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Unveiling Heterogeneity: Innovations and Challenges in Single-Vesicle Analysis for Clinical Translation

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ABSTRACT

Extracellular vesicles (EVs) are key mediators of intercellular communication, carrying diverse molecular cargo that reflects the dynamic physiological and pathological state of their source cell. While analyses of the entire vesicular population (bulk EV) have advanced our understanding of their roles in health and disease, these approaches often obscure the heterogeneity inherent in EV populations. Emerging single-vesicle analysis technologies offer unprecedented resolution, enabling the identification of individual EV subpopulations and their distinct molecular signatures. Such approaches, combined with digital platforms, can now analyze individual molecules from single EVs, including single-molecule features such as protein, mRNA, double-stranded DNA and single-stranded DNA. This perspective explores the transformative potential of single EV technologies in clinical diagnostics and therapeutic applications. We highlight key advancements including microfluidic platforms, super-resolution microscopy and AI-driven data analyses, that are shaping and advancing the field and its applications. With the development and advancement of clinically viable single EV technologies, we are beginning to appreciate the complexity and abundance of cell type and specific EVs. We further discussed the challenges of sensitivity, specificity, standardization and scalability hindering these technologies' broad acceptance and feasibility in clinical translation. This perspective paper originates from discussions at the Chinese Society of Extracellular Vesicles (CSEV) annual meeting, held in Guangzhou, China, on 16 November 2024. At this meeting, researchers from

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various fields of EV research, with a particular emphasis on single EV digital, analytical and quantitative technological platforms, discussed the opportunities and challenges of this emerging single-EV-focused technology. The paper aims to provide a roadmap for integrating single EV technologies into routine EV-research and even clinical practice, paving the way for novel scientific and diagnostic tools, personalized therapies, and a deeper understanding of EV heterogeneity and EV biology.

1 | Introduction

Extracellular vesicles (EVs) are nanoscale particles secreted by nearly all cell types, playing vital roles in intercellular communication by transporting bioactive molecules such as proteins, lipids and nucleic acids (Kalluri and LeBleu 2020; Kowal et al. 2014; Colombo et al. 2014; Zhang et al. 2019). Based on size, EVs can be categorized into small EVs (typically < 200 nm in diameter) and large EVs (usually > 200 nm in diameter) (Welsh et al. 2024). Small EV populations include both small ectosomes and endosomally-derived exosomes. While exosomes are defined by their origin from the endosomal system, ectosomes are derived from the plasma membrane, with each term reflecting the distinct biogenetic pathways of these vesicles. These vesicles reflect their parent cells' physiological and pathological states, making them invaluable for understanding cellular behaviour, extracellular signalling and insights into changes associated with cell phenotype (i.e., disease) (van Niel et al. 2018; Anastasiadou and Slack 2014; Zhang et al. 2021). However, EVs exhibit remarkable heterogeneity in size, composition and molecular cargo, even when originating from the same cell type (Colombo et al. 2013; Willms et al. 2016). This molecular and functional heterogeneity underscores the importance of single EV analysis, allowing researchers to explore the unique roles of individual vesicles often obscured in bulk analyses (Huang et al. 2020).

Single EV studies are transformative, enabling researchers to uncover the inherent heterogeneity of EVs and identify subpopulations with distinct molecular cargo and biological functions, often obscured in holistic analyses of the entire EV population (Ferguson et al. 2022; Lee et al. 2018; Nikoloff et al. 2021). By revealing individual vesicles and their select subsets involved in critical processes such as cancer metastasis, immune modulation and neurodegenerative disease progression, single EV studies provide unique insights into precise roles of these vesicles in health and disease (Ferguson et al. 2022; Marar et al. 2021; Hill 2019). Leveraging advanced technologies like super-resolution microscopy, nanoflow cytometry and single-vesicle sequencing, researchers can achieve high-resolution profiling of EV cargo and surface markers, allowing the detection of rare biomarker-positive EVs and detailed characterization of subpopulations (Zhang et al. 2023; Lozano-Andrés et al. 2023; Kim et al. 2020). These capabilities are pivotal for early disease detection, therapeutic monitoring and understanding intercellular communication, driving innovation in diagnostics, personalized medicine and targeted therapies while advancing our fundamental knowledge of EV biology; namely their origin, composition, trafficking and target cell interaction.

The ability to delineate EV subpopulations advances our understanding of their distinct biological functions and avenues for transformative opportunities in personalized medicine, diagnostics and targeted therapies (Ji et al. 2019). In cancer diagnostics, single EV profiling enables the detection of rare, tumour-derived vesicles in liquid biopsies, enabling the capacity to detect early-stage, small-sized tumours with prognostic accuracy (Liu et al. 2024; Zhou et al. 2020). Tracking disease progression is another key application, as changes in EV cargo can serve as dynamic biomarkers for monitoring therapeutic efficacy or disease relapse (Hinestrosa et al. 2022; Tian et al. 2021). Furthermore, characterizing EV subpopulations in regenerative medicine facilitates the development of vesicle-based therapeutic agents with targeted functions, such as tissue repair or immune regulation.

The collaborative discussions at the Chinese Society of Extracellular Vesicles (CSEV) annual meeting inspired the development of this perspective paper, which captures key insights, highlights emerging trends, identifies critical knowledge gaps and outlines potential directions for advancing single EV analysis. This paper aims to provide a comprehensive perspective on single EV analysis technologies' current state and future potential. It explores recent advances in capture, detection and profiling methods of individual vesicles, discusses their applications in research and clinical settings, and addresses challenges that hinder their broader adoption. By synthesizing these insights, we envisage to inspire innovation and collaboration in the field, ultimately advancing the integration of single EV technologies into biomedical research and healthcare applications.

2 | Advances in Single EV Technologies

While our extensive knowledge of EV biology has traditionally centred around bulk or heterogeneous analyses, recent advancements have underscored the importance of understanding the complexity within EV populations at an individual scale. Bulk analyses have provided invaluable insights into the general characteristics, cargo, biogenesis, tropism and functions of EVs (van Niel et al. 2018; Shah et al. 2018). However, such approaches often mask the distinct subpopulations of EVs (Dixson et al. 2023), each with distinct biogenesis pathways (Mathieu et al. 2019; Ostrowski et al. 2010) (e.g., ESCRT-dependent/independent mechanisms (Ferreira et al. 2022; Trajkovic et al. 2008; van Niel et al. 2011; Baietti et al. 2012; Matsui et al. 2021; Marie et al. 2023)), molecular compositions (e.g., small/large EVs (Jeppesen et al. 2019, 2025, 2023), EV surfaceome composition (Rai et al. 2021) and EV subpopulations (Rai et al. 2021; Kowal et al. 2016; Crescitelli et al. 2021; Karimi et al. 2022; Mizenko et al. 2021), and functional roles (Basso et al. 2024; Wang et al. 2023)). This limitation can obscure rare and specialized vesicle subpopulations crucial for cell-specific or regulatory processes like metastasis, immune response or resistance to treatments (reviewed by Carney et al. (2025)). Emerging single EV technologies are poised to address these challenges by offering unparalleled detail about individual

TABLE 1 | Conventional technologies for EV analysis.

