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Biological Functions and Current Advances in Isolation and Detection Strategies for Exosome Nanovesicles

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Exosomes are nanoscale (≈30–150 nm) extracellular vesicles of endocytic origin that are shed by most types of cells and circulate in bodily fluids. Exosomes carry a specific composition of proteins, lipids, RNA, and DNA and can work as cargo to transfer this information to recipient cells. Recent studies on exosomes have shown that they play an important role in various biological processes, such as intercellular signaling, coagulation, inflammation, and cellular homeostasis. These functional roles are attributed to their ability to transfer RNA, proteins, enzymes, and lipids, thereby affecting the physiological and pathological conditions in various diseases, including cancer and neurodegenerative, infectious, and autoimmune diseases (e.g., cancer initiation, progression, and metastasis). Due to these unique characteristics, exosomes are considered promising biomarkers for the diagnosis and prognosis of various diseases via noninvasive or minimally invasive procedures. Over the last decade, a plethora of methodologies have been developed for analyzing disease-specific exosomes using optical and nonoptical tools. Here, the major biological functions, significance, and potential role of exosomes as biomarkers and therapeutics are discussed. Furthermore, an overview of the most commonly used techniques for exosome analysis, highlighting the major technical challenges and limitations of existing techniques, is presented.

1. Introduction

Exosomes are nanosized (30–150 nm) vesicles released by most cell types and present in different biological fluids such as blood, saliva, and urine.[1–4] These vesicles carry unique cargo containing proteins, messenger RNA (mRNA), and microRNA (miRNA). Exosomes can transfer their cargo to recipient cells, which has been demonstrated to alter the biochemical composition and signaling pathways of the recipient cells.[5,6] Exosomes were discovered nearly 30 years ago. In 1983, two research groups independently reported that transferrin receptors in reticulocytes are associated with small, ≈50 nm diameter-sized vesicles.[7,8] These vesicles are literally jettisoned from maturing blood reticulocytes into the extracellular space. The functions of these small vesicles were unknown until 1996, when it was reported that exosomes are secreted by B immune cells and can stimulate human $CD4^+$ T-cell clones in an antigen-specific manner.[9]

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In 2007, mRNA and miRNA were shown to be present in exosomes derived from mouse and human mast cells.[10] Moreover, this work suggested that exosomes are capable of shuttling RNA between the cells. This significant discovery indicated that exosomes represent a new type of intercellular communication.

Recent studies indicate that exosomes shed from tumor cells may be involved in the metastatic process via the transfer of cancer-specific cargo (i.e., RNAs and proteins) to normal cells.[11–15] It has been reported that breast and ovarian cancer patients contain higher concentrations of exosomes than healthy individuals, suggesting that cancer could increase the overall exosome abundance.[16–18] Due to their ability to represent the metabolic stage of cell/organ origin and their critical role in major pathological processes, exosomes are considered novel and promising biomarkers for a wide range of diseases, including different types of cancer.[19,20] Therefore, accurate isolation, quantification, and analysis of disease-specific exosomes have gained much attention in recent years. Due to the complex nature of the sample matrix and the physicochemical properties of exosomes, accurate isolation of exosomes from bodily fluids poses significant challenges.[21,22] Over the last several decades, various conventional methods such as differential and buoyant density centrifugation, ultrafiltration, and immunological separation have been employed to isolate exosomes.[23–28] Among these methods, differential and buoyant density centrifugation is the most widely used approach to extract exosomes from cell culture media and bodily fluids.[29] Currently, several commercial exosome isolation kits are also available. These kits avoid time-consuming differential steps by precipitating the vesicles with polyethylene glycol or similar components, although the isolation of nonvesicles has also been observed.[30] Despite these advances in detection strategies, routine detection and quantification of exosomes are still challenging and cumbersome,[31,32] partly due to the lack of rapid, sensitive, reproducible and low-cost methodologies.

In recent years, many detection approaches have been extensively developed to analyze exosomes and exosomal cargo for both research and clinical purposes.[33–35] For example, nanoparticle tracking analysis (NTA),[36] enzyme-linked immunosorbent assay (ELISA),[37] flow cytometry,[23] and fluorescence-activated cell sorting (FACS)[38] have successfully been developed for exosome quantification. ELISA is one of the most widely used exosome detection techniques, relying on a sandwich immunoassay between antibodies against the protein enriched membrane of exosomes (e.g., tetraspanin marker of exosome membrane) and a secondary type of antibody marked with horseradish peroxidase (HRP).[39] A range of modified ELISA approaches with improved analytical performance has also been developed. For instance, while conventional ELISA suffers from long assay times, laborious sample loading, reagent addition, washing and incubation steps, a modified ELISA integrated with a lab-on-a-chip microfluidic platform offers a simple, fast, and automated analysis of exosomes.[40] Much attention has also been given to developing new strategies based on microfluidics and electrochemical biosensors.[41–45] Among these, electrochemical biosensor approaches have shown great promise due to their fast, simple, and cost-effective procedures, as well as the requirement for less sample volume. On the other hand, microfluidics-based approaches are well known for improving the overall analytical performance (e.g., significant reduction in the total assay time, minimum consumption of samples and reagents, enhanced sensitivity, etc.) of the method. Moreover, such platforms reduce manual intervention by providing automated processes.[46]

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The aim of this review is to discuss the significance and role of exosomes as intercellular communication vehicles in altering the physiological and pathological conditions of various diseases, including cancer and neurodegenerative, infectious, and autoimmune disorders via transferring RNA, DNA, proteins and lipids between cells. Current advances in exosome isolation and detection strategies will also be discussed alongside their major technical challenges and limitations.

2. Biogenesis of Exosomes

In 1987, the term 'exosome' was coined to describe a group of nanosized (30–150 nm) vesicles that are formed inside endosomes and released into the extracellular environment once endosomes fuse with the cellular membrane.[47] Since then, a series of extracellular vesicles (EV) have been described and classified based on their cellular origin, functions, and/or biogenesis.[48,49] Nevertheless, the nomenclature for different types of EV remains unclear in various reports.[5,50] In accordance with the biogenetic pathway of secretion from cells, extracellular vesicles can be classified into three main categories:[51] (i) exosomes (≈30–150 nm diameter), small vesicles that are released by exocytosis when multivesicular bodies (MVB) fuse with the plasma membrane; (ii) shedding microvesicles, which are vesicles with a diameter of ≈50–1000 nm that are directly shed from the plasma membrane; and (iii) relatively larger apoptotic bodies (1000–5000 nm diameter) that are released by dying cells.

Several mechanisms are involved in exosome biogenesis, which also facilitates protein and RNA cargo sorting to generate exosomes with a particular biochemical composition. As mentioned above, exosomes are of endocytic origin (**Figure 1**A).[5,52–54] An endosome, which is a membrane-bound compartment inside eukaryotic cells, comprises three different compartments: early endosomes, late endosomes, and recycling endosomes. When early endosomes mature into the late endosome state, their location (from the outer cytoplasm to closer to the nucleus) and shape (from tube-like to spherical) are changed.[50] Additionally, intraluminal vesicles (ILV) are formed inside the lumen of the late endosome via an inward budding of the endosomal membrane.[55] These late endosomes are known as MVBs. MVBs have two potential fates: either to fuse with lysosomes or with the plasma membrane. Once an MVB fuses with the lysosome, its contents become degraded inside of the lysosome by hydrolysis. Alternatively, the MVB can fuse with the plasma membrane thereby releasing its ILVs into the extracellular space.[56] These released vesicles are known as exosomes. Recent advances in the exosome field have shown that these vesicles are produced initially as ILVs, but not all ILVs are eventually released into extracellular space as exosomes.[50,52] Exosomes are mainly secreted by two different mechanisms, which constitute release via the trans-Golgi network and inducible release. Several Rab family proteins, including Rab27a and Rab27b, act as key regulators of exosomes secretion.[53] Apart from Rab 27a and 27b, other Rab family members, Rab 35 and Rab 11, have also been shown to regulate the secretion of exosomes by interacting with the GTPase-activating protein TBC1 domain family member 10A-C (TBC1D10A-C).[53] It has also been shown that activation of the tumor suppressor protein, p53, stimulates and increases the rate of exosome secretion by regulating the transcription of various genes such as TSAP6 and CHMP4C.[53]

3. Exosomal Contents

Exosomes contain a variety of molecules such as proteins, mRNAs, noncoding RNAs, DNAs, and lipids (Figure 1B).[39] These species have been collected in an exosome database, which is accessible via ExoCarta (http://www.exocarta.org) and Vesiclepedia (http://www.microvesicles.org).[57] Since 2009, the database has hosted 41860 proteins and over 7540 RNA and 119 lipid molecules. Note that the databases are user submitted, and no quality control of the entry or verification of the authenticity of a claim for an exosomal protein is provided.

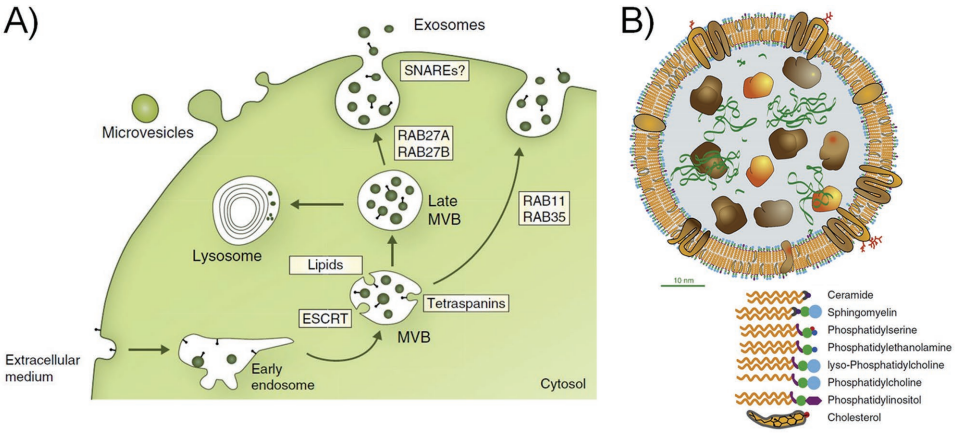

Figure 1. A) Schematic illustration of exosome biogenesis. Exosomes are released from multivesicular endosomes (MVEs) via exocytosis. Exosomes then can either fuse with lysosomes for degradation or be released into the extracellular space by fusing with the plasma membrane. Reproduced with permission.[5] Copyright 2014, Elsevier Inc. B) Typical representation of exosomal cargo. Exosomes are surrounded by a phospholipid bilayer and carry various biological species, including various types of proteins, lipids, mRNA, regulatory RNA, and DNA. Reproduced with permission.[39] Copyright 2012, Elsevier Inc.

