Perspective

For reprint orders, please contact: reprints@futuremedicine.com

Nanomedicine

Measurement and standardization challenges for extracellular vesicle therapeutic delivery vectors

Bryant C Nelson*,[1], Samantha Maragh[1], Ionita C Ghiran[2], Jennifer C Jones[3], Paul C DeRose[1], Elzafir Elsheikh[1], Wyatt N Vreeland[1] & Lili Wang[1]

[1]National Institute of Standards & Technology, Material Measurement Laboratory, Gaithersburg, MD 20899, USA
[2]Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, MA 02115, USA
[3]National Institutes of Health, National Cancer Institute, Bethesda, MD 20892, USA
*Author for correspondence: bryant.nelson@nist.gov

Extracellular vesicles (EVs), such as exosomes and microvesicles, are nonreplicating lipid bilayer particles shed by most cell types which have the potential to revolutionize the development and efficient delivery of clinical therapeutics. This article provides an introduction to the landscape of EV-based vectors under development for the delivery of protein- and nucleic acid-based therapeutics. We highlight some of the most pressing measurement and standardization challenges that limit the translation of EVs to the clinic. Current challenges limiting development of EVs for drug delivery are the lack of: standardized cell-based platforms for the production of EV-based therapeutics; EV reference materials that allow researchers/manufacturers to validate EV measurements and standardized measurement systems for determining the molecular composition of EVs.

First draft submitted: 14 May 2020; Accepted for publication: 10 July 2020; Published online: 4 September 2020

Keywords: drug delivery • exosomes • extracellular vesicles • gene delivery • molecular composition • nucleic acids • protein delivery • red blood cells • reference materials • therapeutics

Extracellular vesicles (EVs) are nonreplicating lipid bilayer particles that are shed by almost all types of eukaryotic [1] and prokaryotic [2,3] cells under both physiological and pathological conditions. Extending in size over the nanometer to micrometer size range, EVs are biological vectors intimately involved in cell-to-cell communication via the lateral transfer of coding and noncoding RNAs, small amounts of DNA, surface and cytoplasmic proteins, phospholipids and soluble small molecules to recipient cells [4,5]. They have been shown, in many fundamental and preclinical studies, to possess low immunogenicity, minimal complement activation potential, low toxicity and excellent biocompatibility [6,7]. EVs released by various bacteria and parasites were first observed 50 years ago [8] and were initially dismissed as artifacts (similarly to what happened to EVs released by the eukaryotic cells, a decade later). Once their cargo was analyzed and determined to contain DNA molecules, their roles in cell-to-cell communication, quorum sensing (adaptation and survival in a new or changing environment) and metabolic cooperation (antibiotic resistance) became better understood [9]. Over the last decade, there has been expansive global interest in the promise that EVs demonstrate for the development of cell-free diagnostics (e.g., liquid biopsies), regenerative medicine therapeutics and next-generation drug-delivery platforms. Of the three major subclassifications of EVs (apoptotic bodies, microvesicles [MVs], exosomes; Table 1), exosomes (approximately 40–160 nm in diameter) [5] have emerged as a promising vector for the delivery of small molecule-, protein- and nucleic acid-based therapeutics. There exists considerable overlap in both the size range and in the type of cargo (biomolecular composition) found in the different EV subclassifications (Figure 1). As an important point of clarification on nomenclature, The International Society of Extracellular Vesicles (ISEV) endorses "'extracellular vesicle' (EV) as the generic term for particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate, e.g., do not contain a functional nucleus" [10]. Within this broad class of particulate structures, researchers may further define

Future Medicine

Table 1. Distinguishing characteristics of the major subclasses of extracellular vesicles.

Vesicle types	Diameter (nm)	Origin	Cargo
Exosomes	Approximately 40–160	Endosomes	mRNA, miRNA, DNA, cytoplasmic and membrane proteins including receptors and MHC molecules
MVs	Approximately 50–1000	Plasma membrane	mRNA, miRNA, DNA, cytoplasmic and membrane proteins including receptors
Apoptotic bodies	Approximately 500–2000	Plasma membrane	Similar to the contents of exosomes and MVs, but also includes histones, mitochondria, etc

MHC: Major histocompatibility complex; MV: Microvesicle.

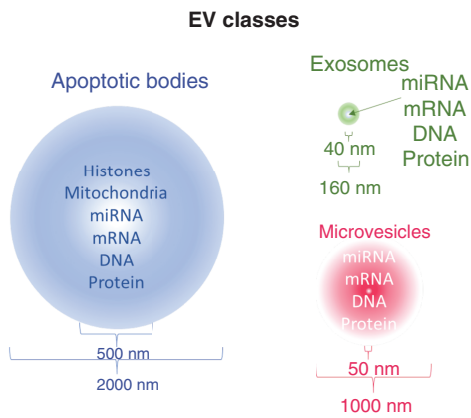

Figure 1. Illustration of the size and compositional makeup of the three major subclasses of extracellular vesicles. There exists considerable overlap among the three subclasses in terms of both size and biomolecular content. Color shading shows the distribution of particles sizes in a given class (sizes are to scale).
EV: Extracellular vesicle.

the specified entities with operational descriptors such as particle size, particle density, biochemical composition (i.e., which protein markers are enriched or depleted within or on the cell surface) and/or cellular origin.

Since the original usage of the term 'exosome' to describe DNA fragments that were transferred between *Drosophila* cells [11], the usage and context of exosome has changed to mean generally a type of EV that originates via the endosomal processing system of a cell [12]. Absolute identification and characterization of exosomes in therapeutic samples is challenging due to the fact that exosomes are similar to MVs (~50–1000 nm in diameter) [5] in many physicochemical and functional aspects, even though exosomes are released from cells into the extracellular space by endosomal processing via multivesicular bodies, while MVs bud directly from the plasma membrane into the extracellular space. There do not currently exist any definitive markers or analytical techniques that can discriminate between exosomes and MVs after they reach the extracellular space. It is even more problematic because the nominal size ranges of exosomes and MVs overlap and there exists no agreement on the upper and lower size limits for either entity. It has been suggested, but not verified, that the term exosome as a general descriptor for therapeutic EVs was popularized by industry focus-group testing as to what terminology would be the most appealing from a marketing perspective for therapeutic and/or diagnostic EVs [13]. Thus, we would strongly suggest to researchers working and publishing in the EV field that the standard terminology for engineered, diagnostic and/or therapeutic/drug delivery EVs not be 'exosomes' but rather 'EVs' with appropriate operational (e.g., size, density, cell type origin) and functional descriptors (e.g., cargo composition). For the purpose of this article on protein a-nd nucleic-acid delivery applications, when we refer to exosomes we mean therapeutic EVs preparations or isolates that are enriched in both exosomes and small-sized MVs (≤150 nm in diameter) as described in a recent report [6].

Landscape of protein- & nucleic acid-based drug delivery using therapeutic EVs

Delivery of peptide- & protein-based therapeutics via therapeutic EVs

Therapeutic EVs are important mediators of intercellular communication between cells near and far and can potentially be utilized, depending on their native composition, as intrinsic targeting agents (vectors) for the delivery of protein- and nucleic acid-based therapeutics to recipient cells via selected pathways (Figure 2) [14]. Therapeutic EVs also have a burgeoning presence as vectors for the delivery of small molecule therapeutics but those applications have been discussed and reviewed elsewhere [15] and are not part of this brief article. The biological function and clinical potential of therapeutic EVs are heavily dependent upon their protein content [16]. The proteins in

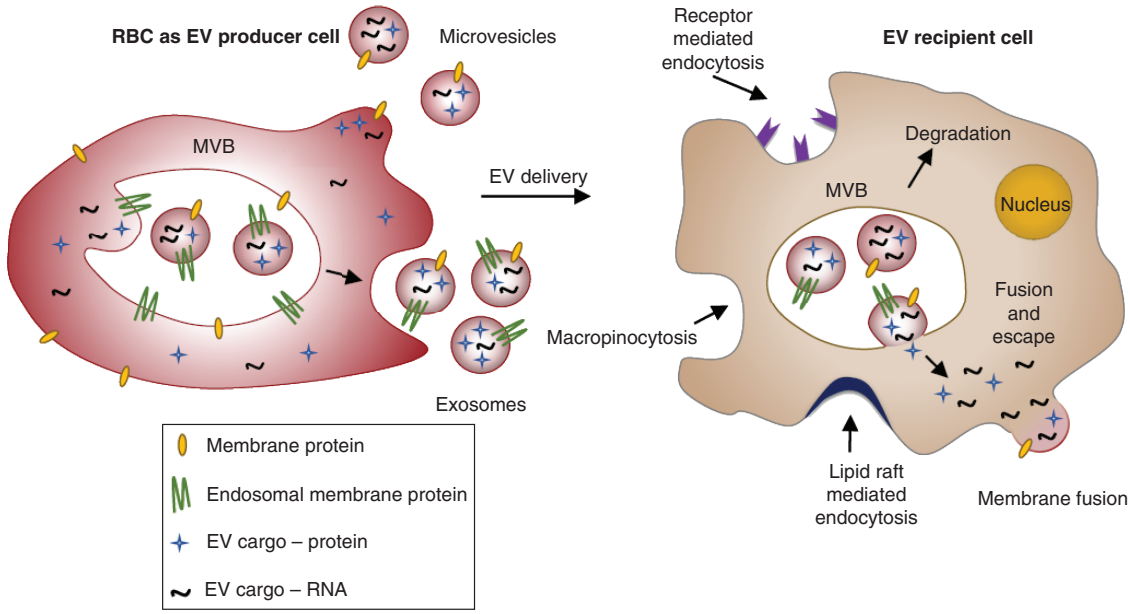

Figure 2. Production of therapeutic extracellular vesicles (exosomes + small microvesicles) from a prototypical red blood cell source for use in drug delivery and other therapeutic applications. EVs are nanoscale particulate structures, not always spherical, that are intimately involved in intercellular communication through the exchange of biological cargo. Both endogenous and exogenous protein and nucleic acid-based therapeutic cargo may be transferred from producer cells to recipient cells via various endocytotic and membrane fusion processes.
EV: Extracellular vesicle; MVB: Multivesicular body; RBC: Red blood cell.

