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Unveiling the Complex World of Extracellular Vesicles: Novel Characterization Techniques and Manufacturing Considerations

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Extracellular vesicles (EVs) function as potent mediators of intercellular communication for many in vivo processes, contributing to both health and disease related conditions. Given their biological origins and diverse functionality from correspondingly unique "cargo" compositions, both endogenous and modified EVs are garnering attention as promising therapeutic modalities and vehicles for targeted therapeutic delivery applications. Their diversity in composition, however, has revealed a significant need for more comprehensive analytical-based characterization methods, and manufacturing processes that are consistent and scalable. In this review, we explore the dynamic landscape of EV research and development efforts, ranging from novel isolation approaches, to their analytical assessment through novel characterization techniques, and to their production by industrial-scale manufacturing process considerations. Expanding the horizon of these topics to EVs for in-human applications, we underscore the need for stringent development and adherence to Good Manufacturing Practice (GMP) guidelines. Wherein, the intricate interplay of raw materials, production in bioreactors, and isolation practices, along with analytical assessments compliant with the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines, in conjunction with reference standard materials, collectively pave the way for standardized and consistent GMP production processes.

Key Words: *Extracellular Vesicles; Bioreactors; Cell Communication*

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INTRODUCTION

Extracellular vesicles (EVs) are membrane-bound structures, released by cells into the extracellular space. There are three main EVs—exosomes, microvesicles, and apoptotic bodies. Exosomes receive the most attention because their relatively smaller particle size, 30-150 nm, makes them better suited structures for in vivo drug delivery applications.[1,2] Since exosomes also have been implicated in the pathogenesis of various diseases, including cancer, neurodegenerative disorders, and infectious diseases,[3,4] many studies aim to explore their usefulness as biomarkers for disease diagnosis and prognosis, as well as potential targets for therapeutic intervention of diseases.[5]

1. Exosome biogenesis

Exosome biogenesis initiates within the endosomal system, maturing from the late endosomes, also called multivesicular bodies (MVBs) that contain intraluminal vesicles (ILVs) with cargo molecules such as proteins and nucleic acids.[6] In order to let ILVs enter the MVBs,[7] late endosomes need to successively pass through four types of endosomal sorting complexes that are required for transport (ESCRT) 0-III, facilitating MVB formation, vesicle budding, and protein cargo sorting.[8] The fate of an exosome is determined by how an MVB fuses with a membrane, whether it be via the Ras-related 27 (Rab27) or Snap receptor (SNARE),[6,9] which are proteins responsible for expelling an exosome to the outside membrane. Without these interactions and ex-

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pulsions, MVBs simply get degraded as the endosomes transition into lysosomes. That is, the exosome vs. lysosome selection process is determined by the Rab proteins and their effectors that coordinate the consecutive stages of tethering events between a vesicle and its target membrane.[10]

2. Cellular uptake

Secreted EVs facilitate intercellular communication by transporting their cargo contents from the donor cells to the recipient cells, where they are taken up.[11] There are three major EV cellular uptake mechanisms, endocytosis, fusion, and receptor-ligand binding,[12,13] with the primary mechanism being endocytosis,[14] which includes pinocytosis, phagocytosis, and receptor-mediated endocytosis. Pinocytosis allows extracellular fluids and small solutes to enter the cell through an inward pinching-off of the plasma membrane, phagocytosis occurs through a similar process but enables larger solutes and solid particles to enter the cell.[15,16] Receptor-mediated endocytosis recognizes EVs through their cell surface receptors and the process ultimately transports them to early endosome structures.[17] EVs also can enter cells via fusion processes, diffusing across the membrane via a concentration gradient.[18] A third route is receptor binding—the recipient cells release specific signals to facilitate EV uptake upon ligand recognition.[19] The internalized EVs follow the endosomal pathway to be received by the nucleus or degraded in the lysosomes.[12]

3. Functions and applications

EVs facilitate intercellular communication across many bodily fluids, for example, blood, breast milk, urine, and such, which suggests a prominent but complicated role in normal physiological regulation and response processes, as well as in disease progression across different tissues.[20] EVs are also considered to be biomarkers,[21] drug delivery vehicles,[22,23] and immunomodulators.[24] For instance, immature dendritic cell-derived EVs may decrease the production of cytokine molecules involved in the regulation of T-cells that cause active tissue rejection, thereby enabling successful immune tolerance in organ transplantation.[25,26]

THERAPEUTIC APPLICATIONS

As their various functions, with underlying mechanisms of action, are becoming better characterized, it is increasingly evident that EVs can be viable treatment options for a variety of disease conditions. According to the data sourced from ClinicalTrials.gov (search bar: "other item," keyword: "exosomes," exclusion: "trials without FDA-defined phases"), the applications of exosome materials in clinical trials is growing. For example, in comparison to our previous summary of the clinical trial space in 2021,[27] the current number of cancer-related trials is still significantly large (21/59), as are trials investigating other conditions, such as diabetes mellitus, psoriasis, cutaneous ulcer, chondroma, sarcoma, and endocrine system diseases. Additionally, representing infectious disease treatment opportunities, the urgency of managing the pandemic afforded the opportunity for ten COVID-19 trial studies to get underway.

