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Advances in Analytical Technologies for Extracellular Vesicles

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Sensitive early detection, along with effective treatments and solid postoperative monitoring remain the grand challenges in fighting against human diseases, especially

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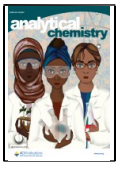

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cancer.[1] In addition to the conventional diagnostic tools such as magnetic resonance imaging, endoscopy, tomography, tissue biopsies, *etc.*, liquid biopsy combined with molecular biomarker analysis is emerging as a paradigm-shifting path that could make cancer diagnosis minimally invasive, more accessible, and more informative to guide clinical treatment.[2] Among various detection targets in the liquid biopsy, extracellular vesicles (EVs) are one of the rapidly evolving and promising candidates, because not only do they exist in almost all the body fluids with relatively higher abundance and stability compared to others such as circulating tumor cells and cell-free DNA but also their various cargoes represent insightful physiological and pathological states of diseases in individuals.[3−5]

EVs are diverse cell-released membrane vesicles sized from the nanoscale to the microscale. EVs were incipiently described as the cell waste carriers for the regulation of cellular health, which is not completely farfetched though,[6] they are more recently recognized to be functioning as the messengers for intercellular communication, conveying informational cargoes including proteins, nucleic acids (NAs), and lipids that can be exploited as biomarkers for diagnosis and longitudinal clinical tracking of diseases.[4,7,8] Furthermore, using various engineering approaches, EVs have also been exploited in therapy with advantages such as innate biocompatibility, high physiological stability, and cell-selective fusion.[9,10] Over the past decade or so, the EV study, ranging from basic biological functions[11] to the analytical methods[8] and clinical applications,[12−14] have witnessed explosive expansion. As represented by Figure 1, a literature search from Web of Science using the keyword of "extracellular vesicles" displays an exponential increase in the overall publication particularly in recent years. Commercially, the first EVs (exosomes)-based cancer diagnostic product was marketed in 2016 in the USA.[15]

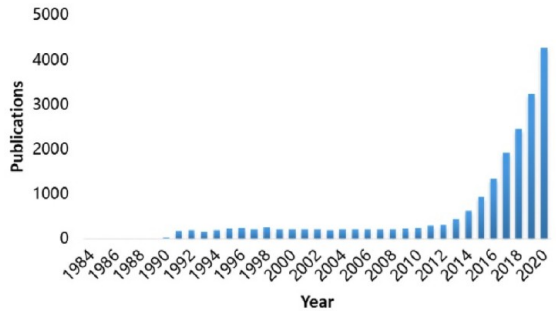

Figure 1. Number of publications returned from a search on Web of Science with the key word "Extracellular Vesicles".

EVs exist in almost all body fluids and are highly heterogeneous,[5] preanalytical factors such as the viscosity and the volume of the samples, the fat and protein content, as well as the gender, ethnicity, age, and dietary habits may affect the amount, purity, and yield of EVs.[16] Isolation protocols need to be adjusted by taking these factors into account. Conventional isolation methods regarded as the gold standard, such as ultracentrifugation, are limited by some major drawbacks, motivating the recent development of a variety of isolation methods with enhanced efficiency and purity.[17,18] Moreover, the complex and dynamic nature of EV biogenesis and molecular constituents present unique challenges to the existing analytical tools, including those originally designed for relevant biological objects such as cells. Therefore, it is imperative to improve current methods and develop conceptually innovative technologies to catalyze the advance of basic and applied EV research toward clinical utilities.[8,19−21] In this review, we intend to provide a comprehensive survey of the state-of-art progress in analytical science for EV study, especially exosomes, which leverages the advances in many areas, including EV biology, preanalytical consideration, characterization and analysis, and data science.

EV BIOLOGY

History of Extracellular Vesicles and Classification. Almost 4 decades have elapsed since the discovery of exosomes, and unraveling the secrets of exosomes never ends. The existence of EVs was first demonstrated in 1946 via high-speed centrifugation of human plasma.[22] Later in 1977, the observations by De Broe et al. gave evidence that the spontaneous shedding of plasma membrane (PM) fragments was a common occurrence in viable cells.[23] The term "exosomes" was first introduced by Trams et al. in 1981 to describe the exfoliated vesicles from the cell membrane.[24] However, the true exosomes as we know today was discovered in 1983 when Harding et al. and Johnstone et al. independently revealed that the trafficking and loss of transferrin receptor on the PM of reticulocytes during their maturation into erythrocytes were associated with small vesicles (~50 nm in

Table 1. Major Types of Extracellular Vesicles and Particles[a]

	subtypes	size (nm)	density (g/mL)	biogenesis	biomarkers
extracellular vesicles	exosomes[38]	50−150	1.13−1.19	endosomal pathway	CD63, CD81, CD9, TSG101, Alix
	enveloped virus[37,39,40]	80−400	1.16−1.18	plasma membrane budding, endosomal pathway	viral-encoded proteins, viral RNA
	microvesicles[38,41]	150−1000	1.16−1.19	plasma membrane budding	Annexin A1, cell-specific biomarkers
	apoptotic bodies and vesicles[38,42]	100−5000	1.16−1.28	apoptosis	Annexin V, thrombospondin, C3b
	exomeres	30−50		unknown	heat shock protein 90 (HSP90), HSPA13[38]
extracellular particles	chylomicrons	75−1200	<0.95		apolipoproteins, phospholipids, cholesterol, triglycerides[8,43]
	VLDL	30−80	0.95−1.006		
	IDL	23−27	1.006−1.019		
	LDL	18−23	1.019−1.063		
	HDL	7−13	1.063−1.21		

[a]Very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), high-density lipoproteins (HDL).

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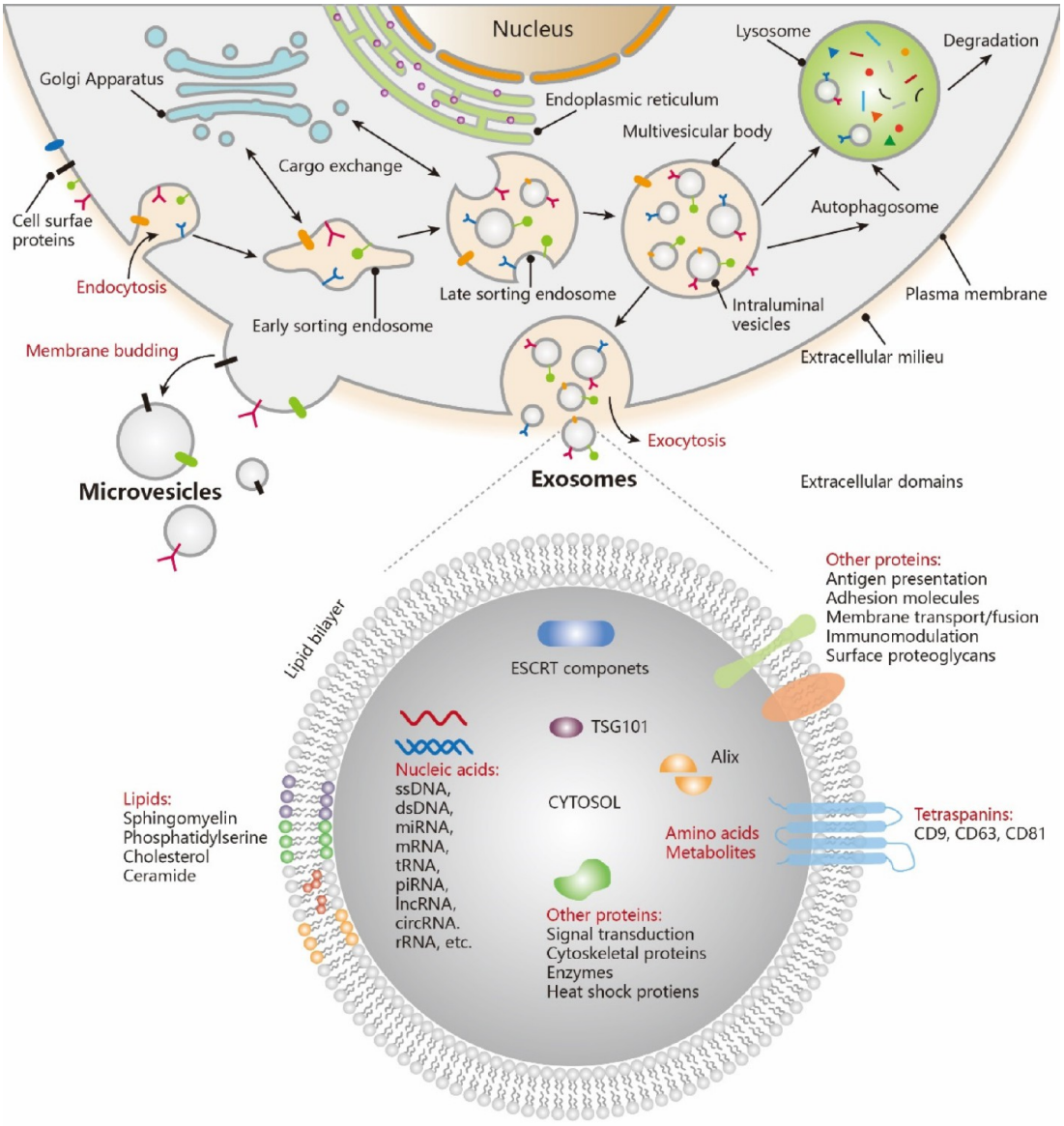

Figure 2. Biogenesis and contents of EVs. Exosomes are generated via the double invagination of the PM, including the formation of early sorting endosomes (ESEs) and late sorting endosomes (LSEs), the generation of ILVs within MVBs, the transportation of MVBs to cytoplasmic membrane, and the fusion of MVBs membrane with the cell membrane. Cargo exchange occurs between ESEs (LSEs) and the trans-Golgi network. MVBs can also fuse with lysosomes or autophagosomes for the degradation and recycling of cellular contents. Microvesicles are derived from the direct outward budding of cellular PM. EVs, including exosomes, transport a vast repertoire of different types of proteins, lipids, NAs, and other small molecules.

diameter) in endosomes, and these vesicles were then released from maturing reticulocytes into extracellular space via endocytosis.[25,26] At that time, Johnstone et al. found that this process was similar to reverse endocytosis, which releases internal vesicular contents rather than internalizes external molecules via membrane structure.[27] So she named these extracellular vesicles as "exosomes" in 1987.[28] "Unfortunately, we neglected to check whether the term had been used before", she recalled in a reminiscent article.[27] In addition, it is worth mentioning that the exosomes discussed in this review should not be confused with the exosome protein that is a molecular complex involved in the RNA degradation.[29]

Our knowledge of EV biology extends explosively since its discovery, followed by the fast development of EV analysis techniques and EV therapeutics.[8,12] Secretion of EVs is found to be a conserved process that occurs in both eukaryotes and prokaryotes throughout evolution.[11] EVs were at first proposed as the units secreted to dispose of the cellular waste and obsolete membrane proteins to maintain the cellular homeostasis,[28,30] which is supported by the subsequent research.[31] Nowadays, they are also recognized as the messengers carrying multicomponent cargos like NAs, proteins, lipids, *etc.* to mediate intercellular communication.[11,32] The recognition of their vital roles in physiological and pathological functions promotes the establishment of various organizations, such as the International Society for Extracellular Vesicles (ISEV) and the American Society for Exosomes and Microvesicles (ASEMV), and also the creation of a dedicated journal, the *Journal of Extracellular Vesicles*.

The ISEV endorses that "EV is the generic term for particles naturally released from the cell that are delimited by a lipid bilayer and cannot be replicate."[33] EVs are a highly diverse

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population of different subpopulations that differ in biogenesis, size, content, and function. EVs can be generally divided into three major categories: microvesicles/ectosomes, exosomes, and apoptotic bodies (ApoEVs), based on their distinct biogenesis processes (Table 1). Exosomes and microvesicles are actively released by living cells, while ApoEVs are shed from dying cells during the process of apoptosis.[34−36] Because of the current lack of reliable and distinct biomarkers to distinguish different EV subtypes, it remains a common practice to classify the harvested EVs based on the widely accepted criteria of size ranges: exosomes (50−150 nm), microvesicles/ectosomes (100−1000 nm), and apoptotic cell-derived EVs (500−5000 nm). Among these EVs, exosomes are of special interest in the scientific community because of their distinct intracellular regulatory biogenesis process,[37] while other EVs like ectosomes and ApoEVs are increasingly arousing the attention of scientists.

Even though microvesicles and exosomes generate at distinct intracellular sites, the overlap in size range, morphology, sorting machineries, and molecular contents poses challenges to the currently available isolation techniques to obtain purified EVs that belong to a particular biogenesis pathway,[11] such as the endosome-originated exosomes or PM-derived microvesicles, unless unique biomarkers of EV subtypes are found or the EVs are being monitored and captured via real-time imaging techniques.[33] The ISEV recommends researchers to use the operational terms for EVs subtypes instead of exosomes or microvesicles, for example, (a) size-based, small EVs (sEVs), medium EVs (mEVs), and large EVs (lEVs) with the definition of ranges; (b) density-based, low, middle, or high density with each range defined; (c) composition-based, CD63+/CD9-EVs or Annexin A5-stained EVs, *etc.*; (d) description of conditions-based, hypoxic EVs; (e) cell origin-based, podocyte EVs.[33] It is noted that while the biology of EVs will be briefly summarized following their biogenesis-based classification, our discussion on the technology development, the major theme of this review, will be based on a relatively broad term of "exosomes" that are increasingly accepted to refer to a heterogeneous mixture of sEVs of less than 200 nm in size.

Biogenesis of EVs. *Biogenesis of Microvesicles.* The biogenesis of microvesicles represents unique mechanisms that are still incompletely understood. Generally, microvesicles are derived from the direct outward budding of the cellular PM, they have a size in diameter that ranges from 100 to 1000 nm and include the cytoplasmic material. The formation of microvesicles is associated with PM remodeling.[44] Phospholipids on the PM distribute asymmetrically, which is called "sideness" of the lipid layer. Aminophospholipids, including phosphatidylserine (PS) and phosphatidylethanolamine, are specifically enriched in the inner leaflet of cellular PM, whereas the external part mainly consists of sphingomyelin and phosphatidylcholine.[44] The trans-bilayer distribution of lipids is controlled by three elements: the flippase, an inward-directed pump; the floppase, an outward-directed pump; and the lipid scramblase which promotes the bidirectional redistribution of lipids. However, the membrane asymmetry can be disrupted by a significant and continuing increase of cytosolic Ca^{2+} following the cell stimulation, which enhances the activities of floppase and scramblase but inhibits the activity of flippase. This process changes the membrane rigidity and curvature, leading to the externalization of PS, the degradation of cytoskeleton by Ca^{2+}-dependent proteolysis, and finally, the release of microvesicles.[44,45]

Microvesicles can also form via direct budding of the PM like budding viruses but in a virus-independent way.[46] During this process, the interaction of tumor susceptibility gene 101 (TSG101) with tetrapeptide PSAP (Proline-Serine-Alanine-Proline) motif of the arrestin domain-containing protein 1 (ARRDC1, located in the PM) leads to the relocation of TSG101 from endosomes to the PM and then the release of microvesicles.[46] This mechanism utilizes endosomal machinery such as the endosomal sorting complex required for transport (ESCRT) to prompt the formation of vesicles. ARRDC1-mediated microvesicles (ARMMs) contain TSG101, ARRDC1, and other cellular proteins but lack known late endosomal markers.[46,47] In addition, researchers have discovered that microvilli can function as vesicle-generating organelles,[45,48] and protein−protein crowding on the membrane surface alone could be sufficient to stimulate the bending of the PM.[45,49]

Biogenesis of Exosomes. Exosomes are generated via the double invagination of cellular PM (Figure 2), and several key steps are needed to complete the biogenesis of exosomes: the formation of endosomes, the generation of intraluminal vesicles (ILVs) within multivesicular bodies (MVBs), the transportation of MVBs to the PM, and the fusion of MVBs membrane with cellular PM. In the first invagination process, inward budding of PM leads to the formation of early sorting endosomes (ESEs) which contain cell-surface proteins and soluble proteins existing in the extracellular milieu. In addition, endoplasmic reticulum (ER) and trans-Golgi network can also contribute to the formation and the content of ESEs.[50−52] ESEs give rise to late sorting endosomes (LSEs) which move their locations from the outer cytoplasm to the vicinity of nucleus and change from the tubelike shape to the spherical shape during maturation.[53] Then the second inward invagination of the LSEs membrane leads to the formation of intracellular MVBs with intraluminal vesicles (ILVs) inside. MVBs can fuse with lysosomes or autophagosomes, leading to the degradation of their contents that could further be recycled by the cells. Alternatively, MVBs will be transported to and fuse with the PM by which ILVs are finally secreted to extracellular space as exosomes with a diameter range from 50 to 150 nm (100 nm on average) through exocytosis.[52] The protein topology in the exosomal membrane owns the same orientation as that in the PM due to the double inward invagination.

The formation of exosomes involves particular sorting machineries among which the ESCRT was first discovered and has been extensively elucidated.[54−56] The four ESCRT complexes act in a stepwise manner and play distinct roles: ESCRT-0 complex recognizes and clusters ubiquitinated proteins in the endosomal membrane; ESCRT-I and ESCRT-II complexes are responsible for the membrane local budding with sorted cargoes; and ESCRT-III drives subsequent scission of ILVs into MVBs lumen.[57] Though ESCRT-III is necessary for the fission of the ILVs, cargo clustering and membrane budding can exist in an ESCRT-independent manner.[52] Tetraspanin proteins are directly involved in the ESCRT-independent ILVs formation and cargo sorting.[58] Tetraspanin-enriched microdomains are ubiquitous specialized membrane platforms enriched in exosomes.[58] Clustering of several cone-shaped tetraspanins (such as CD81) with cholesterol accommodated could induce inward budding of

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tetraspanin-enriched microdomains.[59] CD63 is found to participate in the sorting of pigment cell-specific melanocyte protein (PMEL, a component of melanosomes) to the ILVs in melanocytes.[60] In dendritic cells, the recruitment of major histocompatibility complex-II (MHC-II) to ILVs depends on the incorporation into the CD9-enriched microdomain.[61] In addition, in a mouse oligodendroglia cell, ceramide was found to contribute to a spontaneous negative curvature on the limiting endosome membranes and facilitate the inward budding.[62,63] Activation of G protein-coupled sphingosine 1-phosphate (S1P) receptor on MVB membranes by a constant supply of metabolite S1P is critical for the sorting of cargo molecules into ILVs.[64] The biogenesis of exosomes is complex and the underlying mechanisms, in particular the selective sorting of molecules, remain largely unknown, which motivates the development of better analytical tools for specific isolation and molecular characterization of exosomes.

Molecular Compositions of EVs. EVs contain a broad array of proteins, lipids, and NAs which not only provide insights into the mechanisms of EV biogenesis but also endow EVs with various biological functions and clinical significance (Figure 2). Exosomal proteins embrace transmembrane proteins, lipid-anchored membrane proteins, peripherally associated membrane proteins, and soluble proteins in the exosome lumen.[65] For example, certain tetraspanin proteins, such as CD81, CD9, and CD63, are highly enriched in exosomes and thus have become the most commonly used exosomal markers.[66] Lipid-anchored proteins, such as the C-terminal glycosylphosphatidylinositol-anchored proteins, are also decorated on exosomes' surface, among which glypican-1 was arguably identified as a biomarker for the early diagnosis of pancreatic cancer.[67] Exosomes also carry outer and inner peripheral surface proteins, and their main roles are involved in signaling and the scaffold web, respectively.[65] ALG-2-interacting protein X (Alix), an exosomal scaffolding protein, along with TSG101, are commonly used as markers for exosome identification.[8] The exosome surface is also abundant in extracellular matrix proteins, and the inner membrane is enriched with molecular chaperones such as several members of the HSP70 family.[65] In addition to these general markers, growing results show that exosomes are also enriched in proteins from the cells of origin that may reflect their physiological and pathological status and thus constitute a "disease signature", which will be discussed in greater detail below.