therapeutic EVs may exist as membrane proteins, transmembrane proteins or be encapsulated as soluble entities in the hydrophilic core of the vectors. Some proteins are involved in cell adhesion and binding while others are essential for cell-to-cell communication. It should also be noted that the endogenous homing ability of EVs to specific organs and tissues depends on their surface proteins [14]. Due to the heterogeneity of therapeutic EVs and EV biogenesis [17], it is probable that different therapeutic EV subpopulations will contain variable amounts, ratios and types of proteins. Thus, for therapeutic-protein drug delivery applications, it is crucial to understand and characterize the EV vectors and the EV vector subpopulations, as well as their protein compositions for specific therapeutic applications [6,18] via the use of appropriate reference materials (RMs) and analytical characterization techniques as discussed in later sections. These types of analyses should be performed before and after loading exogenous therapeutic peptides and/or proteins cargos into the EV vector.

Loading peptide & protein cargo into EVs

There are essentially two main modes of loading peptide and/or protein cargo into therapeutic EVs: donor or producer cells can be genetically engineered to express therapeutic peptides or proteins through, for example, transfection with a DNA vector (DNA plasmid) or therapeutic peptide or protein cargo can be fused to a protein that is native (e.g., flotillin, CD9, CD81, etc.) to the therapeutic EV architecture; or secreted therapeutic EVs can be physically loaded with cargo *ex vivo* by incubating, sonicating, extruding, electroporation, freeze-thaw cycling or permeabilizing the EV membranes with saponin or through the use of some combination of the previously listed techniques [19]. After successful loading, therapeutic EVs are highly suitable for efficient delivery of both soluble cytoplasmic and poorly soluble membrane proteins to recipient cells. This suitability is primarily based on therapeutic EVs' inherent homing to selected recipient cells and on the protection from enzymatic degradation that the lipid bilayer membrane provides to surface-bound or membrane-enclosed protein cargo. The native membrane protein domains and microdomains on the surface of therapeutic EVs are also conducive to stabilizing membrane protein therapeutics in their native conformations so that the proteins retain their biological functionality [6]. It is important to note that biomimetic lipid-based vectors are also being explored as potential alternatives to EVs for the delivery of therapeutic peptides and proteins [20–22]. These types of rationally designed vectors allow for discrete structural control that may lead to enhanced sustained release of peptide and protein therapeutics. A recent overview

Table 2. Selected peptide-based therapeutic cargos delivered via therapeutic extracellular vesicles.

Delivered cargo	EV producer cell	EV target cell/tissue	EV delivery	Therapeutic application	Ref
Peptides					
1. MAGE-A3, MAGE-A4, MAGE-A10, MAGE-3DPO4 tumor antigens	Lung cancer patient dendritic cells (monocytes)	Patient immune cells	Subcutaneous and intradermal injection	Cancer immunotherapy for stimulation of $CD4^+$ and $CD8^+$ T cell response	[23]
2. MAGE-3 tumor antigen	Skin cancer patient dendritic cells (monocytes)	Patient immune cells	Subcutaneous and intradermal injection	Cancer immunotherapy for stimulation of $CD4^+$ and $CD8^+$ T cell response	[24]
3. TRP2 peptides (melanoma antigens)	Serum-derived	Immune cells (A264.7 macrophages, DC2.4 dendritic cells)	Cellular incubation	Cancer immunotherapy for activation of proinflammatory cytokines (TNF-α, IL-6)	[25]
4. Enkephalin neuropeptide	Bone MSCs	Rat hippocampal neurons and rats with cerebral occlusions	Cellular incubation and vein injection	Neurological recovery after ischemic stroke	[26]

EV: Extracellular vesicle; MSC: Mesenchymal stem cell; TRP2: Tyrosinase-related protein-2.

Table 3. Selected protein-based therapeutic cargos delivered via therapeutic extracellular vesicles.

Delivered cargo	EV producer cell	EV target cell/tissue	EV delivery	Therapeutic application	Ref
Proteins					
1. HSP60, HSP70, HSP90	Hepatocarcinoma cell lines (HepG2; PLC/PRF/5)	K562, HepG2 cells	Cellular incubation	Cancer immunotherapy for induction of NK cell response	[27]
2. Neprilysin endopeptidase (CD10)	Human adipose tissue-derived MSCs	N2a cells	Cellular incubation	Alzheimer's disease therapy for degrading β-amyloid peptide (Aβ)	[28]
3. Catalase	RAW A264.7 macrophage cells	PC12 neuronal cells, mouse brain	Cellular incubation and intranasal administration	Parkinson's disease therapy across the BBB via oxidative stress reduction	[29]
4. mCherry red fluorescent protein, Bax, IκB, Cre recombinase	HEK293T embryonic kidney cells	HeLa cells, mouse brain	Cellular incubation and ventrolateral brain injection	Platform for efficient intracellular delivery of functional proteins	[30]
5. Transferrin (Tf) & lactoferrin (Lf) iron carrier proteins	THP1 monocyte cells	J774, CHO-TRVb, HepG2, THP1, spleen macrophages, etc	Cellular incubation	Platform for utilizing Tf and Lf for targeted intracellular delivery of therapeutics	[31]
6. TRAIL surface membrane protein	MSCs	11 different rTRAIL-sensitive or resistant cancer cell lines	Cellular incubation	Selective induction of apoptosis in cancer cells	[32]
7. Saporin (ribosome-inactivating protein)	HeLa cells	CHO-K1 cells	Cellular incubation with electroporation	Platform for intracellular protein therapeutic delivery using cell-penetrating-peptide-modified exosomes	[33]
8. RFP and GFP, luciferase	HEK293 cells	U87, HepG2, L929, iPS11, iPS15 cells	Cellular incubation	Platform for delivery of therapeutic membrane proteins via VSVG pseudotyping	[34]
9. Ovalbumin	293F cells	293T, HeLa cells & C57BL/6J mice	Cellular incubation and mouse tail injections	Cancer immunotherapy for stimulation of $CD4^+$ and $CD8^+$ T cell response	[35]
10. SIRPα (membrane protein)	HEK293T cells	HT29 tumor cells	Cellular incubation	Cancer immunotherapy targeting of CD47 on tumor cells through delivery of SIRPα	[36]
11. Cas9	Vero and CHO cells	HuH7, HeLa cells	Cellular incubation	Intercellular delivery of functional CRISPR/Cas9 system	[37]

BBB: Blood–brain barrier; EV: Extracellular vesicle; GFP: Green fluorescent protein; NK: Natural killer; RFP: Red fluorescent protein; SIRPα: Signal regulatory protein alpha; TRAIL: TNF-related apoptosis-inducing ligand; VSVG: Vesicular stomatitis virus glycoprotein.

of selected peptide and protein therapeutic cargos that have been delivered to cells or tissues using therapeutic EVs are described in Table 2 (peptides) and Table 3 (proteins).

Therapeutic applications of peptide & protein-loaded EVs

The majority of the therapeutic areas described in current reports focus on applications related to cancer immunotherapies, neurodegenerative diseases, anticancer therapeutics and advancements in intracellular and membrane protein delivery for regenerative medicine. For example, Park and coworkers isolated exosomes from fetal bovine serum and loaded them with melanoma peptide antigens for the *in vitro* stimulation of antitumor immune responses [25]. In this study, fetal bovine serum-derived exosomes were loaded (via saponin incubation) with both tyrosinase-related protein-2 (TRP2) peptides and with monophosphoryl lipid A (MPLA) to produce the EXO-MPLA-TRP2 vector. The MPLA was utilized as an adjuvant to boost the immune response. The vector was subsequently shown to be efficiently uptaken by both A264.7 macrophages and DC2.4 dendritic cells and to elicit strong proinflammatory cytokine (TNF-α and IL-6) responses.

Liu and coworkers isolated exosomes from bovine mesenchymal stem cells (MSCs) and loaded them with enkephalin in order to promote neurological recovery in the brain after ischemic stroke [26]. Enkephalin is a neuropeptide involved in regulating nociception in the body and in this study the authors tested the biological effects of the enkephalin-loaded exosomes using both *in vitro* (neuronal cells) and *in vivo* (rats with cerebral artery occlusion) models. Transferrin was also incorporated (via transfection of the MSCs) into the delivery vector (tar-exo-enkephalin) because it was known that the transferrin receptor is highly expressed on blood–brain barrier cells after a stroke. Enkephalin by itself was shown to significantly decrease the levels of p53 and caspase-3 and to inhibit apoptosis in neuronal cells. The (tar-exo-enkephalin) vector was demonstrated to significantly decrease the levels of p53 and caspase-3 in rat cerebrospinal fluid and to increase the proliferation and density of the neurons (neuron regeneration) in brain sections of rats with artery occlusions.

