Title

Harnessing extracellular vesicle heterogeneity for diagnostic and therapeutic applications

Journal

Nature Nanotechnology, 20(1)

ISSN

1748-3387

Authors

Carney, Randy P

Mizenko, Rachel R

Bozkurt, Batuhan T

et al.

Publication Date

2025

DOI

10.1038/s41565-024-01774-3

Peer reviewed

Published in final edited form as:

Nat Nanotechnol. 2025 January ; 20(1): 14–25. doi:10.1038/s41565-024-01774-3.

Harnessing extracellular vesicle heterogeneity for diagnostic and therapeutic applications

Randy P. Carney[1,3,✉], **Rachel R. Mizenko**[1,3], **Batuhan T. Bozkurt**[1], **Neona Lowe**[1], **Tanner Henson**[1,2], **Alessandra Arizzi**[1], **Aijun Wang**[1,2], **Cheemeng Tan**[1], **Steven C. George**[1,2,✉]

[1]Department of Biomedical Engineering, University of California, Davis, Davis, CA, USA.

[2]Center for Surgical Bioengineering, Department of Surgery, University of California, Davis, School of Medicine, Sacramento, CA, USA.

[3]These authors contributed equally: Randy P. Carney, Rachel R. Mizenko.

Abstract

Extracellular vesicles (EVs) are diverse nanoparticles with large heterogeneity in size and molecular composition. Although this heterogeneity provides high diagnostic value for liquid biopsy and confers many exploitable functions for therapeutic applications in cancer detection, wound healing and neurodegenerative and cardiovascular diseases, it has also impeded their clinical translation—hence heterogeneity acts as a double-edged sword. Here we review the impact of subpopulation heterogeneity on EV function and identify key cornerstones for addressing heterogeneity in the context of modern analytical platforms with single-particle resolution. We outline concrete steps towards the identification of key active biomolecules that determine EV mechanisms of action across different EV subtypes. We describe how such knowledge could accelerate EV-based therapies and engineering approaches for mimetic artificial nanovesicle formulations. This approach blunts one edge of the sword, leaving only a single razor-sharp edge on which EV heterogeneity can be exploited for therapeutic applications across many diseases.

Extracellular vesicles (EVs) are composite biomaterials formed from a rich membrane of lipids, sterols, membrane proteins and glycans surrounding an aqueous compartment that contains proteins, nucleic acids, metabolites, cytokines and other soluble mediators capable of impacting biological function[1]. EVs exhibit numerous inherent qualities that make them highly desirable for the delivery of nanodrugs. Some exhibit enhanced retention in circulating biofluids[2] and can be immune-tolerant[3,4], avoiding phagocytic clearance for enhanced half-lives in circulation[5]. Others appear to have organotropic character to target specific cells within distant tissues (although only a few precise mechanisms have been

✉ **Correspondence and requests for materials** should be addressed to Randy P. Carney or Steven C. George. rcarney@ucdavis.edu; scgeorge@ucdavis.edu.

Author contributions

R.P.C and R.R.M. wrote the paper and prepared the figures. R.P.C., R.R.M., A.W., C.T. and S.C.G. conceptualized the work. B.T.B., N.L., T.H., A.A., A.W., C.T. and S.C.G. draughted sections of the paper and edited them. N.L. and R.R.M. performed experiments.

Competing interests

The authors declare no competing interests.

identified so far[6]), impacting cell communication[6–8], migration[9,10], differentiation[11,12] and proliferation[13,14]. EVs mediate cellular stress response[15] and are rapidly absorbed by most cells via active entry and fusion[16,17]. Their nanoscale dimensions permit passive tissue uptake via the enhanced permeability and retention (EPR) effect by tumours through leaky vasculature[18] (although the extent and utility of the EPR effect for reaching the deep tumour is debated[19]). Some are reported to cross endothelial barriers, including the blood–brain barrier (BBB)[20–22], although specific mechanism(s) remain largely unknown. They provoke potent functional responses, which include suppressing antitumour immunity[23], altering the tumour microenvironment[24] and inducing angiogenesis[25]. They are also malleable and suitable for genetic manipulation or exogenous loading, piggybacking on their inherent qualities and engineering new ones[26,27].

Despite this potential, approaches to effectively use EVs for clinical therapeutics have not yet materialized—there is currently no EV formulation approved by the US Food and Drug Administration, although favourable safety profiles have been confirmed in several studies[28]. Many hurdles towards clinical translation have been identified, including gaps in uniform quality control and poor alignment with regulatory guidelines[29]. Here we focus on a critical hurdle upstream of those barriers—intrinsic EV heterogeneity—which is defined as the differential distribution of molecules across single EVs.

EV heterogeneity means that individual vesicles do not have all of the chemical or physical traits ascribed to the bulk, suggesting that each vesicle may also not have the functional properties that are ascribed to the bulk. Given that a single vesicle’s ability to perform a given function depends on its precise composition, only a population of EVs with a specific chemical composition may be responsible for a particular function. As EVs produced even from a single cell have compositional heterogeneity, functional subpopulations may exist regardless of the homogeneity of the source. To better describe this phenomenon throughout this Review, we use the term subpopulation heterogeneity to include both inter-subpopulation heterogeneity (compositional and functional differences amongst EVs from various sources) and intra-subpopulation heterogeneity (differences in EVs within subpopulations derived from the same source), specifying ‘inter-’ and ‘intra-’ where relevant. For example, a central claim of the potential utility of EVs is that they have long circulation half-lives compared with free drugs or liposomal counterparts. However, some studies have reported EV half-lives of less than 10 min, whereas EV subpopulations expressing CD47 are much longer[30]. Thus, heterogeneity in molecular composition (that is, CD47[30]) and other features, such as EV size[31], lead to diversity in spatial distribution and thus biological response. These issues are further exacerbated when considering the additional impact of EV isolation or enrichment platforms on EV heterogeneity[32]. Many examples of such highly disparate reports of EV function can be found in the literature, further highlighting their inherent heterogeneity. As such, we posit that intrinsic EV heterogeneity is currently the critical barrier for translational therapeutics.

In this section we have highlighted the known features of EV subpopulation heterogeneity and its impact on functional performance in therapeutics. Next, we review existing technologies, including their advantages and disadvantages, that are capable of characterizing EV heterogeneity with single-vesicle resolution. Finally, we outline three

cornerstones by which to address the EV heterogeneity barrier: (1) the need to carefully implement complementary methodologies for adequately characterizing EVs; (2) the need to establish standardized functional assays for comparing EV subpopulations in the context of a given application; and (3) the need to leverage the growing field of mimetic EVs with defined characteristics. Taken together, these steps will lead to identifying and exploiting defined mechanisms of action (MOAs) and greatly accelerate the development of EV therapeutics, for both endogenous sources and engineered formulations.

EVs exist in highly heterogeneous subpopulations

EV preparations are intrinsically heterogeneous, with each vesicle containing varying concentrations and combinations of molecules. Failure to account for this may compromise scientific investigation and hamper clinical translation (although synergistic beneficial effects may also be gained). Whereas many reports have indicated inter-subpopulation functional differences arising from EVs isolated across different sources, few have investigated the intra-subpopulation variance of EV composition within a given source of EVs. EV subpopulation heterogeneity can be categorized into (1) physical parameters, such as size[33], density[34] or viscoelasticity[35], or (2) molecular composition, for example, as commonly demonstrated by antibody-based catch-and-release for segregation by surface marker[36,37]. Many reports have explained functional differences in EV subpopulations according to either diameter or composition, suggesting that EVs have the potential to perform highly specific functions[38–41].

Size

Size is one of the major determinants of EV composition. Even among enriched isolates from a single source, EVs exhibit a log-linear decrease in concentration over a size range of approximately 30 nm to more than 150 nm, with a mode in the 40–100 nm range for most EV sources[42–44]. Whereas bulk analyses have uncovered thousands of EV-associated biomolecules, single-EV analysis reveals that each vesicle carries only a small subset[45]. The discrepancy in relative stoichiometry is greatly exaggerated by size (Fig. 1a): a 150 nm vesicle has 25-fold more surface area and 125-fold more volume than a 30 nm vesicle—potentially trafficking thousands more proteins and other cargo. Even a moderately large 50 nm vesicle has threefold the surface area and fivefold the volume of a 30 nm vesicle.

Considering that differences in concentration and composition impact potency, it seems reasonable to posit that EVs of different sizes from a single source would exhibit differential biological functions. Indeed, some functional aspects of EVs are known to vary with size, including cell uptake[33] (which is also cholesterol-dependent[46]). Concerning membrane protein composition, smaller EVs (<50 nm) are differentially enriched compared with larger ones[47]. CD9 and CD63, two commonly analysed EV tetraspanins, share disparities in expression depending on EV biogenesis pathway (exosome versus endosome). Our own data (from the quantitative cryogenic electron microscopy (cryo-EM) of EVs isolated from HEK293 cells via size-exclusion chromatography (SEC)) indicate that >70% of EVs by count and >25% of the EV surface area exist in a subpopulation of <80 nm in diameter (at the limit of light-scattering-based resolution), and that protein composition (using hybrid

interferometric reflectance imaging–fluorescence microscopy) varies substantially with size (Fig. 1b,c).

This observation has interesting implications for the biological function(s) of EV subpopulations. As the diameter decreases and the surface-area-to-volume ratio increases, is the biological function dictated by the surface cargo rather than the luminal cargo? Similarly, is there a lower size of EV for which the luminal cargo is negligible? A recent study has suggested that under a limiting surface-to-volume ratio (that is, a diameter of <200 nm), EVs exhibit a prevalence of membrane-associated proteins and thus may be considered to be more surface-active than luminal-active, as compared with larger EVs (>800 nm) or large oncosomes (>1,000 nm), whose function may be based on their dominating luminal cargo[48].