Additionally, different exosome isolation methods can copurify nonexosomal artifacts, which are not largely described in these databases. A more recent community database, EVpedia, attempts to incorporate some of this additional information.[58]

3.1. Proteins

The protein content of exosomes reflects their origin in endosomes and varies depending on their parent cell type.[59,60] All exosomes from diverse cell types carry a few common sets of proteins, such as (i) transmembrane proteins (e.g., CD9, CD63, and CD81) from the tetraspanin family; (ii) programmed cell death 6-interacting proteins (PDCD6IPs), which participate in programmed cell death;[61] (iii) tumor susceptibility gene 101 (Tsg101) proteins, which are involved in sorting and transporting exosomes;[62] and (iv) major histocompatibility complex (MHC) class II molecules.[63] Exosomes also contain other proteins that depend on their cell type of origin. These proteins include cytoskeletal proteins (e.g., actin and tubulin), membrane transport proteins, heat shock proteins (e.g., HSP60, HSP70, and HSP90), and annexins (used for regulation of cytoskeletal changes in membranes and membrane fusion mechanisms).[64,65] In addition, exosomes contain surface proteins, which are involved in intracellular signaling, such as Wnt proteins that activate the Wnt signaling pathway in target cells.[66,67] Furthermore, exosomes contain various enzymes, particularly GTPase from the Rab family, which promotes the fusion of membranes,[53,68] and metabolic enzymes, such as peroxidase, pyruvate kinase,[69] and lipid kinase-1. All the exosomal proteins that have been identified to date are found in the cytosol, plasma membrane or in membranes of endocytic origin. These proteins were not found to consist of proteins of nuclear, mitochondrial, endoplasmic-reticulum or Golgi-apparatus origin. Moreover, proteomic studies of exosomes demonstrated that exosomal proteins are not necessarily obtained from the plasma membrane during fusion. Further analysis showed that exosomes lack abundant cell-surface proteins such as Fc receptors in dendritic cell (DC)-derived exosomes, CD28 and CD40 L proteins in T-cell-derived exosomes, and transferrin receptors in B-cell-derived exosomes.[50]

3.2. Nucleic Acids

The observation of mRNA and miRNA in exosomes secreted by mast cells sparked great interest in biology. In vitro experiments showed that some mRNAs present in exosomes could be translated into proteins in target cells. These observations showed that exosomes have roles in the intracellular transfer of genetic information. One of the most interesting findings was that not all mRNAs present in a cell end up in exosomes, and there are apparently selective mechanisms that control the specific loading of RNA species into exosomes. Originally, the presence of nucleic acids in exosomes derived from mast cells was observed. Further studies have shown that RNAs are also present in the exosomes of various types of cells such as dendrite cells, tracheobronchial cells, B- and T-lymphocytes, lung, esophageal, and stomach cancers cells, and pancreatic adenocarcinoma cells.[70–73] Although many recent fundamental studies have shown that exosomes contain mRNAs, miRNAs, and other noncoding RNAs, the mechanisms that control the specific loading of RNA species into exosomes are still not well understood. Recently, a mechanism involving the encapsulation and exportation of exosomal miRNAs was identified by showing short sequence motifs, which were overrepresented in miRNAs (EXOmotifs), that guide their loading into exosomes.[74] The heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) specifically binds exosomal miRNAs through the recognition of these motifs and controls their loading into exosomes. It was also suggested that hnRNPA2B1 might be a key player in miRNA sorting into exosomes and provides a better understanding of how miRNAs are loaded into exosomes and exported from cells.

3.3. Lipids

In recent years, various studies have shown that besides proteins and nucleic acids, exosomes also carry certain types of lipids, which play an important role in maintaining the biological activity of exosomes.[75–77] An exosome database, ExoCarta, summarizes a total of 194 lipids that have been found in various exosomes types. These lipids include ceramides (implicated in the differentiation of exosomes from lysosomes), cholesterol, other sphingolipids, and phosphoglycerides with long and saturated fatty-acyl chains. It was also shown that exosomes might deliver prostaglandins to target cells. However, the lipid composition of exosomes does not represent the parent cell.[78] For instance, compared with parent cells, exosomes are more enriched in sphingomyelin but not in cholesterol. The phosphatidylcholine content was decreased, but enrichment was noted in desaturated molecular species, as in phosphatidylethanolamines. Moreover, lyso(bis)phosphatidic acid was not enriched in exosomes compared with the parental cells.

4. Exosome Functions

Exosomes are shed by most cell types, circulate in different bodily fluids (e.g., urine, blood, and saliva) and transfer their cargo to recipient cells. These vesicles play a significant role in various pathological conditions, such as different types of cancer,[79,80] neurodegenerative diseases,[81] infectious diseases,[82] pregnancy complications, obesity[83–85] and autoimmune diseases[86] (**Figure 2**). Moreover, several studies have indicated that exosomes are associated with inflammation, coagulation, angiogenesis, and apoptosis.[39,87] Exosomes play a role in intercellular communication between cells by interacting with target cells via endocytosis.[88–90] Additionally, it has also been shown that exosomes play an important role in cancer development.[91–95] Tumor-derived exosomes can transfer oncogenetic cargo and modulate the genetic expression of recipient cells, thereby playing a crucial role in the progression, survival and metastasis of tumor, and drug resistance.[9,37,60,96] Exosomes are actively involved in the remodeling of the extracellular matrix, followed by the promotion of angiogenesis, thrombosis, and proliferation of tumor cells.[97,98] Studying the intercellular communication among tumors of highly malignant brain glioblastoma

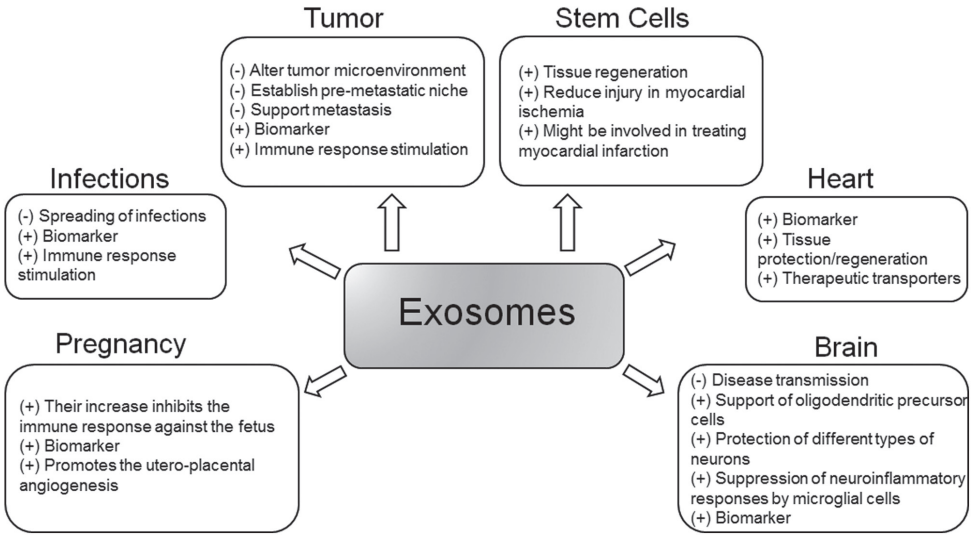

Figure 2. Biological functions of exosomes in various human tissues.

multiforme (GBM) in a mouse model revealed that exosomes mediate hypoxia-dependent intercellular signaling. Considering that hypoxia is a key regulator of tumor development and aggressiveness, it was suggested that exosomes play a prime role in tumor vascularization and hypoxia-mediated inter-tumor communication during cancer progression.[97] Exosomes are also a key player in harboring premetastatic niches.[80,98–100] The formation of premetastatic niches is one of the major events in cancer metastasis, which provides a platform for tumor cells to colonize in a distant tissue for the initiation of metastasis.[101,102] Exosomes are actively involved in the development of these premetastatic niches.[98] It has also been reported that exosomes, along with cytokines and other soluble mediators, are involved in the transport of bone marrow-derived cells to a premetastatic niche to enable the establishment of a tumor microenvironment.[101,102] Furthermore, exosomes were also found to maintain a role in tumor survival via allowing the tumors to design an immune-escape mechanism.[95]

Exosomes from tumor cells inhibit lymphocyte proliferation.[103] Interesting findings suggest that various infectious pathogens (e.g., viruses) can take advantage of exosome properties to infect cells. Exosomes from immature dendritic cells can mediate HIV infection by transferring the virus particles to CD4+ T cells via the endosomal pathway, and in this way, the virus avoids detection by the innate immune system.[104] Moreover, cells infected with the Epstein-Barr virus (EBV) release functional EBV miRNA in exosomes, which is taken up by both neighboring and outlying cells, may spread the infection.[82]

Another area of interest regarding the potential role of extracellular vesicles and mainly the exosomes is during pregnancy. As such, exosomal signaling is an integral signaling pathway that mediates the communication between the maternal and fetal circulation during gestation.[105,106] During gestation, the human placenta secretes exosomes into the maternal circulation from as early as 6–7 weeks of pregnancy.[105] Interestingly, the release of exosomes from placental cells is regulated by factors that include both oxygen tension and glucose concentration[107–110] and correlates with the placental mass and perfusion.[107] The concentration of exosomes that originate from placental cells increases progressively during gestation in maternal circulation.[107] The concentration of placenta-derived exosomes is higher in pregnancy complications such as gestational diabetes[85] and preeclampsia[83] than in normal pregnancy during the first trimester of pregnancy; therefore, profiling placental exosomes that are present in maternal circulation at early gestation may be used to classify women at risk to develop these pregnancy complications.