Technique	Resolution	Notes
Nanoparticle Tracking Analysis (NTA) (Dragovic et al. 2011)	~50 nM	Tracks Brownian motion to estimate size and concentration; lacks molecular specificity
Dynamic Light Scattering (DLS) (Caputo et al. 2021)	Effective for particles >100 nm; poor resolution in polydisperse samples	Measures size distribution based on light scattering; biased by larger particles
Conventional Flow Cytometry (Görgens et al. 2019, Navarro et al. 2024)	>300 nm	High-throughput analysis; limited sensitivity for small EVs without enhancement
Western Blotting (Kowal et al. 2016)	Detects specific proteins; semi-quantitative	Commonly used for EV marker validation; not suitable for single EV analysis

vesicles versus traditional technologies (Tables 1 and 2) (Banijamali et al. 2022; Bordanaba-Florit et al. 2021; Lennon et al. 2019; Spitzberg et al. 2023). Single EVs can be analyzed using both low- and high-throughput approaches to detect molecular profiles for each vesicle (Zhou et al. 2020).

There is immense interest in understanding EVs at high resolution, particularly for their diagnostic potential. Single EV analysis holds immense potential for enhancing diagnostic sensitivity and specificity, particularly through high-resolution profiling of select EVs relative to a broader EV population (Li et al. 2022). Recent advancements in multiplexed measurements have led to new understanding in the inherent heterogeneity of putative tumour cell-associated proteins within EVs (Ferguson et al. 2022). Advances in EV multiplexing tools (i.e., combining rapid, multiplexed and highly sensitive EV-based cancer biomarker analysis) have led to new fundamental insights in EV biology and heterogeneity and the potential for increasing diagnostic specificity (Spitzberg et al. 2023; Ferguson et al. 2022; Reynolds et al. 2023). These technologies enable researchers to identify cells of origin and the frequency of mutated onco/tumour suppressor proteins within single EVs. By enriching and isolating vesicles in this individual manner, combined with high resolution molecular profiling, provides new knowledge in understanding cell type-specific EVs with greater resolution. This provides a detailed view that has challenged the field to enable comprehensive and accurate analysis of EVs.

2.1 | Advances in Isolation and Enrichment Technologies

Microfluidic technology has emerged as a pivotal advancement in single EV analysis, offering high-throughput platforms capable of processing minimal sample volumes (~20 μL) at cohort-level scale (Bordanaba-Florit et al. 2021). These platforms enable precise manipulation of fluids at the microscale, allowing for the efficient isolation of EVs from blood, urine or cerebrospinal fluid (Kang et al. 2020) (reviewed by Sun et al. 2025). High-throughput microfluidic systems are designed to separate EVs from other contaminants or particles, such as lipoproteins or protein aggregates, with precision (Hassanpour Tamrin et al. 2021). From a diverse range of biofluids, source volumes and complexity in sample types, this capability enhances the ability to capture and detect select types of EVs (i.e., from cell source) or select molecular cargo on EVs (i.e., lectin–glycan recognition) from heterogeneous population of EVs (Zhou et al. 2024). Such technology platforms are crucial for downstream analyses that require accurate profiling of EV cargo, including proteins, modified proteins (i.e., glycoproteins), nucleic acids and lipids (Anastasiadou and Slack 2014; Reynolds et al. 2023; Ko et al. 2021; Wu et al. 2025). Additionally, microfluidics enable the integration of various analytical techniques within a single platform, such as real-time monitoring, imaging, and molecular analysis, allowing for the efficient processing of large sample sets (Reynolds et al. 2023; Cheng et al. 2021; Jiao et al. 2024). This combination of speed, scalability, and minimal reagent consumption positions microfluidics as a transformative technology, enabling high-sensitivity and high-precision single EV analysis for clinical diagnostics and therapeutic development.

2.2 | Multiplexed and Multimodal Detection of Single EVs

Recent advances in multiplexed and multimodal detection technologies have enabled comprehensive profiling of single EVs, integrating various molecular and physical parameters to reveal their heterogeneity and complexity (Bordanaba-Florit et al. 2021). The development of high-resolution detection technologies (e.g., sensitive multimodal detection, signal-amplifying approaches and multi-omics profiling) has been pivotal in advancing the ability to profile single EVs with exceptional detail. Techniques such as high-resolution fluorescent confocal microscopy and nanoscale multiplex flow cytometry allow for in-depth analysis of EVs at the level of individual particles, offering insights into their size, concentration, surface markers and molecular cargo. These

TABLE 2 | Technologies for capturing and analyzing single EVs.

Technology	Advantages	Key features	Resolution/dynamic range
Microfluidics (Bordanaba-Florit et al. 2021, Reynolds et al. 2023; Reynolds et al. 2023, Cheng et al. 2021; Jiao et al. 2024)	- High-throughput isolation with minimal sample volume - High purity and precision - Integration of multiple analysis techniques	- Processes volumes as low as 20 µL - Compatible with blood, urine and cerebrospinal fluid	Nanoscale; dynamic range depends on channel design
Nano-flow Cytometry (nFCM) (Liu et al. 2022; von Lersner et al. 2024; Lees et al. 2022)	- Simultaneous analysis of size, concentration and markers - Sensitive to small EVs (<100 nm)	- Multiplex capability with fluorescent markers - Detects surface and luminal cargo	~30–100 nm resolution; dynamic range: 10^5–10^8 EVs/mL
Super-resolution Microscopy (Verweij et al. 2021, Lim et al. 2024, Saftics et al. 2023; Verweij et al. 2019; Ma et al. 2024)	- Visualizes nanoscale differences in EV structure - AI-enhanced algorithms improve resolution and data interpretation	- Tracks EV subpopulations - Captures dynamic structural variations	- Structured Illumination Microscopy (SIM): ~100 nm laterally, ~300 nm axially - Stimulated Emission Depletion (STED) Microscopy: ~20–50 nm laterally, ~50–100 nm axially - Single-Molecule Localization Microscopy (SMLM): ~10–30 nm laterally, ~20–50 nm axially
Surface-Enhanced Raman Scattering (SERS) (Liu et al. 2024; Penders et al. 2021)	- Detailed biochemical characterization - Differentiates EVs based on molecular composition	- Gold nanopyramid substrates enhance signal - Correlates with proteomics data	Nanoscale (10–100 nm); high dynamic range for spectral data

innovations are helping researchers explore the complex world of EVs with unprecedented precision.