1. Sources for producing therapeutic EVs

Therapeutic EVs have been primarily produced using autologous (those secreted from the patient's own cells) and allogeneic (for example, plant-derived EVs) approaches. Although it is reasonable to emphasize using autologous EVs for human applications, since they appear to be more compatible with a given patient's immune system, they are effectively limited to that single patient.[28] Therefore, sourcing EVs for many patients, say from a single batch of material, appears to be best suited for allogenic sources. As will be discussed in the next sections, there are a number of potential allogeneic sources to consider.

1) Autologous EVs: Autologous EVs as therapeutics and as delivery vehicles, for example after the addition of a novel cargo like a cytotoxic chemotherapy agent, appears to have unique advantages, including minimizing potential immune responses that could lead to the neutralization of antibodies against a particular EV. Work by Li et al.[29] illustrated this potential advantage by utilizing autologous EVs loaded with gemcitabine, the first-line chemotherapeutic drug for pancreatic cancer in their study of mice with Panc-1 (the pancreatic cancer cell line xenografts). Based on tumor growth measurements, and levels of related biomarkers in the blood, and histopathological changes in organs, they demonstrated efficient delivery and drug accumulation at the tumor site without significant weight loss during or after treatment, indicating the potential efficacy and safety considerations of this type of approach. The work also highlights the potential power of using tumor-derived EVs as effective drug delivery vehicles that uniquely target the tumor microenvironment for enhanced therapeutic efficacy.

Other EV-based cancer immunotherapies[30] include the ongoing clinical trial NCT05559177 that focuses on development of personalized chimeric EV vaccines for bladder cancer patients by using tumor cells from the lesion site and the patient's dendritic cells or macrophages from peripheral blood. This particular study aims to assess the safety and tolerability of the vaccine through multiple administrations including an emphasis on range finding the maximum tolerated dose (MTD) and dose-limiting toxicity (DLT) range for future clinical trials and studies.

2) Allogeneic EVs: Allogeneic EVs, the EVs from donated sources, such as those isolated from blood contributed at blood-banks, are readily available and provide relatively plentiful numbers of EV particles, wherein single donors or pooling from multiple donors can be utilized. Depending on the intended use and indication, this type of EV sourcing model provides a more streamlined processing and analytical characterization package to supply multiple patients or the repeated dosing of a single patient, for example. Allogeneic EV productions have the potential to be scal-

able and mass-produced, again, enabling the treatment of many patients, and further reducing the need for individualized production processes.

However, in spite of resourcing advantages, allogeneic EVs potentially can be problematic in that their somewhat "foreign" composition may elicit an anti-drug immune response; albeit the extent of a response may depend on a given disease state.[31-33] Efforts to understand this potential challenge have primarily come from in vitro and in vivo studies in animal-based immunology models. In one such study with mice, researchers found that allogeneic exosomes could be captured by antigen-presenting cells (APCs) and that the APCs subsequently displayed donor major histocompatibility complex (MHC) molecules on their surfaces, consistent with allogenic materials being immunogenic.[33] The authors also showed that exosomes could directly bind to mice alloreactive T cells; that is, via T-cell receptors when the exosomes displayed both allogeneic MHC class II and costimulatory molecules. These direct T-cell binding interactions, however, did not stimulate an immune response in this in vitro setting unless the T-cells were also cultured with the APCs, suggesting a more complex process may be involved in triggering a full immune response to them. For example, when the exosomes were introduced to an in vivo setting, wherein the full complement of immune cells exist, they not so surprisingly activated the T cells, thereby sensitizing the mice to the exosome alloantigens, however, rather surprisingly, this only occurred when the exosomes were delivered in an inflammatory environment. More specifically, the researchers intraperitoneally administered allogeneic EVs from spleen antigen-presenting cells, either alone or in combination with complete Freund's adjuvant (a vehicle that stimulates an inflammatory response). Mice only exhibited inflammatory T cell responses when treated with both allogeneic EVs and the complete Freund's adjuvant. The authors surmised that innate inflammation, caused by the adjuvant, promoted donor MHC cross-dressing of antigen-presenting cells and subsequent activation of alloreactive-T cells. They further surmise that in the absence of inflammation, direct binding of the allogeneic exosomes to T cells could be suppressing the T cell response instead of actually activating it.