5. Potential Use of Exosomes as Biomarkers and Therapeutics Agents

Over the past years, several studies have demonstrated the diagnostic and therapeutic potential of exosomes and exosomal content (i.e., nucleic acids and proteins) in many diseases including cancer as well as cardiovascular, neurodegenerative and infectious diseases.

5.1. Proteins Associated with Exosomes as Biomarkers of Onset and Disease Progression

As discussed in Section 3.1, exosomes contain a variety of proteins including proteins involved in exosome biogenesis (e.g., Alix, Tsg101 and ESCRT complex) and endosomes (e.g., annexins and flotillin); tetraspanins (e.g., CD9, CD63, CD81, and CD82) and heat-shock proteins (Hsp70 and Hsp90) (**Table 1**).[108] In a previous study, the presence of exosomal protein markers in human prostate and breast cancer cell lines was tested, and it was found that all the samples tested positive for CD9 and CD81.[109] Recently, a number of studies have established the role of exosomal proteins as diagnostic biomarkers of breast cancer.[110–112] For instance, anti-CD24 and antiepithelial cell adhesion molecule (EpCAM)-coupled

Table 1. Exosomal proteins for clinical applications.

Biofluid	Disease	Exosomal protein content	Reference
Plasma	Prostate cancer	Survivin	[138]
	Melanoma	CD63, caveolin-1, TYRP2, VLA-4, HSP70, HSP90	[11]
	Glioblastoma	Epidermal growth factor receptor VIII	[77]
	Ovarian cancer	CD24, EpCAM, CA-125, TGF β1, MAGE 3/6	[163]
	Breast cancer	EDIL3, Fibronectin	[110,111]
Serum	Pancreatic cancer	Glypican-1	[115]
	Colorectal cancer	CD147, CD9	[109]
	Glioblastoma	EGFR, CD63, EGFRvIII	[169]
	Breast cancer	Survivin	[113]
	Prostate cancer	Survivin	[138]
Urine	Prostate cancer	PCA-3, TMPRSS2:ERG, PSA	[120]
	Bladder cancer	EGF, α subunit of Gs, resistin, retinoic acid-induced protein 3	[121]
Cell culture medium	Ovarian cancer	L1CAM, CD24, ADAM10, EMMPRIN, claudin	[122]

magnetic beads that are used to isolate exosomes in immune-affinity techniques showed that exosomal CD24 can work as a breast cancer-specific marker.[112] Another ELISA-based recent study demonstrated that both EDIL3 and fibronectin in the exosome population can serve as promising biomarkers for the early detection of breast cancer.[111] In addition to breast cancer, exosomal proteins have also been reported to play a diagnostic role in other cancers such as prostate, bladder, ovarian, pancreas and colorectal cancer.[108,113,114] For example, significantly higher amounts of exosomal survivin were detected in patients with prostate cancer than in healthy subjects.[127] The study was further extended to show that alternative splice variants of survivin were also elevated in the plasma of breast cancer patients, suggesting its strong role as a potential breast cancer biomarker.[113] Another exosomal surface protein known as glypican-1 has been found to be exclusively present in the serum of pancreatic cancer; however, the protein is not present in samples that are derived from benign pancreatic diseases.[115] This finding strongly suggests that exosome derived glypican may work as a potential biomarker for pancreatic cancer.

Exosomal proteins have also been found to provide a diagnostic role in hepatic diseases. For example, it has been reported that exosomal CD81 was increased in patients with chronic hepatitis C compared with healthy controls and cured patients. This study also correlated CD81 with inflammation and fibrosis and suggested that exosomal CD81 may be used as a potential biomarker for the diagnosis and prognosis of hepatitis C.[116] Several other exosomal proteins have been found to be potentially associated with the diagnosis of neurological disorders such as glioblastoma-specific epidermal growth factor receptor vIII (EGFRvIII) in glioblastoma;[79] EGFR, EGFRvIII, and TGF-beta in brain tumor;[117] exosomal amyloid peptides and phospho-tau (Thr-181) in Alzheimer's disease;[80,118] and α-synuclein in Parkinson's disease.[119]

5.2. RNAs within Exosomes as Potential Biomarkers for Onset and Disease Progression

Despite being available in most biological fluids, miRNA as a cancer biomarker is still waiting to be widely used in routine clinical application. This is reportedly due to their poor specificity and irregular reproducibility in different physiological and pathological conditions.[123] Additionally, RNA is generally unstable at room temperature for ribonuclease (RNase)-associated progressive RNA degradation. Recently, Witwer[124] has provided a comprehensive review on specificity and reproducibility issues associated with miRNA-based diagnostics. In another review, Haider et al. studied 416 circulating miRNA biomarkers in 57 noncancerous diseases and identified that miR-16, –155, –21, –126, and –223 biomarkers were associated with at least 10 noncancerous conditions, although these miRNAs have been considered as cancer-specific markers.[125] Therefore, applications of miRNA as cancer biomarkers are reportedly under careful consideration. However, several studies have suggested that these biological pitfalls and challenges can be overcome by considering exosomal miRNA as the target biomarker.[123,124] This is because exosomal miRNAs are exclusively protected from RNase-dependent degradation and thus can be stably detected in circulation. Thus, exosomal miRNA is a potential candidate for an ideal biomarker in clinical diagnostics.

Since 2007, after the first report on exosomal miRNA, an increasing number of studies have demonstrated the role of exosomal miRNA in several diseases, mostly in cancer (**Table 2**).[126] For example, in 2009, the circulating levels of exosomes and its cargo (exosomal small RNA and miRNAs) in patients with lung adenocarcinoma and healthy subjects were evaluated for their diagnostic potential. This study found a similarity between the circulating exosomal miRNA and the tumor-derived miRNA patterns, which suggested that circulating exosomal miRNA somewhat represents the tumor miRNAs and thus may be useful as diagnostic and prognostic markers of lung adenocarcinoma.[127] It has been suggested that exosomal miRNAs can improve the current diagnosis strategies for prostate cancer. Note that the widely used prostate-specific antigen (PSA) tests for the early detection of prostate cancer have long been under debate for the associated high false-positive results. In this regard, prostate cancer specific exosomal miRNA may be proven useful in the development of a highly robust and specific diagnostic method. Several studies reported that RNase resistant miR-141 and miR-375 remained stable in circulation and could be used as a specific diagnostic marker for prostate cancer.[128,129] Since, exosomal miR-141 and miR-375 are highly stable inside the protected layer of exosomes, they may provide clinical relevance for prostate cancer diagnosis. Exosomal miRNAs were also reported for use as diagnostic biomarkers in esophageal squamous cell cancer (ESCC).[19] In addition to cancer, these miRNAs have also demonstrated a diagnostic role in cardiovascular and renal diseases.[34,130]

5.3. Exosomes as Therapeutics Agents

Over the past few years, several exosome-based therapeutic approaches (e.g., vaccine development, tissue regeneration

Table 2. Exosomal miRNA for clinical applications.

Biofluid	Disease	Exosomal RNA content	Reference
Plasma	Ovarian cancer	miR-21, -141, -200a, – 200b, -200c, -203, -205, -214	[27]
	Prostate cancer	miR-141, miR-375	[21,22]
	Breast cancer	miR-21, -1246	[140,141]
	Lung cancer	miR-17, -3p, -21, -20b, -223, -301, let-7f, -19a, -19b, -30b, -20a, 30e-3p, -378, -379, -139-5p, -200b-5p, -151a-5p629, -100, -154-3p	[142]
	Esophageal cancer	miR-21, miR-1246	[19,143]
	Liver cancer	miR-34a, -125b, -21	[155]
Cell culture medium	Gastric cancer	Let-7 family miRNAs	[71]
	Colorectal cancer	mRNAs	[144]
	Hepatocellular carcinoma	miR-584, -517c, -378, -520f, -142-5p, -451, -518d, -215, -133b, -367	[145]
	Prostate cancer	miR-4258, -221, -193a-3p,30e, -1297, -129, -21, -485-3p	[146]
	Lung cancer	miR-133b, -98, -181a, -21	[147]
	Glioblastoma	miR-1469, -320b, -320c, -191, -222, Let-7a, -923, -1308, -2185, -351-5p, -25, -939, -30c, -422a, -221, -487a, -335, -4329	[148]
	Breast cancer	miR-16, -1246, -451, -20	[149]
Urine	Renal fibrosis	miR-29c, CD2APmRNA	[34]
	Pancreatic cancer	miR-17-5p, -21	[19]
Serum	Ovarian cancer	miR-21, -141, -200a, – 200c	[27]
	Prostate cancer	miR-141, -107, -375, -574-3p	[150]
	Pancreatic cancer	miR-17-5p, -21	[151]
	Breast cancer	miR-200a, -200c, -205, -101, -372, -373	[152]
	Glioblastoma	miR-21, -574-3p, Snc RNA (RNU6-1)	[153]
	Colon cancer	miR-4772-3p, let-7a, -1229, -1246, -150, -21, -223, -23a	[154]
	Liver cancer	miR-34a, -125b, -21	[155]
Peripheral blood	Lung adenocarcinoma	miR-17-3p, -21, -106a, -146, -155, -191, -192, -203, -205, -210, -212, -214	[127]

therapy, drug delivery, and gene silencing) have been developed and some of them were tested in phase II clinical trials.[39] The first clinical trial (phase I) to develop a vaccine against metastatic melanoma patients using autologous DC-derived exosomes (DEX) was reported in France in 2005.[131] DEX was engineered with functional MHC-peptide complexes that can activate T-cell immune responses thereby allowing tumor rejection. The study also developed a good manufacturing practice protocol to produce exosomes at a large scale. Under this trial, several of III/IV melanoma patients were immunized with four types of exosome vaccines. Following the vaccination, NKG2D protein expression in natural killer (NK) and CD8T cells of many patients were restored and an increased number of NK cells was observed. The results of the trial demonstrated that DC derived-exosomes from melanoma patients are specifically endowed with NK cell stimulatory capacity in vivo. Morse et al. reported a similar phase I clinical approach that employed DEX immunotherapy in patients with advanced non-small-cell lung cancer (NSCLC).[132] In addition to these two trials, few other clinical studies that utilized exosome vaccines were introduced against colorectal[133] and stage III/IV non-small-cell lung cancer.