Flow cytometry is a powerful tool for analyzing EV at the single-vesicle level, providing critical insights into EV heterogeneity. Löf et al. detected and characterized individual vesicles derived from human plasma by flow cytometry through the use of multiplex and multicolor in situ proximity ligation assays (in situ PLA), which enabled each detected EV to be recorded over background noise using a conventional flow cytometer (Löf et al. 2016). Further, Liu et al. analyzed EV DNA at the single-vesicle level by nano-flow cytometry (nFCM), to demonstrate that smaller EVs (<100 nm) predominantly carry external DNA, while larger EVs (80–200 nm) enclose DNA within their lumen (these DNA molecules range from 200 to 1200 bp in size) (Liu et al. 2022). Notably, the proportion of DNA-positive EVs stained by SYTO 16 varied from 30% to 80%, depending on the cell type, indicating that individual EVs can contain less than one DNA molecule per EV. Notably, Welsh et al. discussed the detection limits of EVs using flow cytometry; providing estimates of the lower limit of detection corresponds to the presence of approximately 200 Phycoerythrin (PE) molecules per EV. Such insights imply that EVs with fewer than 200 PE-conjugated antibodies may be below the detection threshold of conventional flow cytometers (Welsh et al. 2023). Von Lersner et al. presented 'EV Fingerprinting', a single-vesicle flow cytometry method that resolves EV heterogeneity by analyzing particle diameter, membrane order and cargo (von Lersner et al. 2024). Using fluorescence and dimensional reduction, this study identified distinct cell-derived EV subpopulations based on their differing genesis mechanisms, to demonstrate selective effects of Rab27a knockdown and CD63 overexpression on EV biogenesis (von Lersner et al. 2024). The method provides high-resolution insights into EV diversity and the heterogeneity in EV biogenesis and composition. However, flow cytometric analysis of EVs, and the proteins present on their surfaces was still hampered by the small size of the EVs, the limited number of antigens present, and their low refractive index (Görgens et al. 2019). Therefore, development of flow cytometry-based technologies, such as surveying higher orders of protein species and antigens on large sets of EVs in parallel in order to assess their heterogeneity and conservation, are still required to improve the detection of individual EVs.

The super-resolution microscopy approach can advance the understanding of vesicle heterogeneity and supports applications in diagnostics, therapeutics, and vesicle biology (Verweij et al. 2021). Lim et al. demonstrated the integration of AI-enhanced deep-learning algorithms with super-resolution fluorescence microscopy for high-throughput single-vesicle analysis, overcoming the limitations of traditional clustering methods (Lim et al. 2024). Saftics et al. introduced Single Extracellular Vesicle Nanoscopy (SEVEN), which combines affinity isolation with

super-resolution imaging to characterize EV subpopulations from human plasma (Saftics et al. 2023). SEVEN quantifies EV number, size, shape, and molecular content, detecting EVs from just 0.1 μL of plasma. The method demonstrated utility in identifying disease-specific EV characteristics, such as cancer-enriched subpopulations with distinct size and molecular profiles, compared to healthy controls. Moreover, Verveji et al. demonstrated the live visualization of single endogenous EVs in zebrafish embryos with CD63-pHluorin, offering key insights into advances in real-time *in vivo* imaging, localization, and distribution of single EVs (Verweij et al. 2019).

Moreover, single-molecule fluorescence imaging, super-resolution mapping, and surface-enhanced Raman scattering (SERS) allow detailed characterization of EV cargo, structure, and dynamics at the single-vesicle level (Ma et al. 2024). Using gold nanopyramid substrates and size-exclusion chromatography, Liu et al. correlated SERS spectral fingerprints with proteomic data, revealing distinct biochemical compositions across different EV size fractions (Liu et al. 2024). By employing single particle automated Raman trapping analysis, Penders et al. generated detailed compositional spectra for EVs, allowing accurate differentiation between cancerous and non-cancerous origins with over 95% sensitivity and specificity (Penders et al. 2021). Using dSTORM imaging performed with superresolution microscopy, Li et al. explored the single-molecular localization for the signature protein at single urinary EVs, yielding proportions of EV subpopulation characterized with different combination of protein markers (Li et al. 2025). Additionally, Surface Plasmon Resonance (SPR) has emerged as a powerful tool for real-time detection of EV interactions with specific ligands (Yang et al. 2020). This technique allows for the assessment of binding kinetics and affinities, aiding in identifying biomarkers associated with diseases.

The integration of single EV analysis with multi-omics approaches enables a detailed comprehensive molecular analysis at a specific EV level. Excitingly, such strategies enable multiple quantitative detections (Li et al. 2020), including key applications in cell secretions from single cells using high-throughput living single-cell multi-index profiling platforms (Wang et al. 2022).

Comparable to single-cell analysis, researchers have valued omics characterization of single EVs to understand the molecular detail of EV subpopulations (Saftics et al. 2023; Schoger et al. 2023; He et al. 2024; Smith et al. 2015). The composition of these EV subpopulations provides key biological insights into elucidating the various biogenesis and functional relationships between an EV's formation and in cell function, cellular communication, and cell interaction. This strategy demands groundbreaking technologies capable of accurately quantifying very low-level biomolecules. Mass cytometry, which uses metal-isotope-tagged antibodies to label targets of interest, enables simultaneous measurements of ~50 proteins or protein modifications in millions of single cells (Lun et al. 2025). Protein-focused techniques enable high-throughput analysis of proteins and EV subtypes using barcoded antibodies and droplet microfluidics technologies (Banijamali et al. 2022; Reynolds et al. 2023; Ko et al. 2021). Ko et al. introduced an antibody-based immunosequencing method that employs droplet microfluidics to compartmentalize and barcode individual nanometre-sized EVs, enabling multiplexed protein measurement through sequencing of antibody-DNA barcodes (Ko et al. 2021). Moreover, Lun et al. presented a signal amplification technology, termed Amplification by Cyclic Extension (ACE), implementing thermal-cycling-based DNA in situ concatenation in combination with 3-cyanovinylcarbazole phosphoramidite-based DNA crosslinking to enable signal amplification simultaneously on >30 protein epitopes (Lun et al. 2025). The proximity barcoding assay (PBA) provides a digital platform for simultaneously characterizing hundred-plexed proteins on single EVs (Wu et al. 2019). This technique involves attaching unique DNA-encoded tags to individual antibodies for protein detection, followed by an EV barcode hybridization reaction and PCR amplification to construct a library for sequencing. Application of this approach, urinary EVs subpopulations were profiled with unique protein signature, revealing the heterogeneity of urinary EV (Li et al. 2025). Additionally, recent advances in single-EV profiling techniques for RNA analysis have demonstrated their potential in revealing EV heterogeneity and their cellular origins. The number of RNA molecules per EV is generally very low, and in most cases, less than one copy per EV on average (Chevillet et al. 2014). RNA-based methods, such as single-EV sequencing and SEVtras (He et al. 2024; Luo et al. 2022), utilize platforms such as 10× Genomics and droplet-based single-cell RNA sequencing to profile RNA cargos at single EV resolution and estimate EV secretion activity. Furthermore, an ex vivo microfluidic chip system may be integrated with such multiplex technologies to expand research possibilities and provide deeper insights into EV functions and therapeutic applications (Hyung et al. 2023).