In spite of these potential immunogenic concerns, clinical trials designed to assess the optimal dosage and potential side effects for allogeneic EVs are progressing, and the investigations signify the heightened interest and therapeutic potential of allogeneic EVs as consistent treatment options. For example, trial NCT04173650, designed to evaluate the safety and effectiveness of AGLE-102, an EV product sourced from allogeneic normal donor mesenchymal stem cells (MSCs), is being evaluated for treating the lesions associated with Epidermolysis Bullosa (EB). Other likeminded investigations include dosage optimization and side effect profiles for a pancreatic cancer treatment (NCT03608631), and safety and efficacy considerations in their treatments of COVID-19 (NCT04798716 and NCT05116761), osteoarthritis (NCT05060107), and Alzheimer's disease (NCT04388982).

3) Plant-derived EVs: Edible-plants are also potential sources for exosome-like nanoparticles, termed edible -pant derived exosome-like nano particles (EPDENs). They share structural and functional similarities with human and other animal derived EVs, and EPDENs from different sources can have different biological effects, which may facilitate their unique applications.[34] For example, fruit-derived EPDENs effectively modulated Wnt/T-cell factor 4 (TCF4),[34] a critical factor in gut homeostasis and immune tolerance, and the ginger-derived EPDENs activated nuclear factor-erythroid factor 2-related factor 2 (Nrf2),[34] a key regulator of the HO1 gene in macrophages, leading to anti-inflammatory and antioxidative responses. These findings suggest that EPDENs from different plant sources can communicate with mammalian cells in the gut, particularly intestinal macrophages and stem cells.

Despite the number of studies that have demonstrated EPDENs can reduce inflammation,[35,36] influence the healing process,[37] and support the growth of beneficial intestinal microbiota,[38] clinical trials using EPDENs are not yet common, but interesting investigations are in the works.[39] For example, Trial NCT01668849 evaluates the ability of grape EVs, given to the subjects as grape powder, to reduce the incidence of oral mucositis during radiation and chemotherapy treatment for head and neck tumors. Another trial, NCT04879810, focuses on comparing the symptoms of inflammatory bowel disease in patients treated with either EVs alone or combined with curcumin (NCT04879810). The study utilizes incidence of blood in stool to validate the sample size. By combining ginger EVs with curcumin, they aim to reduce symptoms by at least 30% compared to using curcumin alone. In spite of these efforts, there currently is no comprehensive database detailing the components of EPDENs obtained from the various sources. As each plant type apparently has unique molecular and mechanistic characteristics, a classification system of their makeups seems warranted.

MANUFACTURING

Manufacturing any biological therapeutic, biological delivery vehicle, or such for human administration certainly comes with significant concerns around the safety and efficacy of the materials being administered. Notably, the produced materials are expected to have batch-to-batch consistency, and validated testing procedures that are performed to illustrate production uniformity in terms of safety and efficacy. Although EVs have proven their versatility and capacity for consistent drug delivery applications, no matter their source, they have an intrinsic heterogeneity (non-uniformity) in structure and content, in that a pool of EVs, derived from the same source, do not all have identical molecular compositions and sizes. It may be that some level of heterogeneity is significant to a respective mode of action, and that needs to be assessed, but alternatively some

compositions may actually detract from the desired functionality of the pool of compositions and sizes. Consequently, achieving some level of uniformity in content and control around reproducibility of different batches is desired. What constitutes uniformity and quality in the production of EVs, therefore remains a challenge to assess. However, because of the intrinsic heterogeneity of EVs, the manufacturing and purification processes can significantly influence EV quality,[40] building a consistent manufacturing process for their uniform production is necessary to pursue. Robust processes also are needed to facilitate pre-clinical development activities too. With these considerations in mind, the ensuing discussion will focus on good manufacturing practices, bioreactors, Minimal Information for Studies of Extracellular Vesicles (MISEV), raw materials, and reference materials (or standards) for assisting manufacturing.