Exosomes were also reported to have therapeutic potential in tissue regeneration. Lai et al. showed that purified exosomes derived from mesenchymal stem cells (MSCs) significantly reduced the infarct size in a mouse model of a myocardial ischemic injury.[98] Recently, MSC-derived exosomes were employed for the treatment of pediatric refractory graft-versus-host disease (GvHD).[134] The utility of MSC-derived exosomes in GvHD treatment further triggered its potentiality against other diseases such as type 1 diabetes.[135] Due to this therapeutic success, regulatory approval was sought in Canada and New Zealand for the clinical use of MSC-derived exosomes in the treatment of pediatric GvHD.[136] MSC-derived exosomes were also found to accelerate the functional recovery from stroke[137] and brain injuries[138] in animal models, which could be explained by improved post-therapeutic neurogenesis and angiogenesis. In addition to protein and miRNA, exosomes can be engineered to express small interfering RNA (siRNA). Note that synthetic siRNAs can be introduced into cells to activate RNA interference (RNAi) that can silence the gene. Thus, an exosome that is engineered with siRNA can be therapeutically used to silence disease specific genes, as evidenced by a study where the tumor gene was knocked out in vitro using exosomal siRNA.[139]

6. Isolation of Exosomes

Since the discovery of exosomes, several conventional techniques have been used to isolate exosomes from bodily fluids.

These techniques include differential and buoyant density centrifugation, ultrafiltration, immunological separation, and commercial exosome isolation kits.

6.1. Differential and Gradient Density Centrifugation

Centrifugation-based techniques are considered the gold standard for exosome isolation.[9,29,145] These methods do not require technical expertise or complicated sample pretreatment steps. Due to these benefits, ultracentrifugation has been the most commonly used method to extract nanosized vesicles from cell culture media and bodily fluids.[29,156–158] There are two types of ultracentrifugation: differential centrifugation and density gradient ultracentrifugation. Differential centrifugation usually requires multiple steps including a low-speed centrifugation ($300 \times g$ for 10–15 min) step to remove cells and apoptotic debris, followed by a high-speed spin to eliminate larger vesicles and finally high-speed centrifugation at $100\,000 \times g$ to precipitate the exosomes. These different centrifugation steps are performed because the sedimentation completely relies on the vesicle density and the distance the exosomes or vesicles can travel. Thus, smaller EV particles at the bottom of the tube are pelleted at low speed, whereas a high-speed spin is required to sediment the larger particles near the top of the tube.[159] Therefore, the top of the tube contains the larger vesicles, with possible coprecipitation of protein aggregates, apoptotic bodies, and other types of EVs, which cannot be separated from the tube. The coprecipitation results in less sample purity and contamination of exosomes with other particles. One possible solution is the use of multiple resuspending and recentrifuging steps of each pellet in a buffer solution (e.g., phosphate-buffered saline (PBS)) to remove some of these impurities, although this step alone cannot perform absolute separation. One of the better alternatives could be the use of a sucrose density gradient with centrifugation steps. This method is based on separating the vesicles according to their different flotation densities, which eventually allows the vesicles to float upward into an overlaid sucrose gradient. Therefore, this method allows the proteins or impurities to be pelleted at the bottom of the tube, which can easily be removed to enable aggregate-free separation of exosomes.[29,160]

6.2. Filtration

Filtration-based techniques have been recently introduced to isolate exosomes.[161] Although filtration was introduced as an independent method, it is currently used in combination with ultracentrifugation to replace the first two spins of the differential centrifugation protocol. The main principle of the ultrafiltration method is to separate the resuspended particles, depending on their size and molecular weight.[162] Thus, the filtration step can eliminate dead cells and large debris, whereas the ultracentrifugation step provides further purification of the filtered samples. A filtration protocol for exosome isolation from urinary samples using a nanomembrane concentrator was reported.[163] Their approach can enrich exosomal proteins from small urine volumes. Recently, another group described a microfiltration isolation method using low-protein-binding-size exclusion filters for the isolation of urinary biomarkers.[25] This method used a hydrophilized polyvinylidene difluoride membrane to extract exosomes from fresh urine samples. The efficiency of this method was also validated by liquid chromatography-mass spectrometry, immunoblot analysis, and electron microscopy. Although, compared with the ultracentrifugation method, the filtration method is relatively simpler and faster and does not rely on specialized equipment, it could still be affected by the loss of exosomes due to the trapping of the exosomes in the pores of filters.[164] Additionally, the force applied to pass the sample through the filter membranes may result in the damage, deformation and break up of large vesicles.[165] One possible solution for the recovery of the exosomes entrapped in filter membranes could be the use of a membrane with low exosomal protein-binding properties. The force driven step can also be avoided via a centrifugation step to prevent the deformation of exosomes.

6.3. Immunological Separation

Numerous proteomic studies of the molecular composition of exosomes have revealed the presence of various proteins on the exosomal membrane.[64,166–168] These proteins can be ideal markers for immune-isolation of exosomes due to immunoaffinity interactions between the proteins (antigens) and antibodies.[29] Recent advances in the exosome isolation field have shown that antibody-coated magnetic beads can be effectively employed to isolate exosomes from antigen-presenting cells. In this regard, choosing a proper exosome membrane marker is one of the most important steps in these immunoassays. It was shown that members of the tetraspanin family, such as CD81, CD9, and CD63, that are present on the membranes of exosomes can be used for efficient immunocapture.[23,169–171] Unlike other conventional techniques for exosome isolation, antibody-coated magnetic beads can be used for direct exosome isolation from bodily fluids, which reduces the time-consuming centrifugation steps. A method for isolating breast cancer-specific exosomes that uses magnetic beads coated with antibodies against the tumor-specific HER-2 (human epidermal growth factor receptor 2) protein found in exosomes was also reported.[172] The isolation efficiency of this method was successfully confirmed by FACS analysis. Taylor and Gercel-Taylor isolated circulating tumor-derived EpCAM-positive exosomes using anti-EpCAM-coated magnetic beads.[18] Zarovni et al. used antibody-functionalized magnetic beads for exosome isolation from both cell culture and plasma samples.[158] They have shown that the capture efficiency of their immunoassay is close to that of the ultracentrifugation method. The advantage of the immunological isolation technique is the associated high specificity due to the use of the antibody, although this high selectivity and specificity can result in a low exosome yield compared with physical separation-based methods. More recently, another immunological method was reported.[173] A lipid nanoprobe system was used to isolate exosomes from serum-free cell-culture supernatant and blood plasma. First, the lipid bilayer of exosomes was labeled with biotin-tagged 1,2-distearoyl-snglycero-3-phosphethanolamine-poly (ethylene glycol). Next, the labeled vesicles were collected by NeutrAvidin-coated magnetic submicrometer particles for subsequent extraction and analysis of the exosomal

cargo. The method was successfully applied to the analysis of exosomal DNA derived from 19 stage-IV NSCLC patients, which allowed the detection of mutations in KRAS (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue) codons 12 and 13 and EGFR (epidermal growth factor receptor) exons 19 and 21. These approaches are also limited by the requirement of costly and diverse antibody-antigen combinations and the isolation procedures. Moreover, the immune-affinity-based capture can only separate exosomes from cell-free samples. Note that during clinical sample analysis, the tumor heterogeneity in a real sample can affect the efficiency of immune capture. Another considerable issue is the use of elution buffers, which are required to release the exosomes from immunocomplexes. This is because unfavorable conditions (i.e., pH alteration, the presence of detergents and reducing agents, etc.) in the buffer can affect exosome functionality via membrane permeability disruption. For these reasons, immunological separation is not yet suitable for large-scale analysis of exosomes.[89]

6.4. Exosome Precipitation

Polymer-based precipitation methods usually involve mixing the biofluids of interest with a polymer solution under optimized salt concentration and low-temperature conditions, followed by an overnight incubation at 4 °C. Then, the precipitated exosomes are recovered by low-speed centrifugation and resuspended in PBS for further applications. The most commonly used polymer for this approach is polyethylene glycol (PEG). This method has been routinely used for the precipitation of various biomolecules, viruses, and other small particles.[174–176] Commercially available isolation kits also use PEG to isolate exosomes from cell culture media and bodily fluids. Several studies indicate that commercial isolation kits (e.g., the Total Exosome Isolation Kit from Invitrogen, Aus and the ExoSpin Exosome Purification Kit from Cell Guidance Systems, USA) provide a simple methodology for the efficient isolation of exosomes from clinical samples via avoiding the need for long differential centrifugation and the precipitation steps used in conventional isolation technologies.[29,30] These kits are also compatible for assaying exosomes in bodily fluids, including serum, plasma, urine, and cerebrospinal fluid, and culture media. One comparative study reportedly found that the commercial kits were more efficient in isolating exosomes from urinary samples compared with differential ultracentrifugation and nanomembrane concentrators.[161] Recently, the extraction performance of four exosome isolation techniques: ultracentrifugation, two sedimentation isolation kits (e.g., the Invitrogen Total Exosome Isolation Kit and the ExoSpin Exosome Purification Kit), and the density gradient method (PureExo Exosome Isolation Kit from Fisher scientific, USA)[177] using liposomes as a model vesicle system, was evaluated. After extraction of the liposomal vesicles from serum-free cell culture media, the samples were characterized by tunable resistive pulse sensing (TRPS) (**Figure 3**). The ExoSpin and Invitrogen kits resulted in the highest yields (between 2×10^{11} and 3.5×10^{11} particles mL^{-1}), whereas both the ultracentrifugation and PureExo methods generated yields that were approximately two orders of magnitude lower, with concentrations between 1×10^{9} and 1.5×10^{9} particles mL^{-1}. Although exosome isolation kits offer many advantages, such as low sample volume, simplicity, and cost-effectiveness, they are limited by their low specificity due to the coisolation of the nonexosome material. Apart from these potential nonexosomal contaminants, the precipitated samples contain polymer molecules, which is unsuitable for some detection/quantification methods such as mass spectrometry.