However, a major limitation in current EV research lies in the unresolved complexity of EV subpopulations, including challenges in defining their number, identifying EVs that are pro- or inhibitory in function, or can direct the tropism and internalization to specific cells, or provide a therapeutic benefit. These seminal and diverse limitations in our knowledge of EV heterogeneity underscores the need for advanced, standardized methodologies combined with technologies in accurate detection and profiling.

2.3 | Bioinformatics Analyses and Integration of Artificial Intelligence (AI)

Bioinformatics and the integration of AI are becoming indispensable tools in the analysis of single EVs, particularly for their application in clinical diagnostics and therapeutics. Bioinformatics tools complement AI-enhanced data analyses and modelling by integrating data from multiple sources, such as genomic, transcriptomic, and proteomic information, to create comprehensive profiles of individual EVs. For example, He et al. exemplifies how AI-driven bioinformatics can transform single-EV studies, particularly in integrating transcriptomic data for vesicle profiling (He et al. 2024). Such integration enables a deeper understanding of the molecular mechanisms underlying tumour progression and facilitates the identification of key therapeutic targets within EVs (Ferguson et al. 2022).

Future advancements are also likely to integrate AI with cutting-edge imaging technologies, enabling ultra-high-resolution visualization and more sophisticated insights into EV heterogeneity

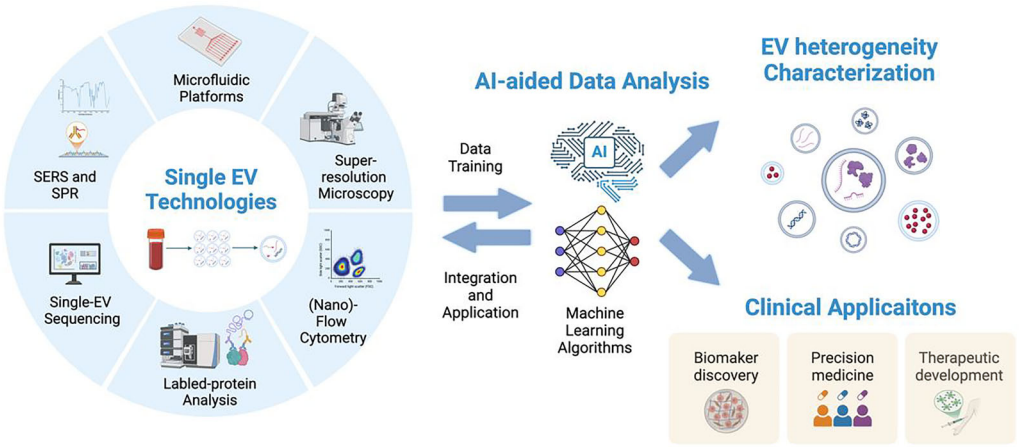

FIGURE 1 | An overview of single extracellular vesicle (EV) technologies, their integration with AI-aided data analysis, and their applications in EV heterogeneity characterization and clinical research. AI indicates artificial intelligence; SERS, surface-enhanced Raman spectroscopy; SPR, Surface Plasmon Resonance. This figure was created using BioRender (https://biorender.com/).

and function. For example, Lim et al. integrated deep-learning algorithms with super-resolution fluorescence microscopy to classify single EV subpopulations, enabling automated, high-throughput analysis and uncovering nanoscale differences in vesicle morphology and marker distribution that were previously undetectable (Lim et al. 2024). AI-enabled computational analysis efficiently processed thousands of high-resolution images, quantifying attributes like eccentricity (0.5366 ± 0.2) and diameter (132.43 ± 67 nm) (Kapoor et al. 2024). AI-driven algorithms, especially deep learning, improve image processing, feature extraction and vesicle classification. AI also supports high-throughput data analysis from the above-mentioned techniques, reducing measurement variability and making single EV studies more reliable by enhancing the accuracy and efficiency of single EV characterization. These capabilities, and their rigorous standardization, assessment and context-based applications, are providing new advances for sensitive and refined detection of low abundant markers, and optimizing therapeutic strategies (Figure 1).

3 | Implications of Single EV Research for Clinical Applications

Single EV research is transforming the fields of biomarker discovery, personalized medicine and therapeutic development by providing unprecedented insights into EVs' molecular and functional heterogeneity.

3.1 | Biomarker Discovery

EVs are secreted into biofluids such as blood, urine and cerebrospinal fluid, carrying molecular cargo reflective of their cells of origin. This makes them valuable carriers of disease-specific biomarkers. However, EV populations are highly heterogeneous in size and content, and this poses a significant challenge for conventional bulk analysis, which averages signals across millions of vesicles (Kapoor et al. 2024).