1. Good manufacturing practice

Manufacturing materials that are intended for human administration, at an industrial scale, requires adherence to Good Manufacturing Practices (GMP), which are established in the various guidances of respective international regulatory agencies and relevant International Council for Harmonisation (ICH) guidelines. GMP practices ensure that pharmaceutical products like EVs are consistently produced and controlled according to quality standards. GMP regulations[41] cover all aspects of the manufacturing process, including facility design and operation, personnel training, equipment calibration and maintenance, documentation, quality control testing, and product release.[42]

To comply with GMP guidelines, manufacturers need to implement measures to reduce the risk of contamination, processing and analytical characterization measurement errors, and other issues that can affect safety and efficacy. GMP for EVs covers three key areas: upstream (the cell cultivation process), downstream (the purification process), and quality control. Some specific requirements include

- GMP-compliant cell culture systems,[43] such as serum-free or xeno-free media, to minimize the risk of contamination or variability;
- Validated and standardized methods for EV isolation (e.g., ultrafiltration);[44]
- Extensive characterization and quality control testing of the EV product—particle size, concentration, purity, identity of the origin, potency, and safety;
- Documenting all aspects of the manufacturing process and quality control testing, and maintaining a comprehensive record-keeping system; and
- A robust quality management system, including procedures for deviation and complaint handling, change control, and risk assessment.[42]

2. Bioreactor for EV production

EVs are secreted in limited numbers from cells into the culture media, consequently, bioreactors are widely used to scale up their numbers.[43,45] The general approach is to provide a controlled and scalable environment for cell cultivation that facilitates EV secretion, as well as that bioreactors are straightforward to operate in compliance with GMP guidelines.[46] From a technical perspective, there are dynamic monitoring systems that provide real-time control of critical parameters,[45] such as temperature, pH, oxygen, and nutrient levels, thus ensuring consistent and reproducible production. The automatability of bioreactors provides for advantageous labor costs and productivity considerations, and their self-containment minimizes potential contamination risks. In addition, bioreactor manufacturing processes are typically scalable and reproducible in a manner that can support the potential increases in production needs that often come with growing clinical demands in the run up to commercial scale launch.

3. Minimal information for studies of extracellular vesicles (MISEV)

EVs are generally characterized by the presence of transmembrane/lipid-bound proteins and cytosolic proteins, which are often involved in cargo sorting, membrane fusion, and endocytic pathways. MISEV is a set of guidelines developed to standardize the reporting of EV research, and provides a framework for reporting key aspects of EV research, including isolation, characterization, and functional analysis. For example, positive and negative protein markers have been endorsed and are organized into four categories:[47] (1) transmembrane or lipid-bound EV protein, (2) EV cytosolic protein, (3) intracellular but not associated with the plasma membrane or endosomes (i.e., relatively less abundant in exosomal EVs than in cells), and (4) extracellular but not typically EV-associated proteins. Additionally, EVs should be characterized using at least one negative protein marker that is not typically found in EVs to exclude non-EV contaminants during isolation and analysis. These negative markers include apolipoproteins A1/2, B (APOA1/2, APOB), and albumin (ALB).[48-50] These general characteristics are important for understanding and isolating EVs for downstream applications, including biomarker discovery and therapeutic development.

4. EVs derived from different raw materials

When considering the use of EVs for therapeutic purposes, it is important to recognize the source and quality of raw materials, which directly impact the EV size, shape, composition, and biological activity. EVs can be categorized by their raw material sources[51,52] as conventional (human/mammal) and non-conventional (non-human/non-mammal). Non-conventional EVs can be further subdivided into plant-derived and animal product-derived—bacteria, fungi, and parasite. EVs from different raw materials, which exhibit different protein and RNA profiles, may impact their functions and potential applications. For example, EVs isolated from the milk of lactating women had a unique RNA profile,[53] including high levels of miRNAs involved in immune regulation and infant development.[54] MSC-derived EVs have been found to have immunomo-

dulatory and regenerative properties primarily through their interaction with immune cells,[55] making them potential candidates for therapeutic use in inflammatory diseases or tissue repair. On the other hand, tumor-derive EVs may contain oncoproteins and promote tumor growth and metastasis. In addition to the cell culture media, common raw materials for production include milk,[56] algae,[56] and plants,[57] which can be processed using ultracentrifugation, precipitation, ultrafiltration, and size exclusion chromatography.[40] The recovery yields for milk and algae are ca. 10^9-10^{11} EVs/mL.[58,59] The yields vary for different plant species[57] and cell types as well as their growth conditions.[43-45] Overall, it is important to carefully consider the raw materials when designing EV production processes.