NAs in EVs (EV NAs) occupy another large cargo, they are encapsulated within EVs via specific sorting mechanisms,[68,69] and so far, many types of DNA and RNA have been identified including ssDNA,[70] dsDNA,[71,72] miRNA,[73,74] mRNA,[75] long noncoding RNA (lncRNA),[75,76] circular RNA (circRNA),[75,77] transfer RNA (tRNA),[78] piwi-interacting RNA (piRNA),[79] ribosomal RNA (rRNA),[80] small nuclear RNA (snRNA),[80] small nucleolar RNA (snoRNA),[80] long intergenic noncoding RNA (lincRNA), *etc.*[80] EV NAs not only convey the information about the cellular origin and physiological and pathological functions, but also they are protected by the lipid bilayer of exosomes from being degraded by enzymes in body fluids,[81] which endows EV NAs the potential to be promising and stable biomarkers in liquid biopsies. Many researches have revealed the value of EV NAs as biomarkers for the diagnosis of disease, such as the circular RNAs in immune-mediated demyelinating disease;[82] lncRNAs in colorectal cancer;[83] miRNAs in Parkinson's disease,[84] in renal fibrosis,[85] in thyroid nodules,[86] in central nervous system diseases,[87,88] in colorectal cancer,[89,90] in ovarian cancer,[91] in prostate cancer,[92] and in depression;[93] combined miRNA−piRNA signature in Alzheimer's disease;[79] tRNA in osteoporosis;[78] and mitochondrial DNA in ovarian cancer.[94]

EVs also contain a variety of lipids whose concentration and distribution may not only provide the clues about the mechanisms of EV biogenesis but also potential biomarkers for disease diagnosis. The thickness of a lipid bilayer membrane is around 5 nm,[95] which indicates that lipids occupy roughly a quarter of the volume of the sEVs with a diameter of 100 nm. The study of EV lipidomics reveals the enrichment of lipid compositions such as cholesterol (CHOL), sphingomyelin, glycosphingolipids, and phosphatidylserine from cells to exosomes, a similar mole percent of phosphatidylethanolamine, and a lower mole percent of phosphatidylcholine and phosphatidylinositol.[96] CHOL along with sphingolipids are the key components of specific membrane domains called rafts that are involved in several transport and sorting mechanisms,[97] and the high content of these lipids in EV membrane proves the presence of lipid rafts during EV biogenesis. Also, the levels or formation of lipids such as ceramide, diacylglycerol, and phosphatidic acid which have the smallest head groups are important for the generation and release of EVs.[97] Besides, lipid compositions may keep the stability of EVs in different extracellular environments and facilitate their interaction with recipient cells.[98] EV lipids have been identified as potential biomarkers such as the phosphatidylserine in ovarian malignancies,[99] the lipid signatures in prostate cancer,[100] and the C16:0 sulfatide in multiple sclerosis.[101] Compared to EV protein and NA biomarkers which have been largely investigated, the potential of EV lipids used as biomarkers for diagnosis, however, has not been explored in detail. Further research on EV lipids may discover more valuable information.

In addition to proteins, NAs, and lipids that have been the major targets of extensive studies, other exosomal constituents, although less explored, may also provide insights into EV biology and clinical relevance. For instance, glycan canopy is presented on the outermost surface of exosomes, and protein glycosylation may provide a unique source of molecular biomarkers.[102,103] Therefore, better characterization of these biochemical analytes will provide useful cross-omics information to facilitate the studies of EVs' roles in cellular functions and disease pathology.

To facilitate the comprehensive characterization of exosomal contents, several databases have been established, including the exoRBase (http://www.exoRBase.org), a database of circRNA, lncRNA, and mRNA derived from RNA-seq data analyses of human blood-derived exosomes,[104] the EVmiRNA (http://bioinfo.life.hust.edu.cn/EVmiRNA#!/), an EV miRNA database,[105] and Vesiclepedia (http://www.microvesicles.org), a compendium of RNA, proteins, lipids, and metabolites in EVs.[106] These databases are constantly updated to share new discoveries and allow researchers to grasp the complexity of EVs.

Heterogeneity of Exosomes. As discussed above, different subtypes of EVs, albeit originated via distinct biogenesis pathways, to some extent have overlapping properties. Meanwhile, exosomes also display heterogeneity in their sizes, contents, functions, and biodistribution. For instance, using density gradient centrifugation, researchers revealed the presence of two distinct subpopulations of

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exosomes, termed lower density exosomes (LD-Exo, mode size 117 nm) and higher density exosomes (HD-Exo, mode size 66 nm), which differ in size, protein, and RNA composition. LD-Exo and HD-Exo were also observed to have different effects on recipient cells and they were speculated to be derived from different types of ILVs.[107] Similarly, by applying the asymmetric flow field-flow fractionation (AF4), two discernible exosome subsets have been identified: large exosome vesicles (Exo-L, 90−120 nm) and small exosome vesicles (Exo-S, 60−80 nm), and another population of nanoparticles termed "exomeres" (~35 nm) has also been discovered.[108] It was reported that NAs, proteins, glycans, and lipids are differentially packaged into these vesicles. Apart from the disparity in cargo composition, cargo distribution across the population of exosomes differs significantly. Quantitative and stoichiometric analysis of exosomal microRNAs revealed that, on average, more than 100 exosomes are required to observe one copy of a given type of miRNA, and most exosomes do not carry any copy, even for abundantly expressed miRNAs.[109] Moreover, the organ and tissue origins of exosomes, whether they are tumor cell-derived or not, constitute other levels of the heterogeneity and complexity of human exosomes.[110] These nonuniformities in size, contents, and origin finally result in the difference of exosomes in their biological functions, such as cell survival, apoptosis, or immuno-modulation,[7] which also drives the development of advanced technologies for more comprehensive and precise identification and understanding of exosomes with a high level of complexity.

PREANALYTICAL CONSIDERATIONS OF SAMPLE COLLECTION AND HANDLING

Exosomes and other EVs have been confirmed to be present in almost all the body fluids since first discovery,[5] including blood,[111−113] pleural effusions,[114,115] aqueous humor,[116,117] breast milk,[118,119] ascites,[103] amniotic fluid,[120] semen,[121] saliva,[122,123] nasal secretions,[124] cerebrospinal fluid,[125,126] bronchoalveolar lavage,[127] synovial fluid,[128,129] bile, urine,[130,131] cell culture-conditioned media,[132,133] *etc*. These EVs containing proteins and NAs provide a rich resource to identify biomarkers for disease diagnosis. Given the complexity and dynamic nature of biological fluids, the measurements of concentration, composition, and biofunction of sEVs may be impacted by the sources, collection, storage, and pretreatment of samples. Therefore, minimizing preanalytical variations is a critical aspect of the analytical science for EV studies to achieve rigorous and reliable characterization of EVs in relation to their biological functions and clinical significance. As the EV research grows rapidly, the importance of the preanalytical phase of EV analysis receives increasing attention and has been discussed in several comprehensive review articles and the ISEV guidelines.[5,33,134,135] Herein, we will briefly highlight some of essential preanalytical considerations associated with several commonly accessible biological sources for sEV studies.

Cell Culture Conditioned Media. EVs derived from cultured cell lines are frequently used as model systems in EV research. Cell culture conditioned medium presents a relatively controlled sample matrix for EV isolation. Nonetheless, EV yields and purity may be directly impacted by several factors that need to be considered, including serum-derived EVs, cell apoptosis, and cell contamination.[136] Generally, cells are cultured with human serum or fetal bovine serum (FBS) which contain abundant EVs. The use of serum-free culture media is one way to avoid the contamination from serum-derived EVs, but such an abrupt change in the culture conditions (serum starvation) is known to cause major stress to cells, which alters cellular metabolism, behaviors, and proliferation, thereby resulting in the changes in the secretion and molecular contents of EVs.[137−140] An alternative method is to use EV-depleted FBS that can be prepared by mostly using the ultracentrifugation-based protocols to deplete EVs.[141] These processes are time-consuming (e.g., >16 h ultracentrifugation), vastly increase the cost, and cannot completely remove FBS-derived EVs.[142] Thus, efforts have been invested in further optimization and the development of new FBS EV-depletion methods to improve the depletion efficiency and consistency across laboratories.[143,144] While EV-depleted FBS has been widely used, increasing evidence reveals that serum EV depletion can also change the viability, proliferation, phenotypic profile, and biological activities of cells in culture.[142,145−149] Given the current technical limitations in EV depletion and largely unknown effects of FBS-derived EVs in cell culture, the concerns on the continued use of EV-depleted FBS have been raised and it has also been suggested to move toward serum-free medium alternatives, such as the growth factors.[144]

Microbial contamination is another factor to be considered. For instance, mycoplasma is the most frequent contamination in the tissue culture laboratory.[150,151] Microbial contamination will cause cell apoptosis, leading to the release of numerous apoptotic vesicles and cell debris. On the other hand, it is very difficult to remove small microbial contaminants with similar sizes and/or density to EVs, such as mycoplasma and viruses, by ultracentrifugation and other size-based isolation methods.[152] Thus, it is important to carefully test whether cultured cells are infected by microbes or viruses before EV isolation. In addition to the interferences from exogenous EVs, the studies of EVs may also be affected by the vesicles of various sizes and membrane debris formed by apoptotic cells. It is challenging to separate these apoptotic entities from living cell-derived EVs by ultracentrifugation, in which large EVs or cell debris could be broken into small particles by mechanical forces. Thus, it is necessary to verify the viability of cultured cells to minimize the interference from apoptotic vesicles.

Taken together, careful considerations of culture conditions and isolation methods should be taken when analyzing and using EVs isolated from cultured cell lines in functional studies. Effects of the change in cell culture conditions must be examined along with other experimental variables to mitigate their interferences and permit a more relevant understanding of differential biogenesis dynamics, cargos, and functions of EVs derived from specific cell types.

Blood. Blood is one of the most abundant sources of EVs as well as one of the most complex biofluid matrixes for isolation and detection of specific EVs associated with diseases, such as tumors. A plethora of methods have been established to isolate EVs from the blood.[153−155] For biomedical studies of EVs, blood collection is normally performed following the standard venipuncture protocols for which 19 to 22-gauge needles are commonly used. The possible influence of venipuncture needs to be considered. Mechanical forces arising from venipuncture, such as contact, shear forces, and pressure, may promote the activation of platelets and the release of platelet-derived EVs.[156] The vacuum pressure applied to blood might cause hemolysis and produce a large quantity of erythrocyte-derived EVs.[157] Therefore, 21-gauge or larger needles and butterfly needles are preferred over small-diameter needles in order to

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reduce the effects of platelet-activating and the release of EVs derived from blood cells.[158] Another important aspect of blood collection to be considered for EV analysis is the selection of anticoagulants. Widely used anticoagulants for blood collection include ethylenediaminetetraacetic acid (EDTA), heparin, sodium fluoride/potassium oxalate, and sodium citrate with or without additives such as dextrose or theophylline, which have differential effects on the properties and molecular analysis of blood-borne EVs. For example, heparin has been reported to be least compatible with EV studies compared to other anticoagulants, because of its negative effects, including PCR inhibition,[159] binding with EVs, and enhancing EV release by monocytes, platelets, and erythrocytes.[160,161] Recent research reported that the concentration of EVs in blood treated by dextrose, EDTA, or citrate alone decreased because the calcium chelators facilitate quick association between sEVs and platelets.[162]

Theoretically, blood samples should be immediately transported to the laboratory without any shake. However, blood samples are always delayed for certain reasons and it is practically impossible to avoid any agitation using a hospital transportation system. These two factors might result in changes in EV concentration and compositions in the blood due to platelet activation. It has demonstrated that the physical impact of the transport system would active platelets and cause a constant release of platelet-derived EVs within several hours following blood collection.[163] To minimize the influence of transportation, Lacroix et al. developed a special container that allowed the sample tubes to be transported vertically to significantly reduce the impact of agitation.[164] Storage of blood samples is another vital factor that needs to be considered because sample collection and processing often occur at different locations. Researchers have attempted to evaluate the influence of freeze−thaw cycles and storage temperature on the concentration, size, and cargos of EVs.[165,166] Plasma samples may maintain the best structure and functions at −80 °C or in liquid nitrogen.[167] The thawing should not occur on ice but at 37 °C or room temperature, because the microparticle recovery rate in samples thawed on ice shows a significant decrease compared with the original samples and the samples thawed at 37 °C or room temperature.[168] Avoiding multiple freeze−thaw cycles is essential to decrease the number of platelet-derived EVs and to preserve the integrity of EVs.[165] In order to decrease the platelet-derived EVs, a large diameter needle, for example, the 21G needle, should be used to withdraw blood; the anticoagulants could be sodium citrate, EDTA, and CTAD (citrate-theophylline, adenosine, dipyridamole); the blood should be centrifuged to obtain the plasma immediately.[169] The influences of storage and transport conditions on EV isolation and quality are very complicated and still need to be further investigated in order to establish a standardized protocol.

Plasma is prepared from whole blood by a quick centrifugation process at room temperature to remove platelets, cell debris, and other blood cells.[170] The viscosity of blood in patient samples with specific diseases will be different from that of normal blood samples, and it should be noticed that viscosity has been shown to affect the sEV pelleting efficiency.[171] Thus, the viscosity of different blood samples should be normalized by diluting with buffer before centrifugation. Compared to plasma, serum contains more sEVs released from platelet during the clot formation process.[172] The numerous platelet-derived EVs might interfere with downstream protein and NA analysis and lead to confounding results. Platelet-derived EVs could be a desirable specimen, if they are associated with specific diseases.[172] Although serum is an appreciated sample for EV analysis, plasma is still the major sample used in most research.

Pleural Effusions and Ascites. The composition of pleural effusion and ascites is complicated, including lymphocytes, mesothelial cells, macrophages, tumor cells, proteins, NAs, *etc.*[173] Pleural effusions and ascites are usually discarded as spent liquor; however, recent studies indicate that pleural effusions and ascites might be great sources for liquid biopsy.[174,175] It has been demonstrated that pleural effusions and ascites contain abundant sEVs which could provide potential diagnostic biomarkers for various cancer types, including advanced pancreatic cancer, gastric cancer, and lung cancer.[103,115,175−177] Compared to blood samples, pleural effusion and ascites offer some advantages that could benefit isolation and molecular analysis of disease-derived EVs. First, the lesions in the chest and abdomen could be exposed in the environment of pleural effusion and ascites such that damaged tissues or cells secreted EVs directly into the pleural effusions and ascites.[178] This makes pleural effusion and ascites a unique biospecimen enriched with disease-derived EVs for lung cancer.[179] In addition, the biological and biomedical properties of sEVs in pleural effusion and ascites were found to be similar to that in blood samples.[180] The collection and storage of pleural effusion and ascites are anticoagulant-free, which avoids the effects of anticoagulants required for blood sampling on EV properties and downstream bioanalysis. Moreover, a practical benefit of pleural effusion and ascitic fluids is their clinical availability because the volume of pleural effusion and ascites collected from patients is usually large, ranging from dozens to hundreds of milliliters providing an abundant supply for EVs. Since pleural effusion and ascites contains plenty of cells, after collection, they should be centrifuged to remove cells and cell debris for future EV isolation and analysis. Then the centrifuged ascites can be stored at −80 °C for the following experiments. However, it should be noted that microbes might also appear in pleural effusions and ascites because of infectious diseases in some cases.[181,182] It is also important to test whether microbes exist in pleural effusion and ascites.

Tear Fluid. Several studies have found a large number of sEVs present in tear fluid by the manners of affinity recognition and centrifugation.[183,184] The potential applications of tear fluid-derived EVs as a noninvasive liquid biopsy have been demonstrated by exploring EV biomarkers for diagnosis, monitoring, and prognosis of eye diseases, such as uveal melanoma, glaucoma, and neovascular age-related macular degeneration.[183−185] An attractive advantage of tear fluids for EV analysis arises from the simplicity and noninvasiveness of the sample collection process. The Schirmer test strip is a convenient procedure for tear sample collection that patients can perform at home. The intact Schirmer strips can be directly transferred into phosphate buffer saline (PBS) buffer to extract sEVs.[116] To improve the efficiency of EV extraction, the Schirmer test strips could also be cut into small pieces and shaken in PBS buffer, followed by ultracentrifugation for EV isolation and purification.[186] The tear fluid strip samples can be stored at −80 °C for downstream bioanalysis.[186] Despite the advantages in sample collection, the relatively small volume of tear fluids available from individuals could pose a significant obstacle to the research and clinical utilities. This limited sample availability calls for the development of better methods

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for efficient EV isolation and integrated EV analysis directly from tear fluids.

Saliva. Saliva, which contains lysozyme, proteins, amylase, *etc.*, is a complex fluid secreted by multiple glands, such as sublingual, submandibular, parotid, and other small glands distributed in the oral cavity.[123] Analysis of sEVs in saliva provides a new strategy to develop diagnostic tests for specific detection of oral cancer and other oral diseases as saliva pervades in the mouth and directly contacts with oral tissues.[123] Salivary fluids show fewer proteins and other biological contents and are much less complex compared with other commonly used clinical specimens, such as blood and ascites,[187,188] which eases the specific isolation and biomarker detection of salivary sEVs.

Saliva collection is an easy, simple, and noninvasive process, which can be done by patients themselves at home. However, an inherent problem lies in the difficulty in standardization and normalization of salivary sample collection because the oral environment is dynamically changing,[189] and salivary contents can be affected by numerous factors including food, beverage, tobacco smoking, and even physical activities.[190] It may be a better practice to refrain from drinking, eating, and smoking at least 1 h prior to sample collection.[191] In addition, the donors should not carry out any dental stimulation 1 day in advance and even not brush their teeth in 1 h before sample collection.[5] Moreover, the oral disease should also be checked before sample collection. All these situations should be taken into consideration when collecting saliva samples.

sEVs have been usually separated from saliva using the ultracentrifugation and gel filtration-based methods.[122,123,192] It should be considered to remove some major pollutants in saliva samples including bacteria that exist in the oral cavity; squamous epithelial cells shed from the tissue that lines inside the mouth and food debris by centrifugation or filter.[5] The purified samples can be stored at −80 °C for as long as 1 year without obvious changes in concentration and diameter of sEVs. However, it was reported that freeze−thaw cycles could affect the concentration of sEVs.[193]

Urine. Urine provides another uniquely useful bodily fluid for sEV analysis because it can be obtained in large quantities in a completely noninvasive way. Urine-borne sEVs originate from a variety of cells in the kidney and urinary tract and have been separated by different methods, including ultracentrifugation, immunoaffinity capture, and size exclusion chromatography.[130,131,194,195] Several studies have demonstrated its potential for clinical diagnosis of kidney, bladder, and prostate diseases.[196,197] For urine sample collection, several special considerations need to be taken to mitigate sampling-induced bias and variation. First, the concentration of sEVs in urine needs to be normalized.[198] In comparison to the relatively constant blood volume in the human body, urine volume is variable and unpredicted, which means that the concentration of sEVs in urine varies with the volume of urine. One possible solution is to select an internal reference as control. For example, uromodulin, separated along with sEVs, is a choice of internal reference to the concentration of sEVs in the urine.[199] Second, urine components are seriously influenced by diet, so diet control is necessary prior to urine collection each time. Just like other biofluid samples, concentrated urine samples should be best kept at −80 °C for long-term storage.[200]

For the isolation of EVs from urine, some biological issues that should be taken into consideration. Uromodulin (also called Tamm-Horsfall protein) produced by the kidney is the most abundant protein in normal urine. Uromodulin can be copurified along with EVs by ultracentrifugation, affecting the purity and quantification of isolated EVs.[199] It has been reported that using reducing reagent and size exclusion chromatography could address this problem.[201,202] Certain exosomal proteins, such as Na−K−Cl cotransporter isoform 2, were found to degrade without protease inhibitors during the isolation of urinary EVs.[196] Therefore, the use of proper protease inhibitors is recommended for sample preparation for sEV analysis.