In general, studies for understanding the influence of size within small EVs (for example, <200 nm) are rare, primarily due to the limited technical capacity to fractionate EVs with meaningful resolution. SEC can be used to modulate the lower range of EV sizes enriched by changing the resin pore size (35 nm versus 70 nm lower size cut-off), yet decreasing the diameter recovered increases the contaminating protein. Such trade-offs often mean that the downstream assessment of functional performance, ascribed to a particular EV size fraction, is problematic.

EV fractionation by size has been demonstrated using asymmetric flow field-flow fractionation (AF4)[38], resulting in the coarse enrichment of large EVs (90–120 nm), small EVs (60–80 nm) and even smaller EV-like particles known as exomeres, which are non-membranous particles with a size of <50 nm. Enriched exomeres can be further pelleted via ultracentrifugation to differentially isolate supermeres (that is, supernatant of exomeres)[49]. Recent biochemical analysis of such fractions has indicated notable differences in composition and function across these subpopulations[50,51]. Nonetheless, more work is needed, both in technical platform development towards high-resolution size fractionation and in applying techniques to better define the localization of molecules across particles of various sizes.

Molecular composition

Membrane.—The EV membrane is the primary interface, regulating immune clearance, tropism, endothelial trafficking and more. Recent compelling work has suggested that the central function of EVs in intercellular communication is not via the delivery of intraluminal content but rather through direct membrane contact, with EVs acting as a surrogate for contact-dependent cell signalling[52]. Yet, how functional surface moieties are distributed across EVs remains largely undefined, as the co-presence of multiple molecules on a single vesicle is difficult to measure. We recently reported the identification of distinct molecularly defined EV subpopulations from the variable co-expression of common EV tetraspanins with several tumour markers (CD24, EpCAM and HER2)[53], underscoring and the importance of carefully selecting pull-down moieties for diagnostic application. Other recent evidence suggests that single EVs may be only sparsely decorated with functional molecules[54].

EV isolation methods also impact heterogeneity. For example, compared with EVs exposed to high, damaging shear forces during ultracentrifugation, EVs isolated via SEC have increased functionality to induce ERK phosphorylation in the context of angiogenic potency[55]. The influence of isolation-driven heterogeneity on downstream function may be insufficiently studied. For example, EVs exist across a range of densities (~1.09–1.29 g ml^{-1})[34], yet no functional studies compare potency or other endpoints across this range.

Core.—For synthetic drug-delivery vehicles, physico-chemical properties are typically confined to a narrow range to maximize potency and minimize variance across preparations[56]. For EVs, such variance in loaded cargo is largely ignored. Furthermore, co-localization of functional surface molecules correlated with internal payloads has rarely been explored at the single-vesicle level, although such approaches are under development[57,58]. The efficiency of drug encapsulation across EVs, with respect to size or the density of a desired surface marker, are mostly unknown, despite the use of surface expression to dictate the EV fate[6] and function[52]. For example, EVs navigate the extracellular matrix in a composition-dependent manner, mediated by the presence of molecules found only on a subset of EVs within a given source[59]. Targeted delivery may be highly impacted, depending on the co-localization of such integrins with the desired functional molecules.

Considering the substantial EV heterogeneity and limitation of the methodologies that are currently available for its assessment, we propose three primary cornerstones (with associated challenges) in the context of improving therapeutic applications.

Cornerstone 1: implementing complementary single-EV methodologies, with calibration and caution

The first step in addressing EV heterogeneity is to accurately characterize their properties, ideally at single-molecule resolution on single EVs. Modern single-particle platforms include NTA, nanoscale flow cytometry (FCM)[60], hybrid interferometric reflectance imaging–fluorescence microscopy[61], RPS[62], super-resolution microscopy (SRM)[63,64], electron microscopy (EM)[65,66] and single-particle spectroscopy[67]. Each of these technologies has distinct advantages, disadvantages and detection limits in terms of molecular or size sensitivity thresholds (Table 1)[68].

Some interesting trends are noted. No single method has the ideal combination of speed, throughput, cost, single-vesicle resolution, limit of detection, phenotyping ability and ability to be calibrated. Whereas NTA can detect the presence of some contaminants via fluorescence modes on some models, it is fundamentally limited by the low scattering efficiency of biological nanoparticles, with a practical lower-size detection limit of ~70–90 nm for most EVs[69]. As such, NTA measurements are intrinsically biased towards larger EVs, potentially missing more than half of particles below its detection limit (Fig. 1b,c). Laser trapping Raman spectroscopy offers molecular fingerprints of EVs, and when combined with light scattering can provide concurrent sizing but is similarly limited by the low refractive index and low scattering of EVs[70]. RPS improves on the lower size limit, down to ~65 nm, but typically offers no molecular information (although the new ARC platform combines fluorescence with RPS). Single-particle-resolved fluorescence platforms

can deliver multiplexed analysis but struggle to detect fewer than several dozen epitopes per particle, and thus may not have the sensitivity to sufficiently characterize EVs. Similar limitations in the lower limit of scatter detection and epitope sensitivity by fluorescence detection impede single-particle FCM[60]. SRM enables spatial resolution down to 20 nm, and can provide information on the morphological, physical and chemical composition of single EVs[64]. However, SRM relies on specialized dyes and chemical conditions, and EVs must be immobilized and fixed. EM has the sensitivity to directly probe the entire size range of EVs but fails to deliver molecular information, except when combined with gold nanoparticle labelling, although this approach is laborious, low-throughput and only enables limited multiplexibility[71].

Despite the limitations of existing technologies, innovations are being rapidly reported for addressing many of these gaps, including single-molecule fluorescence detection[72] and EV droplet sequencing[58], which may deliver robust protein analysis at single-vesicle and even single-molecule resolution. Whereas these technological developments are often driven by EV diagnostics[73], they could be adopted for characterizing therapeutic formulations.

Critically, single-particle technologies are sensitive to subsets of a given size and/or concentration range of EVs; therefore, careful consideration must be taken to ensure parity across instruments[44]. It is not likely that the same single EVs can be compared across techniques, making calibration for direct comparison essential. Currently, different techniques exhibit various forms of internal calibration, depending on the variable measured. In FCM, high-dynamic-range detectors enable calibration using a Mie scattering model to determine the lower limit of detection[74,75]. NTA can provide calibrated data that relate mean squared displacement to diameter and mean number of events to concentration, but its limit of detection cannot be reliably calibrated due to low-dynamic-range camera-based detectors. As a result, the lower limit of detection for NTA fluctuates, making cross-platform comparison unreliable. The reported diameter provided by hybrid interferometric reflectance imaging–fluorescence microscopy (that is, ExoView/Leprechaun) is also obtained via a calibration performed by the manufacturer, albeit under the assumption of a specific EV refractive index (which is known to vary across EVs[76]).

Challenge 1: EV enrichment technologies lag behind EV characterization tools

In general, leaps in technology to enrich or sort subpopulations of EVs have developed more slowly than characterization tools. As technologies to measure the accurate distribution of desired functional molecules across single vesicles at single-molecule sensitivity are developed, platforms for sorting and enriching EVs on the basis of this information must follow. This is a major hurdle for the therapeutic application of EVs, where trillions of EVs are typically needed to provoke a response in vivo. With their customizable designs, microfluidic devices have demonstrated enhanced EV yields, purification capacities and contaminant separation compared with traditional methods[77]. These features enable the purification of EV populations via biophysical characteristics and functional surface

cargo. More work is needed to develop such platforms for efficient, high-throughput, multi-parametric EV isolation.

For routine EV characterization, ideal workflows would be to collect calibrated data from multiple instruments to report concordance[75]. Relevant size ranges and fluorescent sensitivities should be measured and used to contextualize concordance across the measurements. For EV concentration, the context of particles that exceed a given technique's limit of detection is often difficult to assess. Where possible, units of EV measures should be transformed to standard units—for example, the conversion of fluorescence arbitrary units to molecules of equivalent soluble fluorochrome (or MESF) and diameter values reported in nanometres—by calibrating measurements against NIST-traceable standard materials (MESF-calibrated fluorescent beads, refractive index standards and so on)[74].

Cornerstone 2: standardized functional assays for EV subpopulations

Functions of interest for EV therapeutics are (1) high cellular uptake[78,79], (2) cell/tissue tropism[6], (3) immunomodulation[78,80], (4) endothelial barrier crossing[21,81], (5) protective effects (for example, neuroprotection)[82,83], (6) regenerative properties (for example, angiogenesis)[84–87] and (7) cell death/proliferation[29]. Critical gaps that contribute to the barrier of EV clinical translation have been described extensively[29,88–92], and include the difficulty in assessing purity from co-isolates and a poor understanding of MOAs. In recent years, there have been substantial improvements[93], in part associated with a field-wide movement towards reproducibility, which includes efforts such as the minimal information for studies of extracellular vesicles (MISEV) guidelines[94,95] and EV-TRACK[96]. Despite this, dose–response studies[80,97], non-conditioned media controls[98,99] and detergent-treated controls[100] remain under-used.

