2 and MMP-9 downregulation	[14]
miR-1290	Suppressed cytokine signaling 4 (SOCS4)	[15]
miR-520a-3p	Suppressed the EOC cell proliferation, invasion, and EMT and induced cell cycle arrest in vitro	[16]
miR-21	Negatively regulated the expression of the tumor suppressor PTEN resulting in proliferation, invasion, migration, and hypoxia resistance	[17]
miR-383-5p	Inhibited cell proliferation and decreased Ki67 and PCNA expression	[18]
miR-200a-3p	Promoted the proliferation, colony formation, and invasion of ovarian cancer cells by decreasing the expression of PCDH9	[19]
miR-200b, miR-200a and miR-429	Targeted inhibitor of growth family 5 (ING5) and caused cell transformation and tumorigenesis	[12]
miR-200a	Enhanced ovarian cancer cell migratory and invasive abilities by targeting PTEN	[20]
miR-141 and miR-200a	Targeted p38α and modulated the oxidative stress response	[21]
miR-145	Inhibited glutamine metabolism by targeting c-Myc	[22]
miR-32	Inhibited the proliferation, migration, and invasion by targeting B and T lymphocyte associated (BTLA)	[23]
miR-30a-5p and miR-200a-5p	Targeted FOXD1 in turn increased proliferation of ovarian cancer cells	[24]
miR-221	Targeted BMF and promoted the cell proliferation	[25]
miR-127-3p	Regulated BAG5 in EOC and exerted tumor-suppressive effect	[26]
micro-143-3p	Targeted RALBP1and increased apoptosis of ovarian cancer cells	[27]
miR-200	Targeted E-cadherin transcriptional repressors ZEB1 and ZEB2 and reduced EMT transition	[28]
miR-200c	Directly targeted class III beta-tubulin (TUBB3) caused increased sensitivity to paclitaxel	[29]
miR-21	Targeted Akt2-dependent pathway that is activated by hypoxia and promoted tumor resistance	[30]
miR-29a	Repressed anti-correlated genes DNMT3A and DNMT3B and decreased OC cell viability	[31]
miR-30a	Targeted FOXL2 leading to upregulation of BCL2A1, IER3 and cyclin D2 lead to tumorigenesis	[32]

Table 2
Liquid biopsy-based miRNA detection for different diseases with their associated sensitivity and specificity for the assay.

Detection method	Source	miRNA(s)	Sensitivity/specificity	Type of Disease	Reference
Comprehensive microarray analysis	Serum	miR-17-3p and miR-1185-2-3p	Sensitivity = 90 %, Specificity = 90 %	Prostate Cancer	[37]
RT–qPCR	Serum	miR-19a-3p, miR-223-3p, miR-92a-3p and miR-422a	Sensitivity = 84.3 %, Specificity = 91.6 %	Colorectal adenocarcinoma	[38]
Hydrolysis probe–based stem–loop quantitative reverse-transcription PCR (RT-qPCR)	Serum	miR-10a, miR-22, miR-100, miR-148b, miR-223, miR-133a, and miR-127-3p	Sensitivity and specificity values: miR-10a, 81.2 % and 80.0 %; miR-22, 88.6 % and 86.0 %; miR-100, 63.8 % and 81.0 %; miR-148b, 66.4 % and 87.0 %; miR-223, 83.2 % and 83.0 %; miR-133a, 65.1 % and 83.0 %; and miR-127-3p, 78.5 % and 87.0 %	Esophageal Squamous Cell Carcinoma	[39]
RT–qPCR	Serum	miR-1, miR-20a, miR-27a, miR-34 and miR-423-5p	–	Gastric cancer	[40]
RT–qPCR	Serum	miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505	–	Hepatocellular carcinoma	[41]
Microarray	Serum	miR-6799-5p, miR-6075, miR-4294, miR-6880-5p, miR-125a-3p, miR-4530, miR-6836-3p, and miR-4476	Sensitivity = 80.3 %, Specificity = 97.6 %	Pancreatic and Biliary-Tract Cancer	[42]
RT–qPCR	Plasma	miR-17-3p and miR-92	Sensitivity = 89 %, Specificity = 70 %	Colorectal cancer	[43]
RT–qPCR	Serum	miR-17-92a	–	Colorectal cancer	[44]
RT–qPCR	Serum	miRNA-126	Sensitivity = 80 %, Specificity = 60 %	Malignant mesothelioma	[45]
Low-density microarray real time PCR	Serum	miR-191, miR-101, miR-25, miR-26b, miR-335, miR-433, miR-223, miR-29a and miR-516	–	Pleural malignant mesothelioma	[46]
Microarray signature profiling and polymerase chain reaction (PCR)	Urine and blood	miR-210, miR-10b, and miR-29c	Sensitivity = 95.2 %	Bladder cancer	[47]
Microarray and RT–qPCR	Urine	miR-99a and miR-125b	Sensitivity = 86.7 %, Specificity = 81.1 %	Bladder Cancer	[48]
RT–qPCR	Serum	miR-20a	–	Prostate cancer	[49]
Microarray and RT–qPCR	Urine	hsv1-miR-H18 and hsv2-miR-H9-5p	Sensitivity = 66.5 %, specificity = 74.1 % for hsv1-miR-H18 and sensitivity = 70.2 %, specificity = 72.0 % for hsv2-miR-H9-5p	Prostate Cancer	[50]
Microarray and RT–qPCR	Plasma	miR-16, miR-107, miR-130a and miR-146a	–	Breast cancer	[51]
qRT-PCR	Serum	miR-15a, miR-16-1, miR-29c, miR-34a, and miR-155	Sensitivity and specificity values: miR-15a,80 % and 76 %, ; miR-16-1; 94 % and 51 % , miR-34a;100 % and 70 % and miR-155; 83 % and 65 %.	Large B cell lymphoma	[52]
Solexa sequencing and qRT-PCR	Serum	miR-155-5p, miR-10a-5p, miR-93-5p, miR-129-5p, miR-181b-5p and miR-320d	–	Acute Myeloid Leukemia	[53]
qRT-PCR	Serum	miR-335	–	Pediatric acute myeloid leukemia	[54]
qRT-PCR	Plasma	miR-20a	–	B-cell chronic lymphocytic leukemia	[55]
qRT-PCR	Cerebrospinal fluid (CSF)	miR-10b and miR-21	–	Glioblastoma and metastatic brain cancer	[56]
qRT-PCR	Plasma	miR-92	Sensitivity = 89 % and specificity = 70 %	Colorectal cancer	[43]
qRT-PCR	Plasma	miR-181a	Sensitivity = 90.0 % and specificity = 87.5 %	Pediatric acute lymphoblastic leukemia	[57]
qRT-PCR	Serum	miR-133a, miR-23a-3p, miR-1, miR-346 and miR-320b	Sensitivity = 100 % and specificity = 83.3 %	Fibromyalgia Syndrome	[58]

functional involvement in the development of cancer, as well as their stability and cancer-specific dysregulation in tissues and bodily fluids, the prospect of using miRNAs as diagnostic biomarkers in liquid biopsy has grown progressively appealing. For circulating cell-free RNAs including RNAs present inside extracellular vesicles, various non-coding RNAs, mRNAs, and miRNAs, plenty of isolation techniques have been developed based on either their biological or physiological characteristics [7]. Research interest in understanding exosome functions and how to employ them for liquid biopsy-based disease diagnosis and prognosis has grown recently.

Exosomes have been demonstrated to transmit both endogenous and exogenous miRNAs to non-infected cells surrounding a malignant region and are secreted by normal as well as cancerous cells. These are small and extracellular vesicles (EVs) that measure just 30–100 nm in diameter, have a bi-lipid membrane, and are phospholipid-rich lipoprotein particles that resemble membranes of red blood cells in their makeup [8]. Exosomes can be produced either by the conventional pathway or directly, with the former involving the inward invagination of the plasma membrane and the latter having the direct outward extension from the plasma membrane forming exosomes. Human saliva, plasma, and milk exosomes have all been discovered to contain measurable quantities of RNA, including mRNA [9]. However, exosome isolation raises a number of difficulties, including the inability to discriminate between malignant and healthy exosomes and their similar size to other vesicles found in bodily fluids. The most prevalent exosome isolation techniques involve ultracentrifugation, differential centrifugation, polymer-based precipitation size exclusion chromatography, and ultrafiltration [10].