5. Reference materials/standards

Reference materials are essential for validating analytical methods, inter-laboratory comparisons, and quality control in research. EV reference materials are characterized by their known size, concentration, and composition.[60] They can be produced synthetically or by harvesting from a variety of unique but defined sources, including cell culture, blood, and urine.[61] However, the production requires careful optimization of the isolation protocols to ensure the reproducibility and stability of the material.[41] The International Society for Extracellular Vesicles (ISEV) has recently established guidelines[47] for the production and validation of EV reference materials, including recommendations for the characterization of their size, concentration, and composition using techniques such as nanoparticle tracking analysis, transmission electron microscopy, and mass spectrometry. These guidelines aim to facilitate the development of standardized and reliable reference materials for EV research and clinical applications. Table 1 summarizes some of the commonly utilized EV reference materials. Liposomes,[62] silica,[63] and polystyrene[63] beads are commonly used as standards to calibrate vesicle flow cytometry (VFC) analysis via fluorescence for estimating the EV particle size and concentration.[64] Additionally, noisome,[65] a revised version of the liposome with similar bilayer lipid structure, nano-dimension size, and refractive index can also be used in VFC analysis. Compared to liposomes, niosomes are made with non-ionic surfactants and cholesterol instead of phospholipids. Niosomes are often more stable and might be easier to manufacture. Hollow organosilica beads (Hobs)[66] are also used for size gating in flow cytometry investigations. The trackable recombinant EV (rEV),[61] based on HIV-1 virus particles, enables the quantification and tracking of EVs in vitro and in vivo. This is achieved through using the gag-EGFP fusion protein, allowing sensitive and distinct tracking of rEVs.

ISOLATION

Over the past decade, remarkable progress has been made in the development of EV isolation technologies, including ultracentrifugation,[67] ultrafiltration,[68] precipitation,[69] immunoaffinity capture,[70] and size-exclusion chromatography.[71]

- Ultracentrifugation is the gold standard and is effectively used in 80% of EV isolation processing operations.[72] The method separates sample components based on density and does not require elaborate sample preparation and is inexpensive, except for the initial instrumentation costs. However, the approach is time-consuming (2 hours-days) and achieves only moderate purity, ca. 10^7-10^9 particles/μg protein).[70,72,73]
- Ultrafiltration utilizes membranes with extremely small pores (~ 100 nm diameter) to isolate EVs. The method is rapid (seconds-30 minutes/filtration),[74] and allows for high throughput operations. Depending on the demands, the throughput of ultrafiltration can reach up to hundreds of liters per day. However, the applied pressure in filtering the materials can damage EVs via shear stress, and result in losses due to membrane adhesion and membrane blockage from the accumulation of particles.[74] Therefore, the yield is compromised in this approach.
- Precipitation methods use volume-excluding hydro-

TABLE 1. Reference materials for EV characterizations

Name	Properties	Characterizations	Synthesis	Ref.
Liposomes	Particle size	FS, NTA, FC, FFC, DLS, TEM, DSC, NMR	Thin-film, injection, and emulsification	62
Silica nanoparticle	Particle size and concentration	NTA, FC	Stöber, sol-gel	63
Polystyrene nanoparticles	Particle size and concentration	NTA, FC	Emulsion polymerization, seed-mediated growth	63
Niosome	Particle size, concentration, and proteins	DLS, NTA, TEM, FC, FTIR, DSC	Thin-film, and fluorescent labeling	65
Hollow organosilica beads	Particle size and concentration	TEM, FC, NTA, TRPS, MRPS, DLS, and SAXS	Hard template sol-gel method	66
Recombinant EV (rEV)	Particle size, and concentration	VFC, NTA, ELISA, western blot	Endosomal sorting complex required for transport (ESCRT) pathway	61

philic polymers, such as polyethylene glycol (PEG), and dextran, which sequester water molecules to force less soluble components to phase separate.[75] For example, biological materials, including EVs and proteins, are excluded from the solvent regions occupied by the polymers and are concentrated until their solubility is exceeded, at which point precipitation occurs. This method results in higher yields, but in significantly lower purity. Compared to ultracentrifugation, the precipitation can only achieve $<10\%$ of the respective purity value.[76]

- Immunoaffinity isolation targets surface markers via the antibodies immobilized on substrates such as magnetic beads, chromatography column resins, etc.[77] The approach can achieve a higher purity than the aforementioned methods that use intrinsic physical properties (e.g., particle size). But immunoaffinity capture is limited by antibody availability and an exhibited lower capacity, 0.5-3 mL specimens.[70] Furthermore, a long incubation time (e.g., 12 hours) might be needed. For instance, the Dynabeads (Thermo Fisher) protocol requires two 12-hour incubation periods—one for conjugating the antibodies and another for bead capture.[76] The need for these extended incubation periods stems from the large bead size (≥ 1.0 μm) used in the technology,[78] wherein the low intrinsic solution mobility (poor diffusion) of the beads along with their low surface area-to-volume ratio leads to slow binding and assembly interactions.[79]
- Size-exclusion chromatography (SEC) separates biomolecules based on differences in their hydrodynamic radius as they pass through an unreactive, low-adsorption resin consisting of a porous matrix of beads packed in a column.[80] SEC preserves vesicle integrity and biological activity and results in high yield. However, the approach also leads to significant dilution, notably if they are viscous samples, so an additional enrichment step is often required before their next handling activity.[27]

1. Novel isolation approaches

Despite the availability of various isolation methods, large scale EV manufacturing operations still experience some significant drawbacks. For example, the processing volume may vary depending on the quality of the starting material and the desired purity and yield of the EVs.[72,73] As such, it is essential to optimize the isolation process and validate its scalability to ensure consistent and reliable production at the intended manufacturing scale.[81] Therefore, new approaches have been developed to address the various method limitations. Three specific methods (Table 2)[82-84] that have either been successfully implemented on a large scale or have the potential to be scaled up for manufacturing processes are worth further deliberation.