Potency assays, discussed in multiple reviews in the context of EVs[29,92], are an essential validation step for therapeutics, and are ideally designed to test the MOA of a product[101,102]. Potency assays quantitatively measure the ability of a product to produce a given effect in a disease-relevant manner[29]. Harnessing a specific functional subpopulation may drastically improve the potency of EV therapeutics while decreasing off-target effects. However, there is no clear pipeline for how to reproducibly prove a connection between a physico-chemical subpopulation and the function of EVs. Standardization of a matrix of tests to identify the relationship of a physiochemical property of an EV with a specific MOA and function will be essential in the preparation of natural and synthetic EV-based therapeutics[29,101]. The goal of such a matrix of tests would be to compellingly confirm that a subpopulation of EVs with defined physiochemical properties exists and can be quantified (that is, dosed), and that these properties are required for functional effects on a target cell. Single-particle analysis techniques may offer insight into the frequency of the subpopulation and the distribution of key molecules in question[81,103].

We outline an ideal, generalized workflow to develop an appropriate MOA study, using many of the controls and experiments recommended in various reviews[29,92,95], while specifically considering EV heterogeneity at the single-EV scale (Fig. 2).

First, to confirm that the potency in a given assay arises from EVs, several controls are recommended, such as surfactant controls, dose–response curves and negative fluorescence or non-conditioned media controls[94,95]. A method to confirm EV interaction with a target cell (that is, confocal microscopy) would be beneficial. Whereas the assay itself should be specific to the function of interest, it is important to ensure that the measure is quantifiable. To this end, functional negative and positive controls for comparing the potency of an EV to a known, non-EV molecule would also aid in standardization[29,101]. For example, if an EV-based therapeutic was designed to stimulate angiogenesis, a potency comparison with vascular endothelial growth factor (or VEGF) as the positive control would be useful. Finally, the controls and comparisons used to confirm the MOA of a given physiochemical property should be chosen carefully.

As protocols to enrich EVs via target physiochemical properties are lacking, indirect approaches are often used to correlate specific molecules to a given function. These include (1) using multiple endogenous populations of EVs with different physiochemical properties, (2) engineering endogenous EVs, (3) comparing EVs from genetically modified cells[104] and (4) comparing EVs with synthetic/mimetic counterparts. As each has unique limitations, it is necessary for researchers to consider how these limitations may impact their conclusions.

Additional features for an ideal functional assay, as broken down into the following topics, should be considered.

Multiple and complementary assays

We recommend using at least two complementary assays to produce compelling results. For example, it has recently been reported that CD47 expression on EVs limits phagocytic clearance[78]. Whereas fluorescent EVs from CD47 knockout cells were quickly cleared from circulation, EVs from a cell line producing high amounts of CD47 had an increased number of circulating EVs that could be recovered. Complementarily, blocking CD47 using a functionally blocking antibody increased the signal of EVs within circulating monocytes, suggesting that CD47 expression blocked phagocytosis. In this manner, functional conclusions based on multiple methods with different limitations are less likely to result from a confounding variable associated with using any single method.

Cell sources

Examining EVs enriched from different cell sources remains one of the most common practices in correlating EV properties with function. Frequently, this is a top-down approach, first identifying the cells that produce EVs with a given function, and then comparing those EVs to identify similar physiochemical features[6,105]. Similarly, some studies have used an effective ‘control’ population[29] of EVs by comparing the EVs from cells of similar lineage or source with key functional differences to identify, first, whether or not EVs influence a specific biological function and, then, the key features that differentiate the EV populations[21,106–108]. Although these studies are informative and associate a function with an EV type or subpopulation, a clear MOA often remains speculative.

Dosing

Differences in size and molecular composition between EV populations confound equivalent dosing by particle count, protein amount or a combination of these. Instead, by defining a specific MOA, the relevant molecules themselves could be used as the dosing method. The quantification of particle numbers is confounded by the detection limits of most equipment. In addition, no standard method of labelling EVs for any studies that require fluorescence (that is, in vitro uptake or biodistribution) exists. Many commonly used dyes, such as membrane stains (PKH67, MemGlow, DiR), soluble dyes (CFSE) or fluorescently tagged antibodies, have bias towards specific EV locales or molecules and could be unknowingly labelling EV subpopulations[109,110]. Finally, success in vitro has not always been a good indicator of success in vivo.

Blocking, knockout and knock-in

Removing or blocking specific physiochemical components can be used to identify functional molecules[59,78,95,111]. Chemical treatment can also be used to remove certain components of EVs (that is, trypsinization for cleaving extracellular domains of surface moieties[78]). Multiple studies have examined the knockout, knock-in or mutation of molecules to identify functional differences in EV subpopulations[78,112,113]. However, these studies frequently cause unintended effects outside the specific pathway or molecule[95,114] and rely on specific biogenesis pathways.

Finally, comparing EVs with synthetic counterparts can elucidate the functional contribution of EV physiochemical features. For example, EVs as drug-delivery vehicles have been compared to liposomes by incorporating lipids that mimic the bilayer of EVs[78,79]. With the advent of newer technology, proteoliposomes may also offer an option for synthetic comparisons, especially for organotropic uptake[115,116]; however, these vehicles have yet to be directly compared with EVs. As described in the following section, methods to artificially generate EV mimetics largely ignore vesicle heterogeneity.

Challenge 2: heterogeneity of EVs is not considered in clinical therapeutics

Clinical applications of EVs are already well underway, yet heterogeneity has rarely been considered, leading to poor rigour and reproducibility, and potentially eroding confidence in EV-based therapeutics. Very few descriptions of EV clinical trials acknowledge EV subpopulations. Trials that claim a specific EV biomolecule as being responsible for its therapeutic effect typically provide bulk assays to show the presence of the molecule of interest in their EV samples. Only a single currently active EV therapeutic trial has detailed single-EV characterization[117], via CD24-expressing EVs from an engineered cell line. However, FCM data were only used as a qualitative confirmation of CD24 on the EVs and did not include any methods for purifying or otherwise enriching CD24-expressing EVs. Improving trial design using the workflow described in the previous section will undoubtedly improve the performance, consistency and impact of clinical studies.

Cornerstone 3: engineered nanovesicles to complement and benchmark EVs

The third and final cornerstone is the leveraging of synthetic artificial nanovesicles (ANVs). ANV applications are on the rise for addressing the heterogeneity of endogenous EVs and the relatively inefficient harvest rates from cultured cells[118–120]. ANVs are typically produced in higher yields, such as via cell fragmentation[121,122], which can achieve between 100-fold[123] and 250-fold[124] higher yields than native EVs. However, high-yield purification may not translate to the functional purity and homogeneity of samples.

ANVs are typically synthesized using liposomes as the base chassis[125], via sonication, extrusion, double emulsion, microfluidic devices or pH jumping[126–132]. Other methods combine liposomes and native EV to incorporate lipids or proteins and thus create semisynthetic or hybrid ANVs[133]. Microfluidic methods that produce ANVs result in more uniform populations[129]. Other methods, such as AF4 (ref. 134), are yet being developed for reducing the size heterogeneity of liposomes post-synthesis. The lipid composition depends on the therapeutic application and can introduce an additional degree of heterogeneity[135]. Regardless of the preparation method, labelling method or lipid composition, a degree of heterogeneity in size or composition is commonly present[135].

EV-mimetic liposomes are often functionalized with Peptides, proteins, or other molecules via covalent conjugation[136,137]. For example, the bacterial cytolytic toxin ClyA has been attached to bacteria-derived vesicles using a SpyCatcher/SpyTag pair and a SnoopCatcher/SnoopTag pair[138]. Yet these studies rarely consider the heterogeneity of protein distribution across single-engineered vesicles.

Other examples of closely related structural nanoparticles, such as solid lipid nanoparticles, need little leveraging, exemplified by the global rollout of the COVID vaccines manufactured largely by Moderna and Pfizer/BioNTech. These ribonucleic acid/lipid conjugate biomaterials share a nanoscale dimension with EVs but do not offer the additional functionality provided by ANVs (for example, active targeting, immune privilege and structural features such as membrane proteins, glycans and aqueous lumen). The complexation of solid lipid nanoparticles with EVs for creating artificial hybrid nanoparticles with combined functions has been proposed but not yet reported[139].

There is a distinct opportunity to use ANVs to probe some of the long-standing questions regarding the interplay between EV size and composition (Fig. 3). ANVs can be designed for the engineering of various EV membrane and core content features while systematically varying the diameter, or vice versa. The approaches can be scaled more easily than with native EVs and produced in more homogeneous formulations. In addition to such use as positive controls for functional output, they can similarly be applied as reference standards, to benchmark characterization platforms or other assays.

Challenge 3: engineering and characterizing the heterogeneity of ANVs

ANV characterization suffers the same limitations as described above, that is, low scattering efficiency and challenges of single-molecule sensitivity. As with the discussion for EVs above, ANVs would benefit from adopting the current single-particle analysis or microfluidic-based isolation or enrichment platforms that are being developed for EVs for downstream functional testing. However, ANV formulation is a bottom-up process, whereby the physiochemical features of the nanoparticle are introduced starting from a blank state. The field is currently struggling with methods for the facile and reliable introduction of a single protein or function into the ANVs. In this regard, engineering and characterizing their heterogeneity is poorly appreciated and investigated.

In addition, given that the precise molecular composition of active EV subpopulations within a heterogeneous batch is ill-defined, it remains a challenge to synthesize mimetic ANVs. It is unclear whether the current methods for bottom-up ANV synthesis can achieve the complexity needed to mimic EVs, such as multiple disparate membrane proteins or complex glycans with controlled spatial distribution across single vesicles. These remain open questions that need to be addressed if the field is to move forwards.

Outlook

The rapidly evolving field of EVs and ANVs stands on the precipice of substantial breakthroughs. There are, however, prominent challenges to be addressed.