■ ISOLATION AND ENRICHMENT OF EVS

Not only are extracellular vesicles heterogeneous in sizes, origin, and molecular constituents, they are also present in different complex biological fluids containing varying amounts of contaminants, such as lipoproteins, extravesicular protein complexes, and other protein aggregates, which may confound analytical results. The purity of isolated exosomes or other EV subtypes is critical to better understanding their biological mechanisms and their broad applications in many fields, such as diagnostics and therapeutics. At present, several critical challenges hamper rapid advances in the fundamental and applied research of EVs.[154,203] To date, there is a lack of standardized methods for rapid, efficient, and unbiased isolation of EVs from various biofluids. Currently, many of the isolation methods are costly, labor-intensive, and/or time-consuming, limiting their adaptability to clinical utilities. Furthermore, well-defined molecular markers are still lacking to distinguish different EV subtypes and to isolate EVs associated with diseases against host cells. Over the past few decades, cheerful progress has been made in EV isolation and purification, thanks in part to a deeper understanding of the biochemical and physicochemical characteristics of sEVs. There are four major types of classical separation methods including ultracentrifugation, size-based filtration, polymer-based precipitation, and immunoaffinity. New fluidic systems are also being developed to improve separation efficiency and to better meet the downstream analysis requirements. Since there have been many published articles reviewing the classic EV isolation techniques,[154,203] here we will briefly summarize these conventional isolation approaches with their advantages and limitations and update the recent progress in developing new EV isolation methodologies.

Centrifugation-Based Methods. EV subtypes appear to possess different, although overlapped, size ranges and density distribution intervals. Based on these differential physical properties, it is straightforward to use the centrifugation methods to separate, enrich, and purify various EV particles from complex biological samples. Two typical modes of ultracentrifugation are commonly practiced in EV isolation according to actual research needs, differential ultracentrifugation and density gradient ultracentrifugation.

Differential Ultracentrifugation. Since 1989 when Johnston adopted differential ultracentrifugation to isolate exosomes from the culture medium of reticulocyte,[204] it has become one of the most extensively used separation techniques and regarded as the gold standard approach until now.[130,154,205,206] This technique employs the sequential sedimentation of small EV particles by centrifugation depending on the differences in their sizes (<200 nm) and buoyant density (∼1.08−1.19 g/mL) than the contaminants. For instance, sEVs can be separated from a variety of lipoprotein particles based on the difference in density, e.g., VLDL, IDL,

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and LDL (<1.063 g/mL), or the difference in size for HDLs with a similar density range (1.063−1.21 g/mL) but smaller sizes (∼7−13 nm).[43] Because of the overlap in size and buoyant density, differential ultracentrifugation yields a mixture of exosomes and microvesicles which are collectively referred to as small EVs (sEVs). When used to isolate sEVs, an ultracentrifugation-based protocol typically involves several centrifugation steps with increasing speeds: (1) low speed at 300−400*g* to remove cells and bulky cell fragments; (2) medium speed at 2000*g* to remove cell debris and larger vesicles; (3) high speed at 10 000*g* to sediment biopolymer aggregates, apoptotic bodies, and other components with higher density; and (4) ultrahigh speed at 100 000−200 000*g* to pellet smaller EVs, followed by resuspension in the buffer and repeated ultracentrifugation to improve the purify. The efficiency of ultracentrifugation for EV isolation can be affected by many factors, including the centrifugation parameters (e.g., rotor type, speed, and duration) and the properties of samples (e.g., viscosity and volume). Therefore, when implementing an ultracentrifugation workflow, these factors should be taken into account in order to optimize the yield and purity of sEVs and to standardize the protocol for consistent results. While repeated ultracentrifugation can improve the purity by reducing the amount of impurities cosedimented with EVs, it will also reduce the yield due to the loss and damage of EVs. Other separation techniques, including nanofiltration or size exclusion, can be combined with ultracentrifugation to obtain better purified or size-defined EV fractions.[206] However, like the repeated centrifugation, adding additional stages in the EV isolation workflow will also decrease the EV yield.

Density Gradient Ultracentrifugation. To address the major disadvantage of ultracentrifugation of low purity and to resolve the individual EV subgroups, density gradient ultracentrifugation (DGUC) has been adapted to separate the mixture in a medium with a continuous or discontinuous density distribution according to experimental requirements. The choice of gradient materials can affect the performance of DGUC for the isolation of EVs in different biofluids.[207] Sucrose and iodixanol are the most commonly used media to produce the density gradient for EV fractionation.[208] While sucrose has been used widely, iodixanol may offer some advantages. Iodixanol has a lower viscosity, which eases operation and reduces centrifugation time. In addition, iodixanol has lower osmotic pressures than sucrose at a similar density and is iso-osmotic at high densities, which helps preserve the integrity and functionality of EVs. Because of its metabolic inertness and nontoxicity, EVs isolated in the iodixanol media can be directly used in downstream in vitro and in vivo functional assays without the need for additional steps for media removal. Lastly, it was reported that iodixanol could provide better separation resolution of EVs from other particles than sucrose.[209] Paolini et al. recently compared four commonly used sEV preparation methods and reported that DUGC yielded the purest sEV samples suitable for downstream applications, while sEVs obtained by conventional differential ultracentrifugation or one-step precipitation kits were heavily contaminated with residual matrix components.[210] Standard DUGC protocols have been modified to further improve the EV purification performance. For instance, a preconcentration step by ultracentrifugation was implemented to pellet EV particles on a high-density liquid cushion (e.g., 60% iodixanol) rather than the bottom of the centrifuge tube, followed by the purification by DGUC.[211,212] This method termed cushioned-DGUC was reported to maximize sEV recovery and better preserve their integrity and bioactivity compared to conventional protocols and has been recommended in the minimal information for studies of extracellular vesicles (MISEV) guidelines.[33] In recent years, DGUC-based methods have gained increasing popularity in EV isolation and purification for biological studies of their properties and functions. Despite their advantages in improving EV purity, these methods demand sophisticated facilities and result in a considerably lower yield of EVs and a complicated and excessively long isolation process (up to 2 days).[33,213,214] These shortcomings hinder their adaptation in clinical settings.

Polymer-Based Precipitation Methods. The polymer-based precipitation technique utilizes polymers to grab water molecules surrounding sEVs to provide a hydrophobic environment, forcing the less-soluble components of sEVs to aggregate together and precipitate out of the solution, thereby allowing the collection of sEVs in a short time via relatively low-speed centrifugation.[215] Since Rider et al. first used polyethylene glycol (PEG) to enrich EVs,[216] it is now the most commonly used polymer for exosome precipitation among various hydrophilic polymers.[217] Recently, Shtam et al. presented an approach to isolate the exosomes using the SubX molecules that can bind clusters of phospholipids on the exosome surface to aggregate them directly in biological liquids. Although this method is simple and obtained relatively pure populations of exosomes, the yield rate is rather low (10 times lower than UC).[218]

In recent years, various commercial sEV extraction kits based on polymer precipitation have appeared on the market, such as SBI Exosome kit, Thermo Fisher's Total Exosome Isolation kit, Hansa BioMed's ExoPrep Exosome Purification Kit, *etc*. They can quickly and conveniently separate high-quality sEVs from blood samples. These kits do not require special equipment and have high extraction efficiency and purification effect, so they are favored by more and more researchers. However, some problems still exist, low purity and recovery, more heteroprotein (false positive), uneven particle sizes, production of hard-to-remove polymers, damage to sEVs caused by mechanical forces or chemical additives, and so on. To further improve the polymer-based sEV preparation, combining with two or more techniques is required.

Size-Based Separation Methods. *Size-Exclusion Chromatography (SEC).* SEC is a commonly used size-based separation technique that uses columns filled with porous polymer microspheres to separate molecules or particles based on their size. Commercial columns such as the "SmartSEC Single for EV Isolation" from System Biosciences, and the "qEV" from iZON are also available to simplify sEV isolation. Compared with centrifugation-based methods, SEC has its unique advantages: EV particles being separated by SEC experience much smaller shear forces, which is beneficial to preserving the integrity and natural biological properties of EVs. Moreover, SEC is simple, scalable, and economical. Currently, SEC is a widely accepted technique to prepare purified and enriched sEV samples from various bodily fluids, including blood and urine.[154,219,220] Despite its popularity in EV research, the shortcomings of SEC for EV preparation are also obvious. The purity of sEVs isolated by SEC is barely satisfactory compared to ultracentrifugation and a large number of lipoproteins and non-sEV particles of similar sizes cannot be removed from sEVs by SEC.[221] The yield and purity of sEV isolation by SEC depend on the pore size of gel

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microspheres, column structures, chromatographic elution settings, and sample loading volume.[222] The balance between purity and recovery rate needs to be considered when optimizing the SEC separation protocols to meet the needs of the desired applications. The combination of SEC and other isolation technique, such as ultrafiltration, may help improve the purity of isolated EVs and preserve their biophysical and functional properties as well.[223]

Ultrafiltration. Ultrafiltration is another frequently used size separation approach for the rapid EV preparation which uses nanoporous filtration membranes of given particle size cut-offs to selectively isolate vesicular particles of interest based on their sizes. Small molecules will be filtered to the other side of the membrane, while those molecules with higher relative molecular mass, presenting larger sizes more than the aperture of the membrane, will be trapped on the ultrafiltration membrane. In contrast to the ultracentrifugation method, ultrafiltration is much more facile and does not require special equipment, dramatically shortening processing time and gaining great popularity among various sEV isolation methods. The commonly used ultrafiltration membranes aperture is 0.8 μm, 0.45 μm, 0.22 μm, *etc.*, which can be used to collect sEVs with different sizes.

Liu et al. reported a nanoporous membrane-based sEV isolation chip called ExoTIC, the sample such as plasma, urine, and lavage passes through the modular microfluidic filtration unit, and the sEVs can be enriched and purified in the size range of 30−200 nm, enabling size-based sEV sorting from heterogeneous sEV populations.[224] Hyun-Kyung et al. reported an Exodisc system that integrated sample loading, double-filtration, and EV recovery modules in a single disklike microfluidic chip; it can separate EVs in the size range of 20−600 nm from cell-culture supernatant or cancer patients' urine within 30 min automatically, achieving a 95% recovery rate.[225] Recently, Dong et al. also integrated a double-filtration unit into the microfluidic chip, effective isolation, and enrichment of sEVs from 20 to 200 nm were achieved with a low sample consumption (only 20 μL).[226] The double filtration unit integrated with microfluidic platforms endows microfluidic filtering with a bright future for sEVs isolation and enrichment.

However, sEVs may be lost due to the adhesion of sEVs on the filtration membrane. The pressure and shear force during filtration may cause deformation and damage of sEVs. To address this issue, recently, Jang et al. reported an efficient pre-enrichment approach using polyphenol-assisted biomolecule (−)-epigallocatechin-3-gallate (EGCG) for EV isolation. EVs adhered to EGCG to form EV aggregate bundles which were collected by filtration using a much lower pressure and shear force. They demonstrated that the EGCG-assisted filtration method isolated EVs more efficiently with higher purity compared with commercial EV isolation kits-ExoQuick Exosome Precipitation Solution (System Biosciences) and Total Exosome Isolation Reagent (Invitrogen).[227]

Ultrafiltration offers several advantages including simple equipment and operation and time efficiency, and it does not affect the biological activity of sEVs. However, the shortcomings are obvious. The undesired impurities may block the filtration pore, resulting in short membrane life and low separation efficiency. Besides, adhesion also occurs when sEVs are trapped on the membrane, resulting in a reduced yield. However, the tangential flow filtration technique presents an ideal solution to address these problems.

Tangential Flow Filtration. Tangential flow filtration (TFF) is also named cross-flow filtration, and particles pass through the permeable membrane by the manipulation of hydrodynamic flow force, and permeate is put off to the side, while retentate can be recirculated back for repeated filtration during the procedure. TFF differs from the conventional dead-end filtration as the fluid passes parallel to the filter rather than being pushed through a membrane perpendicularly, avoiding filter cake formation, thus less vulnerable to get clogged. TTF has been widely used in biopharmaceutical and food industries,[228,229] and now it becomes another emerging technique for sEV separation. Wang et al. made a micropillar array by using the conventional microfabrication technique, and then porous silicon nanowires were etched onto the micropillars' sidewalls to obtain the ciliated structure. The nanowire forest preferentially trapped sEVs and filtered out proteins and cell debris. This microfluidic filtration chip realized multiscale separation of sEVs with diameters of 40−100 nm.[230] Recently, Busatto et al. utilized TFF for the highly efficient isolation of sEVs from large-volume samples. They compared the ability of TFF and ultracentrifugation to process large volumes of cell culture media and found that TFF can isolate sEVs with a higher yield, improved batch-to-batch consistency, and fewer contaminants (Figure 3A).[231]

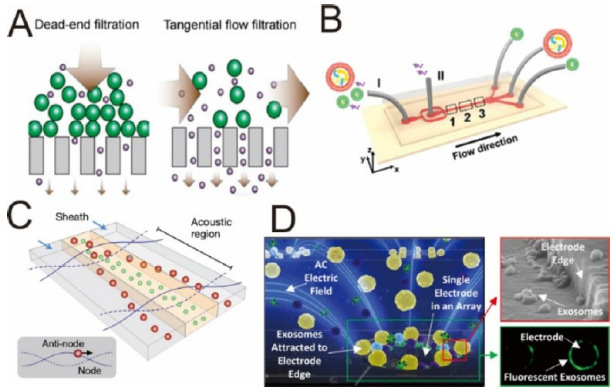

Figure 3. EV isolation technologies based on physical properties. (A) Schematic illustration of the tangential flow filtration (TFF), which is different from dead-end filtration. Reproduced from Tangential Flow Filtration for Highly Efficient Concentration of Extracellular Vesicles from Large Volumes of Fluid, Busatto, S.; Vilanilam, G.; Ticer, T.; Lin, W. L.; Dickson, D. W.; Shapiro, S.; Bergese, P.; Wolfram, J. *Cells* **2018**, *7*, 273 (ref 231). Publisher, Multidisciplinary Digital Publishing Institute (MDPI). (B) Schematic of the viscoelasticity-based microfluidic system for exosome separation from large EVs. Reproduced from Liu, C.; Guo, J.; Tian, F.; Yang, N.; Yan, F.; Ding, Y.; Wei, J.; Hu, G.; Nie, G.; Sun, J. *ACS Nano* **2017**, *11*, 6968−6976 (ref 234). Copyright 2017 American Chemical Society. (C) Acoustic nanofilter system to isolate EVs. Under the acoustic radiation pressure, small particles are transported to nodes of the acoustic pressure region, and large particles moved faster as the acoustic force is proportional to the particle size. Reproduced from Lee, K.; Shao, H.; Weissleder, R.; Lee, H. *ACS Nano* **2015**, *9*, 2321−2327 (ref 240). Copyright 2015 American Chemical Society. (D) Alternating current electrokinetic (ACE) microarray chip to rapidly isolate exosomes. The AC electric field lines (blue line) could form the DEP high-field to make EVs converge onto the edges of the microelectrodes. Reproduced from Ibsen, S. D.; Wright, J.; Lewis, J. M.; Kim, S.; Ko, S. Y.; Ong, J.; Manouchehri, S.; Vyas, A.; Akers, J.; Chen, C. C.; Carter, B. S.; Esener, S. C.; Heller, M. J. *ACS Nano* **2017**, *11*, 6641−6651 (ref 242). Copyright 2017 American Chemical Society.

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Field Flow Fractionation. Field flow fractionation is another separation technique where a force field is perpendicularly applied to the direction of sample flow, causing the separation of the particles present in the fluid, depending on their different sizes and molecular weights under the force exerted by the field. Recently, Zhang et al. reported an asymmetric flow field flow fractionation (AF4) technology for the separation of three nanoparticle subsets from 35 to 120 nm.[108] AF4, using two perpendicular flows, forward laminar channel flow and variable crossflow, to separate these three nanoparticle subsets based on their densities and hydrodynamic properties. This is a vital analytical tool to isolate sEVs and help us to understand the complexities of heterogeneous EV subpopulations. The possible disadvantages are low resolution and bad reproducibility.

Emerging Physical Isolation Technologies. *Viscoelastic Flow Sorting.* Viscoelastic flow sorting is a simple and label-free separation technique, when particles with different sizes pass through the channel in a viscoelastic medium and particle migration occurs under the elastic lift force in a size-dependent manner. This sorting method does not need any externally applied fields, and it can precisely manipulate submicrometer particles in a small sample volume and dramatically simplify the chip design and fabrication.[232,233] Liu et al. designed a viscoelasticity-based microfluidic system using poly-(oxyethylene) as a separation medium to directly separate EVs from cell culture media or serum with high separation purity (>90%) and recovery rate (>80%) (Figure 3B).[234] Two years later, they changed the separation medium and used the viscoelastic λ-DNA and aptamer-mediated approach for the isolation and detection of EV subpopulations at the single-EV level.[235] Still, lack of specificity is the main drawback. Importantly, this method needs to be further expanded in clinical applications.

Deterministic Lateral Displacement (DLD) Sorting. DLD is a continuous-flow particle sorting technique that was first reported in 2004 by Huang et al. to sort particles by utilizing the asymmetric bifurcation of laminar flow around pillar arrays in microfluidic devices.[236] Generally speaking, microfabricated DLD devices are amenable to isolate micron-sized particles such as cells and bacteria because microstructure is easy to fabricate. With the development of nanofabrication technology, DLD now can sort and enrich nanoparticles precisely and efficiently based on their sizes.[237] Wunsch et al. first fabricated a nano-DLD pillar array with gap sizes from 25 to 235 nm, which could sort colloids and sEVs with sizes of 20−110 nm. At the micrometer scale, particles with a diameter larger than the critical diameter (Dc) will move laterally across the array in a bumping mode, while those particles smaller than Dc will follow the laminar-flow direction in a zigzag manner. This work enabled sorting of nanoscale particles at low Péclet (*Pe*) numbers and made a breakthrough in scaling DLD arrays with gaps down to 25 nm.[238] To increase the multiplicity and throughput, later, their group integrated 1024 nanoDLD arrays on a single chip capable of enriching EVs by parallel processing urine or serum samples.[239] This device greatly improved the separation efficiency, and the flow rate was improved to 900 μL per hour; however, 50% recovery yield is not satisfied. The device fabrication is the main difficulty limiting its application, besides, DLD is unable to separate the sEV subtypes and lacks specificity.

Acoustofluidic Technology. Acoustofluidic technology generally uses ultrasound waves produced by acoustic transducers to exert a differential acoustic force on particles according to their mechanical properties such as size, density, and compressibility. In 2015, Lee et al. utilized the acoustic nanofilter system to isolate nanoscale (<200 nm) vesicles from cell culture media. In an acoustic field, the radiation force is proportional to the particle volume, while viscosity tends to drag the small size particles, thus larger particles migrate faster to the pressure nodes when subjected to radiation forces and flow out from sheath streams, and small particles are retained in the center flow as shown in Figure 3C. This label-free and continuous filtration system allows real-time control of the "size cut-off" through *in situ* electronic manipulations, achieving a high sEV separation yield and resolution.[240] In order to make the whole separation process more intelligent, Wu and colleagues developed an integrated acoustofluidic system to isolate sEVs and other EVs directly from undiluted blood samples in an automated fashion.[241] The chip system consists of two modules: a microscale module that first removes larger blood components, followed by a EV subgroup separation module. This label-free and contact-free manner offers a unique and versatile approach to quickly and efficiently isolate sEVs from complex body fluid without pretreatment. This technology basically does not change the biological or physical characteristics of sEVs and avoids the disadvantages of some current separation technologies, such as long analysis time, low separation yield, and uncertain sEV integrity. However, it is also obvious that the sEV subtypes cannot be isolated, and the specificity and purity cannot be guaranteed.