7. Challenges in Exosome Analysis and Potential Solutions

The major issue in the clinical application of exosomes is the lack of consistent and specific methods to isolate and detect an enriched population of exosomes (e.g., tumor-derived exosomes) among other nonspecific exosomes and EVs present in circulation. Because of increasing interest in the exosome research field, there is an urgent need for an efficient and reliable tool for the isolation of specific exosomes. Evidently, precise exosome isolation remains cumbersome due to several

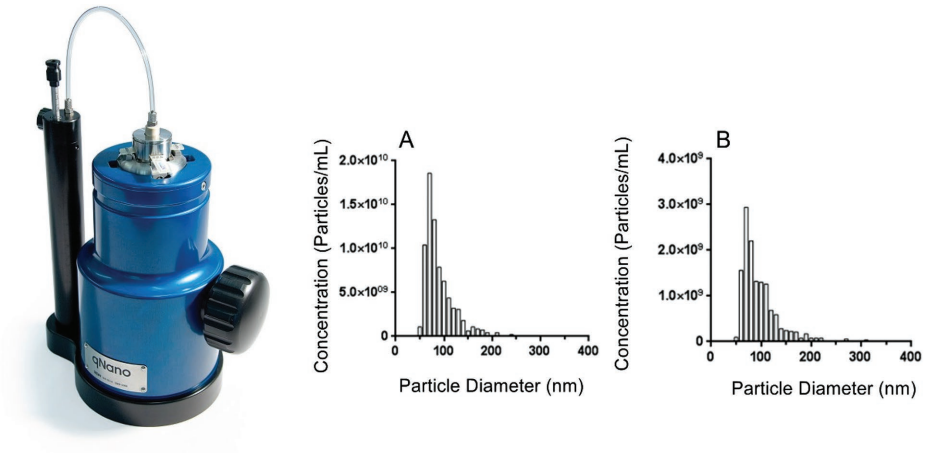

Figure 3. Photograph of the qNano analyzer and concentration of exosome population derived from A) HER-2(+) BT-474 and B) HER-2(–) MDA-MB-231 breast cancer cell lines. Reproduced with permission.[42] Copyright 2017, Wiley-VCH.

technical challenges associated with the currently available isolation and detection techniques. Moreover, there are several biological challenges that need to be considered to develop a reliable method for the analysis of exosomes.

7.1. Technical Challenges

It has been reported that the different preanalytical steps, such as sample collection, storage, use of anticoagulants, and sample processing time, involved in exosome isolation and detection can significantly affect the outcomes of the analysis (i.e., variable outcomes in the analysis).[178] To overcome this issue, the analysis platform should be standardized with regularly calibrated instruments. In this regard, the International Society for Extracellular Vesicles has started providing recommendations for a standardized and evidence-based platform for extracellular vesicle analysis.[159]

One common challenge involved in the sample collection procedure is the presence of impurities, which results from activated platelet-derived vesicles that are mostly due to the physical forces associated with the blood draw. Therefore, standardization of sampling sites, the use of larger needles, and careful blood drawing are suggested to avoid the associated shear stress and resulting platelet activation.[159,179] Another recommendation is to avoid the use of heparin-based anticoagulants in the sample collection tube. This is because heparin competes with primers and/or enzymes for binding to nucleic acids, thereby resulting in a false-negative polymerase chain reaction (PCR) response[180] that affects the analysis and validation of the nucleic acid present in exosomes and other EVs. Heparin was also reported to inhibit the uptake of EV by recipient cells.[181] Therefore, as alternative choices for anticoagulants, ethylenediaminetetraacetic acid (EDTA), sodium fluoride, or sodium citrate in combination with or without different additives such as dextrose have been commonly used in collection tubes. Among these, citrate is generally preferred because EDTA was also found to interfere with the amplification of nucleic acids in EVs (although to a relatively lesser extent compared with heparin).[182]

The discrepancy in the analysis due to inappropriate storage conditions (e.g., freezing) is another hurdle to overcome in exosome analysis. For large-sample analyses, samples are generally collected from distant locations and freeze-stored prior to the analysis, which may affect the quantification.[177,183] Therefore, it is always recommended to use freshly collected samples for exosome analyses.

Over the past several years, many exosome isolation techniques have attempted to address these challenges with little success. For example, differential ultracentrifugation is one of the most widely used methods for exosome isolation but is not convenient in resource-limited settings due time-consuming procedures and high-cost equipment. This technique also frequently suffers from the loss of exosomes and copelleted impurities during the analysis. Immunoaffinity-based techniques provide high selectivity and specificity, but they are limited by the use of costly antibodies, less exosome yield, etc. On the other hand, although exosome precipitation-based methods are relatively simpler and do not require expensive equipment, they are limited by the coprecipitation of exosomes with other extracellular vesicles and protein aggregates. (**Table 3** summarizes the advantages and drawbacks of current techniques for exosome isolation.)

7.2. Biological Challenges

Many genetic, physiological, and environmental factors that are associated with sample heterogeneity can affect exosome analysis. Even in healthy individuals, disease-specific exosomes

Table 3. Comparison of exosome isolation techniques.

Isolation method	Working principle	Advantages	Disadvantages	Reference
Differential centrifugation	The method consists of multiple centrifugation steps to exclude large vesicles and cells debris and precipitate exosomes.	Considered to be a gold standard and reliable method. Allowing the analysis of large sample volumes and multiple samples at the same time.	Time-consuming procedures, high equipment cost, high centrifugation speed resulting in exosome damage.	[9,27,159]
Density gradient centrifugation	Combination of ultracentrifugation with sucrose density gradient.	The method separates low-density exosomes from other extracellular vesicles.	Very high sensitivity to the centrifugation time.	[27,29,160]
Filtration	Ultrafiltration membranes are used to separate exosomes from other vesicles due to size differences.	Filtration allows the separation of big particles from exosomes. During the process, the exosomal population is concentrated by the filtration membrane.	Trapping of exosomes in the pores of the filters and attaching vesicles to the membranes, leading to the loss of exosomes. Additionally, the force applied to pass the sample through the membranes may result in the damage, deformation, and breaking up of large vesicles.	[161–163]
Immunological separation	Exosomes are captured due to interactions between antigens on the exosome surface and prefunctionalized magnetic beads coated with antibodies.	The method isolates exosomes directly from cell culture supernatant or bodily fluids.	The method cannot be applied to large-volume samples.	[169–171]
Polymer-based precipitation	The technique includes mixing of the biological fluid with a polymer-containing precipitation solution, followed by an incubation step and centrifugation at low speed.	Easy to use, does not require specialized equipment, large and scalable sample capacity.	Polymer-based precipitation methods co-isolate nonexosomal contaminants.	[177]

can be present in higher or lower amounts than normal due to different factors such as age, gender, body mass index (BMI), and immunity, which vary from person to person.[159] Therefore, choosing an ideal matched control for a large cohort of heterogeneous samples is a significant challenge (i.e., a control derived from young individuals cannot reliably be used to screen exosomes that are derived from elderly people). More systemic studies are needed to study the effects of sample heterogeneity on the biogenesis, functionality, and quantity of exosomes. Importantly, there is an urgent need to establish a predesigned sample control bank, which contains controls from all possible variants of the target population, such as different ages, races, sexes, physiological conditions, etc.

Although recent progress has improved the isolation efficiency of exosomes from other extracellular vesicles, there are only few reported strategies that describe the efficient detection of disease-specific exosomes in the background of normal exosomes (i.e., exosomes that can be derived from both normal and diseased cells from the same subject).[42,45] It is now widely acknowledged that exosomal cargo, which is encapsulated in the protective layer of the exosome membrane, is a promising source of biomarkers for disease diagnosis and prognosis. This is because the cargo is protected from many harsh conditions inside the encapsulated protective environment of the exosomes (e.g., exosomal miRNA is protected from ribonuclease (RNase) mediated RNA degradation). However, this major advantage of exosomal miRNA may pose a significant challenge, i.e., for the analysis of miRNA, because it needs to be released from the isolated exosomes, which incurs multiple additional complicated steps in the analysis.[184] There are also many fundamental questions still unanswered concerning the functionality of exosomes and their contents.[90] For instance, it is not confirmed whether the transport and uptake of exosomes by distant recipient cells are due to phagocytosis[185] or uptake by selective receptors of distant recipient cells.[186]

8. Detection of Exosomes

ELISA, flow cytometry, FACS, NTA analysis, TRPS, and microfluidics- and electrochemistry-based approaches are the most commonly used methods for the detection and quantification of exosomes. In this section, these developments are discussed.

8.1. ELISA

Over the past decade, ELISA has been widely used for the detection of exosomes.[37,187–191] Generally, for ELISA-based detection, exosomes are directly immobilized on a microwell plate. After blocking the plates with a blocking agent, a recognition antibody (e.g., anti-CD9) is added to the wells for binding to specific antigens (e.g., CD9) present on the exosome surface. Finally, an HRP-linked detection antibody is used for a sensitive (via an enzymatic signal amplification step) and specific readout. A colorimetric substrate (e.g., 3,3′,5,5′-tetramethylbenzidine (TMB)) is used for the assay read-out.[192,193] Logozzi et al. designed a sandwich ELISA to capture and quantify exosomes in cell culture media as well as in plasma samples using the housekeeping proteins CD63 and Rab-5b and the tumor-associated marker caveolin-1.[37] One of the major drawbacks of ELISA-based exosome detection methodologies is the high level of 'biological noise' (i.e., nonspecific adsorption of biomolecules) during the detection of exosomes from complex bodily fluids.