Engineered nanoscale drug delivery-vectors are widely utilized in medical applications for their antioxidant properties and for their ability to scavenge reactive oxygen species [38]. Therapeutic EVs are biological nanoscale drug delivery-vectors. In a recent report, Haney and coworkers [29] described a method for loading the antioxidant protein catalase into the lumenal space of macrophage-derived (RAW A264.7) exosomes for the development of a potential therapeutic modality against Parkinson's disease. These so-called catalase-loaded exosomes 'exoCATs' were efficiently delivered to PC12 neuronal cells *in vitro* and also to mouse brain after intranasal administration. In both cases, exoCATs demonstrated reactive oxygen species deactivation and neuronal protection against oxidative stress. In addition, catalase was inherently protected against endogenous proteases and the exosomal delivery vector was not scavenged by the mononuclear phagocyte system.

As an alternative to viral-vector systems for functional gene editing, Chen and coworkers demonstrated that exosomes could be used for the intercellular delivery of the clustered regularly interspaced short palindromic repeats (CRISPR)/associated nuclease (Cas) system [37]. Using two different *in vitro* models (HuH7 or HeLa cells), the authors showed that the Cas9 protein and single guide (gRNA) could be efficiently exported by endogenous exosomes from CRISPR/Cas9-expressing cells. In concert with the successful exportation of the gene editing components, the authors also demonstrated the overall gene editing functionality of the system. As an example, the functional Cas9 and hepatitis B virus-specific gRNA that was expressed in HuH7 cells was delivered by the HuH7 exosomes into acceptor HuH7 cells and used to cut hepatitis B virus DNA that had been previously transfected into the acceptor HuH7 cells. The authors demonstrated a similar set of experiments and results for Cas9 and human papilloma virus-specific gRNA and its ability to cut HPV DNA in HeLa cells. Even though the delivery of the gene editing components was considered successful, the authors stated that the overall gene editing activity of the system was limited, perhaps due to the low Cas9 levels present in the acceptor cells. These four studies and others described in Tables 2 & 3 illustrate the breadth and potential of peptide and protein-based drug delivery using therapeutic EV-based delivery vectors.

Loading nucleic acid-based (genetic) cargo into EVs

The cellular origin of therapeutic EVs defines their low-immunogenicity and specific biological functions. For instance, EVs derived from antigen presenting cells express major histocompatibility complex (MHC) class I and II molecules that help activate $CD4^+$ and $CD8^+$ T cells during immune induction [39,40]. Glioblastoma cells secrete EVs containing mRNA, miRNA and angiogenic proteins that target microvascular endothelial cells, which ultimately stimulate angiogenesis [41]. Both the nucleic acid and protein content of EVs can be used to detect and identify tumors in patients with prostate, breast and ovarian cancers [42–44]. Most recently, there has been an increasing interest in the application of EVs for the delivery of nucleic acid-based therapeutics. A brief overview of selected nucleic acid-based EV therapeutics is provided in Table 4.

Table 4. Selected nucleic acid-based therapeutic cargos delivered via therapeutic extracellular vesicles.

Delivered cargo	EV producer cell	EV target cell/tissue	EV delivery	Therapeutic application	Ref
miRNA					
1. Let-7a	HEK293	EGFR expressing breast cancer cells	Lipofection	Cancer immunotherapy for breast cancer tissue	[45]
2. miRNA-155 inhibitor	Murine B cells (M12.4)	RAW 264.7 macrophages	Electroporation	Inflammatory inhibition for a variety of diseases	[46]
3. miRNA-15a	RAW 264.7, MH-S, BMDM	Murine macrophages	Calcium chloride transfection and Electroporation	miRNA-based drug delivery	[47]
4. miRNA-26a	Mouse satellite cells	Tibialis anterior muscle	Biogenesis	Treatment of chronic kidney disease complications	[48]
5. miRNA-134	Hs578Ts(i)$_8$	Breast tumors	Lipofection	Triple-negative breast cancer therapy	[49]
6. miRNA-122	Adipose tissue-derived MSCs	HCC	Lipofection	Enhancer of HCC chemosensitivity	[50]
7. miRNA-146b	Marrow stromal cells	Xenograft primary brain tumor	Electroporation	Treatment for malignant glioma	[51]
8. miRNA-159	THP-1 cells	MDA-MB-231 cells	Co-incubation	Therapy for triple negative breast cancer	[52]
9. miRNA-497	HEK293T	A549 cells	Lipofection	Treatment of NSCLC	[53]
siRNA targeting					
1. GAPDH	Dendritic cells	Brain tissue	Electroporation	Therapy for Alzheimer's disease	[54]
2. Huntingtin gene	U87 cells	Cortical neurons	Co-incubation	Therapy for Huntington's disease	[55]
3. HER2	MCF-7 cells	MCF-7	Sonication	Therapy for HER2 positive breast cancer	[56]
4. MAPK1	Human plasma, HTB-177, HeLa cells	Human mononuclear blood cells	Electroporation	siRNA-based gene therapy	[57]
5. BCR-ABL	HEK293T	CML cells	Mass action via biogenesis	Therapy for CML	[58]
6. S100A4	T1 mouse breast cancer cells	Mouse embryonic lung fibroblast	Incubation/extrusion	Suppression of breast cancer metastasis	[59]

CML: Chronic myeloid leukemia; HCC: Hepatocellular carcinoma cell.

The two main strategies for loading therapeutic EVs with a desired genetic cargo are practically identical to the strategies (described previously) utilized for loading protein therapeutics: mass action transfer of cargo during EV biogenesis via overexpression in EV producer cells; or cargo introduction by various biophysical techniques (e.g., lipofection, sonication, electroporation, hydrophobic interaction/incubation, chemical-based methods, etc. [60]) after EV harvest [39,40]. siRNAs regulate the expression of a specific gene while miRNAs are 'master' regulators of gene expression. The primary mode of gene regulation, in each case, is down regulation of targeted mRNAs. Both siRNAs and miRNAs are relatively small RNA species averaging 22–25 nucleotides in length and they can both be easily loaded into therapeutic EVs using a multiplicity of techniques [45–47,54–57]. For example, the method of electroporation destabilizes the EV membrane and allows siRNAs or miRNAs to diffuse into the vesicles. Chemical-based EV transfection methods that rely on the use of calcium chloride or other commercially available transfection reagents have been shown to incorporate miRNA and siRNA into therapeutic EVs for efficient delivery to target cells [47,57]. Though the electroporation method can induce RNA aggregation, it is still widely utilized [46,54].

Loading mRNA, linear DNA or plasmid DNA into therapeutic EVs is much more challenging than loading siRNA and/or miRNA due to therapeutic EV cargo size limitations [61,62]. Lamichhane and co workers have recently shown that linear DNA above 750 bp or plasmids above 4.5 kb exhibit very low loading levels, but general loading efficiency can be improved by using MVs instead of exosomes, resulting in a threefold increase for linear DNA and a fourfold increase for plasmid DNA [62]. Pore size restriction and limitation due to size dependent diffusion reduce the efficiency of large size DNA migration through transient pores generated by electroporation. Considering that MVs are plasma membrane-derived vesicles while exosomes originate from multivesicular bodies inside cells, it is also possible that the MVs have different lipid compositions or responses to electroporation resulting in enhanced

permeability to DNAs compared with exosomes [62]. Loading of miRNA and siRNA can also be accomplished in donor cells by mass action during biogenesis.

Therapeutic applications of nucleic acid-loaded EVs

Ohno and coworkers [45] were able to load miRNA let-7a into exosomes by incorporating it into a human embryonic kidney cell line 293 (HEK293) using lipofection. Through BCR-ABL siRNA transfection of IL3L-HEK293T donor cells, BCR-ABL siRNA was incorporated into exosomes and was able to silence effectively the BCR-ABL gene that was overexpressed in chronic myelogenous leukemia [58]. However, from an industry standpoint, to produce a versatile gene-delivery platform, robust methods for loading therapeutic EVs with specific genetic cargo after EV harvest would be preferred. Our current understanding of the mechanisms of RNA sorting into EVs is incomplete. Even though cellular overexpression of a given RNA is probably reflected in EV isolates, there might also be more specific mechanisms that load RNAs into EVs based on sequence motifs, protein/lipid binding activity, etc., that require further elucidation.

Targeted delivery of therapeutics to specific cell types could both enhance the desired delivery route and avoid off-target effects, representing an important objective of drug delivery. To achieve this goal, the ability to engineer therapeutic EVs provides an opportunity to display targeting motifs on the EV surface to increase uptake by a desired cell type, both *in vitro* and *in vivo*. Alvarez-Erviti and coworkers [54] utilized murine self-derived dendritic cell exosomes to deliver GADPH siRNA and BACE1 siRNA across the blood–brain barrier in mice by fusing the rabies virus glycoprotein to the lysosome-associated membrane protein b (Lamp2b) that binds to the acetylcholine receptor on neurons. The engineered dendritic-derived exosomes were then loaded with GADPH siRNA and BACE1 siRNA by electroporation. The study showed that exosomes loaded with siRNA against BACE1 and the housekeeping gene *GADPH* strongly suppressed target mRNA expression and β-amyloid in the brains of wild-type mice after intravenous injection. Ohno and coworkers [45] reported the delivery of GE11-targeted exosomes containing let-7a miRNA to EGFR-overexpressing breast cancer cells in a mouse xenograft model. Targeting was achieved by engineering HEK293 cells to express the transmembrane domain of platelet-derived growth factor receptor fused to the GE11 peptide that binds specifically to EGFR on cancer cells. The same donor cells were also loaded with let-7a miRNA to produce the GE11-targeted exosomes. The study showed that the exosomes isolated from engineered HEK293 cells transfected with let-7a miRNA successfully inhibited tumor growth in mice.