The deficit of robust and consistent results with numerous reports in the literature that appear to be at odds with one another is a significant barrier to the translation of cell-free miRNAs or exosomal miRNAs in liquid biopsies from laboratory testing into clinical applications. Even when concentrating just on healthy populations, a significant amount of variation among individuals in the quantities of cfmiRNAs exists. The variations in reproducibility and sources, which result from differences in the starting material of the experiment, inadequate number of cohorts, the methods and population chosen for the experiment, the cohort for the control, and the extraction and isolation methods used, are the causes of the discrepancies in the studies.

2. Complexities in exosomal and non-exosomal miRNA isolation from tumor cells

It has been discovered that an array of environmental and physiological parameters, such as cell density, confluency during cell culture and other cell culture conditions, cell stage and type, and the impact of external agents on cell stimulation affect the functional attributes of exosomes. Also, being secreted from different cell types, they have varying sizes and compositions and as a consequence, constitute a multitude of biological activities [33].

Exosomes outperform other liquid biopsy sources by a wide margin. Within 24 h of isolation, they can be stored at 4°C, not altering the quality of the exosomal markers on it and at ultra-low temperatures without negatively impacting their long-term usability [34]. The following benefits derived from employing exosomes as a source of liquid biopsy samples [34].

i. They are highly stable and may be found practically in all physiological fluids because they are encased in lipid bilayers.
ii. Biological information from the original cells is secreted by living cells, making them more representative than cell-free DNAs.
iii. Having cell surface protein markers, it is relatively easy to identify them.
iv. It is considerably easier to isolate them from bodily fluids.
v. Furthermore, compared to other cfDNA sources such as plasma and blood, mitochondrial DNA has been reported to be present in higher copy numbers inside exosomes in cases of epithelial ovarian cancer.
vi. Exosomal DNA has been found to carry more biological information than cfDNA and to be more accurate at predicting prognosis.

Exosome extraction has made extensive use of differential and ultracentrifugation techniques. The physical properties and internal components of the exosome serve as the foundation for the extraction technique, which determines the quality of exosomes. They are typically extracted by size-exclusion chromatography, ultracentrifugation, gradient centrifugation, micro-filtration centrifugation, or based on antibodies to exosomal marker proteins like CD63, CD81, and CD9 [35,36].

To get accurate quantitative and qualitative data on miRNA quantification, it is essential to have a reproducible and efficient method for purifying exosomes. They are becoming more and more recognized as a source of possible biomarkers with diagnostic and prognostic potential. The dependability of their deployment is compromised by the absence of standardization for their isolation, purification, and quantification processes. Exosomal miRNAs in various samples have been analyzed from different biological sources using the liquid biopsy method in a number of earlier investigations enlisted in Table 2.

3. Overview of isolation strategies for exosomes and their associated miRNAs and cell-free miRNAs

Technological variation in circulating miRNA biomarker research is largely due to sample collection, preparation, and storage issues. Additionally, RNA extraction from exosomal and non-exosomal sources requires more effort than a protein-based assay does. One of the most accessible body fluids, serum or plasma from blood, is commonly utilized as a specimen for the collection and analysis of diagnostic and prognostic biomarkers from exosomes. The main problem in the isolation of circulating miRNAs from exosomes, according to several reports, has been the lack of standardized protocols for even the same sample material in an established screening test for clinical application [59]. For the isolation of miRNAs, two common principles are generally adopted: column-based separation and guanidium-thiocyanate or phenol-chloroform, TRIzol-based method. Being associated with various protein complexes and other cellular components inside the cell, the isolation method for miRNA should be carefully chosen, taking into account the method's

complexity and source composition [60]. The method selected for isolation not only decides the quality of the miRNA isolated but is also responsible for isolating specific cell-free microRNAs which may cause anomalies in their expression when isolated using different methods for the sample miRNA. For efficient isolation of cell-free miRNAs, it is necessary to degrade miRNA-protein complexes, deciding the quality and purity of the end product. Lipids and lipoproteins associated with extracellular vesicles have been seen to interfere with the isolation process, which requires to be disrupted while isolation [61,62].

3.1. Isolation of cell-free miRNAs from biofluids

A cell can contain nearly 120,000 copies of mature miRNA molecules [63]. Base modifications and editing of miRNAs have been explained by Torsin et al. [64], where non-coding RNAs (ncRNAs) are shown to be modified by ADAR Dependent, APOBEC Dependent and Non-ADAR, Non-APOBEC editing mechanisms and chemical modifications on 5-Methylcytosine (m^5C), 5-Hydroxymethylcytosine (hm^5C), N6-Methyladenosine (m^6A), Pseudouridylation (Ψ) and Uridylation contributing to the stability of miRNAs by increasing their resistance towards exonuclease degradation. A study by Tsui and colleagues [65], on the stability of endogenous and exogenously added RNA procured from the market has proved that >99 percent of the free exogenously added RNA into the sample were degraded after an incubation time of 15s, whereas plasma/serum RNAs were found to be exceptionally stable confirming their resistivity towards blood exonucleases. There is currently no broadly recognized hypothesis on the existence of a cell-free-miRNA pool. Some miRNA-bearing complexes may be actively released by vesicles such as exosomes [66], whilst others may leak passively from injured apoptotic or necrotic cells [67].

While choosing a technique for isolation, the miRNA composition should be taken care of because it can affect the isolation efficiency. The isolation strategy used for miRNAs has been extremely significant in determining its quality and further miRNA expression analysis, where apart from the extraction strategy, the starting input RNA material plays a key role in providing a reproducible outcome. A lot of research has been done on miRNA expression profiling for disease prognosis, particularly in cases of cancers treated with chemotherapy or surgery [68–71], but less attention has been paid to the isolation and storage methods chosen for the sample miRNA, which frequently causes false-positive results and discrepancies in the study.

Current research and articles do not explicitly state that one RNA separation technique is preferable to another. However, when discussing manual methods of isolation, the TRIzol/TRI reagent followed by ethanol precipitation has been quite popular because they allow for total RNA extraction, allowing researchers to analyze all RNAs from a single sample. miRNA isolation kits by Qiagen, such as miRNeasy Serum/Plasma Advanced Kit (Cat. No. 217204), enable phenol-free isolation and purification of total RNA and miRNAs from all types of animal tissues and cells, and miRNeasy Micro Kit (Cat. No. 217084) from blood serum and plasma samples are widely accepted by researchers for their study since it can purify miRNAs with nucleotides <200 which cannot be effectively separated by classical spin column kits and may lead to their less efficient recovery, other commercially available kits in demand include mirVana™ miRNA Isolation Kit (Cat. No. AM1560) and PureLink™ miRNA Isolation Kit (Cat. No. K157001) by Invitrogen. The Qiagen kit (Cat. No. 217204) is phenol free and can elute miRNAs with just 20 μl sample volume. The contents of the kit Buffer RPL make efficient lysis and Buffer RPP precipitates active contaminants such as proteins from the sample. The spin columns helped to remove unwanted contaminants and total RNA. Whereas, the mirVana miRNA Isolation Kit by Invitrogen (Cat. No. AM1560) enriches small RNAs less than 200 nt in 30 min. This kit works under specialized wash conditions for efficient recovery of miRNAs, siRNAs, shRNAs, and snRNAs. Enrichment methods have an advantage over alcohol precipitation in terms of less time-consuming and more efficient recovery of RNA. Another kit by Invitrogen (Cat. No. K157001) works on the rapid isolation of small RNA in less than 15 min with silica-based two-column system. The RNAs recovered from this kit are ready to be processed for downstream analysis. the use of a two column system enables highly pure elution of small RNA without large RNA contaminants. Skryabin et al. compared different miRNA isolation methods, including mirVana miRNA Isolation Kit and PureLink™ miRNA Isolation Kit. The additional step of miRNA deposition on glass fiber columns made both the kits superior to other methods. Furthermore, the kit employs an extra step for EV lysis in the buffer containing β-mercaptoethanol, making it a more effective kit for high-purity RNA isolation. The highest RNA yield was shown by the miRNeasy Serum/Plasma Advanced Kit, suggesting better RNA isolation and stabilization [72]. Another study by Wright et al. suggested that even though the PureLink RNA kit provides a quick and easy way to isolate RNA, its inability to detect miRNA and the higher variability between samples from fresh and frozen plasma led to less accurate and less consistent results. Moreover, the miRNeasy kit could identify all miRNA and effectively manage the influence of low GC/high ΔG miRNA throughout the extraction procedure than the PureLink kit. Among all, the miRNeasy Serum/Plasma kit performance is superior in terms of sample compatibility; it required the least amount of plasma input and showed the least variation between fresh and frozen plasma samples [62].