8.2. Flow Cytometry

Flow cytometry is a well-established technology for high-throughput analysis and quantification of exosomes.[194,195] The principle of the flow cytometry method is based on the recording of fluorescence and light scattering by individual exosomal vesicles (i.e., nanosized particles) that are present in the suspension. Initially, a single particle suspension is hydrodynamically focused with a sheath fluid to intersect with a laser.[195] Signals are obtained by a forward angle light scatter detector, a side-scatter detector, and multiple fluorescence emission detectors. Then, the signals are amplified and converted to digital form. To acquire information regarding the exosomes that are present in the samples, flow cytometry data are interrogated based on the physical properties of the individual vesicles. However, conventional flow cytometry-based methods have several disadvantages.[195] The major concern is the platform-dependent variation in the analysis, where the data interpretation significantly varies among different laboratories. This variation is because different flow cytometers have different optical setups (e.g., varying laser wavelengths and powers) and different sensitivities. Since exosomes have a lower refractive index than that of the polystyrene beads (PS) of the flow cytometer, the scattered light derived from similar-sized particles is approximately tenfold lower than that of the beads. To resolve this issue, the accurate standardization of the flow cytometry technique is definitely required before it can be employed for exosome detection in clinical applications. Note that dedicated software has already been developed (available at www.exometry.com) for choosing the optimal optical setup and correcting the light scattering differences between reference materials (e.g., PS) and exosomes.

A specialized type of flow cytometry is FACS,[196] which allows the sorting of exosomal vesicles based on fluorescent labeling. This method involves a relatively complex mechanism compared with that of conventional flow cytometry analysis. Using specific antibodies tagged with fluorescent dyes, the target exosomes can be captured and sorted depending on the required parameters. In recent years, both methods have widely been used for the analysis of exosomes.[70,195,197–199] For instance, Rim at el. developed the FACS method for the analysis of exosomes from murine lung-cancer cells.[38] In this method, the initial isolation of the exosomes was performed using CD9- or CD63-antibody-coated magnetic beads. After staining the sample with an exo-fluorescein isothiocyanate exosome staining solution, the analysis of exosomes was performed via FACS. The study reported an increased level of CD63-specific exosomes in LA-4 lung-cancer cells. Clayton et al. used the flow cytometry method to show the expression of the B-cell marker CD20 on B-cell exosomes.[23] First, the isolation of exosomes based on immune-magnetic extraction by anti-HLA-DP, DQ, and DR antibodies was conducted, and

then a subsequent analysis was performed by flow cytometry. Several groups reported that 500 nm is the cutoff value for the precise identification of nanosized particles using previous generation flow cytometers.[193] Recently, a new generation of flow cytometers has been reported, which enables the detection of vesicles smaller than 200 nm.[200] Nevertheless, capturing the relatively smaller vesicles by flow cytometry remains challenging. Flow cytometry also requires expensive equipment, which is not suitable for analyzing exosomes in resource-limited settings.

8.3. Nanoparticle Tracking Analysis

In recent years, fluorescence readout methods have been successfully used for analyzing exosomes. Among the fluorescence methods, NTA is the most widely used tool for the characterization of the concentration and size of exosomes due to its simplicity and ability to capture vesicles within the diameter range of 50–1000 nm.[201] In NTA, a laser beam interacts with the exosomal particle. The scattered light of the particle is captured by a charge-coupled device camera and then analyzed by image processing software. The NTA software tracks the individual vesicles moving under Brownian motion and relates this movement to a particle size using the Stokes–Einstein equation. This tool has been used by various groups for exosome-based research. Dragovic et al. investigated and compared the efficiency of NTA and flow cytometry methods using human placental exosomes.[36] Their findings suggest that NTA can measure the size of biological vesicles as small as ≈50 nm with a greater sensitivity than that of several existing approaches. NTA is also capable of analyzing relatively larger amounts of vesicles compared with electron microscopy and atomic force microscopy.[36] Despite this reliable performance in fundamental research, NTA has substantial limitations for detecting exosomes in clinical samples.[30] These limitations are due to the lengthy procedures involved in data acquisition. Specifically, flow cytometry can analyze 1000 particles in less than a second, whereas NTA typically takes 10 min. Long analysis time also causes the bleaching of the fluorescent dye (i.e., the exosomes are stained with common fluorescent dyes, such as green fluorescent protein or antibodies that are conjugated with fluorescein isothiocyanate), whereas flow cytometry does not have bleaching issues because the readout is obtained in a short time (≈1 s) before the bleaching of the dye occurs. Additionally, this tool cannot analyze the biochemical composition of exosomes.

8.4. qNano (Tunable Resistive Pulse Sensing)

Recently, a new tool, which is based on the TRPS principle and commercialized as qNano, has been developed for the quantification of nanosized particles.[202] The instrument uses tunable pores manufactured in a polyurethane membrane to detect the passage of nano- or microsized particles by a drop in the ionic current measured across the pores. The flexible nature of the pore membrane allows the real-time optimization of the pore size. Lobb et al. used TRPS to obtain measurements for the exosome concentrations after isolating the nanosized vesicles using various protocols.[203] More recently, Sina et al.[204] and Yadav et al.[42] reported the use of TRPS for the characterization of breast cancer-derived exosomes prior to quantifying them with surface plasmon resonance and electrochemical readouts, respectively. qNano has shown great promise as a reliable tool for accurate exosome quantification. This method provides a quantitative analysis of vesicle samples with particles in the size range from 70 nm to 10 μm and performs real-time monitoring of ionic current flow across the pore, which enables the detection of individual nanovesicles in mixed suspensions. However, TRPS does not provide any information about the origin of exosomes.

8.5. Electrochemistry-Based Approaches

Electrochemical detection is highly suitable for biomolecular analysis due to its inherent advantages, such as high sensitivity and specificity, compatibility with miniaturization, simplicity, and relatively low-cost detection.[205–210] After the clinical success of electrochemical glucose sensors, electrochemical systems for biomolecular analysis have received significant attention and have become the focus of interest for many research groups. In electrochemical detection, a recognition element (e.g., antibody) interacts with the target exosomes to selectively recognize antigens that are present on the exosome surface. An electroactive signal transducer is incorporated to obtain a measurable electrochemical signal to quantify the amount of exosomes.[43,44] In most cases, electroactive molecules are tagged with a detection antibody (highly specific to the exosome) and used as an electroactive signal transducer. The detection is read via voltammetry (i.e., cyclic voltammetry, linear sweep voltammetry, differential pulse voltammetry, square wave voltammetry, and stripping voltammetry), amperometry, and impedimetric techniques.

Over the past several years, several electrochemical assays have been developed for the detection of exosomes. Recently, Jeong et al. developed an integrated magneto-electrochemical sensor (iMEX) for exosome analysis.[41] The iMEX platform utilizes two main steps: magnetic selection and electrochemical detection (**Figure 4**I). First, magnetic beads, coated with antibodies against tetraspanin proteins (e.g., CD63, CD9, and CD81), are used for exosome capture and labeling. Next, the captured exosomes are detected via electrochemical sensing. The entire assay was completed within 1 h and consumed only 10 μL of sample. The blood samples collected from ovarian cancer patients were tested and used to demonstrate that the iMEX device can be applicable in a clinical setting. Yadav et al. reported an electrochemical method to directly quantify the disease specific exosomes that are present in cell culture media.[42] Their approach has a two-step design. Bulk exosome populations are first captured using a generic antibody (i.e., tetraspanin biomarker, CD9). The subsequent detection of the cancer-specific exosomes within the captured exosomes is then performed using a cancer-specific antibody. This method used a HER-2 antibody as the cancer-specific antibody to quantify HER2-postive breast cancer-derived exosomes (Figure 4II). This method has shown good sensitivity, with a detection limit of 4.7×10^5 exosomes μL^{-1}. Doldan et al. developed another electrochemical sandwich approach for exosomes determination.[45]

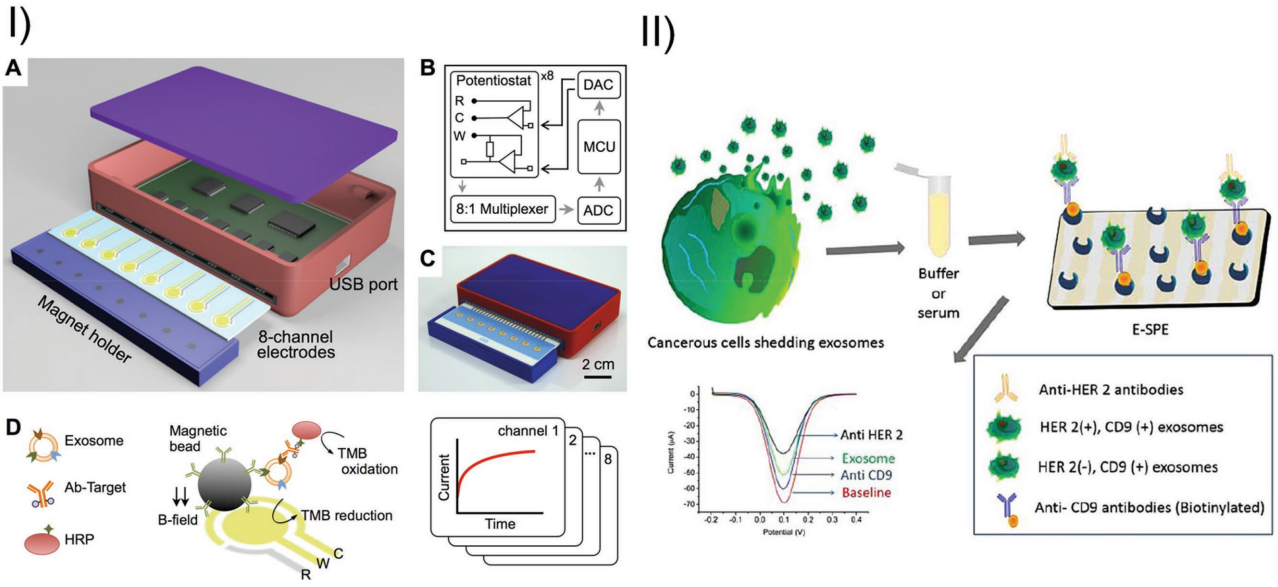

Figure 4. Schematic representation of the I) integrated magnetic-electrochemical exosome (iMEX) platform and II) sandwich assay for the detection of disease-specific exosomes. Reproduced with permission.[41] Copyright 2016, American Chemical Society. Reproduced with permission.[42] Copyright 2017, Wiley-VCH.