Because of the exceptional biocompatibility of EVs, it is highly probable that engineered therapeutic EVs for the delivery of nucleic acid cargos will be translated to clinical use. Their production source will likely be either immune cells [63] or mesenchymal stem cells [64] and they will be artificially optimized by incorporation of specific cargos and targeting motifs. The success of their clinical applications critically relies upon overcoming significant challenges associated with EVs, such as large-scale production, isolation/purification and characterization, donor cell choice for the production and possible interaction between incorporated exogenous cargo and preexisting EV content from donor cells [60,65]. These challenges and probable solutions will be discussed in detail in the following sections.

Cell-based platforms for the production of EV-based therapeutics

Current status of cell-based EV production platforms

Recent studies have focused on EVs and their cargo as functional, stand-alone structures, involved in cell-to-cell communication during normal and pathological conditions. Due to the paucity of information regarding EV biogenesis, their targeting to and uptake by recipient cells, their use as therapeutic entities is currently limited [66], but the great potential for EV therapeutics generated via native cell biogenesis is a rapidly expanding field [67]. Beyond tissues easier to access such as the liver, the small size and distinctive trafficking patterns of EVs may make them well-suited for therapeutic targets inside the central nervous system or to tumors where perfusion of conventional biological agents is limited [68,69]. Progress in the areas of new isolation modalities, characterization of EV biology and the ability for rapid engineering of EVs to deliver cargo selectively to target cells and tissues are key to the maturation and translation of EV therapeutics to the clinic. Although the field of EV therapeutics remains in very early, nascent stages [70], there are already 94 clinical trials in the active and/or recruiting phases of clinical development (www.clinicaltrials.gov; search term = exosomes; access on 5 January 2020).

Both eukaryotic and prokaryotic cells release EVs either constitutively or upon stimulation. The native ability of eukaryotic and prokaryotic cells to generate and release EVs is being leveraged to manufacture EVs as therapeutic agents or delivery vectors from cell platforms. Cell-derived therapeutic EVs may be produced *ex vivo*: EVs isolated

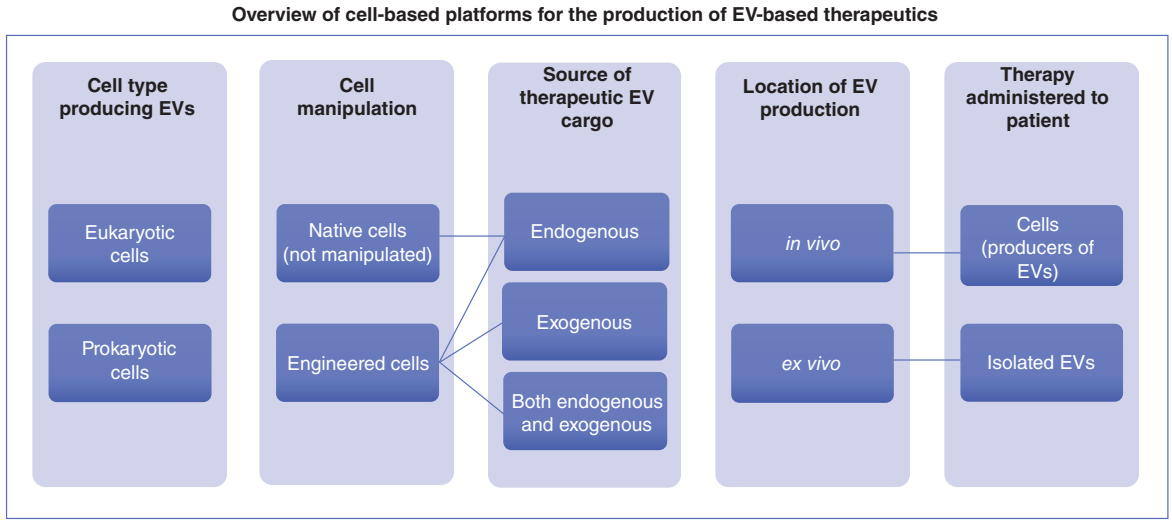

Figure 3. Schematic overview of cell-based platforms for the production of extracellular vesicle-based therapeutics. EV: Extracellular vesicle.

from cells outside the body then introduced into a patient, or *in vivo*: EVs released from cells once cells are within a patient. Eukaryotic cell derived EVs have demonstrated therapeutic promise through administration of natural EVs, such as those derived from MSCs [68,71]. The gut microbiome represents a particular type of EV-mediated cooperation between identical and different species of bacteria, as well as competition and collaboration with host cells [72]. As gut bacteria have been shown to modulate the initiation and growth of gastrointestinal (GI) cancers, as well as the response of the host to GI tumors [73], bioengineered gut bacteria, with an ability to sense the gut environment and respond by releasing EVs, are strongly positioned as a potential Trojan horse releasing EVs that contain specialized cargo to modulate local inflammation to help eradicate GI tumors and/or promote health and homeostasis. Bioengineered eukaryotic and prokaryotic cells are also able to produce EVs containing exogenous nonnative biomolecules of medicinal value, such as genome editing components (e.g., CRISPR/Cas protein and/or RNA) [74–76]. These are exciting developments that bode well for the clinical utility and burgeoning therapeutic application of EVs. Figure 3 shows a generalized schematic outlining the main components of cell-based platforms for EV therapeutic production.

Focus on red blood cells for the production of therapeutic EVs

Both *ex vivo* and *in vivo* cell platforms present the opportunity to leverage a variety of single cell types or mixtures of cell types to manufacture single EVs or mixtures of EVs to be used as therapeutics. One notable advantage of *in vivo* cell platforms for EV therapeutics is the potential for administered cells to engraft in a patient and provide a durable therapy over long periods of time or even possibly a lifetime. In effect, the cells used for *in vivo* EV therapeutics primarily function as vehicles for EV biogenesis and EV release/delivery. One of the eukaryotic cell types recently proposed as an effective EV (exosome) delivery platform is red blood cells (RBCs). Circulating RBCs are distinct among all body cells, having a cytoplasm devoid of organelles, endo/exocytic pathways, DNA, or protein synthesis. Hence, the RBC cytosol is an ideal carrier for drugs, biologicals or genome editing machinery as the cargo is shielded from the immune surveillance of the host. In addition, the intended therapeutic target(s) of the cargo are likely not present in circulating RBCs and therefore unlikely to impact the cell functions. Finally, RBCs generate EVs which upon fusion with target membranes transfer their contents into the cytosol, avoiding the lysosomal compartment [77]. As aging RBCs are gradually removed from circulation and degraded in the lysosomal system of splenic resident macrophages, loaded RBCs would also be safely removed from circulation and the undelivered cargo enzymatically destroyed, decreasing the likelihood of distressing the phagocytic cells [78]. Given these remarkable and unique characteristics and the facts that RBCs taken from a patient are nonimmunogenic and generate large daily volumes of EVs following circadian activation of the complement system [77,79], it is justifiable to believe that a patient's own RBCs would be a highly suitable autologous therapeutic delivery platform. Although the use of RBCs as delivery platforms seems to be straightforward, there are currently surprisingly few companies

attempting to use them. One company is developing novel gene therapies (including genome edited gene therapies) that utilize EVs produced from RBCs. The foundational work behind this revolutionary platform was published in 2018 [76].

Challenges to the establishment of robust cellular platforms for EV production

Several EV and specifically exosome-focused diagnostic and therapeutic solutions have emerged in recent years. A recent news feature by M Zipkin summarizes the products in development as of December 2019 and potential moves to the marketplace by these companies [70]. To be successful, cell-derived therapeutic EVs will need to be manufactured at sufficient levels and throughput to achieve therapeutic doses of high purity, as well as contain the appropriate cargo and surface molecules to make the exosome an effective medicine for the intended patient [80]. Broadly speaking, the production and necessary quality control considerations for EV therapeutics share some attributes of biological therapeutics and nano-enabled drug products and other attributes of cellular therapeutics. At present, the regulatory handling of EV therapeutics in the USA has been presented as falling under the purview of the US FDA's Center for Biologics Evaluation and Research. Following the report of serious adverse events related to "unapproved products marketed as containing exosomes", the FDA has issued public safety warnings to caution that, as of the date of the warning on 9 December 2019, "There are currently no FDA-approved exosome therapeutic products." [81].