Dielectrophoretic (DEP) Separation. DEP refers to the displacement of dielectric particles in an inhomogeneous electric field due to the polarization effect. The phenomenon is related to the electrical properties of the particles, environment, and the applied electric field. Some separation techniques based on DEP have been developed for the isolation and enrichment of sEVs. Alternating current electrokinetic (ACE) is one of them; Ibsen et al. developed an ACE microarray chip device to rapidly isolate and recover glioblastoma sEVs from undiluted human plasma samples with a small plasma sample (30−50 μL).[242] The dielectrophoretic (DEP) separation force generated by the AC electric field made the nanoparticles attracted to the DEP high-field regions around the ACE microelectrode edges, and other large particles are pulled into the DEP low field regions between the electrodes, while ions or small molecules in solution remain relatively unaffected by the DEP field as demonstrated in Figure 3D. Then they integrated an AC electrokinetic microarray chip to achieve the capture and analysis of exosomes and other EVs directly from whole blood, plasma, or serum without pretreatment or sample dilution.[243] Recently, Shi et al. reported a borosilicate micropipet dielectrophoresis to rapidly isolate sEVs from cell culture media and biofluid samples in 20 min.[244] Ramanathan et al. reported an alternating electrohydrodynamic field to generate nanoscaled fluid flow that enhanced the specificity, and they achieved highly specific capture and detection of multiple sEV targets.[245] This method can directly separate untreated samples with the advantages of less sample volume and shorter analysis time. However, a high-voltage power supply is required.

Thermophoresis Technology. Thermophoresis is a phenomenon where particles move from a high to a low-temperature area due to the effect of the temperature gradient. The Sun group utilized the characteristic of thermophoresis to establish the thermophoretic aptasensor for the detection of

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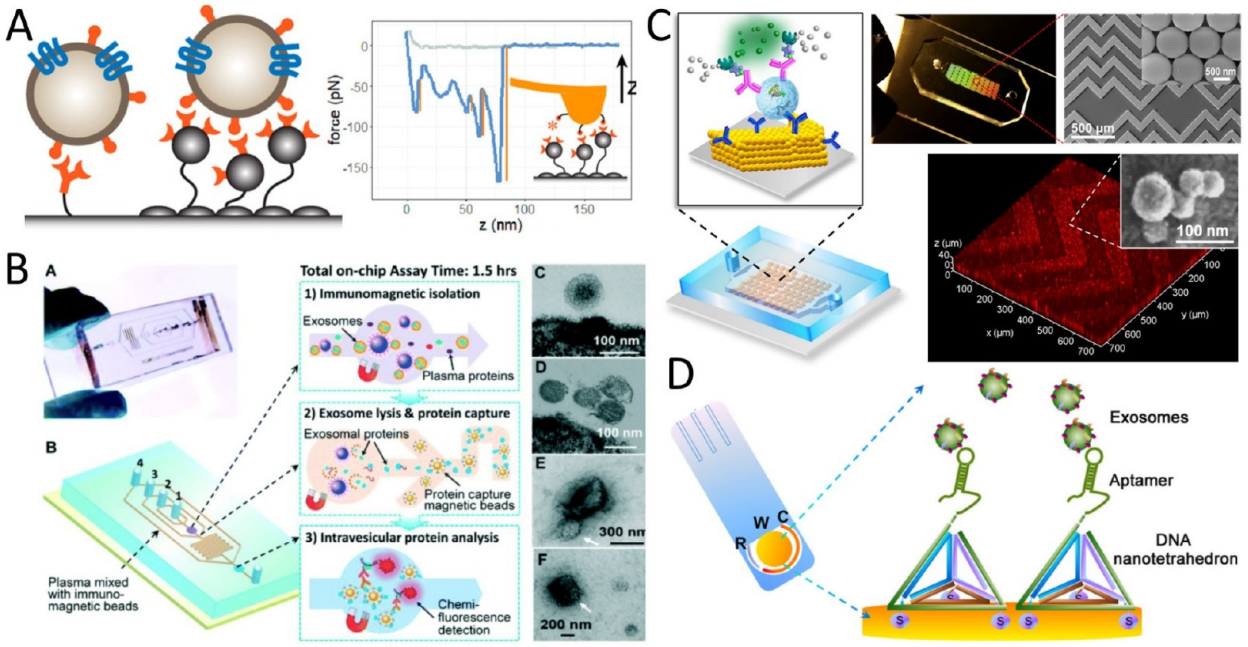

Figure 4. EV isolation techniques based on affinity binding. (A) Schematic of enhanced immunoavidity capture through dendrimers (gray circles). AFM force spectroscopy was used to characterize the binding avidity. Reproduced from Poellmann, M. J.; Nair, A.; Bu, J.; Kim, J. K. H.; Kimple, R. J.; Hong, S. *Nano. Lett.* **2020**, *20*, 5686−5692 (ref 249). Copyright 2020 American Chemical Society. (B) Integrated microfluidic exosome analysis platform containing a cascading microchannel network for multistage exosome analysis. Reproduced from ref 133 by the author: He, M.; Crow, J.; Roth, M.; Zeng, Y.; Godwin, A. K. *Lab Chip* **2014**, *14*, 3773−3780. Publisher: The Royal Society of Chemistry. (C) 3D nanostructured microfluidic herringbone chip fabricated by the designed colloidal self-assembly strategy. Modified from ref 113 by the authors: Zhang, P.; Zhou, X.; He, M.; Shang, Y.; Tetlow, A. L.; Godwin, A. K.; Zeng, Y. *Nat. Biomed. Eng.* **2019**, *3*, 438−451. Publisher: Macmillan Publishers Ltd. (D) Aptamer-based nanotetrahedrons for direct capture of exosomes through three thiol groups immobilized on the gold electrodes. Reproduced from Wang, S.; Zhang, L.; Wan, S.; Cansiz, S.; Cui, C.; Liu, Y.; Cai, R.; Hong, C.; Teng, I. T.; Shi, M.; Wu, Y.; Dong, Y.; Tan, W. *ACS Nano* **2017**, *11*, 3943−3949 (ref 251). Copyright 2017 American Chemical Society.

sEVs. In this work, samples were placed in the microchamber and were locally heated with a laser, due to the interplay of thermophoresis, diffusion, and convection induced by localized laser heating; sEVs rapidly moved toward the laser spot center, while free aptamers and proteins of small sizes could not be enriched by the laser heating, leading the size-dependent accumulation of EVs.[246] Based on the same principle, recently, Yang group combined the aptamers with the separation-free thermophoresis and proposed a HOLMES-ExoPD-L1 method for cancer detection and immunotherapy prediction.[247] The thermophoresis system is homogeneous, low cost, and noninvasive to separate sEVs in a raw sample. After combining with fluorescence labeling technology, it can be potentially translated into clinical cancer diagnostics. The major drawback of this method is that it requires additional labeling; therefore, appropriate markers need to be selected. Besides, the purity and recovery rate of this method are not very satisfactory.

Affinity Binding-Based Isolation. *Immuno-Affinity.* With the help of proteomic profiling, numerous exosomal markers have been found on the surface of exosomes (like CD9, CD81, CD63, *etc.*).[154] The interaction between these protein biomarkers and specific antibodies (or exosome receptors and their ligands) is the basic principle of the immuno-affinity technique. sEVs could also be derived from cancer cells and carry cancer-specific surface proteins, and the immuno-affinity approach can specifically separate cancerous sEVs, which holds great potential in the liquid biopsy.

In recent years, scientific results have demonstrated that antibody-coated magnetic beads, plates, and chromatography matrixes can effectively isolate cancer-specific sEVs from body fluids using the immuno-affinity method.[248−250] These methods are easy, fast, specific with high purity, and promising to be directly translated into diagnostic platforms. In addition, some commercially available immunoaffinity separation kits such as Exosome Isolation Kit CD81/CD63 (Miltenyi Biotec) and Exosome-human CD63 isolation reagent (Thermo Fisher) also have been developed to isolate the specific subpopulation of sEVs.

Conventional immuno-affinity capture is easy and fast; however, capture efficiency needs to be improved because of limited surface area, what's more, the routine laboratory procedure is reagent-consuming, labor-intensive, and not conducive to multiplicity and high throughput. In order to address this issue, micronanostructures and nanomaterials combined with microfluidic chips have been developed. Recently, Poellmann et al. reported a nanostructured multivalent dendrimers polymer surface for the highly effective capture of tumor exosomes through a multivalent binding effect based on the immunoaffinity reaction. The binding sites to exosomes were greatly improved by grafting the multivalent dendrimers and PEG on the plate surface; thus, high capture efficiency was obtained (Figure 4A).[249] He et al. integrated immunomagnetic exosome capture, lysis, protein immunoprecipitation, and sandwich immunoassays on one chip and achieved selective subpopulation isolation and quantitative detection of exosomes from plasma.[133] The sample were first premixed with antibody-labeled magnetic beads to enrich the exosomes, lysis buffer was then introduced to lyse the captured exosomes, then antibody-labeled magnetic beads were injected from two side channels to capture the released intravesicular

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Table 2. Summary of EV Isolation Methods

method	sample type	assay time	sample volume	recovery	purity	clinical application	isolated EV size (nm)
differential ultracentrifugation (DU)[43,204−206]	cell culture, blood, urine, saliva, tear fluid, pleural effusion and ascites	3−9 h	mL−L	2−80%	low	no	20−500
density gradient ultracentrifugation (DGUC)[207−212,214]	cell culture, blood, urine, saliva,	16−90 h	μL−mL	10%	high	no	20−400
precipitation[215−218]	cell culture, blood, urine, saliva	0.3−12 h	μL−mL	90%	high	yes	20−300
size-exclusion chromatography (SEC)[154,219−222]	blood, urine, ascites, saliva,	0.3 h	μL−mL	40−90%	low	yes	20−300
ultrafiltration[224−227]	blood, urine, saliva, ascites	0.5−1 h	μL−mL	10−80%	high	yes	20−300
tangential flow filtration (TFF)[228−231]	cell culture, blood	0.1−1 h	μL−mL	NA	NA	no	60−100
field flow fractionation[108]	cell culture, blood	1 h	μL−mL	NA	NA	no	35−120
viscoelastic flow sorting[232−235]	cell culture, blood	<30 min	μL	80−95%	high	yes	30−200
deterministic lateral displacement (DLD)[237−239]	serum, urine	1−3 h	μL	∼50%	98.5%	no	30−200
acoustofluidic[240,241]	cell culture, blood, saliva	<30 min	μL	>80%	NA	yes	50−300
dielectrophoretic (DEP)[242−245]	cell culture, blood, saliva	15−60 min	μL	NA	NA	yes	50−200
thermophoresis[246,247]	cell culture, blood	10 min	μL−mL	NA	NA	yes	<250
immuno-affinity[111,113,133,249,250]	cell culture, blood, urine, saliva, tear fluid, pleural effusion, ascites	<1 h	μL−mL	40−95%	medium	yes	30−300
aptamer-affinity[251,252]	cell culture, blood, urine, saliva	<2 h	μL−mL	∼80%	NA	yes	60−200
membrane affinity binding[254]	cell culture, blood	<1 h	μL−mL	93.4%	NA	no	65−235

proteins from the lysate and last sequentially introduced detection antibodies and chemifluorescence reagents for sandwich immunoprotein analysis (Figure 4B). The whole on-chip assay can be completed in less than 1.5 h with plasma sample volumes as low as 30 μL.[133] Zhang et al. developed a graphene oxide/polydopamine (GO/PDA) nanointerface in a microfluidic chip, GO/PDA greatly increased the surface area and thus improved the efficiency of exosome immunocapture.[111] Recently, Zhang et al. constructed a 3D nanostructured herringbone isolation platform that can effectively promote microscale mass transfer, increase surface area, and enhance the antibody immune binding efficiency and speed. This work achieved high sEV capture efficiency and enabled the quantitative detection of low-level sEV subpopulations in blood plasma (Figure 4C).[113] The methods described above save the sample volume, analysis time, and simplify the protocols, which are suitable for the detection of clinical samples, and offer significant advantages for cancer diagnostics and prognosis.[250]

Aptamer-Based Isolation. Besides immuno-affinity methods, some researchers also use specific aptamers for sEV capture. For example, Zhang et al. constructed a DNA aptamer-based magnetic isolation system that enables rapid capture and nondestructive release of EVs in 90 min with an isolation efficiency of 78%.[248] Aptamer-anchored DNA nanostructures have also been designed for the capture and sensitive detection of exosomes on one electrochemical substrate in a controllable manner (Figure 4D).[251,252] As demonstrated in Figure 4D, aptamers were built into DNA nanotetrahedron (NTH) to form a pyramidal-like structure and then immobilized via three thiol groups onto the gold electrodes for the direct capture of exosomes in suspension. The individual aptamer strands standing on NTH not only decreased the hindrance effect but also maintained spatial orientation for better biomolecular recognition, thus significantly improved the capture performance. Different from antibody-based isolation, the aptamer-based isolation method is cost-effective and can achieve nondestructive release. Furthermore, it can also be easily used for signal transformation and amplification for downstream analysis of sEVs.

Membrane Affinity Binding. Enderl et al. first reported a spin column-based procedure for the purification of sEVs from biofluids by using an affinity membrane binding column; now this method has been made commercially available by Qiagen as "exoRNeasySerum/Plasma MaxiKit".[253] Recently, Gao et al. presented a novel strategy for facile sEV isolation from human serum based on specific interactions between TiO_2 and phospholipid bilayers of sEVs. TiO_2 can reversibly bind with phosphate groups that are exposed on the outer surface of the exosome with high specificity, and the binding interaction can also be disrupted by alkaline solvents. Based on this principle, exosomes from culture cells and serum samples were quickly isolated within 5 min with a high recovery rate (93.4%), and the isolated exosomes were applied to downstream proteome analysis.[254] The membrane affinity binding provides an alternative method for sEV separation, although the specificity and purity are less than antibody and aptamer; it is simple, economical, and greatly shortens the sample processing time. However, this method lacks specificity and is not suitable for a wide range of applications.

New methods and techniques are emerging in the field of sEV research, and recent developments in sEV isolation technologies are summarized in Table 2. However, to date, none of the discussed methods lead to perfect sEV isolation, and the different isolation methods introduce variations in the concentration, purity, and size of sEVs as reported. Recently, Gemoll et al. compared the protein composition of plasma sEVs isolated by differential ultracentrifugation with that of the PEG precipitation-based kit and found significant differences in the protein components. However, both differential ultracentrifugation and PEG precipitation can be used to distinguish patients from controls.[255] This study demonstrated that the separation strategy of EVs is not the key factor to determine their clinical application, but the standard, fixed, and reliable separation strategy is. Sometimes, to increase the specificity or purity of sEV isolation, combining multiple methods is a good choice. For example, ultrafiltration is

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accompanied by liquid chromatography, which showed a higher yield of sEVs than ultracentrifugation only and retained the biophysical properties of sEVs.[256,257]

CONVENTIONAL METHODS FOR EV CHARACTERIZATION

Morphological and Biophysical Characterization. Standard microscopic modalities have been essential tools to characterize the physical properties of EVs, such as morphologies, size distribution, and abundance. This section briefly surveys these techniques and discusses the recent progress toward the standardization of these EV characterization methods.

Microscopy Methods. EVs possess a wide range of diameters that can be smaller than the diffraction limit of conventional optical microscopic techniques. Therefore, high-resolution EV imaging and characterization have been mostly conducted using electron microscopy (EM) and atomic force microscopy (AFM) methods. These techniques can be used to directly measure the morphology and size of individual vesicles. Since the first report that observed exosomes in cells,[258] transmission electron microscopy (TEM) has been a popular and well-established method used in EV research because of its superior resolution. A critical step for TEM imaging is the specimen preparation, which requires tedious and time-consuming steps for fixation, dehydration, embedding, and microtome slicing. To enhance the imaging contrast for the lipid membrane structures, samples are usually stained with heavy metal stains such as osmium tetroxide and uranyl acetate. Such extensive sample treatment may induce changes in the morphology of EVs. TEM can also be used for molecular characterization by coupling with immunogold labeling (immuno-EM) to detect the proteins present on EVs. In contrast to TEM, scanning electron microscopy (SEM) scans the surface of a subject with a focused beam of electrons to generate 3D surface topography images. While SEM normally provides lower resolution than TEM, it involves much less complicated and faster sample preparation. Thus, SEM has been recommended as one of the standard methods for the assessment of the morphological features of EVs and the validation of the results of EV preparation.[33] Similar to TEM, however, SEM typically requires sample treatment and imaging operation under vacuum, which may alter EV morphologies and sizes.[259] For instance, TEM and SEM studies of small EVs have observed both round-shaped and cup-shaped structures.[113,260]

Cryo-EM provides a means to avoid the artifacts in EM characterization of EV structures. Unlike SEM or TEM, cryo-EM negates the sample dehydration and chemical fixation and measures the frozen EV samples kept at a very low temperature. Cryo-EM visualization of EVs confirms the characteristic round-shape morphology, indicating its advantage in mitigating the sample distortion effects caused by other EM methods.[259] Another advantage of Cryo-EM is its ability to visualize the membranous structures and lumens of EVs with high resolution. Cryo-EM can be used to directly study the blebbing and shedding of EVs from cells, allowing the comparison of differences between EV populations.[261] Despite the attractive advantages of cryo-EM, its application is still largely limited, owing to the expensive equipment, the challenges in sample preparation, and the requirement of expertise.[262] AFM provides an alternative nanoscale tool for the characterization of the morphology, mechanical properties, and biomolecular components of single EVs.[122] An important advantage of AFM is its ability to measure a sample in its native conditions, avoiding the effects of extensive sample preparation. It uses a mechanical cantilever to scan across a surface, probing the topography of surface structures. In addition to mapping the surface topography, AFM measurements can also provide useful information on substructures and local mechanical and biomolecular properties of single vesicles.[122,263,264]

Dynamic Light Scattering and Nanoparticle Tracking Analysis. Dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) are two widely used techniques for characterizing physical features of EVs in suspension. Both techniques measure the Brownian motion of particles in solution to derive the hydrodynamic diameter of the particles based on the Stokes−Einstein equation.[256,257] However, these two methods differ in the mechanism for particle analysis. DLS, also known as photon correlation spectroscopy, detects the total scattered light from all particles that are illuminated by a monochromatic coherent laser beam. Due to the Brownian motions of the particles, the constructive and destructive interference of scattered light causes time-dependent fluctuations in intensity which can be used to compute the diffusivity and thus the hydrodynamic diameter of the particles. This method is capable of measuring particles of a wide size range from 1 nm to 6 μm. Moreover, it also can provide information about surface charge (ζ potential). Caution should be taken when using this method to measure the size distribution of polydisperse vesicles. Because the scattered light intensity is strongly dependent on the particle size, the measured size distribution can be biased by the larger vesicles present in the suspension, even at a low quantity.[265,266]

Distinct from the bulk scattering measurement by DLS, NTA is an optical video-imaging method that can directly visualize individual particles by detecting their scattered light, thus overcoming the polydisperse problems. The Brownian motion of each particle and the particle number are analyzed with the image tracking software to determine the size distribution and the concentration of the particles in the sample solution. In addition to physical information on EVs, NTA can be used for surface protein phenotyping of EVs through combining with immunostaining and fluorescence detection of EVs.[267] To gain accurate size profiling and quantification of EVs, however, NTA requires careful optimization of the analysis settings, including temperature, camera parameters, and size calibration. Moreover, nanoparticle quantification by NTA largely depends on the particle concentration (i.e., the number of particles detected in the field of view); so this method consumes relatively concentrated samples (10^8−10^9 vesicles mL^{-1}) to ensure the accuracy of quantitative analysis of vesicle concentration.