Unravelling the nature of EVs

One of the central pursuits in the EV landscape is an all-encompassing characterization of these vesicles. Microfluidics has emerged as a promising avenue that provides refined and precise ways of isolating and enriching EV samples. With this advancement, the community is poised to comprehensively understand EV heterogeneity. SRM and FCM are at the forefront of single-vesicle characterization, and offer a window into the intricate composition of EVs. However, to truly grasp the complexities, multi-dimensional techniques may hold the key, enabling us to better assess the diverse biomolecular makeup of these vesicles.

Decoding functionality through assays

Whereas characterization paints a detailed portrait of EVs, functional assays reveal their true impact. The present landscape, unfortunately, sees a range of assays with various standards, leading to fragmented insights. A standardized approach is imperative. This understanding will shape dosing methodologies. Furthermore, the fluorescence labelling of EVs needs a refined approach. The current bias in labelling EVs depends on the choice of dye, such that where the label is sited (for example, a soluble dye such as CFSE in the lumen[140], a membrane-inserting dye such as fluorescent PKH26[141] or an antibody-tagged fluorophore against a particular EV membrane protein[57]) can skew the results[141].This potential bias underscores the importance of a standardized method that facilitates comparison across different laboratories and captures the true chemical, physical and biological properties of EVs.

Bridging the clinical divide

Clinical trials, which should be the gold standard of EV-based therapeutics, often sidestep the issue of EV subpopulations. This gap undermines rigour and reproducibility and threatens the foundational confidence in EV-based treatments.

ANVs

Whereas ANVs promise a solution to the challenges of EV heterogeneity and production limitations, their potential has not been realized. Key milestones include the establishment of techniques for assessing the biomolecular heterogeneity within ANVs and simulating the complexities of EVs in these engineered counterparts. However, the intricacies of engineering ANVs are notable as their synthesis poses its own challenges. Achieving a level of complexity that mimics EVs is a daunting task, but successes in this will mould the therapeutic landscape, determining whether ANVs can stand alongside EVs or serve as adjuncts.

This Review briefly presents a path forwards to strategically overcome vesicle heterogeneity as a major hurdle in the clinical translation of EVs/ANVs for nanotherapeutics. We summarize the current knowledge regarding the differential functional roles ascribed to EV subpopulations, highlighting the lack of focus on particle heterogeneity. The gap in addressing intra-subpopulation heterogeneity within a given EV preparation could explain the lacklustre efficacy of current EV-based drug-delivery systems. Off-target effects that arise from contaminating subpopulations could be potentially crippling their potential impact. We emphasize three concrete cornerstones for addressing heterogeneity, including the implementation of complementary single-EV methodologies, the standardization of functional assays in the context of the distribution of potential mechanistic biomolecules across single EVs and the leveraging of engineered ANVs to replace or benchmark native EVs. We highlight a few major challenges that could serve as focal points for future development (Box 1), including the development of isolation technologies that focus on enriching EVs, according to multiple markers and sizes with high resolution, and improving the approaches for proteoliposome/ANV synthesis. Microfluidic platforms hold promise, but so far have not materialized with sufficient throughput for isolating the yields needed for downstream functional assays or in vivo use. Addressing these bottlenecks will improve EV-based therapeutics, inform ANV engineering approaches and accelerate both EVs and ANVs towards clinical translation.

Acknowledgements

We acknowledge support from the National Institutes of Health, including R01CA241666, R01EB033389, R01EB034279 and R35GM142788. R.R.M. was supported by F31NS120590. N.L. was supported by a funded training grant program (T32HL007013) of the National Heart, Lung, and Blood Institute. T.H. was supported by an NDSEG Fellowship. A.A. was supported by UC Davis MCB T32. This work was supported in part by the UC Davis Comprehensive Cancer Center's Women's Cancer Care & Research (WeCARE) program.

References

1. Yáñez-Mó M et al. Biological properties of extracellular vesicles and their physiological functions. J. Extracell. Vesicles 4, 27066 (2015). [PubMed: 25979354]

2. Midekessa G et al. Zeta potential of extracellular vesicles: toward understanding the attributes that determine colloidal stability. ACS Omega 5, 16701–16710 (2020). [PubMed: 32685837]
3. Zhu X et al. Comprehensive toxicity and immunogenicity studies reveal minimal effects in mice following sustained dosing of extracellular vesicles derived from HEK293T cells. J. Extracell. Vesicles 6, 1324730 (2017). [PubMed: 28717420]
4. Elsharkasy OM et al. Extracellular vesicles as drug delivery systems: why and how? Adv. Drug Deliv. Rev. 159, 332–343 (2020). [PubMed: 32305351]
5. Parada N, Romero-Trujillo A, Georges N & Alcayaga-Miranda F Camouflage strategies for therapeutic exosomes evasion from phagocytosis. J. Adv. Res. 31, 61–74 (2021). [PubMed: 34194832]
6. Hoshino A et al. Tumour exosome integrins determine organotropic metastasis. Nature 527, 329–335 (2015). [PubMed: 26524530] This study provides direct evidence that EVs from tumour cells exhibit specific tropism towards target organs, which is one of the most promising features of EVs compared with synthetic nanocarriers intended for clinical applications.
7. Choi H et al. Biodistribution of exosomes and engineering strategies for targeted delivery of therapeutic exosomes. Tissue Eng. Regener. Med. 10.1007/s13770-021-00361-0 (2021).
8. Wiklander OPB et al. Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting. J. Extracell. Vesicles 10.3402/jev.v4.26316 (2015).
9. Sung BH & Weaver AM Exosome secretion promotes chemotaxis of cancer cells. Cell Adhes. Migr. 11, 187–195 (2017).
10. Kriebel PW et al. Extracellular vesicles direct migration by synthesizing and releasing chemotactic signals. J. Cell Biol. 217, 2891–2910 (2018). [PubMed: 29884750]
11. Hosseini R et al. The roles of tumor-derived exosomes in altered differentiation, maturation and function of dendritic cells. Mol. Cancer 20, 83 (2021). [PubMed: 34078376]
12. Yuan P et al. Neural stem cell-derived exosomes regulate neural stem cell differentiation through miR-9-Hes1 axis. Front. Cell Dev. Biol. 10.3389/fcell.2021.601600 (2021).
13. Huang J, Ding Z, Luo Q & Xu W Cancer cell-derived exosomes promote cell proliferation and inhibit cell apoptosis of both normal lung fibroblasts and non-small cell lung cancer cell through delivering alpha-smooth muscle actin. Am. J. Transl. Res. 11, 1711–1723 (2019). [PubMed: 30972195]
14. Matsumoto Y et al. Tumor-derived exosomes influence the cell cycle and cell migration of human esophageal cancer cell lines. Cancer Sci. 111, 4348–4358 (2020). [PubMed: 32969511]
15. Harmati M et al. Small extracellular vesicles convey the stress-induced adaptive responses of melanoma cells. Sci. Rep. 9, 15329 (2019). [PubMed: 31653931]
16. Mathieu M, Martin-Jaular L, Lavieu G & Théry C Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nat. Cell Biol. 21, 9–17 (2019). [PubMed: 30602770]
17. Bonsergent E et al. Quantitative characterization of extracellular vesicle uptake and content delivery within mammalian cells. Nat. Commun. 12, 1864 (2021). [PubMed: 33767144]
18. Walker S et al. Extracellular vesicle-based drug delivery systems for cancer treatment. Theranostics 9, 8001–8017 (2019). [PubMed: 31754377]
19. Shi Y, van der Meel R, Chen X & Lammers T The EPR effect and beyond: strategies to improve tumor targeting and cancer nanomedicine treatment efficacy. Theranostics 10, 7921–7924 (2020). [PubMed: 32685029]
20. Jakubec M et al. Plasma-derived exosome-like vesicles are enriched in lyso-phospholipids and pass the blood–brain barrier. PLoS ONE 15, e0232442 (2020). [PubMed: 32956358]
21. Morad G et al. Tumor-derived extracellular vesicles breach the intact blood–brain barrier via transcytosis. ACS Nano 13, 13853–13865 (2019). [PubMed: 31479239] This research provides a precise description of how tumour-derived EVs manage to breach the formidable BBB, revealing the role of transcytosis and the endothelial recycling endocytic pathway during the process.
22. Saint-Pol J, Gosselet F, Duban-Deweer S, Pottiez G & Karamanos Y Targeting and brossing the blood–brain barrier with extracellular vesicles. Cells 9, 851 (2020). [PubMed: 32244730]
23. Whiteside TL Exosomes carrying immunoinhibitory proteins and their role in cancer. Clin. Exp. Immunol. 189, 259–267 (2017). [PubMed: 28369805]