Heterogeneity of EV preparations

One central challenge to translating EV-therapeutic products to the clinic is the inherent heterogeneity of EV preparations [82,83]. Cellular preparations, prepared or sorted based on surface receptors, can be produced in a way that selects for 'positive' cells, which are broadly homogeneous with respect to all bearing that specific marker. However, cells are typically classified as 'positive' for a specific marker based on the presence of tens of thousands of copies of specific markers and markers of this density can be readily identified on individual cells with conventional instruments, such as flow cytometers. On the other hand, a 'positive' EV may only contain a single specific marker, which is well below the limits of detection for characterization by flow cytometers available in clinical labs. It is also beneficial and yet still challenging, to have detection capability to infer the internal content of an EV separate from the external or membrane-embedded biomolecules [84]. Adequate measurements to characterize therapeutic EVs would ideally be nondestructive to EVs, high-throughput and quantitative within the required nanometer size range. There is also a limitation on existing relevant control and 'RM' EVs for these measurements that can improve measurement confidence, enable interlaboratory comparability and serve as benchmarks for regulatory applications. Because the spectrum of EVs across the full range of EV sizes (as small as diameter 30–50 nm) cannot be evaluated as individual vesicles, methods for individual vesicle characterizations are a topic of intensive research and development, which if successful would be of substantial benefit to this field.

Dosing considerations

A second central challenge to regulating EV-therapeutic products is defining the relevant unit(s) of activity or dose. When any therapeutic agent is given to patients, the dose is defined in a specific unit to be administered. In protein therapeutics, the dose is defined in units of protein concentration. In cellular therapeutics, the dose is defined in terms of number of cells of a defined type. In EV therapeutics, the measure of 'dose' remains to be determined rigorously, since the field lacks instruments to accurately measure EV concentrations and characterize EV heterogeneity [66,85]. Presently, it is feasible to measure bulk payload, for example, in a similar manner to the measurement of drug cargo encapsulated in liposomes in a liposomal preparation; however, qualitative detection or quantitative enumeration of payload contained in few or single EVs is an unmet need. For *in vivo* cell-mediated EV therapies this is further confounded by the need to measure what features of the EV-releasing cell are indicative that the cell will thrive in a patient and release EVs, as well as measures or indicators of EV release rate over the lifetime of cells. In addition to instrumentation to achieve these measures, development of qualified and standardized biologically relevant cell- and/or tissue-based platforms is required for understanding the functional activity or dose of EVs under given conditions. These platforms will also enable a means of normalized evaluation and comparison of EV activity toward optimization of EV therapeutic handling, formulation and storage.

Challenges for preparation of therapeutic EV reference materials

Technical isolation of EVs hinders development of EV reference materials

The relatively small size of EVs compared with cells has made isolation and down-stream analyses of EVs very challenging. The distinguishing characteristics of the major EV subclasses are shown in Table 1. There is extensive overlap between the different EV subclasses in terms of size, density and protein content [86–88]. Furthermore, there are subpopulations within EVs, with each subpopulation containing different sets of biomarkers [89]. It will be of great benefit to distinguish between the EV populations to evaluate their respective biological activities and their potential in different applications. As pointed out earlier in this article, there is no exclusive marker for differentiating between exosomes and MVs, even though they have different biogenesis pathways [90,91]. Tetraspanins were initially considered as potential markers for exosomes, but these membrane proteins are also found in cytoplasmic and MV membranes [92]. Proteomic analysis of several EV subtypes showed that CD9, CD63, CD81 and several other markers previously considered exclusive exosome markers were also found in MVs [93].

The ideal EV isolation method should give high recovery of EVs that are pure (not contaminated by nonvesicular components) and have integrity and biochemical properties that are intact, thus preserving their biological activity/function [10]. Since EVs share physiochemical properties with various nonvesicular structures present in complex biofluids, one-step isolation is unlikely to generate preparations of sufficient purity and orthogonal combinations of methods based on different EV characteristics such as size and density might be required. EVs are very stable and can be stored at -80°C for several months with minimal loss of activity, but they are sensitive to freeze-thaw cycles [94] and choice of storage buffer [95], which may affect functional and experimental reproducibility.

Current status of EV reference materials

There is a general need for EV RMs to quantify the proportion of the EV population that is phenotyped or counted and the minimum number of detectable proteins per vesicle [10]. The ideal EV RM should have properties representative of prototypical EV preparations to enable researchers to standardize and obtain reproducible EV measurements between instruments and between laboratories as recently described by Welsh and coworkers (arXiv:2004.09428v1 [q-bio.QM]). An RM from national institute of standards and technology (NIST) will have reference values and corresponding uncertainties, where reference values are defined as representing the best estimate of the true values based on available data. These reference values do not meet the NIST criteria for certification but are provided with associated uncertainties that may reflect only measurement precision, may reflect a lack of sufficient statistical agreement among multiple analytical methods or may not include all sources of uncertainty. Both synthetic RMs for instrument calibration, such as polystyrene nanospheres and biological RMs for validation of EV measurements, such as EV suspensions with homogeneous size and biological activity are needed. Presently, the most commonly used materials for quality control are silica or polystyrene nanospheres/beads, virus particles and liposomes, but none of these materials match the properties of actual biological EV preparations, such as cargo size distribution, refractive index or surface functional groups [96]. Biological RMs derived from biofluids or cell cultures are commercially available and may be promising alternatives to nanospheres and liposomes, but they require in-depth characterization. Most of the commercially available RMs have been produced using scalable EV isolation processes, but it is likely that the manufacturers did not follow the MISEV2018 EV characterization guidelines (e.g., the use of multiple characterization techniques) and quantitative as well as qualitative characterization data reported by manufacturers is commonly incomplete and/or insufficient.

Recently, Geeurickx and coworkers [97] have generated recombinant EVs with similar EV properties as potential RMs by transfection of HEK293T cells with gag-EGFP DNA. However, these recombinant EVs need to be validated across different laboratories. We recommend evaluating several RMs, to find an optimal RM that is fit for purpose for the intended research focus, EV sample matrix (conditioned medium vs biofluids) and downstream therapeutic application.

In this section, we have discussed relevant issues related to the needs and requirements for the establishment of EV RMs and we have briefly reviewed the current landscape of potential EV RMs that are both currently available commercially and those being developed in research laboratories. There are several crucial issues that may hinder the production of EV RMs. First, purification methods for isolating pure and intact EVs need significant improvement. Second, methods to assess EV size distributions, biodistributions and concentration with high reproducibility and accuracy need to be established. Finally, the development of RMs for EVs is a laborious and challenging endeavor and it requires extensive collaboration and standardization of analytical methods between laboratories and across organizations. In our opinion, there is much work to be done. At NIST, we are working to overcome measurement

barriers in order to develop EV RMs in collaboration with biotechnology companies and other research institutes and welcome additional input from other researchers in the EV community.

Measurement systems to determine the molecular composition of therapeutic EVs

Techniques for characterizing EVs

Therapeutic EVs share many of the same physicochemical characteristics as other biologically derived delivery vectors, such as adeno-associated virus (AAV), adenovirus, lentivirus and lipid-nanoparticles (LNPs). Each of these particle types contain a therapeutic payload which is most often a specially designed nucleic acid polymer or protein. This cargo is encapsulated in a protein structure (in the case of adenovirus and AAV), lipidic structure (in the case of LNP) or a protein/lipid structure (in the case of EV and lentivirus). Given the similarity in gross composition and basic organization of EVs to other vectors, many of the analytical tools developed for viral vectors and LNPs can be applied to the analysis and characterization of therapeutic EVs. In order to fully characterize a therapeutic EV preparation one needs to measure a maximum of measurands including at least:

- Particle size;
- Particles size distribution;
- Particle number or concentration;
- Particle chemical composition.

It is important that these parameters are well known as variation in any of them may affect the amount of therapeutic cargo delivered in a given dose, change the pharmacokinetics of the preparation and influence its pharmacodynamics. Analytical techniques that can yield information about these characteristics include, but are not limited to:

- Multi-angle light scattering (MALS);
- Dynamic light scattering (DLS; also known as quasi-elastic light scattering or photo-correlation spectroscopy);
- Nanoparticle tracking analysis (NTA);
- Resistive pulse sensing (RPS);
- Small angle x-ray scattering;
- Transmission- and cryogenic-electron microscopy (TEM and Cryo-EM);
- Small angle neutron scattering.

The details of these techniques are beyond the scope of this article; many excellent texts exist in the literature that describe each analytical technique [98–106].

Particle size

EV particle size can be measured with each of the above techniques, with each method having certain advantages and limitations.

Light scattering methods

In the size-regime of most EVs (approximately 40–1 μm in diameter), all light scattering techniques (MALS, DLS and NTA) will measure an average particle size that is heavily skewed to the larger particles in a sample. This is because in this size range, the intensity of the scattered light increases with radius to the sixth power (intensity $\propto$ radius^6). Putting that in context, a particle that has a radius = 200 nm scatters 1,000,000 times more light than the same particle with radius = 20 nm, this results in the scatter from larger particles dominating the scatter from smaller particles.

RPS

RPS is similar to a Coulter counter in its general operating principle but operating on vastly smaller scale. Here the amount of electrically conducting liquid in an orifice and in a short microfluidic channel is temporarily displaced by an individual particle transiting through the orifice/channel. This changes the electrical conductivity of the channel orifice that is (to first order) proportional to the particle's volume. Its major challenges are: the channel/orifice clogging by particles that are larger than the resistive channel; and particles that are sufficiently smaller than the

resistive channel will not displace enough fluid to generate a sufficient signal. Generally, available channel/orifice dimensions are applicable to spherical particles ranging approximately from 50 mm to 10 μm in diameter.

TEM & Cryo-EM

TEM provides a powerful imaging system with high resolution that can detect EVs at the single particle level. TEM combined with immunogold labeling can also be used to characterize the proteins on the surfaces of EVs. However, sample preparation and acquisition may damage EVs and introduce artifacts. Further, TEM cannot be used routinely due to slow collection times and the high cost of the instrument. Cryo-EM provides an alternative to TEM that is less harmful to samples [107], but requires higher EV concentrations and is also slow and expensive. Negative-stain TEM may give an incorrect particle size because of variability in sample processing and operator preferences [108].