Resistive Pulse Sensing. Tunable resistive pulse sensing (TRPS) has recently emerged as an attractive nonoptical technique for simultaneous measurements of the important physical properties (i.e., size, surface charge, and concentration) of vesicles in a suspension based on the Coulter effect.[268−270] TRPS detects the transient changes in the ionic current caused by the vesicles passing through a size-tunable nanopore in an elastic membrane. Thus, both the sizes of nanopores and particles affect the accuracy of TRPS measurements. It was reported that compared to NTA, TRPS appeared to be more efficient to detect larger EV particles (e.g., >150 nm), leading to biased results in size

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distribution.[270] Another practical limitation of this method is that TRPS is susceptible to issues in technical stability, such as nanopore clogging, and in analytical variations, such as background noises due to non-EV particles, which necessitates significant efforts on the optimization and calibration of the system parameters.[271,272]

Routine Biochemical Characterization. In addition to the characterization of physical features of EVs, molecular characterization by multiple, complementary methods is necessary to verify the results of EV isolation. Such quality control analysis is vital to ensure the validity of the studies of biological functions and disease biomarkers associated with EVs. It is important to note that given the fact that our knowledge of EVs is very limited and still expanding, the physical and biochemical features criteria for qualitative identification and quantitative validation of EVs remains rapidly evolving. To facilitate the method standardization and transparent reporting across the field of EV research, the ISEV has proposed and kept updating the guidelines on the experimental measurements for EV characterization.[33,135] This section will succinctly survey the molecular analysis methods commonly adapted for routine assessment and validation of EV preparations based on their molecular contents, highlighting their utilities and limitations.

Measurements of Total Molecular Contents. Total Protein. Proteins represent one of the major molecular components of EVs that play significant roles in the biogenesis and functions of EVs. It has become a routine practice to quantify the total protein level of EVs as a means to assess the yield of EV preparation. However, nonspecific total protein quantification can lead to variable and inaccurate results, owing to coisolated protein contaminants. It has been reported that the total protein level was not necessarily well correlated with the abundance of patient-derived sEVs isolated by standard UC.[111,113] A variety of standard assays have been used to measure total EV protein, including colorimetric assays (e.g., Bradford assay and bicinchonic acid (BCA) assay), fluorimetric assays, and global protein staining on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Both BCA and Bradford assays are commercially available, easy to operate, and capable of measuring a wide range of protein concentrations. Despite these advantages, these two assays are limited to assaying highly purified EV samples and their performance can be affected by the experimental factors. The BCA assay is generally compatible with detergents (e.g., SDS, NP-40, and Triton X-100) and denaturing agents (e.g., urea and guanidinium chloride) but can be interfered with by some chelating and reducing reagents, like EDTA, dithiothreitol, and lipids. On the contrary, the Bradford assay is tolerant of the reducing agents commonly used in bioassays but incompatible with most detergents used in protein sample preparation. Thus, results may vary depending on the actual protocol for EV sample pretreatment prior to the protein assay, such as lysis of EVs with a specific type of surfactant to release the EV protein content.

Total RNA Quantity and Integrity. EVs have been found to carry several types of small and long RNAs.[273−275] Assessing the quantity and quality of EV RNAs remains challenging and has not yet been recommended as a standard approach to verify EV quantification or purity, owing to a number of factors. For instance, as discussed above, the isolation methods largely affect the preparation of EV samples, which determines the quantity and quality of EV-associated RNA extracts and hence the results of downstream analysis results.[213,276] Extracellular RNAs associated with coisolated ribonucleoproteins and non-EV bioparticles, including exomeres and lipoproteins, may result in the inaccuracy of the results.[108,277,278] Moreover, EV RNA is known to be of low abundance and highly heterogeneous in quantity and composition. Current RNA extraction/isolation methods can cause variations in RNA yield and bias in RNA types and thus have a significant impact on the accuracy and reproducibility of quantitative detection and sequencing of EV RNA.[274,279] There is a lack of internal standards for result normalization, such as the housekeeping genes established for cellular RNA analysis. Despite the existing challenges for reliable EV quantification and quality assurance, RNA assessment can still provide useful information and is recommended as an important aspect of EV characterization by ISEV.[33]

Standard RNA quantification assays have been used for EV RNA measurements with variable adaptability, depending on their sensitivity, specificity, and sample volume requirement.[273] Spectrometric assays, such as UV absorbance measurement using a Nanodrop spectrophotometer and Qubit RNA HS assay (Thermo Fisher Scientific), are limited by the low sensitivity and relatively large sample consumption. Microfluidic capillary electrophoresis-based assays, e.g., Bioanalyzer small RNA chip (Agilent Technologies), provide one of the most sensitive methods for RNA detection as well as the ability to resolve RNA fragments by size. Such ability allows a quick check of the quality of a prepared EV RNA sample by examining the size profile of EV RNA and detecting the presence of rRNAs because EV RNA cargo, in general, contains mostly small and fragmented RNAs (e.g., microRNA) and should be devoid of intact 18S and 28S rRNAs that dominate the RNA samples extracted from cells.[274,280,281] Therefore, they are by far the most popular methods for EV RNA assessment, although they have limited performance for reliable RNA quantification and assessing the overall integrity of different EV RNA types, such as long EV RNA species. It is noted that most of these techniques, except the Qubit RNA HS Assay, are prone to DNA contamination and thus pretreatment of samples with DNase is recommended for accurate EV RNA quantitation.

Total Lipids. Lipids are a major molecular constituent of EVs and the analysis of total lipids has been investigated as an optional strategy for the assessment of EV quantity and quality, while the effectiveness and reliability of this strategy remain to be established.[98,282,283] Different methods can be used to measure EV lipids, including the sulfophosphovanilin assay[282] and the fluorescence staining assays based on the lipophilic dyes, such as DiI and DiR that strongly fluoresce when incorporated into lipid membranes.[283] Despite the simplicity and rapid results, these two assays are prone to non-EV contaminants containing lipids (e.g., membrane debris and cellular organelles) and have relatively low sensitivity so that a large amount of EV samples are required. A more sophisticated spectroscopic method, attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR), has been used to characterize EV lipid components, which demonstrated the potential of lipid analysis for differentiating EV subpopulations and assessing the EV purity.[283]

Analysis of Selected EV Proteins. As surveyed in EV Biology, recent systematic studies have led to a growing understanding of the heterogeneity of EVs in sizes, cellular origins, and protein composition,[7,38] which suggest some

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proteins as potential specific markers for different EV subtypes. However, these findings are yet to be validated across different cellular and tissue sources of EVs, disease conditions, and EV preparation, and analysis methods. Thus, currently, there are still no universal molecular markers established for specific EV subtypes, such as MVB-derived exosomes versus other small EVs, for characterization of the quantity and quality of EV preparations. Nonetheless, measurement of some of the EV-associated proteins and major components of non-EV structures commonly seen in EV preparations can still provide very useful information relevant to the performance of bulk EV isolation and purification (Table 1). Such characterization of specific markers has been commonly practiced in the field, using several standard analytical methods.

Western Blotting. Western blotting is an immunological protein analysis technique widely used in biological studies, it has been demonstrated as a powerful tool to specifically detect reportedly EV-associated proteins in purified EV samples.[5,8] It combines size separation by SDS-PAGE with antibody–antigen recognition to confer superior specificity for detection of targeted proteins (Figure 5A). Western blotting can be used to semiquantitatively assess the enrichment of proteins in EVs versus the original cell lines or across different sources (e.g., healthy donors versus patients). In these cases, the lysates of the purified EVs from various samples and the source cell lines to compare with are normally loaded either in the same quantity of EV counts, total protein, or equivalent cell amounts. Western blotting cannot be used to validate whether the proteins are from EVs or not, nor to quantify the concentration of EVs. Besides, it requires large amounts of isolated EVs (normally >10 μg total protein), which may not be suitable for small-volume samples, and a lengthy workflow for analysis (more than 10 h). Nevertheless, Western blotting is still an indispensable analytical tool for the protein characterization of EVs.

Immunosorbent Assays. Enzyme-linked immunosorbent assay (ELISA) is a well-established approach to detect and quantify specific protein analytes and has been extensively used in EV analysis.[8,17] Among different assay formats, a typical "sandwich" assay involves immunocapture of targets in a sample, such as EV lysates, using the capture antibody immobilized on the substrate, followed by detection with a paired detection antibody that is linked to a reporter enzyme for fluorescent/colorimetric signal amplification, as shown in Figure 5B. ELISA can also be adapted to capture intact EV vesicles and detect the membrane-bound proteins. In such cases, antibodies specific to generic EV markers (e.g., CD9, CD63, and/or CD81) or the antigens of interest (e.g., PD-L1) can be used to pull down small EVs or surface molecule-defined subpopulations, respectively.[113,284,285] Immunosorbent EV assays are normally carried out in a 96-well format which can be readily scaled up for high-throughput analysis. In addition, large-scale, multiplexed immunosorbent EV assays using either custom-made or commercially available antibody microarrays have been reported for EV characterization probing surface proteins on EVs or protein contents in EV lysate.[286−288]

Compared to Western blotting, immunosorbent EV assays require a simpler workflow and much less time for sample preparation and analysis, which makes it amenable to large-scale measurements. In general, ELISA needs less sample volume than Western blotting, while providing comparable or better detection sensitivity. However, the analytical characteristics (i.e., sensitivity, specificity, and reproducibility) of sandwich ELISA largely depends on the quality of available antibodies and finding highly specific antibodies with no crosstalk across is not trivial for developing new or multiplexed assays. In addition, ELISA-based detection of EVs directly from biological samples faces a major problem of matrix effects due to nonspecific adsorption of numerous proteins and other biomolecules present in the samples, which requires rigorous optimization of the assay variables for various biospecimens.

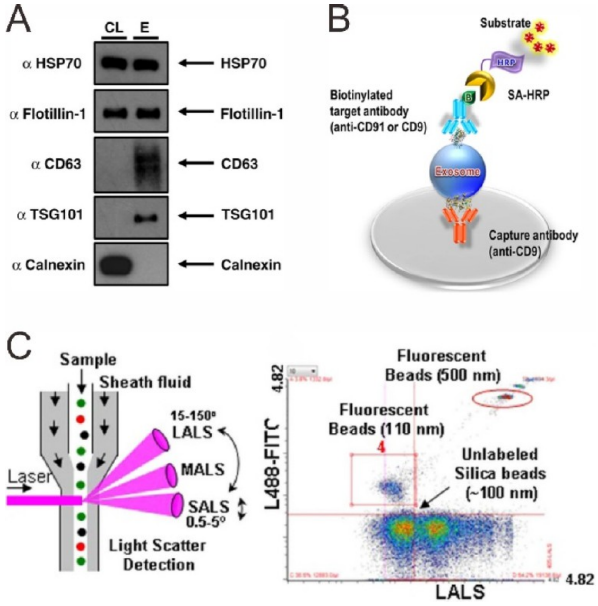

Figure 5. Conventional protein analysis methods for EV characterization. (A) Identification of EV protein by Western blotting. Proteins in the EV lysate are separated by SDS-PAGE, transferred to the membrane, and then immunoblotted to detect specific protein markers. Reproduced from Optimized exosome isolation protocol for cell culture supernatant and human plasma, Lobb, R. J.; Becker, M.; Wen, S. W.; Wong, C. S.; Wiegmans, A. P.; Leimgruber, A.; Møller, A. *J. Extracell. Vesicles*. Vol. *4*, Issue 1 (ref 214). Copyright 2015 Wiley. (B) Sandwich ELISA detection of PD-L1. Anti-PD-L1 antibody is immobilized on the plate as capture antibody to isolate sEVs; then biotin-labeled anti-PD-L1 antibody as detection antibody is used to recognize the sEVs; next, streptavidin-labeled HRP enzyme reacts with detection antibody to catalyze TMB, performing as a signal amplification step. Reprinted by permission from Macmillan Publishers Ltd.: Scientific Reports, Ueda, K.; Ishikawa, N.; Tatsuguchi, A.; Saichi, N.; Fujii, R.; Nakagawa, H., *Sci. Rep.* **2014**, *4*, 6232 (ref 285). Copyright 2014. (C) Automated surface protein profiling of single small EVs by high-resolution FCM. The laser generates three kinds of light scatters, LALS: large-angle light scatter; MALS: middle-angle light scatter; SALS: small-angle light scatter. As a proof-of-concept, fluorescent beads with various sizes can be detected efficiently. Reprinted by permission from Macmillan Publishers Ltd.: Scientific Reports, Kibria, G.; Ramos, E. K.; Lee, K. E.; Bedoyan, S.; Huang, S.; Samaeekia, R.; Athman, J. J.; Harding, C. V.; Lotvall, J.; Harris, L.; Thompson, C. L.; Liu, H. *Sci. Rep.* **2016**, *6*, 36502 (ref 306). Copyright 2016.

Flow Cytometry (FCM). FCM is a powerful tool for phenotypical characterization and sensitive quantification of a variety of biological particles and has been increasingly adapted for EV analysis.[8,289] Briefly, in FCM, particles are carried by a hydrodynamically focused flow to pass through laser beams in a single file and the scattered light or emitted fluorescence signals are detected and recorded to generate the event distribution plots (Figure 5C).

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A major challenge in the application of conventional FCM to EVs is the insufficient detection sensitivity and resolution for EVs owing to their small size, low refractive index contrast against the solution,[290] and low level of protein contents. Conventional FCM is most suitable for the detection of single EV particles larger than ∼500 nm in diameter, while a significant portion of small EVs of <200 nm could be missed or underestimated due to the "swarm detection" which occurs when a group of vesicles are simultaneously illuminated to produce a detectable signal and thus counted as a single particle.[291] In EV analysis by conventional FCM, antibody functionalized microbeads have been employed to enrich EVs on single beads, followed by immunostaining with fluorescent dye-conjugated antibodies for specific detection of protein markers on EVs, such as EpCAM, PDL1, CD41, and annexin A5.[17,292] The bead-based method enabled highly multiplexed EV profiling by using a mixture of uniquely antibody-conjugated beads for simultaneous detection of a set of surface protein markers on EVs by FCM. However, these methods lose the characteristic advantage of FCM for single-particle profiling and provide averaged results across different EV subsets, which may mask molecular features of biological significance.

To overcome these issues, sophisticated FCM instruments with high sensitivity and resolution have been under intensive development to extend the applications to the detection of small EVs.[19,293−296] For instance, a BD Influx flow cytometer[297] and high-sensitivity flow cytometer[298] can discriminate sEVs with a diameter down to 100 nm. Nolan et al. constructed an optimized vesicle FCM to allow enumeration and protein analysis of sEVs.[299] With the use of fluorescent antibodies probing EV membrane proteins (e.g., annexin V and CD61), this platform enabled quantitative detection of specific subpopulations of EVs as small as 100 nm. Recent development of nanoflow cytometry (nFCM) has afforded the abilities for sizing and multiplexed protein profiling of individual EVs as small as 40 nm.[295,296] FCM detection of sEVs can be interfered with by non-EV particles, such as lipoproteins. Imaging flow cytometry (IFC) that combines conventional FCM with fluorescence imaging for postdetection inspection has been used to improve selective detection of small EVs from contaminating particles or background noise.[300]

In addition to the advance in instrumentation, there is a pressing need for the development and standardization of sample preparation, analytical protocol, and data processing. For example, the optical scattering power determined with polystyrene and silica beads of known diameter, concentration, and refractive index have been proposed for calibration of a flow cytometer for size assessment of the smallest detectable single vesicles. This method used an estimated average refractive index for EVs for size determination, which may potentially introduce errors in the results because of the known heterogeneity of EVs' physical and biochemical properties and of different samples that affect their scattering power.[301,302] Multicenter investigations have been initiated to promote the standardization of EV FCM via optimizing analytical protocols and data processing, evaluating various control and calibration reagents, and comparing data obtained with different platforms from different participating groups.[303,304] Despite its unique capabilities for high-throughput, single-EV analysis, the requirement of highly sophisticated instruments and specialty poses a major setback that limits the accessibility and adaptability of FCM to clinical utilities. Future development of innovative FCM platforms, such as microfluidics-based FCM, could open new opportunities for low cost, high-performance single EV analysis.[305]

Characterization of Other EV Molecules. Compared to proteins, other molecules, such as nucleic acids, have been less used for routine biochemical assessment of EV preparations because of their lack of quantitative correlation with and/or specificity for EVs or EV subtypes.[273,274] Nonetheless, other than the measurement of total RNA as described above, quantification of a panel of selected miRNAs and mRNAs (e.g., "housekeeping" transcripts) using highly sensitive PCR-based methods, such as RT-qPCR and digital PCR, may serve as a useful proxy for the quantity control of EV RNA preparation, especially in the case of assessing samples expected to contain a very low amount of EV RNA[109,113,307] or comparing different EV isolation methods. Another critical issue in the RNA characterization of EVs arises from the lack of reliable reference transcripts and the standardized methods for the normalization of quantitative RNA analysis data. Existing reference transcripts that are often used for expression analysis of cellular RNAs, such as "housekeeping" genes, are not necessarily reliable for the normalization of the EV RNA data.[273,308] Some DNA and RNA species might also provide useful negative or positive controls for EV assessment. For example, the detection of dsDNA in exosomes and other sEVs had been reported[309] but was recently shown to be the artifacts of insufficient EV purification that can be largely reduced with improved methodology.[38] These challenges emphasized that a better understanding of exosome biology and more systematic characterization of EV composites is imperative to define reliable nucleic acid markers of EVs and the subtypes.

Other Emerging EV Characterization Methods. Raman spectroscopy (RS) analyzes the scattering spectrum which is different from the incident light frequency to obtain the information on molecular vibration, rotation, and other low-frequency modes of systems, and it is applied to the study of chemical structure, crystallinity, and molecular interactions. RS has been used on sEV analysis by providing the information about the chemical composition.[310] Although RS appears to allow sEV identification, it is still practically challenging to obtain informative spectra due to the chemical complexity of sEVs.[203] Recently, Rasmussen et al. reported a novel characterization method to measure the exosome size and zeta potential simultaneously with a salt gradient in a nanofluidic channel.[311] They designed a nanofluidic device that contained 16 parallel funnel-shaped nanochannels bridging two microchannels, one is for salt solution and the other is for exosome solution. The salt gradient in the nanochannels causes particles and fluids to migrate in opposite directions, and finally, the particles are trapped at an equilibrium position. The spatial distribution of single or a group of exosomes is consistent with their size and surface charge. This method can accurately determine both the particle size and zeta potential for exosomes in a one-step measurement lasting less than minutes, it is also viable for other types of individual particles, ensembles of particles, or even particle mixtures.