24. Yang E et al. Exosome-mediated metabolic reprogramming: the emerging role in tumor microenvironment remodeling and its influence on cancer progression. Signal Transduction Targeted Ther. 5, 242 (2020).
25. Kuriyama N, Yoshioka Y, Kikuchi S, Azuma N & Ochiya T Extracellular vesicles are key regulators of tumor neovasculature. Front. Cell Dev. Biol. 10.3389/fcell.2020.611039 (2020).
26. Richter M, Vader P & Fuhrmann G Approaches to surface engineering of extracellular vesicles. Adv. Drug Deliv. Rev. 173, 416–426 (2021). [PubMed: 33831479]
27. Kim H et al. Engineered extracellular vesicles and their mimetics for clinical translation. Methods 177, 80–94 (2020). [PubMed: 31626895]
28. Lener T et al. Applying extracellular vesicles based therapeutics in clinical trials – an ISEV position paper. J. Extracell. Vesicles 4, 30087 (2015). [PubMed: 26725829]
29. Nguyen VVT, Witwer KW, Verhaar MC, Strunk D & Balkom BWM Functional assays to assess the therapeutic potential of extracellular vesicles. J. Extracell. Vesicles 10, e12033 (2020). [PubMed: 33708360] This study addresses the urgent need for standardized and predictive assays to gauge the therapeutic potential of EVs, ensuring consistent therapeutic efficacy and batch-to-batch reproducibility.
30. Liang X et al. Extracellular vesicles engineered to bind albumin demonstrate extended circulation time and lymph node accumulation in mouse models. J. Extracell. Vesicles 11, e12248 (2022). [PubMed: 35879268]
31. Xu M et al. Size-dependent in vivo transport of nanoparticles: implications for delivery, targeting, and clearance. ACS Nano 17, 20825–20849 (2023). [PubMed: 37921488]
32. Sharma S, LeClaire M, Wohlschlegel J & Gimzewski J Impact of isolation methods on the biophysical heterogeneity of single extracellular vesicles. Sci. Rep. 10, 13327 (2020). [PubMed: 32770003]
33. Caponnetto F et al. Size-dependent cellular uptake of exosomes. Nanomedicine 13, 1011–1020 (2017). [PubMed: 27993726]
34. Bobrie A, Colombo M, Krumeich S, Raposo G & Théry C Diverse subpopulations of vesicles secreted by different intracellular mechanisms are present in exosome preparations obtained by differential ultracentrifugation. J. Extracell. Vesicles 1, 18397 (2012).
35. Mazouzi Y et al. Biosensing extracellular vesicle subpopulations in neurodegenerative disease conditions. ACS Sens. 7, 1657–1665 (2022). [PubMed: 35446554]
36. Mitchell MI et al. Extracellular Vesicle Capture by AnTibody of CHoice and Enzymatic Release (EV-CATCHER): a customizable purification assay designed for small-RNA biomarker identification and evaluation of circulating small-EVs. J. Extracell. Vesicles 10, e12110 (2021). [PubMed: 34122779]
37. Kang Y et al. Isolation and profiling of circulating tumor-associated exosomes using extracellular vesicular lipid–protein binding affinity based microfluidic device. Small 15, 1903600 (2019).
38. Zhang H et al. Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. Nat. Cell Biol. 20, 332–343 (2018). [PubMed: 29459780] This study underscores the heterogeneity of EV populations, introduces the creative use of AF4, two distinct EV sizes and the previously unreported ‘exomeres’.
39. Kowal J et al. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. Proc. Natl Acad. Sci. USA 113, E968–E977 (2016). [PubMed: 26858453]
40. Lázaro-Ibáñez E et al. DNA analysis of low- and high-density fractions defines heterogeneous subpopulations of small extracellular vesicles based on their DNA cargo and topology. J. Extracell. Vesicles 8, 1656993 (2019). [PubMed: 31497265]
41. Lee S-S et al. A novel population of extracellular vesicles smaller than exosomes promotes cell proliferation. Cell Commun. Signal. 17, 95 (2019). [PubMed: 31416445]
42. Emelyanov A et al. Cryo-electron microscopy of extracellular vesicles from cerebrospinal fluid. PLoS ONE 15, e0227949 (2020). [PubMed: 31999742]
43. Arraud N et al. Extracellular vesicles from blood plasma: determination of their morphology, size, phenotype and concentration. J. Thromb. Haemost. 12, 614–627 (2014). [PubMed: 24618123]

44. van der Pol E, Welsh JA & Nieuwland R Minimum information to report about a flow cytometry experiment on extracellular vesicles: communication from the ISTH SSC subcommittee on vascular biology. J. Thromb. Haemost. 20, 245–251 (2022). [PubMed: 34637195]
45. Jeppesen DK et al. Reassessment of exosome composition. Cell 10.1016/j.cell.2019.02.029 (2019).
46. Pfrieger FW & Vitale N Cholesterol and the journey of extracellular vesicles. J. Lipid Res. 59, 2255–2261 (2018). [PubMed: 29678958]
47. Mathieu M et al. Specificities of exosome versus small ectosome secretion revealed by live intracellular tracking of CD63 and CD9. Nat. Commun. 12, 4389 (2021). [PubMed: 34282141]
48. Zendrini A et al. On the surface-to-bulk partition of proteins in extracellular vesicles. Colloids Surf. B Biointerfaces 218, 112728 (2022). [PubMed: 35969923]
49. Zhang Q et al. Supermeres are functional extracellular nanoparticles replete with disease biomarkers and therapeutic targets. Nat. Cell Biol. 23, 1240–1254 (2021). [PubMed: 34887515]
50. Anand S, Samuel M & Mathivanan S in New Frontiers: Extracellular Vesicles Vol. 97 (eds Mathivanan S et al.) 89–97 (Springer, 2021).
51. Wang G et al. Tumour extracellular vesicles and particles induce liver metabolic dysfunction. Nature 618, 374–382 (2023). [PubMed: 37225988]
52. Jahnke K & Staufer O Membranes on the move: the functional role of the extracellular vesicle membrane for contact-dependent cellular signalling. J. Extracell. Vesicles 13, e12436 (2024). [PubMed: 38649339]
53. Mizenko RR et al. Tetraspanins are unevenly distributed across single extracellular vesicles and bias sensitivity to multiplexed cancer biomarkers. J. Nanobiotechnol. 19, 250 (2021).
54. Ferguson S, Yang KS & Weissleder R Single extracellular vesicle analysis for early cancer detection. Trends Mol. Med. 28, 681–692 (2022). [PubMed: 35624008]
55. Mol EA, Goumans M-J, Doevendans PA, Sluijter JPG & Vader P Higher functionality of extracellular vesicles isolated using size-exclusion chromatography compared to ultracentrifugation. Nanomedicine 13, 2061–2065 (2017). [PubMed: 28365418]
56. Mitchell MJ et al. Engineering precision nanoparticles for drug delivery. Nat. Rev. Drug Discov. 20, 101–124 (2021). [PubMed: 33277608]
57. Martel R, Shen ML, DeCorwin-Martin P, De Araujo LOF & Juncker D Extracellular vesicle antibody microarray for multiplexed inner and outer protein analysis. ACS Sens. 7, 3817–3828 (2022). [PubMed: 36515500]
58. Ko J, Wang Y, Sheng K, Weitz DA & Weissleder R Sequencing-based protein analysis of single extracellular vesicles. ACS Nano 15, 5631–5638 (2021). [PubMed: 33687214]
59. Sariano PA et al. Convection and extracellular matrix binding control interstitial transport of extracellular vesicles. J. Extracell. Vesicles 12, e12323 (2023). [PubMed: 37073802]
60. Welsh JA et al. A compendium of single extracellular vesicle flow cytometry. J. Extracell. Vesicles 12, e12299 (2023). [PubMed: 36759917]
61. Deng F et al. Single-particle interferometric reflectance imaging characterization of individual extracellular vesicles and population dynamics. J. Vis. Exp. 10.3791/62988 (2022).
62. Cimorelli M, Nieuwland R, Varga Z & Van Der Pol E Standardized procedure to measure the size distribution of extracellular vesicles together with other particles in biofluids with microfluidic resistive pulse sensing. PLoS ONE 16, e0249603 (2021). [PubMed: 33793681]
63. McNamara RP et al. Imaging of surface microdomains on individual extracellular vesicles in 3-D. J. Extracell. Vesicles 11, e12191 (2022). [PubMed: 35234354] Using advanced direct stochastic optical reconstruction microscopy, this work reports the imaging of spatial microdomains on single EVs, uncovering a previously unappreciated level of structural intricacy and heterogeneity.
64. Saftics A et al. Single Extracellular VEsicle Nanoscopy. J. Extracell. Vesicles 12, 12346 (2023). [PubMed: 37422692] Using SRM, this study introduces an innovative assay that provides in-depth insights into the physical, chemical and morphological characteristics of individual EVs in the context of discerning disease-associated and organ-associated EV subtypes.
65. Höög JL & Lötvall J Diversity of extracellular vesicles in human ejaculates revealed by cryo-electron microscopy. J. Extracell. Vesicles 4, 28680 (2015). [PubMed: 26563734]

66. Musante L et al. Rigorous characterization of urinary extracellular vesicles (uEVs) in the low centrifugation pellet - a neglected source for uEVs. Sci. Rep. 10, 3701 (2020). [PubMed: 32111925]

67. Paolini L et al. Fourier-transform infrared (FT-IR) spectroscopy fingerprints subpopulations of extracellular vesicles of different sizes and cellular origin. J. Extracell. Vesicles 9, 1741174 (2020). [PubMed: 32341767]

68. Kwon Y & Park J Methods to analyze extracellular vesicles at single particle level. Micro Nano Syst. Lett. 10, 14 (2022).

69. Bachurski D et al. Extracellular vesicle measurements with nanoparticle tracking analysis – an accuracy and repeatability comparison between NanoSight NS300 and ZetaView. J. Extracell. Vesicles 8, 1596016 (2019). [PubMed: 30988894]

70. Enciso-Martinez A et al. Synchronized Rayleigh and Raman scattering for the characterization of single optically trapped extracellular vesicles. Nanomedicine 24, 102109 (2020). [PubMed: 31669420]

71. Jung MK & Mun JY Sample preparation and imaging of exosomes by transmission electron microscopy. J. Vis. Exp. 10.3791/56482 (2018).