This approach uses gold electrodes prefunctionalized with α-CD9 antibodies. After spreading the sample onto the electrodes, the captured exosomes are analyzed with another α-CD9 antibody. At the final stage, HRP-conjugated α-mouse IgG antibody is applied. The electrochemical reduction of HRP-oxidized TMB is monitored. The sandwich immunosensor can detect 200 particles per microliter. Kelley and co-workers reported another method where a microfabricated chip was developed and the electro-oxidation of nanoparticles was used for the multiplexed analysis of exosomes. In this study, an electroplated gold layer on the electrodes were used as a sensing platform. Initially, the electrodes were functionalized with thiolated anti-EpCAM aptamers for the efficient capture of epithelial exosomes or microsomes. Then, silver (AgNPs) and copper (CuNPs) metal nanoparticles were conjugated with EpCAM and PSMA (prostate-specific membrane antigen), respectively, to compare the expression level of EpCAM and PSMA on microsomes and exosomes. Linear sweep voltammetry was applied for the oxidation of the AgNPs or CuNPs, which enabled the readout and confirmed the differential presence of surface markers in prostate cancer cells.[43] Recently, Zhou et al. reported the development of an aptamer-based electrochemical biosensor for the quantitative detection of exosomes.[211] In this method, aptamers specific to the exosome transmembrane protein CD63 were immobilized on gold electrode surfaces and incorporated into a microfluidic system. More recently, our group has reported a proof-of-concept electrochemical method to quantify exosomes using quantum dot (QD)-functionalized disease specific antibodies, which achieved a sensitive detection limit of 100 exosomes μL^{-1}.[212] This method leverages the advantages of the signal enhancement capability of a QD-based anodic stripping voltammetric readout. Briefly, magnetic beads that are functionalized with a tetraspanin CD63 antibody were initially mixed into the extracted sample to isolate the total exosome population. Then, breast and colon cancer-related exosomes were quantified by the respective use of CdSe QD functionalized biotinylated breast and colon cancer-associated antibodies. Nitric acid dissolution of the CdSe QDs and a subsequent anodic voltammetric readout were performed to quantify the number of cancer-related exosomes. The current method achieved a several hundred-fold increase in sensitivity compared with existing bioaffinity-based assays, mainly due to the use of QDs as the intrinsic signal amplifying labels. Over the past decade, significant progress has been made in the nanotechnology field to bring new strategies in electrochemical biosensing strategies to exosome analysis. In comparison with conventional methods for exosome detection and quantification, electrochemical techniques are fast, simple, cost-effective and do not require large sample volumes. However, since complicated fabrication steps are involved in biosensor development, the bioconjugation process has to be carefully controlled to ensure assay reproducibility. At the same time, to avoid nonspecific adsorption issues in electrochemical immunoassays, careful attention must be given to signal amplification tags and biomarkers. The integration of electrochemical approaches together with microfluidic platforms can result in an efficient tool for clinical diagnosis, particularly in point-of-care (POC) devices for a wide range of disease detection applications using exosomal biomarkers.

8.6. Microfluidics

Over the last several decades, microfluidic-based technologies have shown great promise in producing novel manipulation techniques for biological applications.[213] Microfluidic devices allow the manipulation of small amounts of samples and reagents in their channels and enable rapid and inexpensive separation and detection of targets. Since 2010, various microfluidics-based platforms have been developed for

efficient exosome analysis. In 2010, Chen et al. reported a microfluidic exosome analysis platform where an anti-CD63 functionalized channel was used for the immunocapture of exosomes from human serum.[31] Anti-CD63 functionalized channel was also used in another method, referred to as the ExoChip. This method utilized a surface-functionalized circular microchamber to capture exosomes, followed by fluorescent carbocyanine dye (DiO) staining for quantification.[35] He et al. developed an approach that enables on-chip immunoisolation and in situ detection of exosomes directly from patient plasma. In this method, isolation and enrichment of circulating exosomes, on-line chemical lysis, protein immunoprecipitation, and sandwich immunoassays assisted by chemifluorescence detection were performed on a single chip. The device was successfully tested to analyze plasma specimens derived from patients with NSCLC.[40] Zhao et al. developed the ExoSearch chip which combines on-chip continuous-flow mixing and immunomagnetic isolation with an in situ multiplexed exosome immunoassay.[214] More recently, Fang and colleagues developed a microfluidic device that comprises two chambers (for collecting the immunomagnetic particles), two circuitous mixing channels, four inlets, and one outlet (**Figure 5**I).[215] First, the sample was

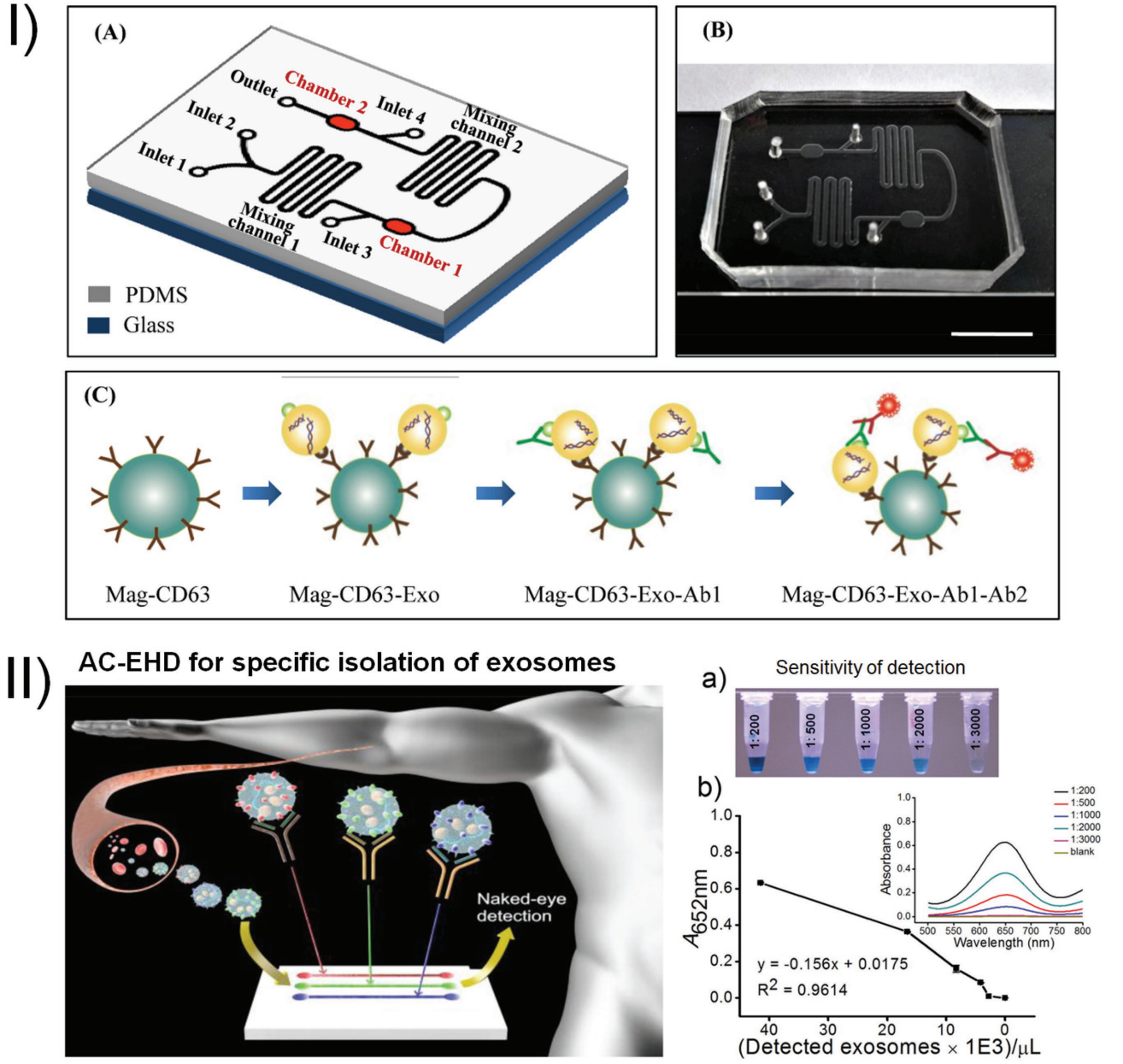

Figure 5. A schematic representation of I) on-chip immunocapture of exosomes. A) Schematic view of the microfluidic chip. B) Image of the chip. The scale bar represents 1 cm. C) Assay steps for the immunocapture of exosomes. II) Multiplexed device based on ac-EHD-induced nanoshearing for the isolation of multiple exosome targets. Reproduced with permission.[215] Copyright 2017, PLOS One. Reproduced with permission.[2] Copyright 2014, American Chemical Society.