By uncovering molecular signatures masked in bulk analyses, single EV analysis allows for identifying rare EV subpopulations carrying disease-specific and cancer-specific biomarkers, even in highly complex biological samples. While pan-EV markers have demonstrated utility, their low specificity can limit the precision of disease detection and monitoring (Ferguson et al. 2022). Single EV technologies address this limitation by identifying distinct molecular features of individual EVs in context of the broader EV population, offering enhanced sensitivity and specificity. For instance, Xu et al. has found that a digital dual CRISPR-Cas-powered single EV evaluation system enables simultaneous detection of surface proteins and internal miRNAs at the single-EV level, achieving high sensitivity (as few as 214 EVs/μL, 474.8 copies/μL of DNA and 551.8 copies/μL of RNA) and 92% diagnostic accuracy in distinguishing breast cancer patients from healthy donors, thus demonstrating strong potential for clinical EV-based diagnostics (Xu et al. 2025). Reynolds et al. introduced a novel microfluidic-based double digital assay, enhanced with tyramide signal amplification, which enables the absolute quantification of rare proteins in single EVs, such as PD-L1, thereby advancing the profiling of prognostic and diagnostic cancer biomarkers (Reynolds et al. 2023). Kim et al. developed a platform for isolating and profiling EV subpopulations by size (large, medium and small EVs) and labelling them for single-EV protein analysis using only minimal sample volumes (10 μL of plasma) (Kim et al. 2020). Using this approach, they found that HER2 expression was highest in small EVs (20–100 nm) from breast cancer cells, while PSMA was most enriched in large EVs (200–1000 nm) from prostate cancer cells, with findings confirmed in patient plasma-derived nanoparticles (Kim et al. 2020). As large EVs such as apoptotic bodies, large oncosomes, exophers and migrasomes are generally larger than 1 μm in diameter, identifying distinct large EV subsets in tumour tissues and circulation emphasizes their potential in clinical diagnostics and therapeutic development (Nicolás-Ávila et al. 2020; Ciardiello et al. 2019; Atkin-Smith et al. 2024). Liu et al. demonstrated that the droplet digital ExoELISA platform enables ultra-sensitive and precise quantification of cancer-specific exosomes in patient plasma, highlighting its significant potential for early cancer diagnosis and biomarker discovery (Liu et al. 2018). Tian et al. used nanoFCM flow

cytometry to assess the diagnostic power of CD147-positive EVs in plasma for colorectal cancer, with an area under the curve of 0.932 (Tian et al. 2018). Zhang et al. introduced a single-EV miRNA profiling strategy using TIRF imaging combined with a deep learning algorithm, achieving precise multi-miRNA detection that overcomes EV heterogeneity and achieves 85–100% accuracy in cancer diagnosis and classification using EVs from cell lines and a clinical cohort of 20 cancer patients and 5 healthy individuals (Zhang et al. 2024). Furthermore, techniques analyzing hundreds of millions of single analytes are now uncovering rare circulating EV-centric biomarkers, providing critical insights into early diagnosis and disease monitoring (Ferguson et al. 2022).

3.2 | Potential Therapeutic Development

Single-EV detection technologies can overcome challenges in EV-based therapeutics, including knowledge in their potency and targeted delivery as therapeutic agents—that interact with target cells and intracellular compartments with specificity.

In therapeutic formulation, single EV analysis enables the precise quantification of molecular cargo—such as proteins, RNAs or small molecules—delivered by each vesicle. This is essential for dosing calibration and potency evaluation, especially in the context of engineered EVs designed for drug delivery. For example, Silva et al. found that different EV-sorting proteins (CD63, TSPAN14, CD81TM) vary in their cargo-loading efficiency, with single-vesicle measurements uncovering substantial heterogeneity in cargo distribution and copy number (Zhou et al. 2024). Cao et al. used single EV imaging to evaluate the intracellular fate of miRNA-loaded EVs and found that, despite efficient cellular uptake, only a small fraction of EVs successfully delivered their cargo into the cytoplasm (Lowe et al. 2024). These insights highlight the importance of single EV analysis for verifying functional delivery and optimizing EV design for therapeutic applications.

Moreover, single EV technologies support personalized medicine by enabling the discovery and selection of naturally therapeutic EV subtypes (Rai et al. 2024; Greening et al. 2023). Some vesicles are enriched with bioactive molecules—such as anti-inflammatory microRNAs or immunomodulatory proteins—that influence disease pathways. Techniques like nano-flow cytometry, super-resolution microscopy and single-vesicle omics now allow for the precise functional and phenotypic profiling of such EV subpopulations (Kunitake et al. 2024; Koo et al. 2024; Huang et al. 2023). This facilitates the selection or engineering of EVs with enhanced therapeutic potency, targeting and limiting off target distribution.

Importantly, single EV analysis is advancing how the field of EV therapeutics is evolving, enabling metrics to assess individual EV types, quality control (including composition, quantitative information), guiding subpopulation-specific engineering, and uncovering functional heterogeneity. These capabilities are central to developing next-generation EV therapies for precision medicine, from targeted drug delivery to personalized monitoring and treatment optimization (Rai et al. 2024).

4 | Challenges in Single EV Characterization and Clinical Utility

Because of their nano size and diversity in form and function, challenges remain in how EVs are isolated and current methodologies in their biological and clinical application. Despite the remarkable advancements in the study of single EVs, key challenges currently impede their widespread application and biological and clinical feasibility. These challenges span across biological, technological, and analytical domains, each requiring targeted strategies to overcome. It should be emphasized that some of these challenges extend beyond single EV investigations and are pertinent to generally EV research.

4.1 | Isolation and Analysis Challenges

A key challenge in single EV analyses remains in their isolation and classification. The inherent heterogeneity of EVs, varying significantly in size, origin and cargo composition, complicates this task and poses difficulties in distinguishing EVs from other nanoscale particles, such as lipoproteins and protein aggregates. This issue is not unique to single EV studies but represents a broader challenge across EV research. Impure isolation can substantially confound downstream analyses. For example, exosomes and ectosomes exhibit distinct surface and luminal cargo, which reflect their biogenesis pathways (Meldolesi 2018). Exosomes, originating from the endosomal system, typically carry endosomal proteins, tetraspanins and RNA, whereas ectosomes, derived from the plasma membrane, are enriched in integrins, phosphatidylserine and cytosolic proteins (Rai et al. 2021). Although these vesicle subtypes are expected to harbour distinct cargo profiles and consequently exert different biological functions, their efficient isolation remains challenging due to the difficulty in separating exosomes from ectosomes of similar size within biofluids or conditioned media (Mathieu et al. 2021).

Despite ongoing challenges, efforts have been made to isolate and analyze individual vesicles. For example, Zhang et al. developed a microfluidic platform incorporating graphene oxide and polydopamine nanostructured coatings to achieve ultrasensitive immuno-capture and isolation of single EVs from biological fluids. This system leverages strong surface interactions for efficient vesicle retention while minimizing nonspecific binding, achieving a resolution of 50 vesicles/μL, a 4-log dynamic range, and requiring only 2 μL of unprocessed plasma. While the platform offers excellent sensitivity and minimal sample requirements, its limited multiplexed molecular profiling and lack of integration with downstream analytical tools constrain its broader application in biomarker discovery and clinical diagnostics (Zhang et al. 2016).