■ EMERGING TECHNOLOGIES FOR EV ANALYSIS

Various molecular contents on or within EVs provide an informative insight into the EV study. Many approaches have been used or developed for EV analysis. In this section, we will

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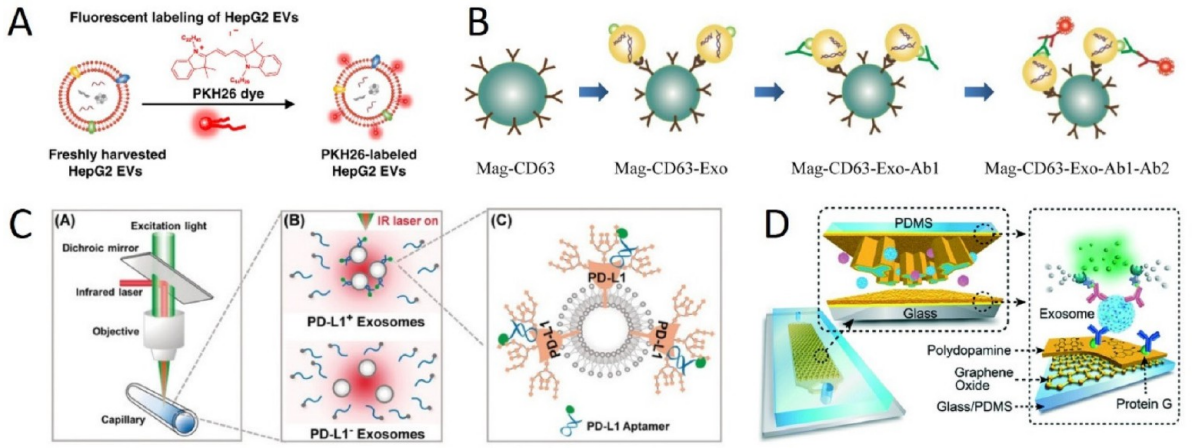

Figure 6. Identification and analysis of EVs by the fluorescence detection. (A) The EVs were stained by the nonspecific lipophilic membrane fluorescent dye of PKH26 dye. Reprinted by permission from Macmillan Publishers Ltd.: Nature Communications, Sun, N.; Lee, Y. T.; Zhang, R. Y.; Kao, R.; Teng, P. C.; Yang, Y.; Yang, P.; Wang, J. J.; Smalley, M.; Chen, P. J.; Kim, M.; Chou, S. J.; Bao, L.; Wang, J.; Zhang, X.; Qi, D.; Palomique, J.; Nissen, N.; Han, S. B.; Sadeghi, S., et al. *Nat. Commun.* **2020**, *11*, 4489 (ref 313). Copyright 2020. (B) The EVs were first enriched on the magnetic beads, then were labeled with detect antibodies. Next, the EVs were stained by the fluorescence-conjugated secondary antibodies. Reproduced from Fang, S.; Tian, H.; Li, X.; Jin, D.; Li, X.; Kong, J.; Yang, C.; Yang, X.; Lu, Y.; Luo, Y.; Lin, B.; Niu, W.; Liu, T. *PLoS One* **2017**, *12*, e0175050 (ref 314). Copyright 2017 PLOS. (C) A homogeneous, low-volume, efficient, and sensitive exosomal PD-L1 (HOLMES-ExoPD-L1) quantitation method by a fluorescence-labeled aptamer. Exosomal PD-L1 was recognized by a fluorescence-labeled aptamer and then sEVs were enriched by homogeneous thermophoresis. Reproduced from Homogeneous, Low-volume, Efficient, and Sensitive Quantitation of Circulating Exosomal PD-L1 for Cancer Diagnosis and Immunotherapy Response Prediction, Huang, M.; Yang, J.; Wang, T.; Song, J.; Xia, J.; Wu, L.; Wang, W.; Wu, Q.; Zhu, Z.; Song, Y.; Yang, C. *Angew. Chem. Int. Ed. Engl.* Vol. *59*, Issue 12 (ref 316). Copyright 2020 Wiley. (D) After the EVs were captured in the chip, biotinylated detection antibodies were used to label the EVs. Then the streptavidin-conjugated β-galactosidase was reacted with the biotinylated detection antibodies to catalyze the di-β-D-galactopyranoside, thus amplifying the fluorescent signal. Reproduced from ref 111 by the author: Zhang, P.; He, M.; Zeng, Y. *Lab Chip* **2016**, *16*, 3033−3042. Publisher: The Royal Society of Chemistry.

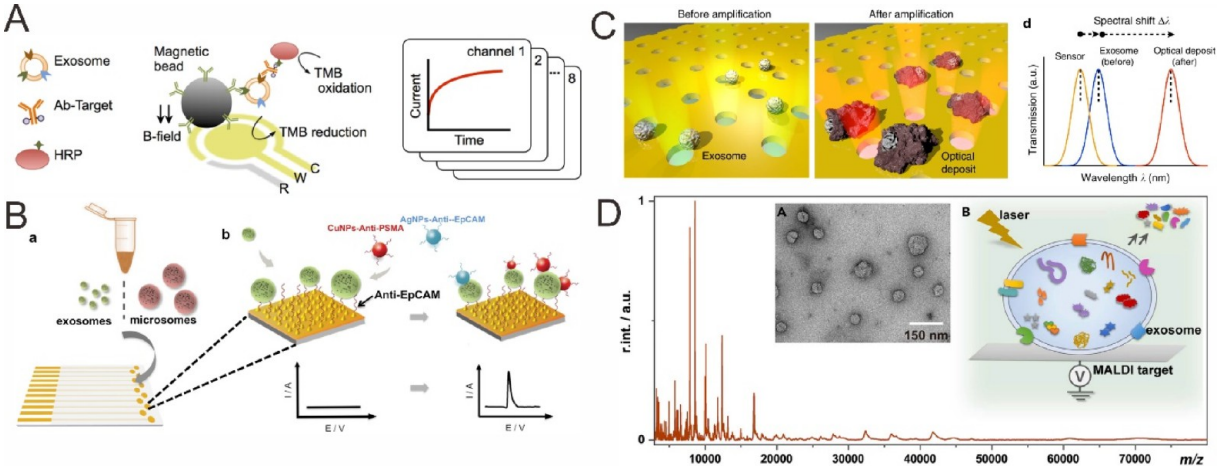

Figure 7. (A) Integrated magnetic−electrochemical exosome (iMEX) platform for the analysis of exosomal proteins. This detection device contains eight channels, and each channel can be immobilized with one kind of antibody to simultaneously detect eight kinds of exosomal protein markers. Reproduced from Jeong, S.; Park, J.; Pathania, D.; Castro, C. M.; Weissleder, R.; Lee, H. *ACS Nano* **2016**, *10*, 1802−1809 (ref 320). Copyright 2016 American Chemical Society. (B) An electrochemical sensor for detection of microsomes and exosomes using metal nanoparticles. The microsomes and exosomes were captured on the gold nanosubstrate, then the Cu nanoparticles conjugated anti-PSMA antibodies and Ag nanoparticles conjugated anti-EpCAM antibodies were used to label the microsomes and exosomes for the electrochemical detection. Reproduced from Interrogating Circulating Microsomes and Exosomes Using Metal Nanoparticles, Zhou, Y. G.; Mohamadi, R. M.; Poudineh, M.; Kermanshah, L.; Ahmed, S.; Safaei, T. S.; Stojcic, J.; Nam, R. K.; Sargent, E. H.; Kelley, S. O. *Small* Vol. *12*, Issue 6 (ref 321). Copyright 2016 Wiley. (C) The amplified plasmonic exosome (APEX) platform for exosome analysis. Based on the nPLEX system, insoluble optical deposits are locally produced through enzymatic amplification after the exosomes were capture on the Au nanohole array. Reprinted by permission from Macmillan Publishers Ltd.: Nature Communications, Lim, C. Z. J.; Zhang, Y.; Chen, Y.; Zhao, H.; Stephenson, M. C.; Ho, N. R. Y.; Chen, Y.; Chung, J.; Reilhac, A.; Loh, T. P.; Chen, C. L. H.; Shao, H. *Nat. Commun.* **2019**, *10*, 1144 (ref 326). Copyright 2019. (D) Multiple protein analysis of intact exosomes by MALDI-TOF MS. The intact exosomes were directly analyzed by MS and an exosomal fingerprint was obtained for qualification and semiquantitation of exosomal proteins. Reprinted from *Chem* Vol. *5*, Zhu, Y.; Pick, H.; Gasilova, N.; Li, X.; Lin, T.-E.; Laeubli, H. P.; Zippelius, A.; Ho, P.-C.; Girault, H. H. MALDI Detection of Exosomes: A Potential Tool for Cancer Studies, pp 1318−1336 (ref 331). Copyright 2019, with permission from Elsevier.

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comprehensively discuss emerging technologies for EV protein and NA analysis as well as for single EV analysis. Lipids are also an important component of EVs; however, compared with the protein and NA analysis, analytical methods of lipids are underdeveloped and thus need to be explored more in the future. We will discuss the lipid analysis in the EV omics studies along with proteomics and transcriptomics.

EV Protein Analysis. *Fluorescence Detection.* Fluorescence label techniques have been broadly used in the field of bioanalysis and clinic diagnosis.[312] Generally, EVs can be labeled with fluorescence by nonspecific lipophilic membrane fluorescent dye to observe and quantify the EVs. For example, the PKH26 dye could be used to stain the EVs for the identification of EVs captured in the microfluidic chip (Figure 6A).[313] However, the nonspecific lipophilic membrane fluorescent dye could not be used for the surface protein analysis of EVs. Therefore, fluorescence-labeled aptamers or antibodies for protein markers are exploited to stain EVs, then the fluorescence-stained EVs are observed under the fluorescent microscope, and fluorescent intensity is recorded for the qualitative and quantitative analysis of vesicular protein markers. Fluorescence detection shows great capability in the identification of vesicular protein makers thanks to the great accuracy and high specificity. Several kinds of protein markers have been identified by fluorescence-labeled antibodies, such as CD63, MHC I, and HER2.[235,314,315] For example, Liu et al. reported a quantitative method of exosomal protein markers using a fluorescence-labeled specific antibody (Figure 6B). They proved the consistency of the HER2 expression level between exosomes and tumor tissues.[314] However, the detection sensitivity of fluorescent molecule-labeled antibodies is limited due to the existence of glycosylation. The size of an aptamer is much smaller than that of an antibody, providing a good choice to overcome the steric hindrance of glycosylation and to improve the detection sensitivity. Yang et al. selected the MJ5 aptamer for recognizing sEV PD-L1 protein, showing 11 times higher sensitivity than the antibody (Figure 6C).[316] They also demonstrated that the expression level of exosomal PD-L1 protein could be an indicator of tumor metastasis. The detection sensitivity of EVs directly stained by fluorescence molecules is always not satisfied due to the low fluorescence intensity. Thus, some fluorescence signal amplification strategies have been developed to further improve the fluorescence detection sensitivity of EV proteins. Zeng et al. used the biotinylated detection antibodies to label the captured EVs, then reacted with the streptavidin-conjugated β-galactosidase to catalyze the di-β-D-galactopyranoside, producing a strong fluorescent signal (Figure 6D). In this way, the limit of detection (LoD) could be as low as ∼50 exosomes μL^{-1}.[111] When combined with the 3D-nanopatterned chip, the LoD was improved to ∼10 exosomes μL^{-1}.[113]

Electrochemical Detection. The electrochemical detection technique is suitable for biological sample analysis by virtue of high sensitivity, good specificity, facility, and low cost. The property of targets can be determined by measuring the change of voltages or currents.[8,17,312,317] Generally, the capture molecules (e.g., antibody and aptamer) are used to bind target EVs, then another detection antibody conjugated with an electroactive signal transducer recognizes the targets to perform electrical signals. So far, many electrochemical detection methods have been developed for the analysis of EV proteins.[113,145,317−319] Lee et al. reported an integrated magnetic-electrochemical exosome (iMEX) detection platform for the rapid and on-site profiling of exosomes (Figure 7A).[320] The iMEX platform integrated eight independent channels, and each channel could be employed for the analysis of different protein markers (e.g., CD63, EpCAM, CD24, and CA125). The iMEX system could simultaneously profile four exosomal protein markers from ovarian cancer samples with a LoD of 10^3 exosomes, indicating the high sensitivity and throughput of the iMEX. Kelley et al. reported a novel method for electrochemical detection of protein markers. The Cu nanoparticles conjugated with anti-PSMA (prostate-specific membrane antigen) aptamers and Ag nanoparticles conjugated with anti-EpCAM aptamers were used to label exosomes (Figure 7B). Owing to the different oxidation potentials of Cu and Ag, exosomal EpCAM and PSMA could be verified based on the potential and quantified based on the intensity of currents simultaneously.[321]

Surface Plasma Resonance. Surface plasma resonance (SPR) is a rapid and label-free method for the analysis of vesicular proteins.[8,312,322] SPR is based on the oscillation of conduction electrons on the metal−dielectric interface which is illuminated by the incident light. Generally, when the targets are bound to the substrate, a spectral shift and intensity change occur, which can be used to quantify the target protein expression levels. Many studies have been published based on SPR techniques,[323−325] and among them, the nanoplasmonic exosome (nPLEX) is a typical technique.[323] The nPLEX platform depends on the optical transmission through periodic nanoholes instead of total internal reflection. The significant superiority of nPLEX is that the detection sensitivity was notably improved since the probing depth matched the exosome size. They designed a 12 × 3 sensing array and integrated this array into a microfluidic chip with multiple channels. Each channel was functionalized with different capture ligands for the recognition of exosomes and, thus, to detect various protein markers simultaneously. The nPLEX demonstrated a superior LoD of 3000 vesicles with a processing time of less than 30 min. The nPLEX system showed great potential in clinical application. Based on the nPLEX platform, Shao et al. developed the amplified plasmonic exosome platform (APEX) to further improve the EV detection sensitivity (Figure 7C).[326] Basically, the biotinylated anti-CD63 antibodies were used to label the captured exosomes. Then the horseradish peroxidase-conjugated with neutravidin reacted with the biotinylated anti-CD63 antibodies and catalyzed the substrate of 3,3′-diaminobenzidine tetrahydrochloride to produce the insoluble optical deposits, thus amplifying the SPR signal. In this way, the detection sensitivity was improved to ∼200 exosomes.

Mass Spectrometry. Mass spectrometry (MS) is a powerful tool for high-throughput protein analysis of biological samples, and it has shown a great capability to detect EV proteins.[153,327−329] Generally, several critical steps are essential to apply MS for the analysis of EV proteins. First, EVs are separated and purified by multiple strategies; then EVs undergo enzyme digestion and peptide purification via peptide OFFGEL fractionation, liquid chromatography (LC), or SDS-PAGE;[8] finally, the purified peptides fragments are applied for MS analysis, and the MS spectrum is used for the identification of peptides by searching the protein database. Multiple kinds of proteins can be verified at the same time from the complex mixture, and the protein quantitation of EVs can also be performed by a tag-labeled manner or spectral counting of the chromatogram intensity. So far, lots of EV proteins have been

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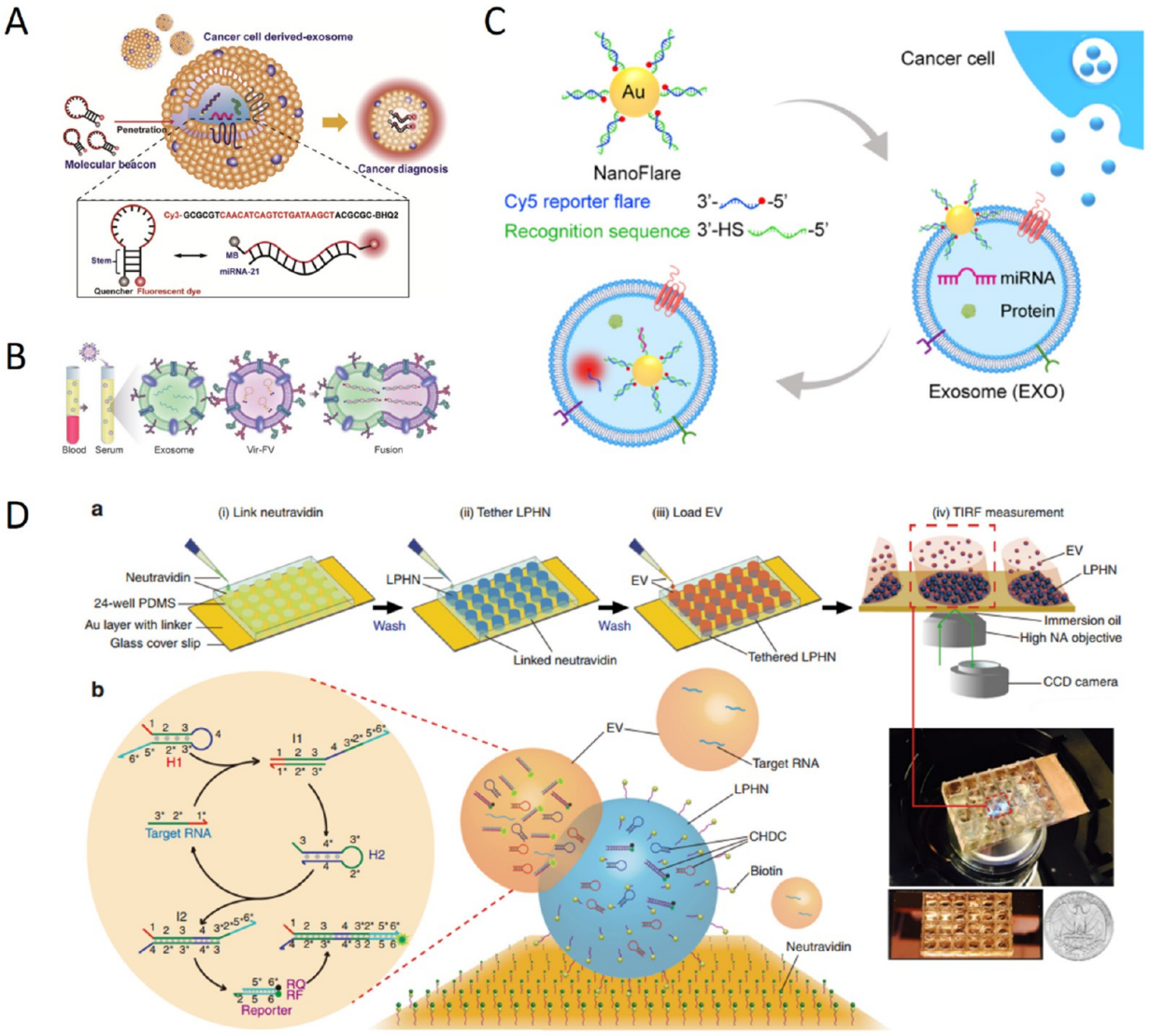

Figure 8. *In situ* detection methods of EV NAs. (A) Exosomal miRNA is detected by molecular beacons that can penetrate the exosomes, fluorescence signal generates when the beacons hybridize with target miRNA. Reprinted from *Biomaterials*, Vol. *54*, Lee, J. H.; Kim, J. A.; Kwon, M. H.; Kang, J. Y.; Rhee, W. J. *In situ* single step detection of exosome microRNA using molecular beacon, pp 116−125 (ref 355). Copyright 2015, with permission from Elsevier. (B) Molecular beacons are encapsulated in the Vir-FV, target miRNA can be identified after the fusion and mixing of exosomes and artificial vesicles. Reproduced from Rapid Detection of Exosomal MicroRNAs Using Virus-Mimicking Fusogenic Vesicles, Gao, X.; Li, S.; Ding, F.; Fan, H.; Shi, L.; Zhu, L.; Li, J.; Feng, J.; Zhu, X.; Zhang, C. *Angew. Chem. Int. Ed. Engl.* Vol. *58*, Issue 26 (ref 358). Copyright 2019 Wiley. (C) *In situ* detection of exosomal miRNA using nanoflares. After the diffusion of nanoflares into exosomes, fluorescence-labeled blocking sequence is displaced by the target miRNA, and then emits a fluorescent signal. Reproduced from Zhao, J.; Liu, C.; Li, Y.; Ma, Y.; Deng, J.; Li, L.; Sun, J. *J. Am. Chem. Soc.* **2020**, *142*, 4996−5001 (ref 359). Copyright 2020 American Chemical Society. (D) A nanoparticle-based biochip that integrates the capture of EVs and *in situ* detection of RNAs in a single step. Cationic lipid-polymer hybrid nanoparticles with DNA circuit confined in are mobilized on the chip surface via biotin−neutravidin interaction, and EVs can be captured and fused by the nanoparticles, which triggers the displacement reaction and generates fluorescent signals detected by a TIRF microscope. Reprinted by permission from Macmillan Publishers Ltd.: Nature Communications, Hu, J.; Sheng, Y.; Kwak, K. J.; Shi, J.; Yu, B.; Lee, L. J. *Nat. Commun.* **2017**, *8*, 1683 (ref 360). Copyright 2017.

confirmed by MS from blood and urine as biomarkers for disease diagnosis.[330,331]

Recently, Girault et al. reported a novel MS analysis method for exosomal proteins by forming exosome-matrix crystals without lysis, as shown in Figure 7D.[331] The intact exosomes were directly analyzed by MS, and the exosomal fingerprint was obtained for the qualification and semiquantitation of exosomal proteins. This approach is a quick and high-throughput manner to measure 48 samples within 1 h, multiple protein biomarkers can be detected simultaneously without any label, and it also allows the discovery of new biomarkers. Despite many advantages of MS, tedious preparatory and processing time seriously limits its clinical application. Besides, the detection sensitivity of MS is inferior to antibody-based techniques. Moreover, it is difficult to quantify the concentration of proteins due to the existence of abundant interfering proteins in biological samples. Nevertheless, MS-based techniques are still a powerful tool for molecular characterization and biomarker discovery in EV research.