1) Super absorbent polymer beads: Super absorbent polymer (SAP) beads[72] utilize EV size differences as the mode of isolation. SAPs are hydrogels with the ability to absorb water weighing several hundred to thousand times their dry weight value.[85] These water absorbing materials have been successfully used for diapers, hygienic products, and in the agriculture and food industry for protection and storage purposes.[86] SAPs absorb water through physical water entrapment via capillary forces and osmosis, which is driven by counter ions attached to the polymer.[87,88] The SAP water channel is 5 nm in diameter,[76] and the beads exclude/concentrate EVs that are larger than these nanoscale channels by absorbing water as well as molecules (e.g., proteins). It has been demonstrated that the SAP beads increased EV concentration 2-fold, from 1.38 to 2.61×10^8 particles/μg protein.[87]

2) Nanoporous membrane chip: Nanopourous membrane chips are designed to isolate EVs based on their size dependency, a function of their respective diffusion coefficients and sedimentation velocities.[83] Bigger particles have greater sedimentation velocities than smaller particles, but the diffusion rates of the smaller particles are faster than for the bigger particles.[89] The nanoporous polycarbonate membrane acts as a filter, smaller particles as well as EVs can pass through the membrane via faster diffusion (no sedimentation) while particles larger than the pores cannot pass, resulting in them settling at the bottom of the inlet chamber. The chip has demonstrated EV isolation from human serum (ca. 150 μL), leading to strong signal for both syntenin and CD63 (western blot). The control experiment via ultracentrifugation showed no signal for the target analytes because the loading amount of 150 μL was not enough. The approach doesn't require extensive user training, so it can be easily used for isolating EVs from various samples. However, an additional pre-processing step is needed to remove the soluble proteins that are smaller than the EVs.

3) Asymmetric depth-filtration: Asymmetric depth-fil-

TABLE 2. Novel EV isolation methods

Method	Mechanism	Specimen	Scale, mL	Yield	Purity, EVs/μg protein	Ref.
Super absorbent polymer beads (SAP)	Particle size	Culture media, urine	12	High (50-70%)	$\sim4\times10^8$	82
Nanoporous membrane chip	Particle size	Culture media, whole blood	≥0.5	High (42%)	Unknown	83
Asymmetric depth-filtration	Particle size and elasticity	Culture media, plasma, urine	10	High (51%)	$\sim10^{10}$	84

tration utilizes pores with tortuous geometry to facilitate isolation.[84] The method immobilizes EVs on the surface and within the depth of the porous medium, and then recovers them by reversing the carrier flow through the filter. This approach is applicable for complex biological fluids like plasma, and can be performed in a single step, and can be scaled up by concurrently processing multiple centrifuge tubes, up to the rotor's capacity. However, harvesting significant quantities of therapeutic EVs from large volumes of growth medium requires purpose-built centrifugation equipment or the use of displacement or pressure-driven flows perpendicular or tangential to the depth-filtration (DF) medium to achieve a higher throughput.[90] A useful feature is that the DF cartridge can be modified to accommodate smaller specimen volume, ca. 5 mL, for the diagnostic applications, for example. Overall, asymmetric depth-filtration provides a simple, fast, and cost-effective workflow for isolating EVs from small biological samples, such as for biomarker detection, and the scalability can enable larger scale manufacturing processes.[84]