Another effort toward single EV isolation and analysis is presented by Lee et al., who developed a multiplexed profiling platform based on high-throughput flow cytometry. This approach utilizes antibody-coated magnetic beads to capture individual EVs and subsequently detect surface protein markers using fluorescent DNA barcodes. The platform allows for the simultaneous detection of multiple proteins (e.g., CD63, HER2, EGFR) at the single EV level, enabling detailed subpopulation analysis and disease-specific biomarker profiling. This method demonstrates

strong potential for non-invasive cancer diagnostics, particularly in distinguishing tumour-derived EVs from background noise in plasma samples. However, the technique relies heavily on specific antibodies, which may limit detection to known markers, and limit detection of EVs with low or variable antigen expression, reducing the utility to identify rare and low abundant EV subtypes from specific cells (Lee et al. 2018).

Current methods, such as differential ultracentrifugation, size-exclusion chromatography, and immunoaffinity capture, are effective for isolating bulk EV populations but limit in their ability to capture single EVs with high specificity. Many benchtop isolation/enrichment methods require large sample volumes (> millilitre levels) and can impart changes in EV integrity or molecular alteration in EV composition. These methods often fail to adequately separate EVs from the surrounding matrix, further limiting their utility in precise EV characterization (Kang et al. 2020; Welsh et al. 2024; Zhao et al. 2021). In contrast, techniques such as microfluidic platforms, flow cytometry, and label-free SERS (surface-enhanced Raman spectroscopy) (Liu et al. 2024), offer greater sensitivity and specificity for isolating and analyzing individual EVs. While these approaches address many of the limitations of traditional methods, they also introduce challenges such as limited multiplexing, sensitivity to surface fouling, and integration with downstream analyses.

4.2 | Standardization and Reproducibility in the Single EV Analysis

Single EV isolation technologies require standardization and characterization. Establishing a framework for standardization and reproducibility in the single EV field is essential to ensure consistent and reliable results across research studies and clinical applications.

To ensure comparability across studies, it is essential to clearly define the source of EVs, such as plasma, serum, urine, or cell culture supernatants, as well as the specific EV subtypes being analyzed, such as exosomes, microvesicles or apoptotic bodies (Théry et al. 2018; Nieuwland et al. 2020). This clarification helps in establishing consistency across experiments and promotes reliable cross-study comparisons (Witwer et al. 2013). Equally important are the pre-analytical variables that can influence EV characterization (López-Guerrero et al. 2023; Barreiro et al. 2021). Standardized protocols should be developed to control for factors like the time to processing, temperature, and methods of EV isolation, including ultracentrifugation, size-exclusion chromatography, and immunoaffinity capture. These steps, when standardized, can minimize batch-to-batch variability and ensure reproducibility of results.

Due to the inherent complexity of EVs, where even minor changes in protein or lipid composition can significantly influence their biology and function, comprehensive characterization requires a holistic approach leveraging -omics technologies. Machine learning models trained on proteomics or lipidomics data from reference products (such as specific EV subpopulations or refined EV types) can be employed to predict their functions, therapeutic potential, and diagnostic utility, enhancing the development and application of EV-based strategies (Rai et al. 2024).

4.3 | Resolution

Resolution limitations in current analytical tools continue to hinder progress in single EV studies. As summarized in Table 1, conventional techniques such as single particle analyses and flow cytometry, while widely used for bulk EV analysis, often lack the precision required to accurately characterize individual EVs. These methods typically fall short in resolving the fine molecular and morphological details necessary to study EV heterogeneity. In contrast, emerging techniques such as microfluidics and nano-flowcytometry, highlighted in Table 2, offer enhanced sensitivity and resolution, making them more suitable for single EV analysis. However, even with these advances, the inability to resolve distinct subpopulations within complex EV mixtures remains a key challenge, particularly when attempting to associate specific EV subsets with defined biological functions or disease states (Serrano-Pertierra et al. 2020).

4.4 | Multiplexing for Ultrasensitive Single EV Detection

Traditional bulk detection methods have been widely used to analyze EVs, but they average signals across large populations, obscuring the underlying heterogeneity and leading to the loss of critical biological information (Zhu et al. 2023). For single EV analysis, surface proteins can be directly detected through immunoblotting (Hartjes et al. 2019), while detecting EV RNAs and other cargos requires accessing the EV lumen. However, using lysing agents to partially disrupt or dissolve the EV membrane to create pores may also risk removing potential surface biomarkers of interest. Traditional protein and mRNA measurement methods, such as Western blotting and RT-qPCR, require large sample amounts (10^5–10^6 EV per biomarker (Lee et al. 2018) and may not accurately reflect the information from all examined EVs. Despite so many ongoing research, a reliable technique for measuring the expression of specific proteins and nucleic acids at the single EV level is still lacking (Zhou et al. 2020). This limitation necessitates the development of ultrasensitive detection methods capable of amplifying signals without introducing significant noise (Cheng 2024).

Single-vesicle analysis offers a more nuanced and comprehensive approach to EV characterization than bulk methods. By examining individual vesicles, it reveals unique molecular profiles and enables the detection of specific biomarkers that may be obscured in population-level analyses. This approach supports advanced investigations, including the colocalization of distinct molecular markers on the same vesicle, the frequency distribution of markers across EV populations, and the identification of discrete subpopulations through dimensionality reduction techniques such as principal component analysis and t-distributed stochastic neighbour embedding. As a result, single-vesicle analysis significantly enhances sensitivity and specificity, providing deeper insights into EV heterogeneity and enabling the detection of rare events and subtle biomarker shifts (Ferguson et al. 2022). While the diverse nature of EVs is now well recognized, most recently highlighted by the International Society for Extracellular Vesicles, the field still lacks precise tools to differentiate between EV subtypes and, ultimately, to characterize individual EVs, which likely carry distinct molecular signatures and biological

functions (Kowal et al. 2016). With its growing recognition, this methodology is increasingly regarded as a critical tool for advancing our molecular understanding of EVs.

Using a modelling approach, Ferguson et al. estimated that single EV analysis (sEVA) could detect pancreatic ductal adenocarcinoma (PDAC) at a tumour volume as small as ~0.1 cm^3, well below the detection threshold of conventional clinical imaging, highlighting the potential of sEVA to enhance diagnostic sensitivity (Ferguson et al. 2022). Such capture strategies rely on specific antibody-based capture, where the quality of antibodies is essential in detecting rare and scant proteins such as mutated variants. Other challenges focus on optimization of microfluidics for nanoflow detection of tumour-specific EVs based on ultrasmall-volume measurements, while concurrently improving direct biofluid analyses (such as from tumour and tissue sources directly) without the dilution that occurs in circulation. Nguyen et al. developed a multiplexed analysis platform capable of simultaneously detecting multiple targets within a single assay. In this approach, gold spherical nanoparticles were employed to amplify signals and enhance the sensitivity of single EV detection. Distinct antibodies were immobilized on the chip surface to capture and classify EVs into subpopulations based on their membrane protein profiles. However, despite achieving highly sensitive multiplexed detection of diverse biomolecule types, the throughput for detecting large numbers of the same biomolecule type remains limited (Nguyen et al. 2022).