EV Nucleic Acid Analysis. As discussed in Molecular Compositions of EVs, EV NAs are stable and promising

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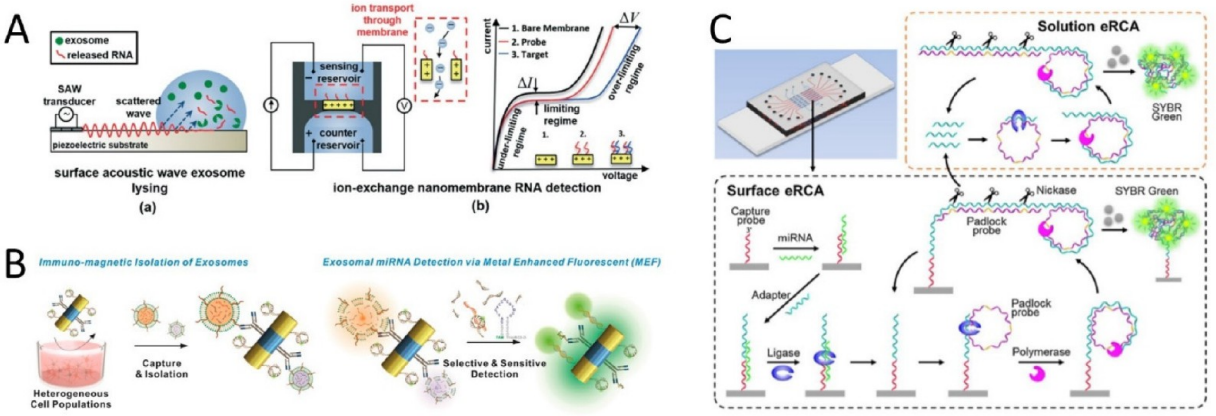

Figure 9. Integrated methods for EV NA analysis. (A) A serial platform containing the surface acoustic wave chip for exosomes lysis and the ion-exchange nanomembrane chip for RNA detection. Reproduced from Taller, D.; Richards, K.; Slouka, Z.; Senapati, S.; Hill, R.; Go, D. B.; Chang, H. C. *Lab Chip* **2015**, *15*, 1656−1666 (ref 362), with permission of The Royal Society of Chemistry. (B) In the multifunctional nanorod, the central Ni part is modified with anti-CD63 antibody and realizes the immunomagnetic capture and concentration of exosomes, the Au parts flanked are modified with specific molecular beacons to capture and detect miRNA after *in situ* lysis of exosomes. Signals are amplified by metal-enhanced fluorescence effects. Reproduced from Lee, J. H.; Choi, J. H.; Chueng, S. D.; Pongkulapa, T.; Yang, L.; Cho, H. Y.; Choi, J. W.; Lee, K. B. *ACS Nano* **2019**, *13*, 8793−8803 (ref 364). Copyright 2019 American Chemical Society. (C) An integrated microfluidic chip for the capture and amplification of exosomal miRNA. Signals are generated via surface exponential RCA. Reprinted from ref 365 by the author: Cao, H.; Zhou, X.; Zeng, Y. Microfluidic exponential rolling circle amplification for sensitive microRNA detection directly from biological samples, *Sens. Actuators, B Chem.* **2019**, *279*, 447−457. Publisher: Elsevier.

biomarkers. For the comprehensive analysis of EV NAs, a variety of technologies, assays, and devices have been leveraged or developed to meet different requirements such as point-of-care (POC), multiplexing, high-throughput, integration, rapid detection, or high sensitivity. Many novel amplification- or nonamplification-based technologies can be directly transplanted to detect EV NAs no matter whether they are designed originally to analyze EV NAs, as long as the lysis of EVs and NA purification step are conducted. In this section, we will mainly introduce the state-of-art methods that directly interact with the NA cargoes in intact EVs (termed *in situ* methods) and the integrated assays for EV NA analysis. The conventional and non-*in situ* EV NA detection methods will also be discussed below.

Conventional Methods. Conventional analytical methods of NAs such as Northern blot, high-throughput sequencing (HTS), microarray, and PCR can be directly used for the EV NA analysis. HTS is a powerful tool to profile NA contents for the discovery of unknown NAs or potential biomarkers and the understanding of EV NA distribution and quantitation.[80,332] The microarray is also a well-established technology that is a relatively easier and cost-effective way compared with HTS for the measurement of gene expression.[333,334] However, the microarray platform needs to be updated or redesigned when any new sequences are introduced to the databases, and it also lacks the ability to discover novel sequences because microarray uses only known sequences as targets, which hinders its widespread use.[333] For the analysis of given target sequences, PCR is the solid choice due to its simplicity, convenience, and quickness compared with HTS and the microarray, albeit of limited throughput.[109,335] Besides the routine protocol such as RT-qPCR for EV miRNA detection, Lee et al. developed a single-step stem-loop based RT-qPCR method in which RNA purification was skipped by using a lysis solution compatible with the subsequent RT reaction. Though this method compromised the sensitivity, it is time- and cost-effective and will support the development of EV miRNA assay in the POC field.[336] In addition, droplet digital PCR has also been leveraged to detect EV NAs such as miRNA,[337] or DNA[338] with enhanced sensitivity and accuracy compared with conventional PCR.

Non-In Situ Methods. With a growing focus on EV NAs as markers of the liquid biopsy, various emerging biosensor technologies such as fluorescence-based methods,[339,340] SERS-based methods,[341,342] electrochemical methods,[343−346] the photonic resonator absorption microscopy assay,[347] CRISPR/Cas system-based methods,[348,349] *etc.*, have been developed, providing many opportunities for realizing the POC testing or personalized medicine. Many reviews are focusing on these technologies.[350−353] These methods enable efficient, rapid, and robust analysis of EV NAs; however, manual NA extraction step is needed, which may increase the labor intensity when analyzing multiple samples. To address the issue, *in situ* and integrated methods have been developed.

In Situ Methods. The Rhee group has developed an *in situ* and single-step approach for miRNA detection in whole exosomes using a molecular beacon.[354] The molecular beacon can directly penetrate exosomes or with enhanced delivery efficiency via the permeabilization by streptolysin O treatment, giving significantly increased fluorescence signals when hybridized with target miRNAs (Figure 8A).[355] Multiplex detection of exosomal miRNAs from breast or prostate cancer cells was successfully achieved using this method in the presence of spiked human serum or urine.[356,357] Furthermore, Gao et al. encapsulated molecular beacons within a virus-mimicking fusogenic vesicle (VirFV), upon contents mixing of VirFV and exosomes in human serum, efficient and rapid detection of exosomal miRNAs was completed within 2 h (Figure 8B).[358] Instead of using a molecular beacon, Zhao et al. utilized the nanoflares for the *in situ* detection of exosomal miRNA (Figure 8C). A nanoflare is a gold nanoparticle functionalized with antisense sequences of specific miRNAs which are prehybridized by a short and fluorescence-labeled reporter sequence. The amplified fluorescence signal was

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realized using the thermophoretic accumulation of exosomes, and direct and quantitative measurement of exosomal miRNAs down to 0.36 fM in 0.5 μL serum samples was achieved.[359]

Similarly, Hu et al. reported a nanoparticle-based biochip that could achieve the capture of EVs and *in situ* detection of RNAs in a single step (Figure 8D). Catalyzed hairpin DNA circuit (CHDC) was first confined in cationic lipid-polymer hybrid nanoparticles (LPHNs) labeled with biotin, and then the LPHNs were immobilized on the chip surface via biotin−neutravidin interaction. After loading the samples, EVs were then captured and fused with LPHN to form the LPHN−EV complex, followed by the transfer and mixing of CHDC with EV-associated RNAs. CHDC was triggered upon target RNA binding and multiple cycles of toehold-mediated strand displacement reactions occurred, generating amplified fluorescence signal by destabilizing the reporter dsDNA. GPC1 mRNA was selected as a target and the fluorescence signal was measured by the total-internal-reflection-fluorescence (TIRF) microscopy. The LoD of EVs from AsPC-1 cells was calculated to be ∼60 EVs per μL. The assay can achieve accurate quantification of GPC1 mRNA in serum EVs from pancreatic cancer patients, highlighting its potential in cancer diagnosis, prognosis, and therapeutic monitoring.[360]

These *in situ* techniques avoid the time-consuming preisolation and NA extraction procedures, preventing the occurrence of degradation and contamination, offering a simple, cost-effective, and fast alternative in EV RNA based diagnosis and prognosis of diseases.[355−359] Nonetheless, the methods introduced above are nonenzymatic, and in the future, various enzymatic amplification approaches, such as the DNAzyme,[361] may be paired with *in situ* strategies for better sensitivity and quicker readout time.

Integrated Analytical Methods. In order to overcome the obstacles of the conventional EV NA analysis methods, such as sample loss, tedious NA isolation, and time-consuming process, the Chang group developed two serial microfluidic devices including an exosome lysis chip based on the surface acoustic wave (SAW), and an RNA sensing platform using the ion-exchange nanomembrane (Figure 9A).[362,363] Cell medium sample from pancreatic cancer cell lines (100 μL) can be directly dropped on the SAW device after a simple centrifugation step and the lysis of exosomes and release of RNA can be done within 30 min. Next, target miRNA-550 can be selectively captured on the surface of the nanomembrane functionalized with the oligonucleotide probes, allowing the accurate determination of the concentration of the target miRNA in 1 h by measuring the current−voltage characteristic. This method displayed a lysis rate of 38% and a LoD of 2 pM with two magnitudes of linear dynamic range. The conjunctive platform provides rapid detection of exosomal miRNA for a cancer study, and diagnosis and can be easily extended for other RNA and DNA targets of interest.[362]

Besides the engineering integration of the exosome analysis, Lee et al. prepared a magneto-plasmonic nanorod sensor for the exosome isolation and miRNA detection (Figure 9B). Generally, the multifunctional nanorod comprises two parts, one is a central Ni component modified with antibodies against CD63 to immunomagnetically capture and concentrate exosomes, the other one is the two end-capping Au components modified with specific molecular beacons to capture and detect miRNA with an amplified signal via metal-enhanced fluorescence effects after the *in situ* lysis of exosomes. This method showed a good linearity ranging from 1 pM to 1 μM and was successfully used to detect miRNA-124 to characterize stem cell neurogenesis in a nondestructive and efficient manner.[364]

Though the methods above provided integrated solutions for EV NA analysis using devices or novel sensors, the sensitivity was compromised due to the nonenzymatic signal detection. In order to fulfill the criteria for early detection of cancer where the targets are usually in trace amounts, Cao et al. developed an integrated microfluidic exponential rolling circle amplification (MERCA) platform streamlining solid-phase miRNA extraction, miRNA-adapter ligation, and a dual-phase exponential RCA (Figure 9C). This method achieved a remarkably low LoD of 10 zmol and can afford highly sensitive detection of down-regulated low-level miRNA such as let-7a in as few as 2×10^6 cancerous exosomes.[365]

Reátegui et al. established a microfluidic platform (^{EV}HB-Chip) for the antigen-specific isolation of EVs and the profiling of tumor-specific RNAs. In this design, a cocktail of antibodies was modified on the herringbone chip to achieve the specific capture of tumor-derived EVs, followed by the *in situ* lysis of EVs and the extraction of RNA from lysates using kits. The whole assay can be completed within 3 h with a LoD of 100 EVs per μL. Using this platform, relatively rare EGFRvIII transcripts can be detected, and by profiling the exosomal RNA from glioblastoma multiforme (GBM) patients' serum and plasma, they identified 54 genes in the EV transcriptomes that were promising panels for disease monitoring as well as the transcripts that hallmarked the subtypes of GBM.[366]

Consideration of EV NA Analysis. To quantify the abundance of candidate RNAs, it is essential to normalize their expression levels to well-established references. Since EV RNAs are selectively enriched from the parent cells, endogenous RNA controls for cellular RNA normalization are not appropriate for EV RNA normalization. Previous studies have used spike-in miRNA control such as the *Caenorhabditis elegans* miR-39/54/238 to normalize RT-qPCR data, which, however, is only capable for the correction of the RNA extraction variations but not the variations from different biological and pathological conditions.[367] Yuan et al. reported a systematic and comprehensive analysis of exosomal RNA profiles considering individuals and genders with varying ages and health conditions, revealing that miR-99a-5p shows the highest abundance and stability among all the exosomal RNAs tested. However, the usage of such miRNA as a reference for exosomal RNA quantification needs further proof.[367] Furthermore, most of the recent EV NA analysis studies focus on the detection of miRNA;, in the future, more efforts could be paid on the analysis of other types of NAs for the more comprehensive reveal of the molecular information and even on the joint detection of EV proteins and NAs in liquid biopsy for higher accuracy.[368,369]

Single EV Analysis. With the proposal and development of precision medicine, ultrasensitive approaches are required to adapt to the early detection of diseases and individualized theranostics. The existing complexity of EVs makes the bulky isolation and detection methods such as UC, Western blot, ELISA, and conventional PCR, *etc.* underqualified to uncover the tiny distinction between individual EVs. Similar to the research field of single cells, the emerging analysis of single EVs is important to reveal the heterogeneity and to prevent the loss of information when using bulky methods. Though, unlike a single circulating tumor cell that can replicate and lead to metastasis, EVs function in a bulky way biologically because a

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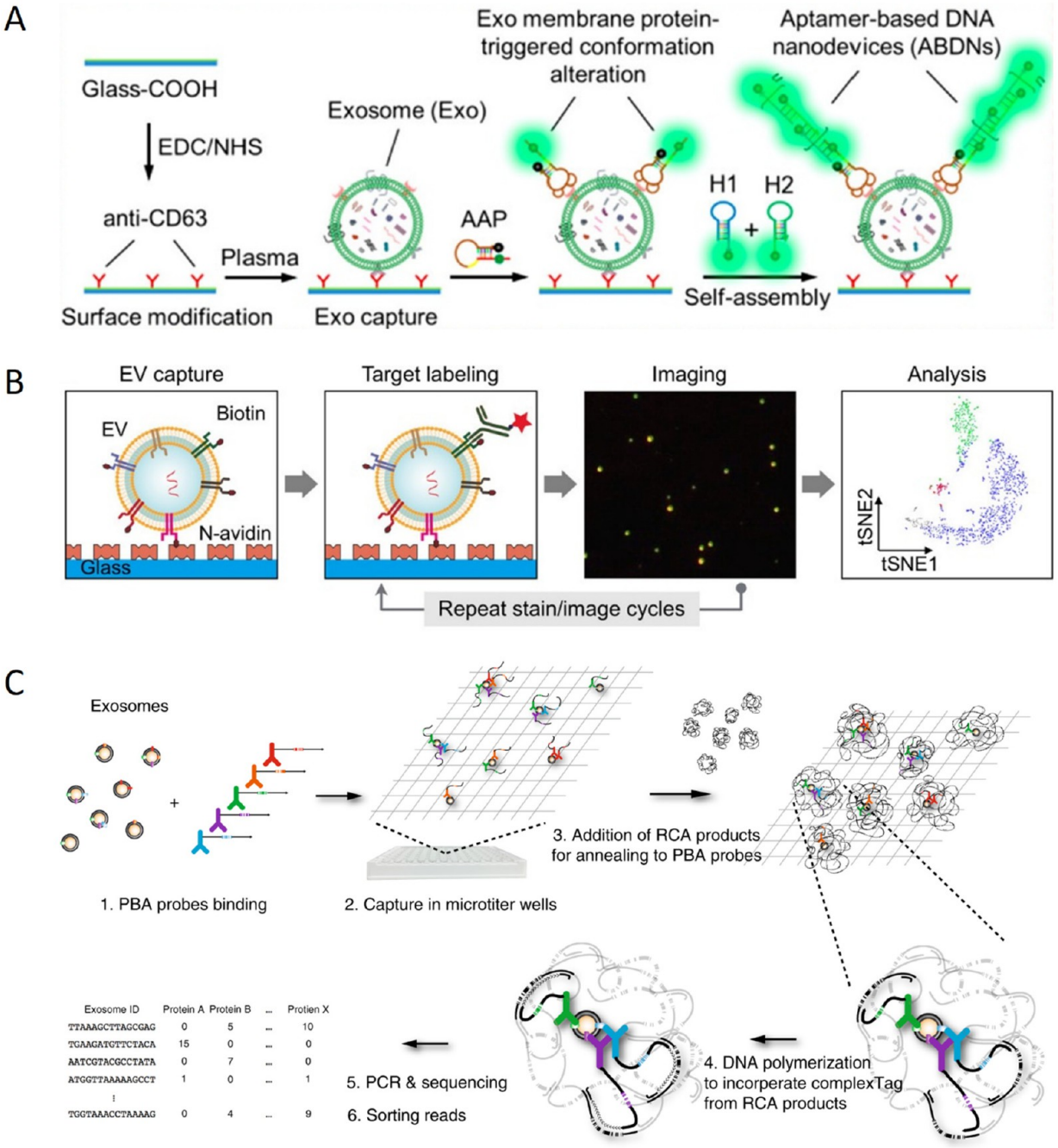

Figure 10. Single EV detection methods. (A) Direct visualization and quantification of single EVs by TIRF. Signals are generated and amplified by the aptamer and the subsequent HCR, respectively. Reproduced from He, D.; Ho, S. L.; Chan, H. N.; Wang, H.; Hai, L.; He, X.; Wang, K.; Li, H. W. *Anal. Chem.* **2019**, *91*, 2768−2775 (ref 377). Copyright 2019 American Chemical Society. (B) The principle of the on-chip immuno-staining and imaging platform to realize the single EV analysis. Biotinylated EVs are mobilized on the glass surface, and then repeated staining and quenching protocol are used for the profiling of a panel of 11 protein biomarkers sequentially. Reproduced from Lee, K.; Fraser, K.; Ghaddar, B.; Yang, K.; Kim, E.; Balaj, L.; Chiocca, E. A.; Breakefield, X. O.; Lee, H.; Weissleder, R. *ACS Nano* **2018**, *12*, 494−503 (ref 379). Copyright 2018 American Chemical Society. (C) The principle of the proximity-dependent barcoding assay (PBA) for the profiling of individual exosomes. Protein composition is barcoded with DNA sequence information and then decoded by next-generation sequencing. The numbers of exosomes, protein expression on individual exosomes, and the abundance of each protein from each exosome can be revealed by the complexTag, the proteinTag, and the moleculeTag, respectively. Reprinted by permission from Macmillan Publishers Ltd.: Nature Communications, Wu, D.; Yan, J. H.; Shen, X.; Sun, Y.; Thulin, M.; Cai, Y. L.; Wik, L.; Shen, Q. J.; Oelrich, J.; Qian, X. Y.; Dubois, K. L.; Ronquist, K. G.; Nilsson, M.; Landegren, U.; Kamali-Moghaddam, M. *Nat. Commun.* **2019**, *10*, 3854 (ref 380). Copyright 2019.

single EV contains few cargoes, and the cancer biomarkers from single EVs are still of great value for researchers to locate a hint of a disease in its very early stage. NTA which has been discussed in Dynamic Light Scattering and Nanoparticle Tracking Analysis and is the most commonly used technology to characterize EVs at the single-particle level, and in the fluorescence mode, quantitative detection of proteins by using fluorescence-labeled antibodies and miRNA contents by using

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the molecular beacons have been achieved.[370,371] FCM is also a powerful tool for the single EV analysis (see Analysis of Selected EV Proteins). Besides NTA and FCM, various labeling methods and label-free methods have also been successfully developed for the single EV analysis,[372] and the emerging techniques will be discussed below.