Specialty methods

Small angle x-ray scattering and small angle neutron scattering are of limited availability due to the requirement of expensive specialized experimental equipment that is not widely available.

Particle size distribution

The highly nonlinear bias of scattering-based techniques can be overcome by placing a chromatography system upstream of the scattering detector(s). This is especially amenable to MALS and DLS instruments that were originally designed to be coupled inline to liquid chromatography instruments. This benefit is twofold: if the chromatography system sufficiently separates the particles, particles entering the scattering detector will be uniform and the size-dependent intensity difference of multiple particles is ameliorated. Thus, the measured size of each fraction will be less biased; and the separation of the particles will give information about its size distribution. The two main techniques used to fractionate EVs are size-exclusion chromatography (SEC) and asymmetric flow field flow fractionation (AF^4). The applicable particle size for SEC depends on the total pore size of the stationary phase with commercially available pore sizes approximately between 100 Å (10 nm) and 2000 Å (200 nm). AF^4 has a wider dynamic range than SEC but requires more complex method development and chromatographic equipment. Current commercial AF^4 instruments can separate particles approximately between 2 and 20 μm in diameter. NTA can measure particle size distribution and is the most commonly used method to measure the size distributions of EVs. However, the reported values are very sensitive to instrument and software settings (illuminated light intensity and detector sensitivity) and particle detection is biased to larger particles due to the extreme size dependence of the particles' light scattering intensity [109]. NTA measures the diffusion constant of individual particles, enabling their hydrodynamic diameters to be calculated, thus background vibration or mechanical noise can lead to large particles appearing to have an artificially large diffusivity, resulting in the software calculating an erroneously small size. Further, NTA has limited ability to distinguish between EVs, protein complexes and/or lipid particles. Finally, RPS can give particle size distributions for particles that are well matched to the resistive channel diameter. This limitation can be somewhat eliminated by analyzing the same sample with channels of differing diameters. Data from each channel-size can then be aggregated to give information about particles over different size ranges.

Particle number or concentration

MALS data can be used to determine particle numbers if the particles are well fractionated (by SEC, AF^4 or another appropriate chromatographic separation methodology) and the particles' shape and optical properties (specifically refractive index) are well established [110]. NTA can also determine particle number information, bearing in mind the caveats noted previously regarding the limitations due to nonuniform scattering intensity [109]. RPS works by providing a series of single particle-by-particle measurements, thus it can be used to determine particle counts subject to the same limitations by which it can determine particle size. Finally, EM techniques can be used to determine particle counts if a sufficient number of particles can be identified and measured to obtain a representative sampling of the original sample volume. Different techniques to measure EV particle numbers currently only agree within an order of a magnitude and activities to harmonize these measurements are just now beginning in earnest.

Particle composition

It is crucial to enumerate and characterize the biomolecular composition (e.g., relative amounts and types) of the various native proteins, nucleic acids and lipid species incorporated within and/or bound to the surface of

EV preparations that will function as either therapeutics or as delivery vectors for therapeutics. From a quality standpoint, it is important to ensure that therapeutic cargo is uniformly distributed among the various EVs in a preparation and that the markers that influence EV pharmacokinetics are sufficiently present on each EV. Techniques to quantitatively measure the composition of single/individual particles are currently lacking. The EV community in general would benefit from development and validation of new techniques to address this weakness. Chromatographic separation technologies with multiple detectors that have a different sensitivity and selectivity for individual constituents of the particle and that can determine the relative amount of each component in an eluting chromatographic fraction have the potential to address this weakness [111]. This type of technology has been successfully applied to the characterization of the protein and RNA contents in AAV particles [112], however for particles larger than approximately 25 nm diameter, the data analysis needs to be extended to account for excessive scattering from large EV particles that could adversely affect concentration measurements in UV and refractive index detectors. Standard techniques such as digital droplet PCR and quantitative PCR can be used to provide quantitative information on nucleic acid content of an EV, with digital droplet PCR being the preferred technique [113]. ELISAs, western blots and mass spectrometric proteomic techniques can verify the presence of a given marker in an EV sample or the presences of a specific type of loaded cargo, but these techniques can only provide semi-quantitative (relative) information about the average amount of markers or cargo among different EV preparations [114].

The importance of developing accurate metrology for therapeutic EVs

It is possible to learn a great deal regarding the importance of sound metrology for EVs if we look at the challenges faced in the development of other biologically produced therapeutic agents. Among the most successful of these has been the family of IgG molecules that serve to block certain biological pathways associated with disease states. In many ways an IgG molecule is much simpler than an EV from a physicochemical perspective; it has a known amino acid sequence and glycosylation pattern and a well-known secondary and tertiary structure. Nonetheless robust analytical tools needed to be developed and optimized to characterize the purity, as well as the stability of the antibody over different stages of storage, handling and transportation and administration to the patient [115–117]. Given the required homogeneity of a high-quality pharmaceutical-grade protein, measurands like molecular size, concentration and composition were relatively simple to measure, thus most of the technical challenges were focused on developing techniques for determining high resolution molecular structure, stability and optimizing the formulation.

Arguably, the next significant class of complex biologic drugs will be gene therapeutics delivered by viral vectors, with several AAV therapeutics currently entering into clinical trials. An AAV system is more physicochemically complicated than an antibody as it is composed of multiple different biomolecular components that work in concert. An AAV is a capsid composed of 60 individual peptides of three different varieties (VP1, VP2 and VP3) arranged with icosahedral symmetry [118]. This viral capsid assembly contains an engineered single-stranded DNA (ssDNA) cargo that is the gene therapy agent. It is recognized that there is heterogeneity in any given preparation with respect to the amount and quality of the ssDNA in the capsid. Thus, the AAV community is just now in the process of determining the best methods for measuring the amount and quality of ssDNA, as well as determining the heterogeneity in a given preparation despite knowing about these issues a quarter of a century ago [119]. The virus particles are remarkably monodisperse in size, but measurement of the particle number concentration, particle size distribution, as well as viral chemical compositions (e.g., how many virus particles have full length ssDNA payload vs truncated ssDNA payload vs no ssDNA payload) remains challenging [120], with different analytical techniques giving significantly different results. In the case of AAVs, the particle size is relatively easy to measure and constant; however as mentioned above, state-of-the-art particle number concentration measurements typically only agree within an order of magnitude. Finally, particle composition is heterogeneous and techniques to measure accurately capsid loading with ssDNA are an area of active research.

Considering therapeutic EVs, we now have a much more complicated system from a physicochemical perspective. There will be variable particle sizes which must be measured and well understood. The inherently broad particle size distribution increases the difficulty in accurately determining the number concentration of the EV particles. Further, there will be surface marker proteins that must be identified and quantified both as ensemble particle averages and on a single-particle basis both within and between therapeutic preparations. Given that there may only be a few individual epitopes on a given EV, current instrumentation lacks the sensitivity to quantify individual surface marker proteins on a single EV particle. Finally, once loaded with the proteins or nucleic acids of interest,