4.5 | Throughput

Throughput constraints further complicate single EV research. Current high-resolution and sensitivity technologies, such as super-resolution microscopy or single-molecule sequencing, are often low throughput and labour-intensive. Conversely, high-throughput platforms, such as traditional flow cytometry, sacrifice resolution and sensitivity for speed.

Efforts are underway to bridge these gaps. For instance, in 2020, Zhou et al. developed a high-throughput nano-biochip capable of simultaneously detecting proteins and mRNA/miRNA in single EVs. The platform features a low sample requirement (~90 μL), and high throughput, accommodating up to 384 samples per run using human liquid biopsy specimens. However, the EV collection and enrichment process involves multiple centrifugation steps at varying speeds and durations. The complex sample preparation and overnight incubation make this procedure time-consuming and labour-intensive. Additionally, the setup for Total Internal Reflection Fluorescence Microscopy (TIRF) is not standard in most labs or hospitals (Zhou et al. 2020). Bridging the gap between throughput and analytical precision remains a significant challenge for the field (Welsh et al. 2024; Park et al. 2024).

4.6 | Data Interpretation

Finally, the interpretation of data from single EV studies presents unique difficulties. The complexity and heterogeneity of EVs generate vast datasets, often requiring advanced computational tools and bioinformatics expertise for meaningful analysis. Integrating multi-omics data, such as proteomics, transcriptomics and lipidomics, at the single EV level further complicates the analytical landscape. Standardized pipelines and accessible tools for data analysis are urgently needed to enable reproducibility and consistency across studies (Shaba et al. 2022; Kapoor et al. 2024; Hendrix et al. 2023).

Addressing these challenges will require a concerted effort from multidisciplinary teams, integrating expertise in biology, engineering, and computational sciences. Tackling these hurdles head-on will pave the way for a more comprehensive understanding of single EVs and their applications in research and clinical settings.

5 | Future Directions

The future of single EV research holds immense promise, with rapid advancements expected across several dimensions.

5.1 | Integration of Multi-Modal Technologies for Comprehensive Single EV Profiling

The integration of multi-modal technologies refers to combining distinct analytical platforms—such as nano-scale imaging (e.g., super-resolution microscopy or electron microscopy) with molecular profiling techniques (e.g., proteomics, transcriptomics or nano-flow cytometry)—to obtain a more holistic view of individual EV. This approach enables researchers to correlate physical properties of EVs (e.g., size, morphology, surface topology) with their molecular cargo (e.g., specific proteins, RNAs), providing deeper insights into their functional heterogeneity. The ability to correlate structural characteristics with molecular signatures in single EVs could lead to unprecedented insights into their biological and pathological functional roles.

For example, Lee et al. demonstrated a method that combined flow cytometry with molecular barcoding to profile multiple surface proteins on single EVs, enabling identification of disease-relevant subpopulations (Lee et al. 2018). Similarly, Görgens et al. employed imaging flow cytometry to simultaneously assess EV morphology and surface marker expression in a high-throughput way at the single-vesicle level. This technique bridges the gap between high-throughput quantitative analysis and detailed visual characterization, enabling a more comprehensive understanding of EV heterogeneity (Lannigan and Erdbruegger 2017). These integrated approaches are particularly valuable in disease research, where subtle changes in EV subtypes may be associated with specific pathological states.

By combining structural and molecular data at the single EV level, multi-modal systems can reveal correlations that are otherwise obscured in bulk analyses, thereby advancing both diagnostic and therapeutic applications of EVs.

5.2 | Enhanced EV Characterization via AI

The role of AI in revolutionizing single EV research is becoming increasingly apparent. AI-powered algorithms could automate

data processing, identify subtle patterns in EV populations, and aid in interpreting complex multi-omics datasets. As deep learning techniques evolve, AI could help to create predictive models for disease progression based on the molecular profile of individual EVs, offering real-time diagnostics and personalized treatment strategies (Zhou et al. 2020). Some AI algorithms have been applied in EV research, such as convolutional neural network (CNN) (Lee et al. 2020), Support Vector Machines (SVMs) (Zhang et al. 2019) and Random Forest (RF) (Burrello et al. 2020).

Lee et al. developed a CNN-based classification model to distinguish tumour-derived EVs using Raman spectroscopy data. Their model was trained on both preprocessed and raw Raman spectra, achieving classification accuracies above 90%, and even 93% without preprocessing. The performance of the CNN was compared to traditional principal component analysis (PCA), showing superior results. This study demonstrates the potential of deep learning for direct, high-accuracy analysis of EVs in label-free spectroscopic applications (Lee et al. 2020).

He et al. developed SEVtras, a novel algorithm designed to identify small extracellular vesicle (sEV)–containing droplets and estimate the sEV secretion activity (ESAI) of individual cells using droplet-based single-cell RNA sequencing (scRNA-seq). Through validation on both simulated and real datasets, SEVtras demonstrated high efficacy in characterizing sEV secretion activity, particularly in distinguishing cell types. The algorithm was applied to four tumour scRNA-seq datasets, revealing that ESAI serves as a valuable indicator of tumour progression, especially in early stages. This work highlights SEVtras' potential to provide deeper insights into cellular heterogeneity and sEV dynamics (He et al. 2024).

5.3 | Standardization and Global Collaboration

As single EV technologies move toward clinical and translational applications, global collaboration between research institutions, clinical laboratories and industry partners becomes increasingly essential. Unlike bulk EV studies, single EV analysis requires highly sensitive and precise methodologies, which are currently limited by a lack of standardized protocols. The development of universally accepted guidelines for isolation, characterization, and data interpretation will ensure reproducibility, enable meaningful comparisons across studies, and facilitate regulatory approval for clinical use. For example, the International Society for Extracellular Vesicles (ISEV) has initiated the MISEV (Minimal Information for Studies of Extracellular Vesicles) guidelines to promote consistency in EV research, emphasizing characterization of single vesicles using at least two complementary technologies to assess heterogeneity (Welsh et al. 2024). Similarly, the EV-TRACK knowledgebase encourages transparent reporting and helps harmonize protocols across labs. These collaborative efforts lay the groundwork for robust and reproducible single EV studies, making standardization a critical future direction for the field. This would accelerate the translation of single EV technology into applications.