72. Andronico LA et al. Sizing extracellular vesicles using membrane dyes and a single molecule-sensitive flow analyzer. Anal. Chem. 93, 5897–5905 (2021). [PubMed: 33784071]

73. Morales R-TT & Ko J Future of digital assays to resolve clinical heterogeneity of single extracellular vesicles. ACS Nano 16, 11619–11645 (2022). [PubMed: 35904433]

74. Welsh JA, Jones JC & Tang VA Fluorescence and light scatter calibration allow comparisons of small particle data in standard units across different flow cytometry platforms and detector settings. Cytometry A 97, 592–601 (2020). [PubMed: 32476280]

75. Nieuwland R, Siljander PR-M, Falcón-Pérez JM & Witwer KW Reproducibility of extracellular vesicle research. Eur. J. Cell Biol. 101, 151226 (2022). [PubMed: 35460959]

76. Kashkanova AD, Blessing M, Gemeinhardt A, Soulat D & Sandoghdar V Precision size and refractive index analysis of weakly scattering nanoparticles in polydispersions. Nat. Methods 19, 586–593 (2022). [PubMed: 35534632]

77. Kanwar SS, Dunlay CJ, Simeone DM & Nagrath S Microfluidic device (ExoChip) for on-chip isolation, quantification and characterization of circulating exosomes. Lab Chip 14, 1891–1900 (2014). [PubMed: 24722878]

78. Kamerkar S et al. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. Nature 546, 498–503 (2017). [PubMed: 28607485]

79. Roerig J et al. A focus on critical aspects of uptake and transport of milk-derived extracellular vesicles across the Caco-2 intestinal barrier model. Eur. J. Pharm. Biopharm. 166, 61–74 (2021). [PubMed: 34077790]

80. Wolf M et al. A functional corona around extracellular vesicles enhances angiogenesis, skin regeneration and immunomodulation. J. Extracell. Vesicles 11, e12207 (2022). [PubMed: 35398993]

81. Banks WA et al. Transport of extracellular vesicles across the blood–brain barrier: brain pharmacokinetics and effects of inflammation. Int. J. Mol. Sci. 21, 4407 (2020). [PubMed: 32575812]

82. Kumar P et al. Neuroprotective effect of placenta-derived mesenchymal stromal cells: role of exosomes. FASEB J. 33, 5836–5849 (2019). [PubMed: 30753093]

83. Yadid M et al. Endothelial extracellular vesicles contain protective proteins and rescue ischemia–reperfusion injury in a human heart-on-chip. Sci. Transl. Med. 12, eaax8005 (2020). [PubMed: 33055246]

84. You B et al. Extracellular vesicles rich in HAX1 promote angiogenesis by modulating ITGB6 translation. J. Extracell. Vesicles 11, e12221 (2022). [PubMed: 35524442]

85. Fan J, Xu G, Chang Z, Zhu L & Yao J miR-210 transferred by lung cancer cell-derived exosomes may act as proangiogenic factor in cancer-associated fibroblasts by modulating JAK2/STAT3 pathway. Clin. Sci. 134, 807–825 (2020).

86. Choi JS et al. Exosomes from differentiating human skeletal muscle cells trigger myogenesis of stem cells and provide biochemical cues for skeletal muscle regeneration. J. Control. Release 222, 107–115 (2016). [PubMed: 26699421]

87. Yang J, Zhang X, Chen X, Wang L & Yang G Exosome mediated delivery of miR-124 promotes neurogenesis after ischemia. Mol. Ther. Nucleic Acids 7, 278–287 (2017). [PubMed: 28624203]

88. Kennedy TL, Russell AJ & Riley P Experimental limitations of extracellular vesicle-based therapies for the treatment of myocardial infarction. Trends Cardiovasc. Med. 31, 405–415 (2021). [PubMed: 32822840]

89. Reiner AT et al. Concise Review: Developing best-practice models for the therapeutic use of extracellular vesicles. Stem Cells Transl. Med. 6, 1730–1739 (2017). [PubMed: 28714557]

90. Paolini L et al. Large-scale production of extracellular vesicles: report on the “massivEVs” ISEV workshop. J. Extracell. Biol. 1, e63 (2022). [PubMed: 38939213]

91. Witwer KW et al. Defining mesenchymal stromal cell (MSC)-derived small extracellular vesicles for therapeutic applications. J. Extracell. Vesicles 8, 1609206 (2019). [PubMed: 31069028]

92. Gimona M et al. Critical considerations for the development of potency tests for therapeutic applications of mesenchymal stromal cell-derived small extracellular vesicles. Cytotherapy 23, 373–380 (2021). [PubMed: 33934807] This road-map article on mesenchymal stromal/stem cells (MSCs) presents an analysis within the context of therapeutic medicine, emphasizing the potential of small EVs over traditional cellular MSC products, with a focus on the intricacies and challenges in quality control and consistent potency.

93. Poupardin R, Wolf M & Strunk D Adherence to minimal experimental requirements for defining extracellular vesicles and their functions. Adv. Drug Deliv. Rev. 176, 113872 (2021). [PubMed: 34284058]

94. Lötvall J et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. J. Extracell. Vesicles 3, 26913 (2014). [PubMed: 25536934]

95. Théry C et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J. Extracell. Vesicles 7, 1535750 (2018). [PubMed: 30637094]

96. Consortium EV-TRACK et al. EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research. Nat. Methods 14, 228–232 (2017). [PubMed: 28245209]

97. Otero-Ortega L et al. Low dose of extracellular vesicles identified that promote recovery after ischemic stroke. Stem Cell Res. Ther. 11, 70 (2020). [PubMed: 32075692]

98. Sjöqvist S et al. Exosomes derived from clinical-grade oral mucosal epithelial cell sheets promote wound healing. J. Extracell. Vesicles 8, 1565264 (2019). [PubMed: 30719240]

99. Karttunen J et al. Effect of cell culture media on extracellular vesicle secretion from mesenchymal stromal cells and neurons. Eur. J. Cell Biol. 101, 151270 (2022). [PubMed: 35987046]

100. Salmond N, Khanna K, Owen GR & Williams KC Nanoscale flow cytometry for immunophenotyping and quantitating extracellular vesicles in blood plasma. Nanoscale 13, 2012–2025 (2021). [PubMed: 33449064]

101. Basu J & Ludlow JW Cell-based therapeutic products: potency assay development and application. Regen. Med. 9, 497–512 (2014). [PubMed: 25159066]

102. Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products (Food and Drug Administration, 2011).

103. Ko SY et al. The glycoprotein CD147 defines miRNA-enriched extracellular vesicles that derive from cancer cells. J. Extracell. Vesicles 12, 12318 (2023). [PubMed: 36973758] This study reveals a fundamental discovery: the glycoproteins CD147 and CD98 uniquely characterize specific EV subpopulations, fundamentally different from the traditional tetraspanin-positive EVs, providing an enhanced specificity in distinguishing cancerous origins.

104. Sung BH et al. A live cell reporter of exosome secretion and uptake reveals pathfinding behavior of migrating cells. Nat. Commun. 11, 2092 (2020). [PubMed: 32350252]

105. Lee EJ, Choi Y, Lee HJ, Hwang DW & Lee DS Human neural stem cell-derived extracellular vesicles protect against Parkinson’s disease pathologies. J. Nanobiotechnol. 20, 198 (2022).