EXTRACELLULAR VESICLE CHARACTERIZATION

In addition to commonly utilized characterizations methods, such as nanoparticle tracking analysis (NTA), western blots, flow cytometry, and mass spectrometry,[91] MISEV also asks for inclusion of single vesicle analysis methods, such as electron microscopy imaging, and atomic force microscopy (AFM).[47,92] The importance of characterization approaches, at single vesicle resolution, stems from the high level of heterogeneity with EVs, which is attributed to their intrinsic content, recipient cells, and cellular origins. Many studies have shown that under a given set of conditions, some types of single-cell forms can secrete a highly diverse population of vesicles in a rather short period of time. For example, Palma et al.[93] found a variety of unique vesicles, containing different miRNAs, being produced from malignant breast cancer cells, however, benign epithelial cells did not show this diversity. Some studies also have indicated that bulk analysis approaches can identify the presence of DNA in some EV subsets; however, the results have been contentious because EV-DNA cannot easily be distinguished from cellular-free-DNA, which includes DNA associated with non-vesicular particles that may co-isolate with EVs.[94] Although different mechanisms of EV biogenesis have been reported[95] the actual manner in which the different mechanistic processes are used or how they are regulated within a single cell, remain largely unknown. As a result, the mechanisms behind the production of heterogeneous distribution of EVs is currently limited, which further emphasizes the crucial need for obtaining precise and highly quantitative characterization data for EVs at the individual vesicle level. Albeit, single vesicle level analysis provides more comprehensive information via higher resolution information, these assays have limitations, such as having low-throughput, being time-consuming and complicated procedures, and often coming with poor reproducibility.[96]

A few emerging single vesicle analytical techniques that show promise in mitigating some of the method limitations, summarized in Table 3,[97-101] are worth discussing. Each method shows various cargo targets, sample preprocessing, a minimum of volume loading and fundamental technique.

1. Novel single vesicle analysis approaches

1) Quantitative single-molecule localization microscopy: Single-molecule localization microscopy (SMLM), also called super-resolution microscopy (SRM), has high spatial resolution compared to traditional microscopy techniques, which exhibit limited spatial resolution (ca. half

TABLE 3. Single vesicle characterizations

Method	Working principle	Pre-processing	Specimen, volume, LoD	Particle size, nm	Surface marker	Cargo molecule	Ref.
Quantitative single-molecule localization microscopy (qSMLM)	Fluorescence- switchable imaging	Size-exclusion chromatography	Plasma, 200 μL	10	CD63	TSG101, CA 19-9	97
Single-molecule nanoscale flow analyzer	Time-dependent fluorescence with high sampling rate (10 kHz)	Size-exclusion chromatography, ultracentrifugation	Semen, 10 μL	35-300	CD63	N/A	98
isExoCD (in situ exosome concentration and detection)	Enzyme-free amplification (CHA), fluorescence	Ultracentrifugation	Culture media, 10 μL, 10^6 EV/mL	30-150	CD63, CD81	microRNA (e.g., miR-21)	99
Droplet-based extracellular vesicle analysis (DEVA)	By encoding the droplet through correlation-based detection among neighboring droplets	Immunoaffinity magnetic separation	Culture media, ≤10 μL, 10^4 EV/mL	50-300	CD81	N/A	100
ExoView	Interferometric reflectance imaging	Ultracentrifugation	Culture media, 35 μL, 10^6 EV/mL	≥50	CD63, CD81, CD9	Protein (e.g., PD-L1)	101

wavelength of light) due to the diffraction barrier.[102] The image from lens-based microscopy, for example, is a point spread function (PSF) rather than a spot with high spatial resolution, resulting in a blurry image. To avoid this PSF issue, separate fluorescent emission signals are processed in time by switching between active 'ON' or inactive 'OFF' states. Fluorophores for SMLM can be divided into several types: photoswitchable, photoactivatable, photoconvertible, and spontaneously blinking. For the optics microscopy, SMLM requires high flame rates to achieve the highest accuracy and minimize acquisition time. Scientific complementary metal oxide semiconductor (sCMOS) cameras have become major contributing cameras to the microscopy methodologies because of their high quantum efficiency and low noise characteristics. Lennon et al. utilized SMLM to quantitate a single vesicle from pancreatic ductal epithelial cells with ca. 10 nm accuracy and determine its biomarker content. Nizamudeen et al.[103] accurately applied DiD dye-based super resolution microscopy to characterize all the lipid-based structures within an EV isolate from mouse mesenchymal stem cells. Verta et al.[104] characterized EVs that expressed the SARS-CoV-2 spike protein on their surface, by SRM. That analysis showed a distribution of spike proteins with a tetraspanin (CD9, CD63 and CD81) co-expression ratio, and using SRM, they confirmed EV size and particle counts.

2) Single-molecule nanoscale flow analyzer: Traditional flow cytometry is a laser-based technology for measuring a particle's physical and chemical properties. The approach has recently made some significant methodology and technology improvements towards characterizing EVs. For example, the technique of mass cytometry uses heavy metal ion-labels to quantitate a high level of multiplexed cellular components,[105] whereas the more common flow cytometry methods use fluorophores to achieve a relatively high sensitivity, and combined with microfluid technologies it can perform high throughput types of analyses. The single-molecule nanoscale flow analyzer, is another high-resolution device. It is used to measure the properties of individual molecules in a liquid that is flowing through a nanoscale channel. The technology has been shown to be a sensitive means of precisely and sensitively detecting various biomolecules, such as DNA and proteins. Andronico et al.[98] demonstrated that the nanoscale flow analyzer and flow sorter could sensitively analyze and sort individual EVs from semen samples, after initial isolation by centrifugation and size exclusion chromatographic methods.