These kits are highly tailored to isolate total RNA from the sample with the enrichment of small RNAs. Because of the more intense signals and lower signal-to-noise ratio, the separation of enriched short RNAs rather than total RNA can be particularly beneficial for microarray and qPCR expression studies.

Although the QIAzol or TRIzol, i.e., acid phenol-chloroform method, is a frequently used approach for miRNA isolation, it has some drawbacks as well, including a longer time requirement and the need for efficient waste management because it calls for hazardous and high-quality reagents. Also, the investigator's skill has a considerable impact on the results. This poses serious challenges for using it on a regular basis.

Another method that has been used for the isolation of miRNA from blood or plasma is by combining guanidine isothiocyanate (Gu) and octanoic acid (OcA). Lekchnov et al. [73], proposed to extract miRNA by OcA/Gu protocol which caused the degradation of protein complexes associated with miRNA and the subsequent precipitation of the miRNAs, and the purification of miRNA is further performed by spin column method. Being a chaotropic agent, Gu itself is not very efficient in isolating miRNA, but when used in combination with another precipitating agent that prevents or degrades protein complexes of miRNA such as octanoic acid, it causes

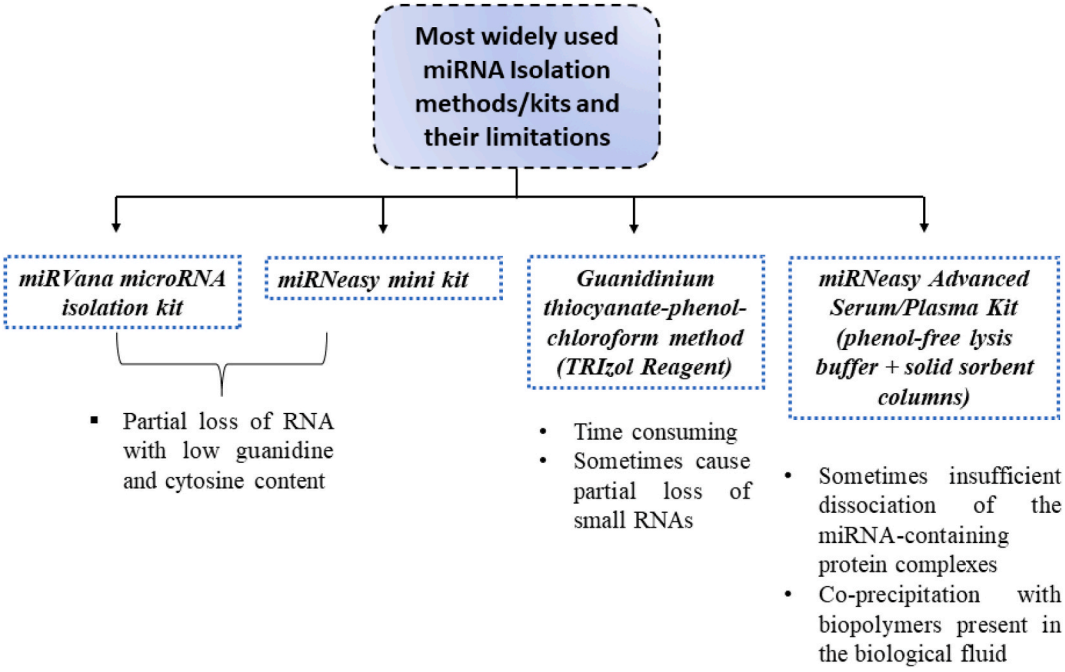

Fig. 1. This schematic representation depicts the most widely used kits and methods by researchers for the isolation of miRNAs from biofluids along with their associated limitations.

the release of miRNA from the complex and its efficient isolation. Not just in blood plasma this method was found to be efficient for isolating miRNAs in urine with high yield. It was 2.29 times more efficient than the acid phenol-chloroform extraction method [73].

For extracting and isolating miRNAs from exosomes or body fluids including blood/plasma sources, few studies favor manual approaches; instead, kit-based procedures are recommended for improved yield and purity of the final product and requirement of less amount of starting material [74,75]. Some of these are the TRIzol/QIAzol Reagent, the miRVana microRNA isolation kit, and the miRNeasy small kit. Although using these kits has significant limits related to the chemicals used and the amount of time required, some of which are mentioned in Fig. 1.

3.2. Isolation of exosomes from biofluids

Exosomes are a good source of cell-free miRNAs and are used predominantly for miRNA analysis. They have consistently been accustomed to liquid biopsy-based techniques for the non-invasive diagnosis of ovarian cancer. Exosomes have been isolated and purified using a number of techniques based on their physical properties and differential solubility, some of which are discussed briefly in the upcoming section; however, it is yet unclear how employing these different methods affects the expression profiling of miRNAs. Considering these manual methods some exosome isolation kits, for example, Total Exosome Isolation Reagent for exosome isolation from cell culture media by Invitrogen (Cat. No. 4478359), ExoQuick® ULTRA and ExoQuick-TC® ULTRA isolating exosomes from blood plasma, serum, and ascitic fluid and tissue culture media respectively by System Biosciences (SBI), ExoSure™ Exosome Isolation Kit by GeneCopoeia and exoEasy Maxi Kit (Cat. No. 76064) by Qiagen, are also available in the market (discussed in Table 3.) and have been employed extensively to save time and get better yield of highly purified exosomes.

3.2.1. Ultracentrifugation based

Ultracentrifugation involves the centrifugation of samples at high speed, causing the separation of large vesicles from small vesicles on the basis of gravitational force [Fig. 2 (a)]. Though it is an extensively employed and acknowledged technique, centrifugation at high speed often causes structural damage to exosomes. Additionally, there is still room for improvement in terms of how well exosomes are extracted from serum without becoming contaminated with proteins. Most of the time, the centrifugation method of isolation involves albumin protein contamination, making it a less successful isolation technique than kit-based isolation techniques like ExoQuick. Cardec et al. compared ultracentrifugation onto sucrose cushion and the precipitation-based ExoQuick kit method to isolate exosomes and came to the conclusion that the former method is not effective or reproducible for serum samples, primarily because serum albumin contaminates the samples [92]. Exosomes were purified from the plasma of ovarian cancer patients by ultracentrifugation in a study by Szajnik and team [93], and exosomal protein content was found to be higher for advanced-stage cancer.

Table 3
Depicts the exosome isolation techniques, their associated limitations and commercial kits available.