106. Hao Q et al. Mesenchymal stem cell-derived extracellular cesicles decrease lung injury in mice. J. Immunol. 203, 1961–1972 (2019). [PubMed: 31451675]
107. Kulaj K et al. Adipocyte-derived extracellular vesicles increase insulin secretion through transport of insulinotropic protein cargo. Nat. Commun. 14, 709 (2023). [PubMed: 36759608]
108. Xie F et al. Breast cancer cell-derived extracellular vesicles promote $CD8^+$ T cell exhaustion via TGF-β type II receptor signaling. Nat. Commun. 13, 4461 (2022). [PubMed: 35915084]
109. Baumgart T, Hunt G, Farkas ER, Webb WW & Feigenson GW Fluorescence probe partitioning between L_o/L_d phases in lipid membranes. Biochim. Biophys. Acta Biomembr. 1768, 2182–2194 (2007).
110. Tertel T et al. Imaging flow cytometry challenges the usefulness of classically used extracellular vesicle labeling dyes and qualifies the novel dye Exoria for the labeling of mesenchymal stromal cell–extracellular vesicle preparations. Cytotherapy 24, 619–628 (2022). [PubMed: 35314115]
111. Welsh JA et al. Minimal information for studies of extracellular vesicles (MISEV2023): from basic to advanced approaches. J. Extracell. Vesicles 13, e12404 (2024). [PubMed: 38326288]
112. Zhang X et al. Proteomic analysis of MSC-derived apoptotic vesicles identifies Fas inheritance to ameliorate haemophilia a via activating platelet functions. J. Extracell. Vesicles 11, e12240 (2022). [PubMed: 36856683]
113. Campos-Mora M et al. Neuropilin-1 is present on Foxp3+ T regulatory cell-derived small extracellular vesicles and mediates immunity against skin transplantation. J. Extracell. Vesicles 11, e12237 (2022). [PubMed: 35676234]
114. Collino F et al. AKI recovery induced by mesenchymal stromal cell-derived extracellular vesicles carrying microRNAs. J. Am. Soc. Nephrol. 26, 2349–2360 (2015). [PubMed: 25901032]
115. Scalise M, Pochini L, Giangregorio N, Tonazzi A & Indiveri C Proteoliposomes as tool for sssaying membrane transporter functions and interactions with xenobiotics. Pharmaceutics 5, 472–497 (2013). [PubMed: 24300519]
116. Sejwal K et al. Proteoliposomes – a system to study membrane proteins under buffer gradients by cryo-EM. Nanotechnol. Rev. 6, 57–74 (2017).
117. Shapira S et al. A novel platform for attenuating immune hyperactivity using EXO-CD24 in COVID-19 and beyond. EMBO Mol. Med. 14, e15997 (2022). [PubMed: 35776000]
118. Gwak H, Park S, Yu H, Hyun K-A & Jung H-I A modular microfluidic platform for serial enrichment and harvest of pure extracellular vesicles. Analyst 147, 1117–1127 (2022). [PubMed: 35212324]
119. Bordanaba-Florit G, Royo F, Kruglik SG & Falcón-Pérez JM Using single-vesicle technologies to unravel the heterogeneity of extracellular vesicles. Nat. Protoc. 10.1038/s41596-021-00551-z (2021).
120. Hromada C, Mühleder S, Grillari J, Redl H & Holnthoner W Endothelial extracellular vesicles—promises and challenges. Front. Physiol. 8, 275 (2017). [PubMed: 28529488]
121. Yang Z et al. Functional exosome-mimic for delivery of siRNA to cancer: in vitro and in vivo evaluation. J. Control. Release 243, 160–171 (2016). [PubMed: 27742443]
122. Ramasubramanian L, Kumar P & Wang A Engineering extracellular vesicles as nanotherapeutics for regenerative medicine. Biomolecules 10, 48 (2020).
123. Jang SC et al. Bioinspired exosome-mimetic nanovesicles for targeted delivery of chemotherapeutics to malignant tumors. ACS Nano 7, 7698–7710 (2013). [PubMed: 24004438]
124. Jo W et al. Large-scale generation of cell-derived nanovesicles. Nanoscale 6, 12056–12064 (2014). [PubMed: 25189198]
125. Bozzuto G & Molinari A Liposomes as nanomedical devices. Int. J. Nanomed. 10, 975–999 (2015).
126. Akbarzadeh A et al. Liposome: classification, preparation, and applications. Nanoscale Res. Lett. 8, 102 (2013). [PubMed: 23432972]
127. Patil YP & Jadhav S Novel methods for liposome preparation. Chem. Phys. Lipids 177, 8–18 (2014). [PubMed: 24220497]

128. Lapinski MM, Castro-Forero A, Greiner AJ, Ofoli RY & Blanchard GJ Comparison of liposomes formed by sonication and extrusion: rotational and translational diffusion of an embedded chromophore. Langmuir 23, 11677–11683 (2007). [PubMed: 17939695]

129. Large DE, Abdelmessih RG, Fink EA & Auguste DT Liposome composition in drug delivery design, synthesis, characterization, and clinical application. Adv. Drug Deliv. Rev. 176, 113851 (2021). [PubMed: 34224787]

130. Jahn A, Vreeland WN, DeVoe DL, Locascio LE & Gaitan M Microfluidic directed formation of liposomes of controlled size. Langmuir 23, 6289–6293 (2007). [PubMed: 17451256]

131. Carugo D, Bottaro E, Owen J, Stride E & Nastruzzi C Liposome production by microfluidics: potential and limiting factors. Sci. Rep. 6, 25876 (2016). [PubMed: 27194474]

132. Genç R, Ortiz M & O′Sullivan CK Curvature-tuned preparation of nanoliposomes. Langmuir 25, 12604–12613 (2009). [PubMed: 19856992]

133. Piffoux M, Silva AKA, Wilhelm C, Gazeau F & Tareste D Modification of extracellular vesicles by fusion with liposomes for the design of personalized biogenic drug delivery systems. ACS Nano 12, 6830–6842 (2018). [PubMed: 29975503]

134. Kuntsche J, Decker C & Fahr A Analysis of liposomes using asymmetrical flow field-flow fractionation: separation conditions and drug/lipid recovery. J. Sep. Sci. 35, 1993–2001 (2012). [PubMed: 22865761]

135. Larsen J, Hatzakis NS & Stamou D Observation of inhomogeneity in the lipid composition of individual nanoscale liposomes. J. Am. Chem. Soc. 133, 10685–10687 (2011). [PubMed: 21688773]

136. Moulahoum H, Ghorbanizamani F, Zihnioglu F & Timur S Surface biomodification of liposomes and polymersomes for efficient targeted drug delivery. Bioconjug. Chem. 32, 1491–1502 (2021). [PubMed: 34283580]

137. Staufer O et al. Bottom-up assembly of biomedical relevant fully synthetic extracellular vesicles. Sci. Adv. 7, eabg6666 (2021). [PubMed: 34516902] This research introduces an exciting synthetic approach based on mimetic EVs with defined compositions, a promising avenue for clinical applications and a platform for dissecting the intricacies of EV signalling mechanisms.

138. Cheng K et al. Bioengineered bacteria-derived outer membrane vesicles as a versatile antigen display platform for tumor vaccination via Plug-and-Display technology. Nat. Commun. 12, 2041 (2021). [PubMed: 33824314]

139. Rodríguez DA & Vader P Extracellular vesicle-based hybrid systems for advanced drug delivery. Pharmaceutics 14, 267 (2022). [PubMed: 35214000]

140. Morales-Kastresana A et al. Labeling extracellular vesicles for nanoscale flow cytometry. Sci. Rep. 7, 1878 (2017). [PubMed: 28500324]

141. Melling GE et al. Confocal microscopy analysis reveals that only a small proportion of extracellular vesicles are successfully labelled with commonly utilised staining methods. Sci. Rep. 12, 262 (2022). [PubMed: 34997141]

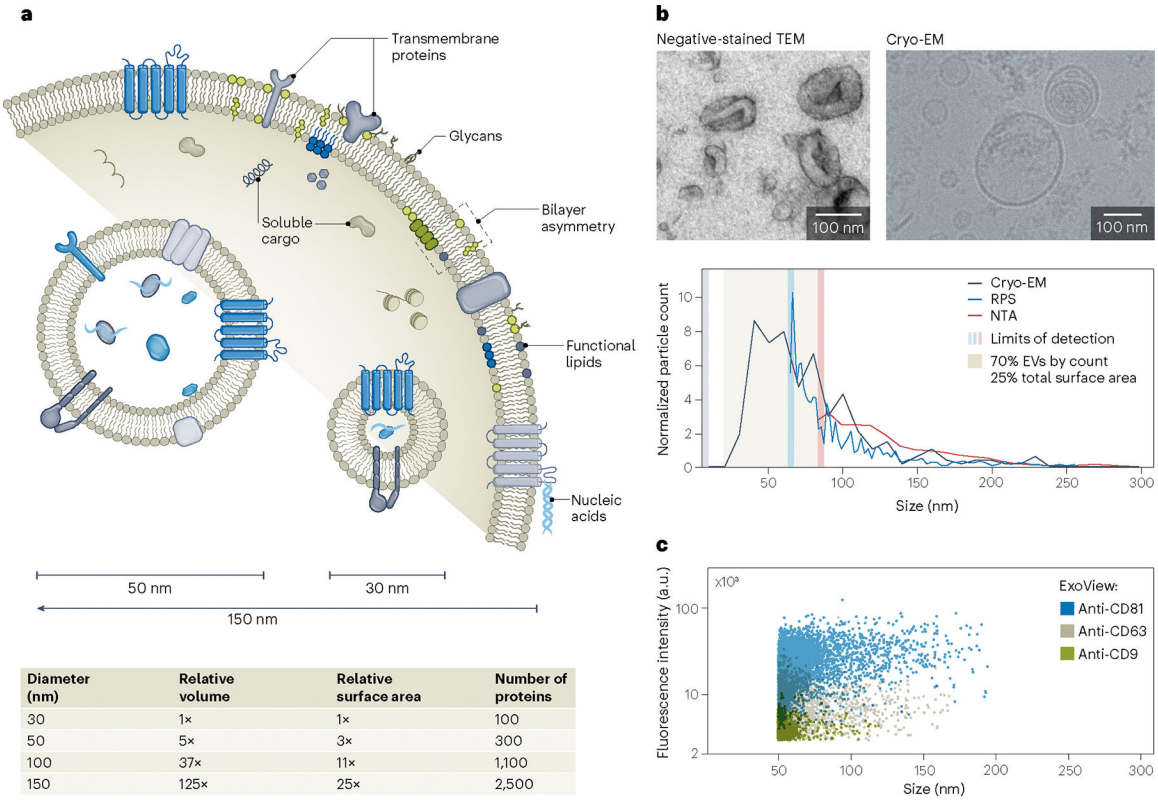
Box 1

Key future milestones

As the research community ventures further into the applications of EVs and ANVs, these milestones may shape the direction and pace of advancements.