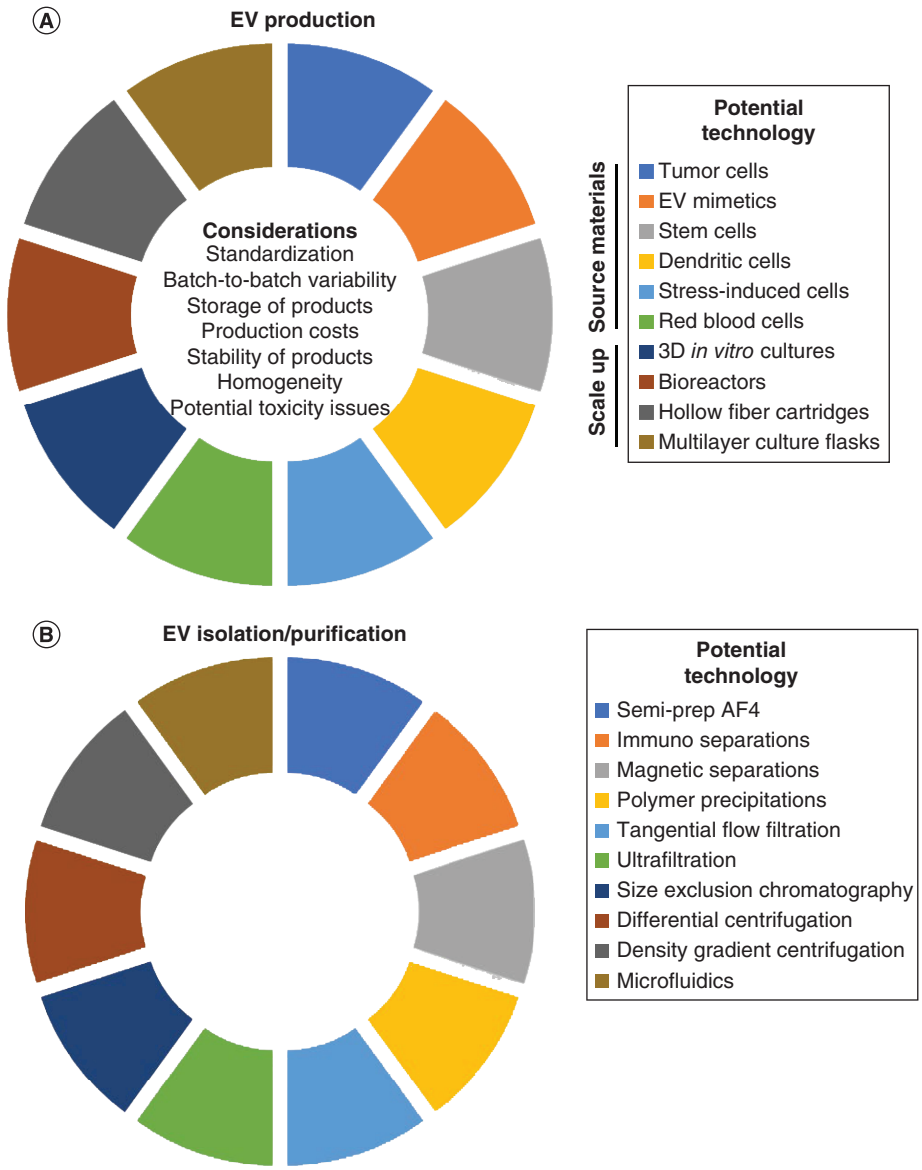

Figure 4. Primary standardization, measurement and technology considerations for extracellular vesicle therapeutics. In order to enable the continued clinical development and viable commercialization of EV therapeutics, both lab-scale and manufacturing-scale research in the areas of EV production, isolation/purification and robust physical and biological characterization are critically needed. **(A–G)** show the potential technology areas and measurement and standardization considerations that impact the current and future potential of EV therapeutics. **(A)** EV production; **(B)** EV isolation/purification; **(C–G)** EV characterization (size and size distribution, morphology, concentration, composition, functional testing). In all Figure panels, applicable technologies are given in each Figure legend.
AF4: Asymmetric flow field flow fractionation; AFM: Atomic force microscopy; Cryo-EM: Cryogenic electron microscopy; DLS: Dynamic light scattering; EV: Extracellular vesicle; FC: Flow cytometry; NTA: Nanoparticle tracking analysis; SANS: Small angle neutron scattering; SAXS: Small angle x-ray scattering; SEM: Scanning electron microscopy; SERS: Surface-enhanced Raman spectroscopy; SP-IRIS: Single particle-interferometric reflectance imaging sensor; TRPS: Tunable resistive pulse sensing.

there will be inherent variability in the type, amount and distribution of the therapeutic cargo within and between biological preparations that must also be measured and understood. Currently there is a paucity of robust and reliable analytical techniques capable of measuring the particle size, particle size distribution, number of particles in a given volume and the amount and homogeneity of the EV cargo. These are all quantities we need to understand very well to ensure that EVs attain their full potential as safe, effective therapeutic agents. Due to the complexity

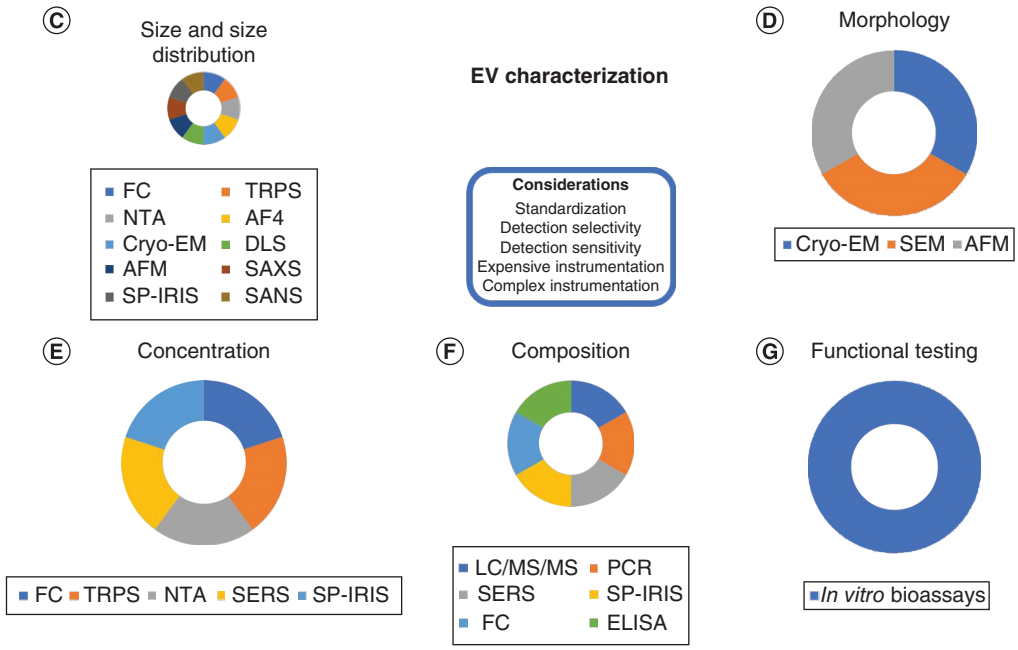

Figure 4. Primary standardization, measurement and technology considerations for extracellular vesicle therapeutics (cont.). In order to enable the continued clinical development and viable commercialization of EV therapeutics, both lab-scale and manufacturing-scale research in the areas of EV production, isolation/purification and robust physical and biological characterization are critically needed. **(A–G)** show the potential technology areas and measurement and standardization considerations that impact the current and future potential of EV therapeutics. **(A)** EV production; **(B)** EV isolation/purification; **(C–G)** EV characterization (size and size distribution, morphology, concentration, composition, functional testing). In all Figure panels, applicable technologies are given in each Figure legend.
AF4: Asymmetric flow field flow fractionation; AFM: Atomic force microscopy; Cryo-EM: Cryogenic electron microscopy; DLS: Dynamic light scattering; EV: Extracellular vesicle; FC: Flow cytometry; NTA: Nanoparticle tracking analysis; SANS: Small angle neutron scattering; SAXS: Small angle x-ray scattering; SEM: Scanning electron microscopy; SERS: Surface-enhanced Raman spectroscopy; SP-IRIS: Single particle-interferometric reflectance imaging sensor; TRPS: Tunable resistive pulse sensing.

and heterogeneity of these physicochemical properties (size and composition) in comparison to other more well-developed biotherapeutic agents, tools and techniques need to be developed or modified to account for this increased complexity.

Future perspective

The potential clinical use of EV vectors for the delivery of proteins and nucleic acid therapeutic cargos is remarkably bright even in the midst and in spite of our still incomplete understanding and characterization of EV biogenesis. Furthermore, it is not clear whether we should continue in our attempts to harness and control the inherent subpopulation diversity that is characteristic of therapeutic EV preparations or just accept that EV subpopulations will be diverse and that such diversity is important for maintaining EV biological function. Notwithstanding these points, incremental technical progress will continue to direct us toward realizing the overarching potential of EV delivery modalities. Figure 4 illustrates and focuses our attention on some of the primary technology tools, measurements and standardization considerations that are relevant for enabling the robust development of EV therapeutic delivery vectors from the point of donor cell evolution through analytical characterization of 'purified' vector populations and subpopulations. In many instances, the available technologies for the production, isolation and characterization of EVs at the bench scale exist, but these technologies are not adequate for high throughput, manufacturing scale processes. It is apparent that technology standardization (RMs, control materials, validated test methods, etc.) is critically needed at all stages of EV vector (e.g., exosome-based therapeutics) development [121]. Alternative measurement approaches or metrological tools that overcome the limitations of the current analytical methods utilized for EV measurements (Figure 4) need to be developed in the future.

It is also important to make a few remarks on potential issues related to the long-term safe use of EV vectors. Regarding potential source materials for EV production, it has been repeatedly demonstrated that tumor cells secrete many more EVs than nontumor cells [122]. Cancer-cell derived EVs have also been shown to be efficacious in the development of cancer vaccines. Thus, cancer cells are potentially a rich source of EV delivery vectors for numerous therapeutic agents. However, cancer cell-derived EVs have also been shown to contribute to aggressive cancer progression and metastasis. So even though cancer cell-derived EVs appear to be a good starting point for the development of EV delivery vectors and are potentially excellent biomarkers for tracking and characterizing cancer progression and cancer treatment outcomes, it is probably wiser to focus on other potential cell-based sources, such as MSCs or RBCs for the production of therapeutic delivery vectors on a large-scale.

Conclusion

In closing, research and development on the use of EVs to deliver peptide-, protein- and nucleic acid-based therapeutics is rapidly expanding from the bench to the clinic. EV based vectors are currently being investigated for the efficient delivery of therapeutics to fight various cancers, neurodegenerative diseases, inflammatory diseases, genetic abnormalities, etc. However, there exists a number of key scientific and measurement challenges that the field will need to address in order to bring commercially viable and safe therapeutics to the market. These challenges include the development of robust cell-based platforms for the production of manufacturing-scale quantities of EVs, the development of EV RMs that enable traceable and reproducible measurements which will allow therapeutic manufacturers to have confidence in the quality attributes of their final products and the development of highly sensitive and selective analytical techniques that will allow EV therapeutic production batches to be reproducible in terms of their molecular scale characteristics and composition.

Executive summary

- Extracellular vesicles (EVs) are nonreplicating lipid bilayer particles that are naturally shed by most types of eukaryotic and prokaryotic cells. The nomenclature that is currently in use for describing certain types of EVs, such as 'exosomes', is ill-defined. Standardization of nomenclature in the EV field is urgently needed.