Method of isolation	Commercial kits available	Constraint associated	Advantages	Advancements in the method	Reference
Ultracentrifugation	–	• Time consuming • Labor intensive • Large amount of starting material is required	• Large volume of samples can be processed • High purity of isolated exosomes	• Combining sucrose gradient ultracentrifugation with successive centrifugations results in isolation of high purity exosomes. • Ultracentrifugation is often integrated with ultrafiltration and size exclusion chromatography to increase purity	[65,76–80]
Ultrafiltration	ExoMir™	Membrane often gets clogged after extensive usage	• Structure remains intact • Faster than other methods • Simple process • No high-tech instruments required	• Tangential flow filtration is preferred over conventional ultrafiltration to get improved recovery rate and purity (recovery rate >80 %). • Cyclic tangential flow filtration (exosomes are isolated in 30–200 nm size range)	[57,81,82]
Polymer precipitation	ExoQuick™ Total Exosome Isolation Reagent from Invitrogen™ PureExo® Exosome Isolation kit miRCURY™ Exosome Isolation kit	• Time consuming • Removal of precipitating reagents is sometimes cumbersome • Reagents often get co-precipitated • Removing bound exosomes from antibodies is difficult	• High yield and purity • Large volume of samples can be processed • Rapid • High recovery rate • Simple procedure	• Charge-based precipitation (negatively charged exosomes interacts with positively charged particles e.g., protamine) • Protamine with PEG gives higher yield of intact exosomes. • Charge neutralization with acetate isolates exosomes efficiently at 0.1 M, pH 4.75.	[54,64,65] [58,63–65, 78,81,83,84] [63] [64,65,85, 86]
Size exclusion chromatography	ExoSure™ Exosome Isolation Kit- combine precipitation and size exclusion chromatography (SEC) Exo-spin™ combines precipitation and size exclusion chromatography (SEC) SmartSEC™ Single for EV Isolation PURE-EVs	Less amount of sample can be processed at a time	• Less complex • Exosome Structure remains intact • Cost effective • Requires low sample volume • Uniformity in exosomal size	• Dichotomic SEC with reusable CL-6B 20 ml columns gives high purity exosomes with less no. of steps and particle/protein ratio of $1.2 \pm 0.9 \times 10^9$ particles/μg protein. • qEV SEC columns from Izon science separates exosomes with 150 μL sample volume within 15 min. These improvised methods reduced pore clogging limitation	[53,56,65, 87–89] [54] [54,65]
Density gradient centrifugation	OptiPrep™	• Time consuming • Labor intensive	• Less exosomal damage • High purity • Gold standard method with comparatively higher purity	Iodixanol-based gradient density centrifugation improves separation of exosome subpopulation	[54,83,84, 90]
Immunoaffinity	Exosome-Human EpCAM Isolation Reagent MagCapture Exosome Isolation kit.	• Expensive • Several elution steps required • Sometimes low yield of exosomes	• Highly specific • Allows enrichment of exosomal subtypes • Fast and continuous process • Double positive exosomes can be separated together	Exosome-Human EpCAM Isolation Reagent by thermofischer (Cat. No. 10618D) enables isolation of EpCAM-positive subpopulation of exosomes.	[55,83,84, 91] [66]

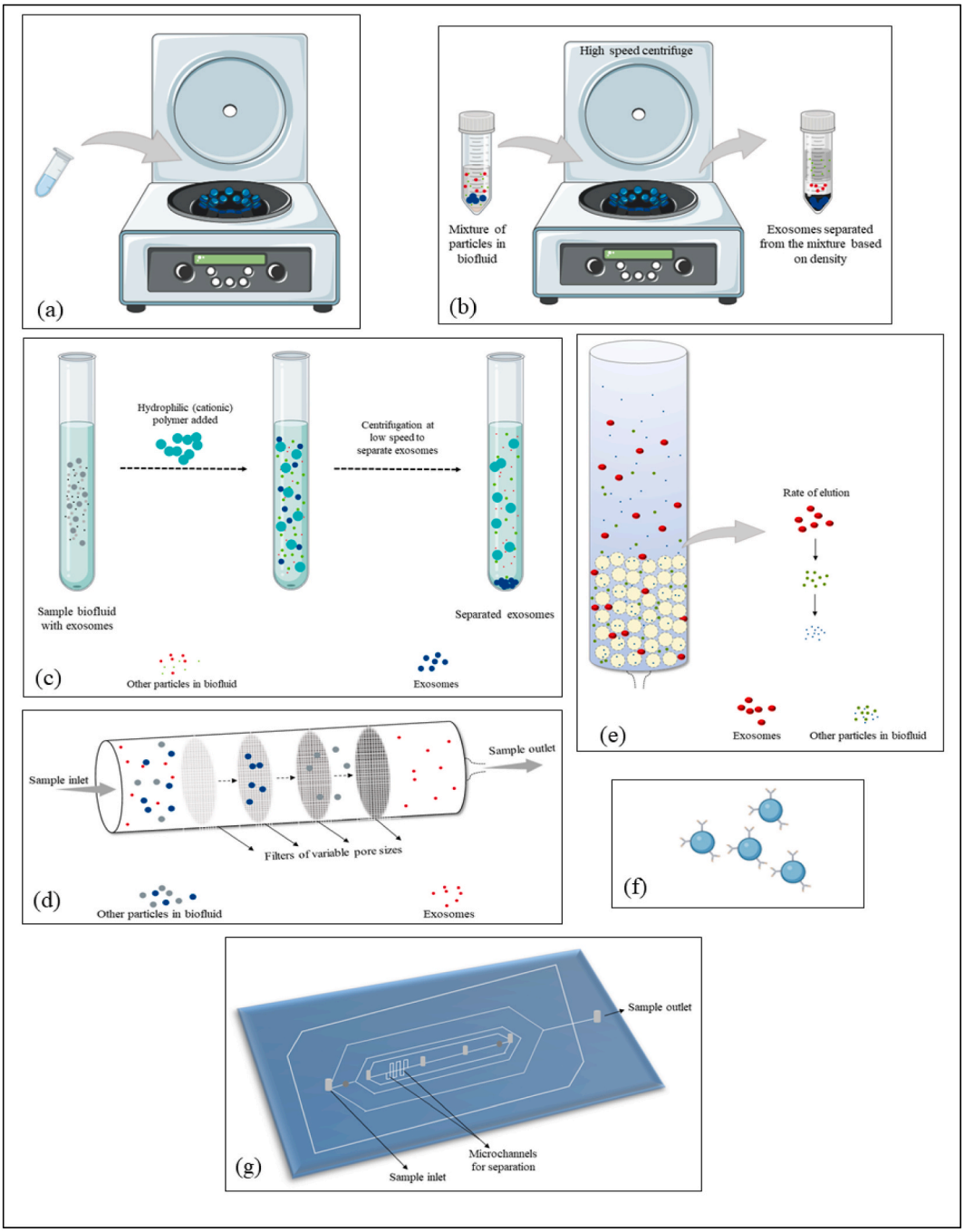

Fig. 2. (a–h) Depicts different techniques for exosome isolation (a) Diagrammatic representation of Ultracentrifugation, (b) Density gradient centrifugation, (c) Polymer precipitation, (d) Ultrafiltration, (e) Size exclusion chromatography, (f) Beads with exosome specific biomarkers for affinity-based isolation and (g) Basic microfluidic chip design for exosome isolation.

3.2.2. Density gradient centrifugation based

Density gradient centrifugation, as another method for isolating exosomes, falls under the broad category of ultracentrifugation. This method distinguishes exosomes from other cellular vesicles not only by their density but also on the basis of their size and shape [Fig. 2 (b)]. Exosomes, which have a density of roughly 1.15–1.19 g/mL, are among the particles and vesicles that separate depending on different densities and move from top to bottom [94]. But this method is often time-consuming, and the diagnosis of small volumes of samples is not well suited, thus making it less feasible for clinical applications. Also, the requirement of types of equipment and the need for the technical ability of the operator makes it less potent for large-scale applications.

When compared to ultracentrifugation by Zhang and group [95], density gradient centrifugation was found to be more comprehensive with better efficiency in extracting exosomes enriching exosome markers and exosome-associated proteins than ultracentrifugation.

3.2.3. Polymer precipitation based

Polymer precipitation relies on the interaction between the exosomal surface antigens and antibodies. Separation can be achieved using water-resistant hydrophilic polymers, which compete for water molecules in the vicinity of the exosomal membrane, such as polyethylene glycol (PEG). The polymer can decrease the solubility of less soluble components, i.e., exosomes, and result in their separation, forcing them out of solution by low-speed centrifugation and further filtration and producing highly pure exosomes with a good recovery rate [As described in Fig. 2 (c)]. Despite not requiring any complicated equipment for the extraction, this approach has certain shortcomings, including extensive information about surface biomarkers, sometimes non-specific protein aggregation contamination, and expensive polymer usage. Also, the removal of precipitating reagents for downstream analysis can be a cumbersome process. Commercial kits based on polymer precipitation approaches like ExoQuick are in use for exosome isolation and enrichment. Ryu and team [96] used three different techniques to isolate exosomes from the breast cancer patient serum. The first technique used only ultracentrifugation, whereas the second and third methods used ultracentrifugation followed by polymer-based precipitation using ExoQuick and Total Exosome Isolation kit respectively. They concluded that separating exosomes from serum samples by ultracentrifugation followed by the use of a polymer precipitation kit could effectively isolate them and give a higher yield of exosomes.