3) In situ exosome concentration and detection, isExoCD: Recent advances in microfluidic technologies have led to platforms that combine isolation and detection approaches enabling analyses focused on pre-concentrated specimens, especially those in in situ conditions. For miRNA analysis, reverse transcriptase real-time quantitative PCR (qRT-PCR) is considered to be the gold standard approach.[106] However, the method requires complicated procedures, well-trained analysts, high-end instrumentation and bioinformatic computational tools.[107] Consequently, Qian et al.[99] developed a simpler, more routine based alternative approach that uses a rapid agarose-based microfluidic chip with enzyme-free catalyzed hairpin assembly (CHA) strategy. This approach uses a Hairpin Probe 1 (HP1) to hybridize with the target miRNA that is present in the EVs, generating a single-stranded sticky end that subsequently hybridizes with Hairpin Probe 2 (HP2) to produce a double-stranded DNA (dsDNA) complex. After release, the miRNA can initiate the formation of multiple HP complexes, leading to a high level of sensitivity in the subsequent analysis, with a limit of detection (LOD) of ~10^6 EV/mL.

4) Droplet-based extracellular vesicle analysis, DEVA: Some biomarkers in biofluid samples only exist at very dilute protein content levels (e.g., those in urine or saliva).[108] Therefore, digital-based approaches have been introduced to help the quantitative analytical analysis, such as in the limit of detection. Yang et al.[100] developed a droplet-based optofluidic platform to quantify specific individual EV subpopulations, and in a high throughput manner. The approach takes advantage of there being multiple proteins of interest on targeted EV surfaces that can be specifically captured and labeled by microbeads. The beads are encapsulated in droplets, which minimize background signal noise, and then analyzed using fluorescence microscopy. By counting the number of fluorescent beads in each droplet, the number of target EVs can be quantified with high sensitivity and specificity. The detection cameras used in the platform can record signals from the microfluidic droplets in a high throughput manner (~20 million droplets/min) and with high sensitivity (LOD=9 EVs/μL).

5) ExoView: The ExoView platform, based on the so called single particle interferometric reflectance imaging sensor (SP-IRIS) and microarray,[101] is a technology which enhances nanoparticle scatter signals by the application of extra layered substrates (normally applied silicon-oxide). Various studies have demonstrated that the SP-IRIS can effectively perform comprehensive high throughput analyses.[109] Tetraspanin proteins that are expressed on the surface of vesicles, which are generally considered to be unique markers on EVs, can be specifically labeled by antibodies targeted to a given isoform of the proteins. This specific labeling approach allows for SP-IRIS based analyses to be performed down to the single vesicle level. Although, different cell types contain different amounts of the tetraspanin isoforms.[110] The Exoview platform has demonstrated that it can characterize different ratios of these surface markers, allowing for differentiation of the small subsets of EVs populations produced by cancer cells, for example.[101] Historically, flow cytometry and SP-IRIS methods have produced inconsistent results between them, but Mizenko et al. have indicated that this lack of consistency may be due to differences in the sensitivities of fluorophores used in their respective approaches. In comparison to high throughput flow cytometry, the SP-IRIS approach has a much lower limit of detection, with a lower limit value approaching ~50 nm, without issues from the diffraction limit.

CONCLUSION

Extracellular vesicles (EVs) have gained prominence as critical mediators of intercellular communication, potential tools for diagnostics, and as therapeutic modalities. However, several challenges must be addressed to harness their full potentials in these uses. Isolating EVs from complex biological samples is a fundamental step toward any of their use, with separation methods, such as ultracentrifugation, ultrafiltration, and immunoaffinity capture, being amongst the most widely used ones. Recent innovations in isolation methods, including super absorbent polymer beads, nanoporous membrane chips, and asymmetric depth-filtration, offer improved scalability and efficiency for large-scale EV production. Ensuring standardized manufacturing practices through adherence to Good Manufacturing Practice (GMP) guidelines, utilization of bioreactors, and careful selection of raw materials is essential to guarantee consistent EV quality. Characterization techniques, such as quantitative single-molecule localization microscopy, nanoscale flow analyzers, and droplet-based analysis methods, are enabling the study of EV heterogeneity at the individual vesicle level. The significance of adhering to the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines for comprehensive reporting is highlighted. This review provides a comprehensive overview of the current state of EV research, offering insights into isolation, manufacturing, characterization, and standardization strategies that will drive the future of EV-based diagnostics and therapeutics.

CONFLICT OF INTEREST STATEMENT

None declared.

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