Digital Methods. Digital approaches have also been leveraged to fulfill single EV detection. Liu et al. developed a droplet digital ExoELISA approach that enabled absolute quantification of cancer-specific exosomes. Exosomes were first captured by CD63 antibodies on the magnetic beads which were subsequently encapsulated into droplets, during this process, only one or no exosome was on the beads and only one or no bead was in the droplets according to the Poisson distribution. Signals were generated via the detection of exosomal surface protein Glypican-1 (GPC-1), and the results confirmed that GPC-1 positive exosomes in breast cancer patients were much higher than that in healthy people and patients with benign breast disease. This method achieves a LOD of 10 enzyme-labeled exosomes per microliter with a linear range of 5 orders of magnitude.[373]

Besides droplet technology, Tian et al. used microchambers to achieve the digital detection of exosomes. The lipid membranes of the exosomes were inserted with biocompatible anchor molecules conjugated with ssDNA, and the antibody-DNA conjugates were used to label the protein markers GPC-1; after modification, the exosomes in amplification buffer were allocated into the microchambers. The two different DNAs were amplified via rapid isothermal nucleic acid detection assay[374] to determine the number of total exosomes and GPC-1 specific exosomes, respectively.[375]

Fluorescence Imaging. Fluorescence imaging techniques allow us to see and analyze single EVs directly. Fraser et al. used a streptavidin-coated coverslip to capture the biotinylated EVs, followed by the labeling with fluorescent antibodies, and then the imaging of single EVs was performed on a BX-63 Upright Automated Fluorescent Microscope (Olympus). Via data analysis, they found that tumor-derived microvesicles occupied less than 10% of all the microvesicles in the plasma from glioblastoma patients and the tumor marker expression was highly heterogeneous among tumor-derived microvesicles.[376]

Li group leveraged the TIRF microscopy to realize the direct visualization and quantification of single EVs (Figure 10A). Exosome samples were added on the anti-CD63-modified coverslip, and the signals were generated and amplified by the aptamer and the subsequent *in situ* hybridization chain reaction (HCR), respectively. The authors proved that PTK-7-positive exosomes from human acute-lymphoblastic-leukemia patients' plasma were significantly higher compared with the healthy controls.[377] They also used this imaging platform to perform the *in situ* miRNA analysis in single exosomes, and the split Mg^{2+}-dependent DNAzyme partially complementary to the self-quenched substrate was used as the fluorescence probe; after its penetration into exosomes and the hybridization with the target miRNA, the DNAzyme was activated and cleaved the substrate with the help of Mg^{2+} to generate a fluorescent signal. Precise stoichiometry of exosome individuals and exosomal miRNAs has been achieved using this assay, and a common tumor marker miRNA-21 in serum exosomes was tested to prove its practicability in monitoring tumor progression and responses to treatment with an LoD of 378 copies per microliter.[361]

For the multiplexed detection of single EVs, Chen et al. developed a method that utilized the DNA points accumulation for imaging in nanoscale topography (DNA-PAINT) and machine learning to identify cancer-derived exosomes. In DNA-PAINT, the fluorescent dye-labeled imager strands bind to the complementary docking strands transiently and then release because of the thermal fluctuation, and the continuous on and off of fluorescence in the focus plane can be captured by TIRF microscopy. The quantitative DNA-PAINT was achieved by plotting the fluorescence intensity blinking versus time, which contained the information about the number of target molecules. In this assay, exosomes were sparsely immobilized on the positively modified glass substrate via electrostatic interaction and then modified with a panel of four specific antibodies which were conjugated with a specific docking DNA strand, respectively. The information on each protein biomarker was obtained sequentially with a washing step of excessive imager strands between each signal acquisition. Using this principle and linear discriminant analysis (LDA), the profiling approach successfully identified exosomes from cancer-derived serum samples and was applied to diagnose pancreatic and breast cancers from unknown samples with 100% accuracy.[378] Compared with analogue profiling, this analysis based on single exosomes minimizes the concentration variations of exosome samples, providing more accurate detection results.[378]

Lee et al. developed an on-chip immuno-staining and imaging platform to realize the single EV analysis (Figure 10B). Biotinylated EVs were captured on the glass surface of a microfluidic device, and then staining and quenching protocols were optimized to achieve the profiling of a panel of 11 protein biomarkers sequentially (three at a time, 46 min per cycle), and t-distributed stochastic neighbor embedding (t-SNE) was conducted to group the EVs, which displayed high heterogeneity in glioblastoma EV subpopulations. Image capture can be done within 1 s, and more than 1000 EVs can be analyzed simultaneously in single image acquisition.[379]

High-Throughput Sequencing Based Methods. The innate capability of some techniques or the overlapping of fluorophore spectra limits the simultaneous profiling of higher orders of protein species.[380] In order to address this limitation and provide a more powerful tool to assess the heterogeneity of EVs, Wu et al. established a proximity-dependent barcoding assay (PBA) to profile 38 surface proteins of individual exosomes. Briefly, the information on protein composition was barcoded and converted to DNA sequence information and then decoded by next-generation sequencing (Figure 10C). In PBA, specific antibodies were first conjugated with DNA oligonucleotides which contained a proteinTag and moleculeTag. Each kind of antibody shared with one proteinTag sequence to identify the target protein qualitatively, and the moleculeTag comprised a random sequence served as the unique molecular identifier (UMI) for the further counting of the number of one specific kind of protein on the surface of exosomes quantitatively. After this conjugation, exosomes were captured sparsely by cholera toxin subunit B (CTB) coated in a 96-well microtiter. In order to barcode individual exosomes, RCA products containing about 200 copies of identical random DNA sequence named complexTag were used to interact with the exosomes captured. PBA oligos were then hybridized with one RCA product particle in a proximity-dependent way, and the same complexTag was incorporated into the PBA oligos that were bound to the same exosome by

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DNA polymerase mediated extension. After sequencing, the numbers of exosomes, protein expression on individual exosomes, and the abundance of each protein from each exosome can be revealed by sorting and counting the complexTag, the proteinTag (sharing the same complexTag), and the moleculeTag (sharing the same complexTag and proteinTag), respectively. By adopting t-SNE, it was found that exosomes from different sources were better distinguished using more surface proteins, and specific protein combinations were identified to sort the exosomes from heterogeneous samples such as seminal fluid, medium of K562 cell line, and serum.[380] Though this method has the ability to provide high-order protein signatures, the procedure is complex and thus the LoD needs further improvement.

Nanoplasmon-Enhanced Scattering. Liang et al. developed a nanoplasmon-enhanced scattering (nPES) assay to quantify tumor-derived EVs at single EV resolution. EVs in a small volume (1 μL) of original plasma samples (diluted by 40-fold) were captured by the anti-CD81 antibody on the silica surface of the sensor chip and then modified with the antibody-conjugated gold nanospheres and nanorods simultaneously, which produced a local plasmon effect and signal increase of scattering. This method displayed a wide linear range (4−5 logs) and with a LoD of 0.23 ng/μL. A pancreatic cancer EV biomarker, ephrin type-A receptor 2 (EphA2) was identified, and the nPES platform for EphA2 was able to distinguish patients with pancreatic cancer from pancreatitis patients and healthy controls, and monitor tumor progress and therapy responses.[381]

Other Methods. Besides the labeling method discussed above, many label-free methods have also been developed such as AFM,[382] TEM, SEM, cryo-EM, Raman spectrometry,[383−385] surface plasma resonance techniques,[386] resistive pulse sensing,[387] and interferometric imaging.[388] All these methods broaden the way to uncover the heterogeneity of EVs, increase the detection sensitivity, and meet the requirements of precision medicine. For comparison, FCM is one of the most promising methods in terms of analysis speed, multi-parameter detection, and commercialization.[372] In the future, single EV detection can also benefit from the multilevel analysis of targets.[372]

Omics Study of EVs. Cargoes of exosomes are selected using complex sorting mechanisms precisely rather than randomly, which reflects the psychological and pathological conditions of our body.[41,389] EV omics or exosomics is a powerful tool to characterize and identify the exosomal cargoes of interest and it reveals the information on biogenesis,[390] biological functions,[391,392] biomarker panels for diagnosis,[392−397] and therapy response.[395,398,399] The study of exosomics includes exosomal proteomics, transcriptomics, and lipidomics.[400] The majority of the exosomics literature focused on proteomics and transcriptomics, and several thousands of exosomal proteins and RNAs have been discovered from various cell types, body fluids,[400] and purification methods also affect the results.[401−403] Several published reviews elaborated the conventional methods for exosomics.[95,333,399] Public comprehensive databases such as EVpedia (http://evpedia.info) and ExoCarta (http://exocarta.org) have been established for sharing and cataloging the data.[400]

Transcriptomics. Next-generation sequencing techniques are commonly used in the studies of exosomal transcriptomics nowadays.[400] The accuracy of the subsequent analysis of exosomal transcriptome not only depends on the isolation methods[404] but also on the library preparation kits.[405] So the choice of methods should be carefully considered according to the research aim by balancing factors such as reads mapped to the genome/transcriptome, RNA transcript diversity, number of genes detected, and reproducibility.[405] Exosomal transcriptomic analysis reveals the information on NA components, improving the biological studies of EVs and the discovery of biomarkers. For example, it is found that the proportion of RNA varies in different body fluids and cancer types.[404] Amorim et al. developed a one-step total transcriptome protocol for the transcriptomic analysis of plasma-derived EVs.[406] They used RNase-III treatment to cut the RNAs into small fragmentation, which allowed the increment of molecules available for ligation to adaptors without amplification of the initial material. Small RNAs that have largely unknown functions have been found, as well as other types of RNAs, indicating the potential of this method to identify the cell-to-cell communication mechanisms and biomarkers.

Lipidomics. Thin layer chromatography (TLC), LC, gas chromatography (GC), and MS are the common techniques for EVs lipidomic analysis.[95,400] Lipidomic analysis is an effective strategy to discover new biomarkers for disease diagnosis, and hundreds of lipids can be analyzed simultaneously. Skotland et al. quantified 107 lipid species in urinary exosomes by high-throughput mass spectrometry and found that the levels of 9 exosomal lipid species in prostate cancer patients were different from those in healthy controls.[100] These lipids species could be indicators to diagnose prostate cancer with 93% sensitivity and 100% specificity, thus demonstrating the diagnostic potential of lipid species. In another research study, Glover et al. studied the exosomal lipidomics in urine samples of hereditary α-tryptasemia patients.[407] They separated more exosomes from hereditary α-tryptasemia patients than those from healthy donors. However, they discovered that the levels of 64 lipid species in hereditary α-tryptasemia patients were significantly lower than those in healthy donors after they analyzed 521 lipid species in the urinary exosomes. These results suggest that lipids participate in many various biological processes and can be useful biomarkers for disease diagnosis.

Proteomics. High-throughput MS-based techniques play a vital role in exosomal proteomic studies, such as the liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS).[398,400] Spectral libraries or novel computational frameworks can be used to analyze the obtained MS/MS spectra.[408] Exosomal proteomics provides detailed information on the protein composition, which will benefit the biological study of exosomes and lead to biomarker discovery.[409] Proteomic techniques have been reported by many excellent reviews.[95,410] Beyond proteomic identification, profiling of post-translational modifications (PTMs) could provide more information in biogenesis, biomarker discovery, and EVs uptake mechanisms.[411] Although MS-based proteomics has made it possible to comprehensively study EV biogenesis and structural features, accurate quantification of proteins, characterization of PTMs, and the development of multiomic workflows to characterize EVs remains a challenge.[203]

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Table 3. Exemplary Applications of Machine Learning in EV Analysis

publication	disease	algorithms	sample	data form/biomarkers	no. of training data	accuracy	groups
Gomez-de-Mariscal et al. (2019)[414]	N/A	residual convolutional neural network, radon transform	cell culture media and ascites of ovarian cancer patients	TEM images	65, 346, 688 single EVs images	segmentation accuracy 0.62−0.74, detection accuracy 0.65−0.81	N/A
Itami-Matsumoto et al. (2019)[415]	occurrence and recurrence of HCC (hepatocellular carcinoma) in patients who achieved an SVR (sustained viral response) with DAA (direct-acting antiviral) therapy	linear support vector machine	serum	microarray analysis/miR-4718, 642a-5p, 6826-3p, and 762	139	85.5%	69 CH (hepatitis C virus infection patients) patients who underwent HCC curative treatment vs 70 CH patients
Ko et al. (2017)[21]	pancreatic cancer	linear discriminant analysis (LDA)	plasma	qPCR/mRNA: CD63, CK18, DCN, Lgals1, Erbb3, GAPDH, ODC1, KRAS	N = 5 per group	100%	stage IV pancreatic cancer vs healthy gender-matched controls
Shin et al. (2020)[416]	lung cancer	residual neural network	cell culture media	SERS	normal cell exosomes: 1025 cancer cell exosomes: 1125	95%	normal cell exosomes vs cancer cell exosomes
Chen et al. (2019)[378]	pancreatic cancer and breast cancer	LDA	serum	DNA points accumulation for imaging in nanoscale topography/HER2, GPC-1, EpCAM, EGFR	N = 7 per group	100%	healthy samples vs breast cancer samples vs pancreatic cancer samples
Zhang et al. (2019)[132]	ovarian cancer	LDA, nonsupervised hierarchical clustering analysis	plasma	ELISA/exosomal surface protein biomarkers: EGFR, HER2, CA125, FRa, CD24, EpCAM, CD9+CD63	15 ovarian cancer patients and 5 benign controls	100%	ovarian cancer samples vs benign control
Zhang et al. (2020)[413]	breast cancer	LDA	plasma	FRET and ELISA/MMP14	22 breast cancer patients and 8 healthy controls	training cohort accuracy: 96.7% independent validation cohort accuracy: 92.9%	patients with stage 0 to III breast cancer vs. age-matched noncancer controls

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SYSTEMS ANALYSIS AND BIOINFORMATICS STRATEGIES

Because of the individual heterogeneity in genotypes, phenotypes, and responses to diseases, a single biomarker or parameter in EVs in most cases cannot effectively predict the status of a disease. To address this challenge, investigators combined multiplexed measurements of different biomarkers to define robust signatures for specific disease states. With the accelerated growth of omics and profiling approaches, analysis of EVs is becoming increasingly information-intensive, making the manual analysis and computation of multiparameters challenging. Machine learning (ML) offers an opportunity to automatically manage a large amount of molecular data using mathematical algorithms; thus, it is being progressively adopted in the discovery of high order interactions among biomarkers for disease diagnosis, prognosis, and precision medicine.[21]

The ecosystem of ML is diverse, ranging from basic approaches, such as logistic regression and support vector machines, to advanced deep learning such as artificial neural networks with many hidden layers that determine the "computational capacity" of the network.[3] One classification which is widely used describes two specific groups of ML: supervised learning and unsupervised learning.[412] In supervised learning, the algorithms are presented with a collection of training data in which the true state of the data is identified, while in unsupervised learning, algorithms look for correlations in data sets with no defined states.[21] Generally, the workflow of canonical machine learning consists of four main steps: data cleaning and preprocessing, featurization, model fitting, and evaluation.[3] Taking supervised learning for example, a collection of labeled samples is input into the data set first with each sample described by measured biomarker signatures. Then the complied data set is segmented into a labeled "training dataset" for building the algorithm and a blinded "evaluation dataset" for the later assessment of its performance via a method called cross validation. Tuning algorithm-specific parameters is important if its output needs to be further improved.[21] The power of a machine learning algorithm is significantly determined by the composition of cohort selection, sample capacity, and data quality. So far, many publications have used ML to process the data for a more intelligent and precise analysis of EV biomarkers (Table 3).

For example, using ML-based data processing, our group has established a robust and translational lab-on-a-chip platform for the integrative analysis of the expression and proteolytic activity of matrix metalloproteinase 14 (MMP14) on EVs to monitor tumor progression and metastasis.[413] In this study, 22 plasma samples from breast cancer patients with stage 0 to III and eight age-matched noncancer controls were used as the training cohort, and the data was processed by LDA. Accurate classification of breast cancer including the ductal carcinoma *in situ*, invasive ductal carcinoma, locally metastatic breast cancer, and the age-matched controls was achieved with 96.7% accuracy in the training cohort (n = 30) and 92.9% accuracy in an independent validation cohort (n = 70), indicating that our platform could provide a useful tool for cancer diagnosis and the monitoring of tumor evolution in a liquid biopsy and, finally, realize personalized therapy. In the future, on-chip sample analysis throughput and detection multiplicity could be improved for a more labor-effective and comprehensive detection of diseases.

Though ML benefits the data analysis in clinical diagnostics, certain concerns, such as the ethical and legal issues[3] and the limited amount of specimens typically accessible owing to the time and cost involved with obtaining samples from clinics or animal models, need to be further addressed.[21]

CONCLUSION AND OUTLOOK

In this review, we surveyed recent advance in the development of new technologies for EV sample preparation, detection, and data processing. Many physical (e.g., acoustic,[240] thermal,[246] electrical,[242] hydro-mechanical,[232] and optical[378]) principles, functional molecules such as aptamers[417] and molecular beacons,[356] and engineering approaches such as microfluidics[132] have been adapted to improve our capabilities to characterize EVs and associated biomolecules. However, there are still some challenges for researchers to solve in the future. For example, EV samples from all kinds of body fluids need different processing procedures and the modularization of sample prepreparation devices that can be connected to the EV isolation platforms would benefit the integration and automation of the protocols for the POC testing and practical clinical use. In addition, microfluidic systems are faster, cheaper, and less reagent and sample-consuming compared with conventional approaches. However, improving the reproducibility of microfluidics is essential before it becomes a common clinical technique. Moreover, leveraging on the emerging analytical technologies and artificial intelligence methodologies, future efforts focused on the systems-level studies of the contents of EVs in real-world samples and EV-mediated cellular interactions would provide unprecedented insights in their biological functions and clinical value for cancer detection and therapeutic applications. Altogether, EV research is a rapidly emerging field and offers exciting opportunities for technology development and scientific discovery, which calls for multidisciplinary research across analytical sciences, engineering, molecular and cell biology, clinical sciences, and data science.

It is interesting to point out the technologies developed for EV analysis may find significant applications in other fields. The COVID-19 disease caused by the severe acute respiratory syndrome coronavirus (SARS-CoV-2) outbreak in 2019 and rapidly spread to result in a global pandemic. Sensitive and reliable detection of SARS-CoV-2 virus poses an enormous challenge to global public health. Besides the gold standard methods RT-qPCR, many state-of-art approaches based on isothermal amplification and CRISPR-Cas technologies have been developed to meet the pressing demand of POC testing.[418,419] There is a long-standing discussion on the relationships between EVs and viruses initially based on their similar biogenesis that involves the intracellular processes (e.g., endocytosis, endosomal pathway, and exocytosis) and some molecular machineries.[420,421] Recent advance in molecular and functional studies of EVs has revealed the interactions between EVs and viruses during viral infection[422] and their similarity in physical properties (e.g., shape, diameter, and buoyant density), biochemical composition, and biological functions.[11,423−425] Both EVs and coronaviruses have proteins and NAs inside protected by a lipid bilayer with various structural and functional proteins anchored,[11,426] which could be used as the biomarkers for capture and detection. Based on such similarity, it was hypothesized that the emerging technologies and methods designed originally for EV analysis can be inherently and rationally utilized for the SARS-CoV-2

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diagnosis. Moreover, because of the overlapping biogenesis pathways, coronaviruses may affect the cargo sorting process of exosomes, and the infected cells may release exosomes that contain viral components, such as human ACE2,[427] SARS-CoV-2 genome,[428] and even the virus assemblies,[429] suggesting a supportive role of EVs in SARS-CoV-2 virus internalization and reinfection/reactivation.[430] Logically, analyzing the virus-derived biomarkers in EVs could pave a way to decipher such a "Trojan horse" strategy of coronavirus infection.

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