3.2.4. Ultrafiltration based

This technique depends on the size and molecular weight of the molecules. It employs membranes with different pore sizes, also known as nanomembranes, to sieve molecules with the desired size [Fig. 2 (d)]. Molecular weight cut-off membranes (MWCO) are generally applied to separate exosomes from cell culture supernatants [97]. Exosomes have been purified with the benefit of non-significant component removal using ultra-purification and further purification using ultracentrifugation [98]. This method of separation offers certain advantages, including the fact that the biological activity of exosomes is not compromised, and the procedure is simple to execute. Although, despite being a very common exosome isolation method, ultrafiltration is not without its drawbacks. For example, the filter membranes employed in this procedure frequently clog and trap vesicles, reducing their shelf life, and the method requires complex equipment, decreasing the effectiveness of isolation. Due to a lack of specificity, this technique is often now recommended for high-end use. Liu et al. [99] employed both ultracentrifugation and ultrafiltration for the collection of exosomes from SKOV3 cell lines and used it for Triptolide drug target by designing a triptolide-loaded exosomes delivery system. Another analysis by Gao et al. compared the yield and purity of isolated exosomes by ultrafiltration and polymer precipitation methods. The exosomes were isolated from adipose tissue conditioned medium using size exclusion-based ultrafiltration and ExoQuick-TC kit. When compared for exosomal yield, the ultrafiltration method is superior but the purity is low. The filtration method maintained the biological properties of the isolated exosomes when compared to the precipitation-based method indicating its efficiency in translating into higher clinical setups [100].

3.2.5. Size exclusion chromatography based

Size-exclusion chromatography (SEC) segregates the components on the basis of their molecular size and the pore size being used for the exosome separation in which smaller molecules take longer to elute because of diffusion into the pores, whereas larger molecules elute first, as shown in Fig. 2 (e). Combined with another method, this approach has been shown to provide high purity with a good recovery rate with less damage to exosomal structures, yet its shortcomings are exacerbated by high equipment prices and several enrichment steps [94,98].

During a study conducted by Baranyai and team [101] they compared two exosome isolation methods simultaneously, Differential Ultracentrifugation and Size Exclusion Chromatography from blood plasma in which the product from centrifugation was found to be extremely contaminated with serum albumin proteins, making it a poor approach for obtaining a pure exosomal preparation. Size exclusion chromatography, on the other hand, had no such contamination problems in this case. When applied alone, however, this strategy has its own efficiency restrictions due to the lack of specificity of the isolated molecules where particles with similar size are often separated together resulting in contamination issues.

3.2.6. Immunoaffinity based

Using an immunoaffinity-based method, exosomes are isolated from a multitude of cell components based on cell surface markers' presence. The most prevalent of which are platelet-derived growth factor receptors (PDGFRs) and transmembrane proteins such as CD81, CD82, and CD9 on the exosomal membrane. Specific antibodies are complementary to the surface receptors and are immobilized on certain carriers like magnetic beads [Fig. 2 (f)], chromatography matrices, and microfluidic chips, which firmly attach to the associated exosomes and lead to very specific and selective extraction of its subpopulation [94]. Although it offers the advantage of isolating specific exosome subtypes and high purity of isolated vesicles, its disadvantages still include extensive cost and lipoprotein contamination, and the method may take long hours to complete [76]. Also, this technique can only be applied to the EVs that have a high degree of marker expression on their surface which can lead to the loss of other miRNA-carrying exosomes and may give biased results.

3.2.7. Microfluidics based

Microfluidics-based separation is the most advanced technique for isolating exosomes with utmost purity and specificity. By combining sensing and analysis on a single chip using microfabrication technology, microfluidics offers miniaturization of the process. Micro- and nano-fabricated channels are made on-chip surfaces to direct small volumes of accelerated fluids and cause high-purity separation of target molecules [33]. Fig. 2 (g) displays a basic design of a microfluidic chip for exosome isolation. Based on the different physical and chemical properties of exosomes, the microfluidics-based isolation of exosomes is further divided into categories such as immunoaffinity-based, acoustic-filed based, and filtration-based approaches [76]. Dorayappan et al. [102], described an in-house microfluidic system for isolating culture media exosomes on the basis of cell surface markers displayed by High-Grade Serous Ovarian Cancer (HGSOC) cell lines in contrast to epithelial cells of normal ovary surface and epithelial secretory cells of the fallopian tube. With the potential to discover new exosome surface biomarkers, this study opened up new possibilities for the early detection of ovarian cancer.

Microfluidics-based systems have been broadly categorized into two main types based on separation approaches: i) on the basis of physical properties, which includes Filtration, Viscoelastic, Acoustic-Wave, Deterministic Lateral Displacement (DLD) and Electrical-Field ii) on the basis of immunoaffinity of exosome surface proteins with antigens or antibodies [81,103]. Other categories include the integrated techniques for microfluidics-based exosome isolation. The basic categorization and principles have been described in Table 4.

(i) Microfluidics based on physical property

The physical property based microfluidic separation mainly includes the separation methods based of particle size. Table 5 represents summary of studies on physical property-based exosome isolation.

(a) Filtration based microfluidics

Filtration-based microfluidics comes with the advantage of non-disruption of exosomal structure, maintaining its functional integrity. The method is relatively simple and more cost-effective. The chip designed for filtration constitutes nano filters also known as nanomembranes or nanowires [104,105]. The filtration method applied in microfluidics can be pressure-driven, electrophoresis-driven, double filtration, multimembrane setups or nanowire filtration. Based on filtration-based studies, it can be analyzed that although a pressure-driven filtration takes less time still, an electrophoretic filtration is more preferred due to its high purity yield.

(b) Viscoelastic based microfluidics

It is a size-based and label-free method of exosome separation. The elastic lifting force, which is proportional to the particle volume, is used to sort particles by size. When deformed, viscoelastic fluids display both viscous and elastic properties. The technique uses fluid elasticity or inertia to propel particle migration laterally into stable equilibrium points inside a microchannel. This directional inertial movement is experienced by Newtonian fluids [106].

(c) Deterministic Lateral Displacement (DLD) based microfluidics

DLD is a passive microfluidic separation technique with no requirement of external force. In DLD, tilted pillars are arranged in such a way that the sample fluid bifurcates between the gaps created in between the pillars [107]. The laminar fluid works with the array to push particles along a path that the device has predetermined. DLD method works in two modes: first is displacement mode where large particles move laterally and second is zigzag mode where smaller particles follow the streamline motion [108].

Table 4
Basic categorization of methods under microfluidics for exosome isolation.

Microfluidics based exosome isolation techniques	
Type of technique	Principle
(i) Based on physical properties	
(a) Filtration based	Separation of particles based on particle size
(b) Viscoelastic flow-based	Separation of particles based on shear force induced on particles
(c) Deterministic Lateral Displacement (DLD) based	Separation of particles based on laminar flow and bifurcations between particles with different radius
(d) Acoustic wave-based	Separation of particles based on acoustic force acting on the fluid which is proportional to particle size
(e) Electrical field-based	Separation of particles based on electric field driven pumping
(ii) Based on affinity	
Immunoaffinity	Separation based on affinity of exosome surface proteins to antibodies functionalized on beads/surfaces or conjugated to particles such as avidin-biotin
(iii) Based on integrated techniques like fluorescence, colorimetric, surface plasmon resonance (SPR) and electrochemical	

Table 5
Studies on exosome isolation by physical property based microfluidic techniques.