- **Advanced vesicle characterization.** Achieving higher resolution and the more accurate characterization of individual EVs—shedding light on their true heterogeneity—will be pivotal. The development of newer techniques or the refinement of existing ones, such as SRM, will be crucial milestones for understanding vesicle nuances. The creation of innovative new approaches to characterize EV heterogeneity will be highly impactful.
- **Standardized functional assays.** Establishing universally accepted functional assays will enhance our understanding of EV behaviour and pave the way for more reproducible research outcomes. The creation of standardized assays is urgently needed to validate the potency and utility of EVs.
- **Clinical trial refinement.** As EV-based therapeutics grow in prominence, an essential milestone will be the broad acknowledgement of EV heterogeneity in the design of clinical trials. This recognition could improve the efficacy, reproducibility and safety of EV applications in clinical settings.
- **Engineering mastery of ANVs.** The coming years will see a focus on producing ANVs that closely mimic the complexity and functionality of natural EVs. Successfully introducing multiple proteins, complex glycans and controlling their spatial distribution across ANVs will be a key achievement.
- **Therapeutic applications and benchmarks.** A substantial milestone will be the use of ANVs, not just as therapeutic agents but also as benchmarking standards against which natural EVs are compared in terms of functionality and characterization.
- **Interplay of size and composition.** Using ANVs to investigate the relationship between EV size and composition will lead to greater insights into their natural function and potential therapeutic applications.
- **Microfluidics for EV and ANV isolation.** Scaling and optimizing microfluidic-based isolation techniques will revolutionize how researchers isolate and enrich specific vesicle populations. Reaching a point where these techniques are commonplace in laboratories worldwide will be a notable advancement.
- **Understanding and mimicking EV biogenesis.** Deepening our understanding of the biogenesis pathways of specific EV subpopulations will be instrumental. This knowledge will subsequently inform the engineering of ANVs, for reliably reproducing the properties of particular EV types.

- **Addressing dosing challenges.** Solving the complex puzzle of EV dosing —whether by particle count, protein amount, specific molecules or other measure—will be crucial, especially as these vesicles move closer to widespread therapeutic use.
- **Collaborative research and knowledge sharing.** Establishing and refining platforms for global collaboration and standardization will ensure that researchers worldwide can build on each other's work, providing opportunities for accelerated breakthroughs in the field.



Diameter (nm)	Relative volume	Relative surface area	Number of proteins
30	1×	1×	100
50	5×	3×	300
100	37×	11×	1,100
150	125×	25×	2,500

Fig. 1 |. EVs exhibit major heterogeneity that currently limits their effective application in drug delivery.

The first cornerstone by which to address EV heterogeneity is to assess it using multiple, complementary single-particle characterization techniques. **a**, EVs are heterogeneous in size, from ~30 nm to more than 150 nm. The smallest and largest EVs are unlikely to exhibit similar functions due to large differences in their volume, surface area and composition (for example, membrane components and soluble cargo). The relative volume, surface area and number of membrane proteins scale for a given vesicle size. **b**, Single-particle sizing (via negative-stained TEM, cryo-EM, resistive pulse sensing (RPS) and nanoparticle tracking analysis (NTA)) reveals high polydispersity, with as much as 70% of EVs by count (and 25% of EVs by surface area) falling below 80 nm, that is, the size detection threshold of NTA. TEM, transmission electron microscopy. **c**, Molecular information measured using single-particle immunofluorescence indicates that common membrane proteins (that is, tetraspanins CD9, CD63 and CD81) vary with diameter.

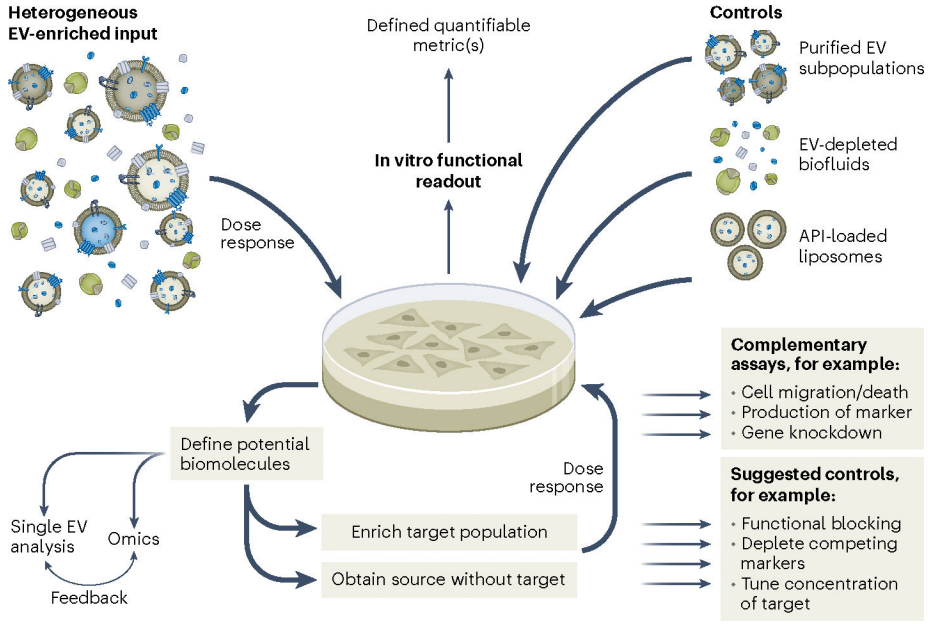


Fig. 2 |. Generalized experimental workflow for determining the EV MOA in the context of single-vesicle heterogeneity.

A second cornerstone by which to address EV heterogeneity is to develop in vitro functional studies towards identifying an MOA. EV-enriched inputs should be dosed in complementary functional assays with defined quantifiable metrics. Bold arrows along the left and centre of the schematic illustrate an idealized path towards defining potential biomolecules (ideally defining their distribution across single particles), enriching target subpopulations with those biomolecules and repeating the assay against EV sources without the target. Various controls that depend on the context of the assay are summarized along the right-hand side of the schematic. Input controls, such as purified EV subpopulations, EV-depleted biofluids or liposomes loaded with the target biomolecule or active pharmaceutical ingredient (API) should be used if possible. To aid in identification of the MOA, functional blocking or other manipulation of the target biomolecule should be performed.

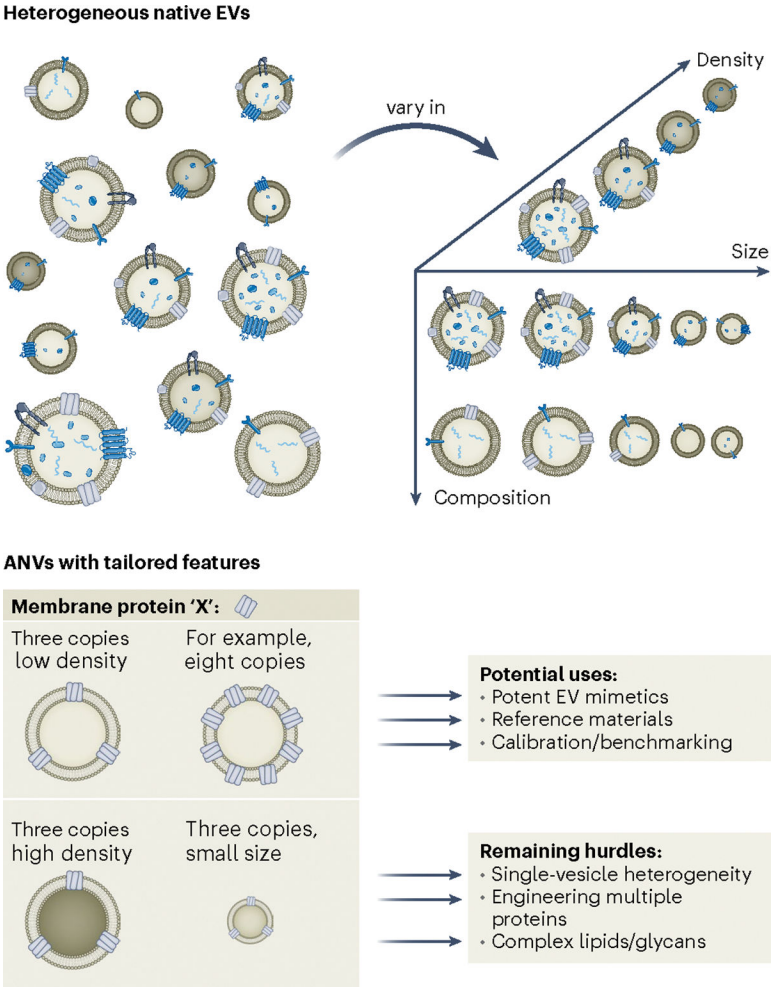


Fig. 3 |. Engineering ANVs to address long-standing challenges that are associated with EVs.
A third cornerstone for addressing inherent EV heterogeneity is to use synthetic ANVs. Whereas native EVs exist in highly entangled mixed populations that vary in, for example, size, density and molecular composition, ANVs can in theory be tailored with highly controlled molecular content (for example, a desired protein 'X'), size and density. Whereas such materials could benchmark or replace/complement EV therapeutics, it remains a challenge to assess ANV heterogeneity, and technical capabilities for synthesizing vesicles that match the complexity of native EVs (for example, multiple membrane proteins per vesicle, complex lipid and/or glycan decoration) lag behind.

Table 1 |

Comparison of single-particle characterization methodologies

Characterization method	Single-vesicle resolution	Difficulty (time+skill)	Approximate lower limit of detection (nm)	Phenotyping	Able to be calibrated
NTA	••	•	70–90	••	••
RPS	••	••	65	None	••••
RPS with immunofluorescence (via ARC platform)	••	••	65	•••	••••
Single-particle FCM	••	•••	90	••••	••••
Laser trapping Raman spectroscopy	••	••••	90	•••[a]	•••
Hybrid interferometric reflectance imaging-fluorescence microscopy	••	•••	50	•••	•
SRM	•••	••••	20	••••	•••
EM	••••	••••	<30	•	••••

A comparison across current commercial platforms with respect to their ability to definitively resolve single vesicles, level of difficulty, lower limit of size detection, degree of molecular information that can be measured and ability to be calibrated using appropriate reference materials. •, very low; ••, low; •••, medium; ••••, high.

[a]Label-free.