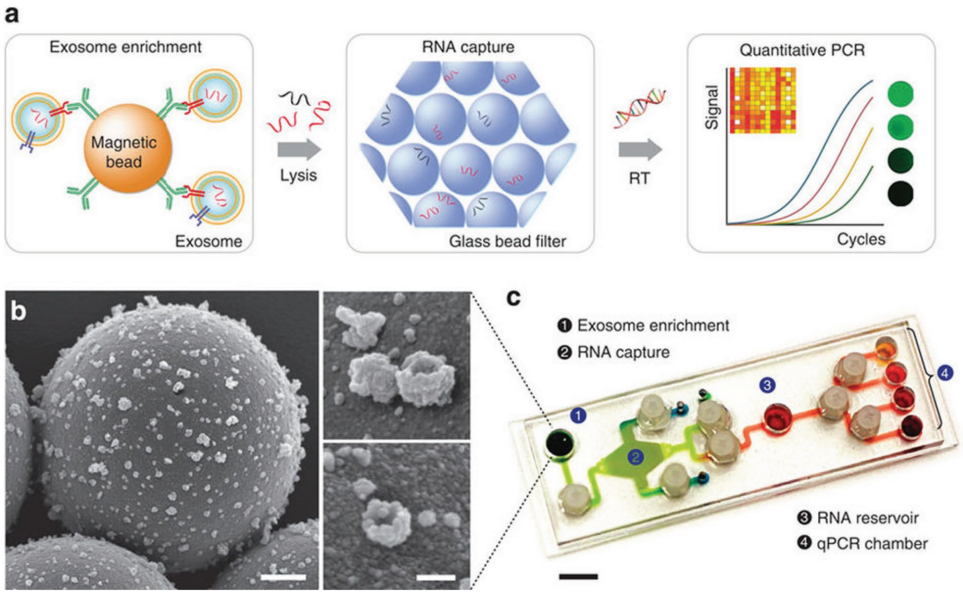

Figure 6. Schematic representation of an immunomagnetic device for exosomal RNA analysis (iMER). Reproduced with permission.[217] Copyright 2015, Macmillan Publishers Limited.

premixed with the capture agent Mag-CD63 to form a Mag-CD63-Exo complex. The complex was passed through inlet 1, while the primary antibody was introduced through inlet 2. This allowed the formation of a Mag-CD63-Exo-Ab1 complex. Then, the fluorescently labeled secondary antibody was introduced through inlet 3 to capture the target exosomes, which were finally examined by an inverted fluorescence microscope. This on-chip sensor was challenged to capture breast cancer specific exosomes in clinical samples, and the results showed that a significantly higher amount of EpCAM-positive exosomes were present in the plasma of breast cancer patients than in healthy controls.

Previously, Vaidyanathan et al. demonstrated a multiplexed microfluidic device for highly specific capture and detection of multiple exosome targets using a tunable alternating current electrohydrodynamic (ac-EHD) methodology, referred to as nanoshearing (Figure 5II).[216] In this approach, the exosomes derived from cells expressing HER-2 and PSA were simultaneously detected via colorimetry using a simple chip. The readout was based on the catalytic oxidation of TMB on the peroxidase-based exosome-antibody immunocomplex (e.g., from an HRP-conjugated detection antibody). The device also exhibited a significant enhancement in detection sensitivity and demonstrated the versatility of using ac-EHD-induced fluid flow through asymmetric microelectrode pairs that were used as a microfluidic component, without the need for active components such as pumps, valves, and mixers. The capture and detection domains were the same, and the simultaneous analysis of multiple target exosomes under ac-EHD-induced fluid flow was achieved.

Shao et al. presented a microfluidic chip that could be used to analyze mRNA levels in enriched tumor exosomes that were obtained from blood (**Figure 6**).[217] Their iMER platform integrates three functional compartments for targeted enrichment of extracellular vesicles, on-chip RNA isolation, and real-time RNA analysis.[217] Several other microfluidic platforms based on the size of the exosomes have recently been demonstrated. For example, Davies et al. fabricated nanoporous membranes in a microfluidic filtration system to isolate vesicles from whole blood with a tunable size cutoff (<500 nm).[218] Wang et al. fabricated a microfluidic device that consists of an array of porous silicon nanowire-on-micropillar structures.[219] The internanowire spacing was tuned within a range of 30–200 nm to create a high density of interstitial sites, which allowed the physical trapping of exosomes. Im et al. introduced a nanoplasmonic exosome sensor (nPLEX), which is based on the transmission of surface plasmon resonances through a periodic lattice of nanoholes that were patterned in a gold film (**Figure 7**A).[33] The nPLEX platform is integrated with a multichannel microfluidic cell for parallel and independent analyses. Shao et al. developed another microfluidic-based device for analyzing exosomes that were derived from glioblastoma (Figure 7B).[220] In this assay, the exosomes, which were inserted onto a microfluidic chip, were labeled with target-specific magnetic nanoparticles and finally detected by a miniaturized nuclear magnetic resonance system.[220] More recently, another microfluidic platform has been developed, which is based on a graphene oxide/polydopamine (GO/PDA) nanointerface.[221] The PDA coating allows an easy coupling of Protein G to the surface to immobilize monoclonal antibodies (mAbs). This approach demonstrated a higher yield signal and lower nonspecific background in comparison with a traditional GO/PEG coating. This improvement could be due to several features associated with thick PDA films such as 3D nanoporous structures, better surface coverage and larger surfaces. This platform successfully analyzed exosomes that were purified from a colon cancer cell line and plasma samples of patients with ovarian cancer.

Since 2010, several microfluidic lab-on-a-chip technologies have been developed to isolate, detect and provide the molecular analysis of exosomes. Due to many advantages such as quick analysis output, high yield and efficiency, low-volume

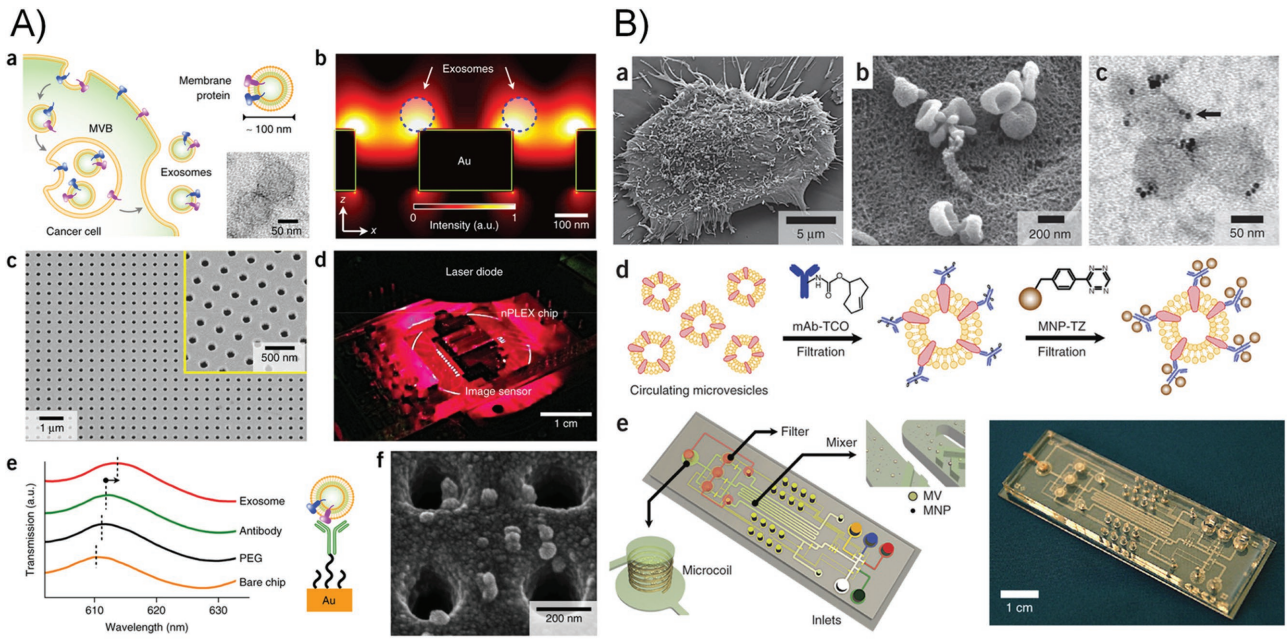

Figure 7. Schematic representation of A) exosomes released from cancer cells and a nanoplasmonic sensor (nPLEX) for label-free detection of exosomes and B) µNMR device for analysis of exosomes derived from human glioblastoma cells. Reproduced with permission.[33] Copyright 2012, Nature Publishing Group. Reproduced with permission.[220] Copyright 2012, Nature Publishing Group.

consumption, automation and functional integration for streamlined exosome molecular analysis, these microfluidic platforms have shown great promise for exosome analysis in clinical applications. However, to implement microfluidic technologies in clinical settings, several hurdles must be overcome. Many microfluidic chips are still not automated and require manual off-chip sample preparations, which are not suitable for real applications. Moreover, this technology requires well-trained specialists to perform the experiments. In this regard, a key point for future consideration of the microfluidic analysis of exosomes should be the translation of a benchtop platform to a robust, user-friendly and automated point-of-care device.

9. Conclusions and Perspectives

We have summarized the biological functions and significance of exosomes and their role as potential biomarkers for various diseases. Additionally, we have thoroughly discussed the recent advances in isolation and detection techniques for exosomes. We have also addressed the major technical and biological challenges of these strategies. Despite progressive advances, it is obvious that none of the existing techniques for exosome isolation are a one-size-fits-all model. The major technical challenge in exosome detection in clinical applications is to specifically detect disease-specific exosomes in the presence of exosomes that are derived from normal cells. Another considerable biological hurdle is the heterogeneity of disease-specific exosomes and the exosomal cargo, which could significantly affect the reproducibility of the analysis. Therefore, the right selection of detection techniques is required, along with the proper choice of exosomal cargo as the established biomarker. Most importantly, for ultimate clinical utility, there is an urgent need for the development of normative sample pools, which contain control samples of healthy heterogeneous populations (i.e., male and female, sedentary versus active lifestyle, young and old, etc.). Furthermore, new assays and technologies should be tested with a sufficiently large population of clinical samples for their robustness, accuracy, and selectivity. We believe that by combining outstanding components of different exosome quantification techniques and by innovative assay design in a multiplexed system that is capable of selective isolation of various exosome subtypes in heterogeneous samples, it will be possible to open a new avenue in exosome detection and related research. Clearly, researchers have continuously attempted to develop such techniques. For example, the recently developed iMEX platform,[41] which consists of a portable eight-channel device, could simultaneously profile multiple exosomal markers within one hour. Moreover, we recently reported a proof-of-concept electrochemical method to quantify exosomes using quantum dots that were functionalized with a disease-specific antibody, which could be extended to the development of a single-assay platform for the simultaneous detection of multiple tumor-specific exosomes.[212] We expect that, in the near future, ongoing effort toward the development of high-performance exosome detection techniques will result in an ideal next-generation platform that can be routinely used for exosome analysis for both research and clinical purposes.

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Conflict of Interest

The authors declare no conflict of interest.

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