Techniques	Sample-type	Size of exosomes	Recovery yield	Reference
Filtration based				
Electrophoresis driven filtration	Whole blood	~150 nm	–	[113]
Pressure-driven filtration	Whole blood		–	[113]
Ciliated nanowires	Liposomes	83–120 nm	–	[104]
Double tangential flow filtration	Blood	30 and 200 nm	77.8 %	[114]
Double-filtration	Cell culture media and urine	30–200 nm	74.2 %	[115]
Exodisc: double filtration	Cell culture supernatant and urine	20–600 nm	>95 %	[116]
Particle property based				
Deterministic Lateral Displacement (DLD)	Urine	20–110 nm	10^4–10^6 particles/min	[117]
Viscoelastic Flow	Cell culture medium	100 and 300 nm	>81 %	[118]
Acoustic-wave	Cell culture media	<200 nm	>80 %	[109]
	Whole blood	~110 nm	>99 %	[117]
	Plasma	10–100 nm	~90 %	[119]
Electrical field	Plasma	50–150 nm	–	[112]
•Dielectrophoresis	–	51 nm–1500 nm	–	[120]
	MCF-7 cells	104.02 ± 6.99 nm	–	[121]

(d) **Acoustic-Wave based microfluidics**

This method applies acoustic waves to separate exosomes from biofluid. Acoustic radiation forces, which are proportionate to the volume of the particles, are applied to the fluid's particles during the separation process. Stokes' law, which is proportional to the particle's radius, will also have an impact on the particle when it is subjected to the acoustic radiation force. Larger particles, therefore, experience stronger acoustic forces, which cause them to further alter their path [76]. Lee et al. isolated exosomes from cell culture media with <200 nm diameter fabricating an acoustic-based microfluidic device. With a >80 % recovery rate, this acoustic system was able to maintain structural integrity and was compatible with a small amount of sample [109].

(e)**Electrical field-based microfluidics**

Microelectrodes in a solution can be subjected to an alternating current (AC) voltage to generate a range of electrokinetic phenomena. When electrophoresis or dielectrophoresis combines with microfluidics, the particles move due to the polarization effect under an electric field and gets separated [110,111]. Separation is based on their electrophoretic or dielectrophoretic potential. Ibsen et al. developed an alternating current electrokinetic (ACE) microarray chip to concentrate exosomes based on their dielectric potential from undiluted human plasma samples of glioblastoma patients. With a minimum 30–50 μL sample volume, the system was able to separate exosomes in less than 15 min of size range 50–150 nm [112].

(ii) **Microfluidics based on immunoaffinity**

Exosomes possess various markers on their surface unique to the parent cell of origin which makes their immunoselection possible. Immunoaffinity-based microfluidics often involve the employment of exosome-specific antibodies immobilized on beads or on the microfluidic chip surface to isolate exosomes with high specificity [122,123]. The most prevalent markers on the exosomal surface, CD63 and CD9, allow targeted and affinity-based separation of exosomes from other vesicles derived from membranes. It has also been demonstrated that heat shock proteins (Hsp90 and Hsp70) and tetraspanins (CD9, CD63, CD81, and CD82) are involved in exosome transport and membrane fusion with target cells during EMT [124,125]. Presently, several microfluidic-based methods have been used to analyze exosomes [Table 6]. For instance, the first immunoaffinity-based system for separating exosomes from serum and cell culture medium was reported by Chen et al., escaping ultracentrifugation and density separation steps, the method employed separation of exosomes from serum and culture medium on anti-CD63 IgG coated surfaces. The method had 95 % exosomal isolation efficiency, which can be successfully exploited to develop a liquid biopsy-based diagnostic assay. With just 100–400 μL serum samples,

Table 6
Summary of microfluidic techniques based on immunoaffinity.

Techniques	Marker	Sample-type	Recovery yield	Reference
Hydrodynamic trapping in microchannels and exosome capture beads	CD63	Culture medium supernatant	NA	[122]
^{HB}EXO-Chip	GPC1	Plasma	75 %	[123]
Anti CD9 immobilized on magnetic nanoparticles (Fe3O4NPs)	CD9	Whole blood	NA	[129]
ExoChip	CD63	Serum	NA	[127]
CD9/CD63 immobilized on gold surface	CD9/CD63	Serum	NA	[130]
CD63 immobilized on magnetic nanoparticles	CD63	Cell culture medium and plasma	NA	[131]
iMER: immuno-magnetic exosome isolation	CD63, EGFR and IgG	Serum	>93 %	[132]

the method could do separation within 1 h. The study also introduced herringbone groves into the fluid channels for efficient mixing and separation of particles and to increase the capture potential [126]. In the year afterwards, Kanwar et al. fabricated a microfluidic chip in the same vein. This study employed ExoChip functionalized with CD63, which was further stained with a fluorescent carbocyanine dye (DiO) to fluoresce exosomes. They were able to isolate exosomes with nearly ~30–300 nm size [127]. Similarly, Sharma et al. isolated melanoma cell-derived exosomes by using antibody 763.74 specific for chondroitin sulphate peptidoglycan 4 (CSPG4), which is present in >80 % of melanomas [128].

Microfluidics has paved its way by being exceptionally practical with a high recovery rate, from needing small amounts of reagent to taking very little time to isolate molecules [133]. Yet, the lack of standardization and extensive testing on clinical samples add to its limitations and hinder the downstream study of the separated exosomes [134]. To address these challenges various research advancements have been made associated with optimization of sample preparation methods and purification protocols. For instance, to address the challenges associated with filtration-based microfluidics systems, Wang et al. designed a microfluidic device made up of ciliated micropillars which formed a porous silicon nanowire-on-micropillar structure. Filtering other cell structures, such as proteins and cell debris, the designed device could potentially trap intact exosomes, which can be recovered by dissolving the nanowires in a PBS buffer. The device was able to separate highly pure vesicles from other components of the biofluid, maintaining their structural and functional integrity [104]. Another novel design by Chen et al. fabricated a 3D scaffold chip with ZnO nanowires to capture exosomes effectively. The nanowires were immobilized with antibodies having an affinity for exosomes, and the captured exosomes were detected colorimetrically by horseradish peroxidase enzyme-labeled antibodies [135]. Based on filtration technique, a chip known as Exosome Total Isolation Chip (ExoTIC) was designed by Liu et al., which was easy to use and produced a highly pure yield of up to ~4–1000-fold higher than that with ultracentrifugation alone for exosomes of 30–200 nm size range. The fabrication involved multistep filtration layers and was able to isolate more than 90 % of exosomes from the sample. The designed ExoTIC chip could isolate exosomes from a sample volume of as low as 10 μL, which might be beneficial for clinical purposes. Also, having an extremely high yield, this could be easily scaled up to large-scale sample processing, unlike other conventional isolation techniques [88].

(iii) **Integrated microfluidics methods**

Although the aforementioned conventional separation techniques are the most popular, they also have some very common limitations associated with them, including a high sample requirement, lack of desired purity, a possibility of exosome damage, and a lengthy time commitment that makes it difficult to keep up with the cutting-edge escalating demands of scientific research. The primary drawback of the various exosome isolation strategies is the co-precipitation of proteins with non-exosomal origin. Therefore, despite the abundance of available techniques, one universal technique that can be used to separate exosomes from samples of any type and source is still needed. However, it has been shown that combining two or more techniques to separate exosomes from complex samples has substantially enhanced the reproducibility and purity of the isolated product, as shown in Table 7. For instance, Cho et al. [97], integrated methods like polymer precipitation, ultracentrifugation, size exclusion chromatography, and ultrafiltration to isolate exosomes from a urine sample and the integration of the methods has shown to improve the purity and increase the yield of the isolated exosomes to a greater extent. However, their low purity and lengthy extraction process render them less trustworthy for lab-to-clinic transition. Fig. 3. Represents different sources for miRNA/exosome isolation for ovarian cancer detection and their isolation via different techniques.