5.4 | Therapeutic Application and EV-Based Drug Delivery

One of the most exciting directions for single EVs analysis is its application in optimizing the development of EV-based therapeutics, including targeted drug delivery systems.

Single EV analysis allows for precise characterization of the molecular content, surface proteins, and cargo of individual EVs, which can be critical for improving drug delivery efficiency. By profiling single EVs, we can identify specific EV subtypes that are best suited for carrying therapeutic agents to targeted tissues or cells (Frolova and Li 2022). Single-EV profiling could enable patient-specific EV signatures, guiding personalized EV therapeutics (e.g., selecting or engineering EV subtypes tailored to a patient's tumour biology or immune landscape) (Hyung et al. 2023; Jung et al. 2024). Many diseases, particularly cancers and neurodegenerative disorders, can lead to the release of EVs that are less abundant than the overall vesicle population in biofluids. These EVs may carry unique molecular signatures, including proteins and nucleic acids (Liu et al. 2024; Candelario and Steindler 2014; Li et al. 2023). By profiling surface proteins and cargo of individual EVs, we can identify EV subtypes with optimal delivery properties (Cai et al. 2024). The ability to design and engineer EVs to deliver specific cargos to desired tissues could revolutionize treatment for cancers, autoimmune diseases, and neurodegenerative disorders, and applications in tissue repair and regeneration, such as EV-based therapeutics in cardiovascular medicine (Rai et al. 2024). This would require overcoming technical hurdles, such as optimizing EV cargo loading and targeting, but the potential impact is vast.

5.5 | Personalized Medicine

Single EV analysis is emerging as a powerful approach with profound implications for personalized medicine. Unlike bulk EV profiling, which averages signals across heterogeneous populations and often obscures clinically relevant subtypes, single EV analysis enables the resolution of distinct vesicle populations with unique molecular cargo and functional signatures. This level of granularity is particularly important because EVs originate from diverse cellular sources and mirror the dynamic physiological or pathological states of their parent cells. Leveraging this property, single EV approaches can identify disease-specific EVs in biofluids at very early stages, supporting earlier diagnosis and timely intervention (Lee et al. 2023). Moreover, real-time monitoring of single EV signatures provides dynamic and individualized feedback on therapeutic responses, offering a valuable tool for adapting treatments to each patient's evolving condition. Such resolution may also leverage advances in imaging and tracking capabilities of single EVs, understanding important aspects from their origin and genesis, cell signalling function, cell interaction, the ability to transverse and interact with membranes, and intracellular fate. Importantly, single EV analysis also enables the quantitative distinction between immunosuppressive and immune-activating vesicle populations, thereby informing the design of more precise immunotherapeutic strategies. Such new knowledge could help leverage select specific source cells for optimal therapy, refine specific EV types for specific applications, and a platform for evaluating specific EV cargos and therapeutic

strategies. By capturing patient-specific molecular information at the nanoscale, single EV analysis has the potential to transform precision diagnostics, prognostics, and treatment decision-making, ultimately advancing the realization of personalized medicine (Braig 2022).

6 | Conclusion

The study of single EVs is an exciting and transformative area of research that promises to reshape our understanding of cellular communication, disease mechanisms and therapeutic approaches—and ultimately the clinical feasibility of EVs. Despite the considerable challenges related to isolation, characterization and data interpretation, the advances made thus far are paving the way for a future where single EV analysis becomes an integral tool in future EV-research, as well as clinical diagnostics and personalized treatments.

To fully realize the potential of single EV research, overcoming technical and biological obstacles is paramount. The integration of cutting-edge technologies, the standardization of methodologies and the use of AI and multi-omics approaches will propel the field forward. Alongside with the development of other related techniques, advances in single-vesicle analysis techniques such as super-resolution microscopy, single molecule localization microscopy, flow cytometry, microfluidic and other sensor designs, will enable researchers to study the heterogeneity of EV populations in greater detail. Paired with advances in sensitivity in omic technologies such as mass spectrometry, cutting edge nanoscopy technologies (Saftics et al. 2023), and liquid-phase separation strategies will enable profiling of key molecular analytes (proteins/RNA species/metabolites/lipids) in EVs and their changes in target cells associated with phenotype/function, which in turn will allow identification of rare markers in body fluids and advanced knowledge in EV form and function.

Such insights will help to identify specific EV subpopulations associated with particular disease states, thereby improving the specificity and sensitivity of circulating EV-based biomarkers (Sung et al. 2025). Ultimately, single EV research cannot only enhance our understanding of disease but also unlock new avenues for precision medicine, offering the possibility of more effective and individualized therapies.

In the future, as technology continues to evolve and interdisciplinary collaborations intensify, the potential of single EVs as diagnostic, prognostic, and therapeutic tools will undoubtedly expand. The future of single EV research is bright, offering transformative possibilities that could significantly impact a wide range of diseases, from cancer to neurodegenerative disorders, and ultimately improve patient outcomes worldwide.

Author Contributions

Ying Zhang: conceptualization, data curation, funding acquisition, investigation, writing–original draft, writing–review and editing. **Xiaotong Meng**: conceptualization, data curation, investigation, writing–original draft, writing–review and editing. **David W. Greening**: conceptualization, data curation, validation, writing–original draft, writing–review and editing. **Yiyao Huang**: conceptualization, validation, writing–original draft, writing–review and editing. **Bo Li**: conceptualization, validation, writing–original draft, writing–review and editing. **Zhigang Li**: conceptualization, validation, writing–original draft, writing–review and editing. **Gang Long**: conceptualization, validation, writing–original draft, writing–review and editing. **Jan Lötvall**: conceptualization, validation, writing–original draft, writing–review and editing. **Linli Lv**: conceptualization, validation, writing–original draft, writing–review and editing. **Ivan Poon**: conceptualization, validation, writing–original draft, writing–review and editing. **Tong Wang**: conceptualization, validation, writing–original draft, writing–review and editing. **Jia Xian Law**: conceptualization, validation, writing–original draft, writing–review and editing. **Tanxi Cai**: conceptualization, validation, writing–original draft, writing–review and editing. **Fuquan Yang**: conceptualization, validation, writing–original draft, writing–review and editing. **Lijuan Yu**: conceptualization, validation, writing–original draft, writing–review and editing. **Lei Zheng**: conceptualization, validation, writing–original draft, writing–review and editing, funding acquisition. **Hang Yin**: conceptualization, funding acquisition, project administration, supervision, writing–review and editing.

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Data Availability Statement

Encourages Data Sharing.

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