(a) **Fluorescence method**

When a substance is exposed to a specific wavelength of light, it undergoes fluorescence, which is the short-lived emission of light with a wavelength longer than the incident light. Fluorescence detection is quick, sensitive and precise with a comparatively simple process. A widely used strategy is to use fluorophore-tagged antibodies to bind to the exosomes that have been captured and then characterize the exosomes using fluorescence microscopy [141]. An efficient exosome isolation method was designed integrating affinity with particle trapping by Tayebi et al. Functionalized microbeads (CD63-Exobead) of 20 μm diameter with biotinylated antibodies specific for exosome surface protein (CD63 antibody) were used to trap exosomes based on the passive hydrodynamic trapping principle. The design made fluorescence quantification of single microbeads, reducing optical interference of the background noise and

Table 7
Integrated microfluidic techniques for exosome isolation.

Technique	Sample	Recovery yield/detection limit	Reference
ExoSD Immunoaffinity + Fluorescence	Serum	>80 %	[136]
PS-ED Immunoaffinity + Fluorescence	Whole Blood	2.5×10^2- 2.5×10^8 particles/μL	[137]
ExoChip Immunoaffinity + Fluorescence	Serum	–	[127]
Graphene oxide/polydopamine (GO/PDA) nano-interface	Plasma	50 μL−1	[138]
Immunoaffinity + Surface plasmon resonance imaging (SPRi)	Cell culture supernatant	–	[139]
Surface plasmon resonance (SPR) + Immunoaffinity	Tumor cell culture medium	–	[139]
Deterministic lateral displacement + Size exclusion chromatography	Serum and urine	~70 %	[140]

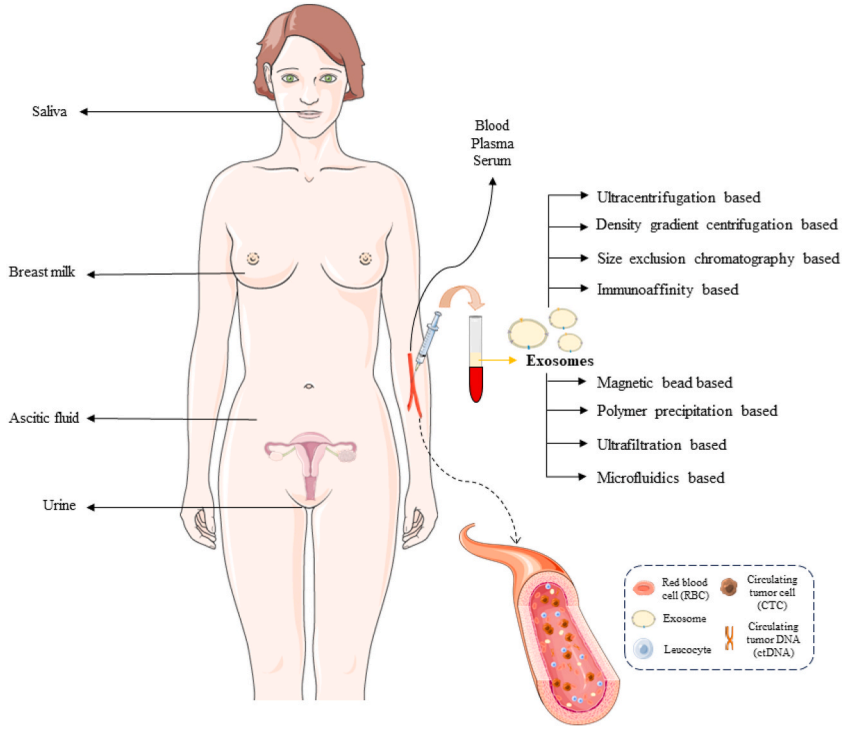

Fig. 3. Schematic representation of exosomal miRNA based liquid biopsy from different sources for detecting ovarian cancer and techniques for their isolation (Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

capturing the average fluorescence density of individual tagged beads. The fluorescence signals were quantified for both CD63-positive and EpCAM-positive exosomes, where CD63-Exobeads outperformed EpCAM-positive exosomes in terms of fluorescence intensity [122].

(b) **Colorimetric method**

The colorimetric detection principle for exosomal content is based on the determination of depth of the solution and color change [138]. Centrifugal microfluidic system integrated with two nanofilters (Exodisc) separated exosomes with 20–600 nm size in 30 min from cell-culture supernatant and urine of cancer patient with >95 % recovery yield. The isolated exosomes by this method showed the highest optical density for CD9/CD81 by sandwich ELISA and a 13-fold greater concentration of isolated vesicles [116].

(c) **Surface plasmon resonance (SPR) method**

SPR measures variations in permittivity brought on by molecules adhering to heavy metal films. The SPR-based exosome isolation devices are portable with high sensitivity and specificity [142]. Based on Localized Surface Plasmon Resonance (LSPR) Thakur et al. designed a self-assembled gold nano-island biosensor to detect exosomes in blood serum and urine of a lung cancer mouse model and A-549 and SH-SY5Y cells with a detection limit of 0.194 μg/ml. This biosensor covered a wide range of detection from 0.194 to 100 μg/ml [143]. Similarly, Zhu et al. designed a surface plasmon resonance imaging (SPRi) based microfluidics system combined with microarray of antibodies against CD9, CD41b and MET immobilized on a glass sensor chip coated with gold to detect and characterize exosomes in tumor cell culture medium. The device established a positive correlation between the level of exosomes and the metastasis by cell lines [139]. Similarly, using CD24 and EpCAM as an exosomal marker, Im et al. have carried out a quantitative analysis of exosomes integrating SPR with microfluidics into a biosensor. The study designed a nano-plasmonic exosome (nPLEX) sensor, which consisted of a metal film with nanohole patterns. When exosome binds to the sensor surface, its refractive index changes which is measured using wavelength shifts ($\Delta\lambda$) in the light spectrum (spectral detection) or intensity change (Δp) at a fixed wavelength [144].

(d) **Electrochemical method**

The electrochemical detection approach has been successfully integrated with microfluidics to separate exosomes in many studies. For instance, Cavallaro et al. developed an affinity-based electrokinetic microfluidic separation system for lung cancer cell line H1975-derived exosomes using surface markers EGFR, CD9, and CD63. The change in streaming current is analyzed with the subsequent

interaction of the marker with exosomes and displayed a very high sensitivity of ~0.4 pM [145]. The electrochemical approach is integrated with microfluidics to make the detection process faster, miniaturized and cost-effective, thus enabling fast processing of the result and sensitive capture of exosomes.

To meet the growing demand for the therapeutic and diagnostic benefits of purified exosomes, standardization and advancements in lab-on-a-chip techniques are necessary, notwithstanding the development of microfluidics-based strategies and the application of various integrated techniques. On this note, many researchers have worked together to address the challenges. For example, in the case of ultrafiltration, clogging of pores is a major limitation of the method. To overcome this drawback, sequential filtration or allowing large particles to pass through beforehand can be given a consideration [146]. For instance, ExoTIC, an exosome total isolation kit, uses nanofilters also called nanoporous membranes, in which, before passing through the 30–200 nm membrane, the biofluid/sample is prefiltered through a 200 nm filter so that large debris is removed, and exosomes are enriched further through sequential filtering [88].

In a similar way, exosome isolation can be fastened by combining nanofiltration with centrifugation. ExoDisc, a double filtration membrane system, processes a sample within 30 min and when used in combination with ELISA (fabricated on the chip) takes 1 h for complete analysis [116]. Likewise, designing microfluidics to improve mixing and enable automated washing, which is not achievable with conventional methods, paves the way for efficient exosome isolation. Taking this into account, Chen et al.'s revolutionary study showed how to use an anti-CD63 functionalized microchannel to extract exosomes from serum. The device could process 10–400 μL of sample at 16 μL/min the vesicles were then isolated with 42–92 % efficiency, and analyzed further [126]. The capturing of exosomes can also be improved by introducing nanostructures to the chip surface while fabrication. The likelihood of exosome interaction and isolation increases with the surface area. For example, Zhang et al. integrated a graphene oxide/polydopamine nano-interface and showed low non-specific protein absorption leading to decrease in protein contamination ultimately resulting in an improved detection limit of 50 exosomes/mL [138]. Kamyabi et al., in order to improve the interaction between the antibody coated pillars (anti- -CD9, -CD63 and -CD81 antibodies) and the EVs, used a zigzag pattern to arrange ~100,000 pillars to isolate tumour-derived exosomes. In 1.5 h, they were able to process 2 mL of plasma and produce 2–14 ng of DNA [147]. Another significant contribution has been made in Deterministic lateral displacement-based microfluidics, where using a massively parallel nano-DLD device with 1.44 billion pillars per chip, Smith and team improvised this technique. It was designed with 1024 nanoDLD arrays integrated into a single chip, processing sample fluids at up to 900 μL h^{-1} in parallel. This resulted in a notable increase in throughput of the method with an improved yield of ~50 %, and the system could separate vesicles from serum and urine at a 15 μL/min flow rate and 1 MPa driving pressure with smaller sample volume input [140].

4. Development of automated standardized assay with required miniaturization for bench-to-bedside translation

Several methods have been adopted till now for exosome separation but with some or the other limitations making them difficult to transform from lab to clinics. Some of the most common isolation techniques involve ultracentrifugation, polymer precipitation and ultrafiltration-based approach but they also have their associated drawbacks which make them difficult to translate to large scale down streaming for clinical applications. Exosomes are frequently challenging to separate and detect in body fluids at a low cost with sufficient specificity and sensitivity due to their small size and sample variability. Therefore, finding a reliable and uniform way to extract exosomes for therapeutic and diagnostic application is essential.

Ultracentrifugation has traditionally been the preferred approach for isolating exosomes, followed by differential centrifugation, which later emerged as the most popular and widely acknowledged technique. In a poll conducted in 2020, more than 80 % of researchers agreed to prefer this approach for their study [34]. Since the isolated vesicles are comparable in size and density to other extracellular vesicles and viruses, these procedures, despite being widely accepted, have also demonstrated significant limitations relating to purity, number of processing steps and yield of exosomes as an end product.

In order to find solutions to these constraints, researchers have used novel approaches such as by combining two or more separation techniques and applying microfluidics-based methods to achieve precise isolation with reduced contamination of other cell components. Because of its inexpensive cost, demand of less experimental time and sample, and enabling high sensitivity for detection, microfluidics makes a suitable way for clinical use, notably in the field of disease diagnosis and prognosis by analyzing microRNAs present in the body fluids.

Despite the fact that microfluidic technologies are frequently asserted to be "cost-effective" substitute to traditional benchtop-scale instruments for exosome isolation when compared to other conventional approaches in use, this classification still remains arbitrary since it is difficult to compile accurate cost data for different microfluidic systems with consistent data. When it comes to extensive clinical testing and their validation in patient cohorts, the microfluid systems are presented to be confined only within lab research settings and to guarantee reproducibility of the system proper standardization of the methods are still under investigation. Even with existing microfluidic methods for isolating exosomes [113,117,148], lab to benchtop translation is still an open subject when not taking healthcare regulations and appropriate validation tests into account.

5. Conclusion and future outlook

Exosomes are likely candidates to function as a source in the diagnosis and prognosis of cancer because it has been constantly discovered that these cells actively release them. They are an excellent picture of the diseased state and pathological condition of the body tissues and any abnormalities that may exist in the cells. However, exosome isolation must be standardized technically in order for exosomal miRNAs to be used in clinical settings, as these can only be used to focus the search for diagnostic and prognostic miRNAs if they can be isolated to high purity repeatedly while still preserving enough material for subsequent analysis. Several isolation

techniques can be combined to overcome this restriction and improve the assay's yield and specificity. When size exclusion chromatography was combined with ultrafiltration to isolate exosomes from a urine sample, higher concentration of exosomes with less protein contamination was obtained in comparison to the extraction done only with ultracentrifugation [149]. The optimization, as well as intra- and inter-laboratory variability of the sample source should be addressed to effectively standardize protocols. Also, the sample quality should be checked prior to exosome isolation to get an insight about the correct isolation procedure adopted and track any external contamination chances. The development and inclusion of kits with cutting-edge techniques can help in the easy standardization of isolation protocols. For instance, a recent development by iZON science brought a new era in the isolation field of exosomes where a qEV Exosome Isolation Kit enabled quick, affordable and highly accurate separation of exosomes with a sample volume of less than 150uL to up to 10 mL just within 15 min employing size exclusion chromatography [150]. Selective isolation of exosomes may help standardize such protocols and maintain their primary functions. Additionally, biofluid-specific functional characterizations of exosomes and established controls should be taken into account while standardizing and optimizing isolation techniques. Prior to implementing these protocols and applying them in clinical settings, it is imperative to identify and define the population of exosomes with homogeneous characteristics and gain more understanding towards exosome biology and their biochemical analysis.

Although liquid biopsy is among the emerging fields and surpasses multiple hurdles possessed by tissue biopsy samples, still while implementing liquid biopsy samples in clinics for disease management various ethical considerations should be taken into account. Chances of false positives and false negative results from both clinical as well as ethical point of view is one of the major concerns related to liquid biopsy detection. As false negative results indicate the absence of disease hence compromising patient's safety and delay in the treatment. Similarly, false positive results could have harmful repercussions on the health if the patient relies on and proceeds with therapy, affecting the patient-physician relationship and putting a sense of trust at risk, raising concerns from an ethical perspective. It might be ethically questionable to subject people to such severe emotional distress. In fact, having the option of early detection, which is associated with higher rates of treatment success, is a very significant opportunity in the battle against disease diagnosis. However, there is a chance that the detection method will yield imprecise results; the ethical and clinical implications for patients from the perspective of informed patient consent, open communication between patient and the physician about the treatment procedures, the associated risks and its repercussions and the physician-patient relationship should be carefully considered. Protecting the ethical rights of patients, the intent of the treatment, the available alternatives, and how it will affect their health should all be discussed beforehand. More trainings should be given to the clinical staff about the statistical data interpretation which should be communicated to patients to avoid misinterpretation of biopsy results and help them take an informed decision.

It is obvious that the existing separation techniques are not ideal despite the significant advancements made. There is no all-round method for isolating exosomes in the current state of the art. More integrated, selective, high-throughput, sturdy, high-recovery-rate and reproducible method will open up a promising new path for exosome-based miRNA detection for cancer diagnosis and prognosis in the years to come along with attaining high-purity exosome isolation integrated with downstream analysis.

Efforts to develop alternative separation and isolation platforms have been sparked by the constraints of current exosomal miRNA extraction procedures, a significant limiting factor in therapeutic applications. In the due to significant overlap in their physicochemical and biochemical properties, and the heterogeneity of exosomes themselves have all been shown to make it difficult to quickly and effectively isolate exosomes with purity and good yield.

In this study, we have outlined the key advantages and challenges of exosome isolation and purification using conventional techniques as well as accepted novel approaches. We have also emphasized the need for standardization of a single approach for exosome separation is necessary, regardless of the biological complexity and source of the exosomes, and how this method's bench-to-bedside translation remains difficult for researchers due to inadequacies in one or more isolation techniques. Additionally, we have discussed how to best solve the problems that these strategies frequently encounter in the future. Exosomes with their associated microRNAs are set to provide a potent platform for cancer detection and its prognosis whose success will undoubtedly represent a significant step forward in the improvement of human health, once the aforementioned issues are resolved.

Ethical approval

Not applicable.

Data availability statement

Sharing research data helps other researchers evaluate your findings, build on your work and to increase trust in your article. We encourage all our authors to make as much of their data publicly available as reasonably possible. Please note that your response to the following questions regarding the public data availability and the reasons for potentially not making data available will be available alongside your article upon publication.

Has data associated with your study been deposited into a publicly available repository?

- No data was deposited into a repository for this study.

Sharing research data helps other researchers evaluate your findings, build on your work and to increase trust in your article. We encourage all our authors to make as much of their data publicly available as reasonably possible. Please note that your response to the following questions regarding the public data availability and the reasons for potentially not making data available will be available

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Has data associated with your study been deposited into a publicly available repository?

- No data was used for the research described in the article.

CRediT authorship contribution statement

Mridula Bhadra: Writing – original draft. **Manisha Sachan:** Supervision.

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.

Acknowledgments

Author **MB** would like to thank the Ministry of Education (MoE), Government of India for the research assistantship.

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