Landscape of protein- & nucleic acid-based drug delivery using therapeutic EVs

- Peptide and/or protein cargo can be loaded into EVs through donor cells that have been genetically engineered to express therapeutic peptides/proteins or through the use of fusion proteins.
- Peptide and/or protein cargo can also be directly loaded into secreted EVs by incubation, sonication, extrusion, freeze-thaw cycling or permeabilizing the exosomal membranes.
- Therapeutic EVs are suitable for efficient delivery of both soluble cytoplasmic and poorly soluble membrane proteins to recipient cells.
- Native membrane protein domains and microdomains on the surface of therapeutic EVs are conducive to stabilizing membrane protein therapeutics in their native conformations so that the proteins retain biological functionality.
- The strategies for loading nucleic acid cargo into therapeutic EVs are very similar to the modes for loading peptides and proteins into therapeutic EVs.
- Both siRNAs and miRNAs are relatively small RNA species averaging 22–25 nucleotides in length and both species can be easily loaded into therapeutic EVs.
- Loading mRNA, linear DNA or plasmid DNA into therapeutic EVs is more challenging than loading siRNA and/or miRNA due to EV cargo size limitations.

Cell-based platforms for the production of EV-based therapeutics

- The small size and distinctive trafficking patterns of EVs may make them well-suited for therapeutic targets inside the central nervous system and/or to tumors where perfusion of conventional biological agents is limited.
- Bioengineered gut bacteria, with an ability to sense the gut environment and respond by releasing EVs, are strongly positioned as a potential Trojan Horse releasing EVs containing specialized cargo that can promote health and homeostasis.
- Both *ex vivo* and *in vivo* cell platforms present the opportunity to leverage a variety of single cell types or mixtures of cell types to manufacture single EVs or mixtures of EVs for releasing EVs as therapeutics.
- One of the eukaryotic cell types recently proposed as an effective EV (exosome) delivery platform is red blood cells because these cells are nonimmunogenic and generate large daily volumes of EVs.
- The production and necessary quality control considerations for EV therapeutics share some attributes of biological therapeutics and nano-enabled drug products and other attributes of cellular therapeutics.
- One central challenge to translating EV-therapeutic products to the clinic is the inherent heterogeneity of EV preparations.
- A second central challenge to regulating EV-therapeutic products is defining the relevant unit(s) of activity/dose.

Challenges for preparation of therapeutic EV reference materials

- There exists no exclusive marker for differentiating between exosomes and microvesicles, even though they have different biogenesis pathways.
- EVs are very stable and can be stored at -80°C for several months with minimal loss of activity, but they are sensitive to freeze–thaw cycles and choice of storage buffer, which may affect their functional and experimental reproducibility.
- The ideal EV reference material (RM) should have properties representative of prototypical EV preparations to enable researchers to standardize and obtain reproducible EV measurements between instruments and between laboratories.
- Both synthetic RMs, for instrument calibration, such as polystyrene nanospheres and biological RMs for validation of EV measurements, such as EV suspensions with homogeneous size and biological activity are needed.

Measurement systems to determine the molecular composition of therapeutic EVs

- Therapeutic EVs share many of the same physicochemical characteristics as other biologically derived delivery vectors, such as adeno-associated viruses, adenoviruses, lentiviruses and lipid-nanoparticles and many of the analytical tools developed for viral vectors and lipid-nanoparticles can be applied to the analysis and characterization of therapeutic EVs.
- To fully characterize a therapeutic EV preparation one needs to measure, at a minimum, the following measurands: particle size, particle size distribution, particle number and particle chemical composition.
- The analytical techniques available for measuring these particle parameters include, but are not limited to, multi-angle light scattering, dynamic light scattering, photo-correlation spectroscopy, nanoparticle tracking analysis, resistive pulse sensing, small angle x-ray scattering, transmission electron microscopy and cryogenic-electron microscopy and small angle neutron scattering.
- Important lessons can be learned regarding the development of methods and tools for characterizing EVs from past experiences with the analytical characterization of IgG molecules and the current challenges in characterizing adeno-associated viruses.
- Currently there is a paucity of robust and reliable analytical techniques capable of measuring the particle size, particle size distribution, number of particles in a given volume and the amount and homogeneity of the EV cargo.

Future perspective

- The potential clinical use of EV vectors for the delivery of proteins and nucleic acid therapeutic cargos is bright even in the midst and in spite of our still incomplete understanding and characterization of EV biogenesis and the inherent molecular cargo and size heterogeneity of EV subpopulations.
- Technology standardization (RMs, control materials, validated test methods, etc.) is critically needed at all stages of therapeutic EV vector development.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

NIST disclaimer

Certain commercial equipment, instruments and materials are identified in this paper to specify an experimental procedure as completely as possible. In no case does the identification of particular equipment or materials manufacturer imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials, instruments or equipment are necessarily the best available for the purpose. This manuscript is a contribution of NIST and therefore is not subject to copyright in the United States.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat. Rev. Immunol.* 2(8), 569–579 (2002).

•• **This is a standard bearer publication in the extracellular vesicle (EV) research field. The authors provide a comprehensive overview of the biology and function of exosomes as well as a brilliant overview of the exosome protein composition.**

2. Kulp A, Kuehn MJ. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu. Rev. Microbiol.* 64, 163–184 (2010).

3. Mashburn LM, Whiteley M. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* 437(7057), 422–425 (2005).

4. De Jong OG, Kooijmans SaA, Murphy DE *et al.* Drug delivery with extracellular vesicles: from imagination to innovation. *Accounts Chem. Res.* 52(7), 1761–1770 (2019).
5. Kalluri R, Lebleu VS. The biology, function and biomedical applications of exosomes. *Science* 367(6478), eaau6977 (2020).
6. Yang Y, Hong Y, Cho E, Kim GB, Kim IS. Extracellular vesicles as a platform for membrane-associated therapeutic protein delivery. *J. Extracell. Vesicles* 7(1), 1440131 (2018).
7. Raemdonck K, Braeckmans K, Demeester J, De Smedt SC. Merging the best of both worlds: hybrid lipid-enveloped matrix nanocomposites in drug delivery. *Chem. Soc. Rev.* 43(1), 444–472 (2014).
8. Kuipers ME, Hokke CH, Smits HH, Nolte-'t Hoen ENM. Pathogen-derived extracellular vesicle-associated molecules that affect the host immune system: an overview. *Front. Microbiol.* 9, 2182 (2018).
9. Bassler BL, Losick R. Bacterially speaking. *Cell* 125(2), 237–246 (2006).
10. Théry C, Witwer KW, Aikawa E *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(1), 1535750 (2018).

•• **A fundamental and comprehensive publication for all areas of EV research and application.**

11. Fox AS, Duggleby WF, Gelbart WM, Yoon SB. DNA-induced transformation in *Drosophila* – evidence for transmission without integration. *Proc. Natl Acad. Sci. USA* 67(4), 1834–1838 (1970).
12. Johnstone RM. The Jeanne Manery-Fisher Memorial Lecture 1991. Maturation of reticulocytes: formation of exosomes as a mechanism for shedding membrane proteins. *Biochem. Cell Biol.* 70(3–4), 179–190 (1992).
13. Witwer KW, Théry C. Extracellular vesicles or exosomes? On primacy, precision and popularity influencing a choice of nomenclature. *J. Extracell. Vesicles* 8(1), 1648167 (2019).
14. Hoshino A, Costa-Silva B, Shen TL *et al.* Tumour exosome integrins determine organotropic metastasis. *Nature* 527(7578), 329–335 (2015).
15. Luan X, Sansanaphongpricha K, Myers I, Chen HW, Yuan HB, Sun DX. Engineering exosomes as refined biological nanoplatforms for drug delivery. *Acta Pharmacol. Sin.* 38(6), 754–763 (2017).
16. Hall J, Prabhakar S, Balaj L, Lai CP, Cerione RA, Breakefield XO. Delivery of therapeutic proteins via extracellular vesicles: review and potential treatments for Parkinson's disease, glioma and schwannoma. *Cell. Mol. Neurobiol.* 36(3), 417–427 (2016).
17. Pegtel DM, Gould SJ. Exosomes. *Annu. Rev. Biochem.* 88, 487–514 (2019).
18. Ferguson SW, Nguyen J. Exosomes as therapeutics: the implications of molecular composition and exosomal heterogeneity. *J. Control. Rel.* 228, 179–190 (2016).
19. Liu CY, Su CQ. Design strategies and application progress of therapeutic exosomes. *Theranostics* 9(4), 1015–1028 (2019).
20. Angelov B, Angelova A, Filippov SK, Drechsler M, Stepanek P, Lesieur S. Multicompartment lipid cubic nanoparticles with high protein upload: millisecond dynamics of formation. *ACS Nano* 8(5), 5216–5226 (2014).
21. Angelova A, Drechsler M, Garamus VM, Angelov B. Pep-lipid cubosomes and vesicles compartmentalized by micelles from self-assembly of multiple neuroprotective building blocks including a large peptide hormone PACAP-DHA. *ChemNanoMat* 5(11), 1381–1389 (2019).
22. Rakotoarisoa M, Angelov B, Espinoza S, Khakurel K, Bizien T, Angelova A. Cubic liquid crystalline nanostructures involving catalase and curcumin: bioSAXS study and catalase peroxidatic function after cubosomal nanoparticle treatment of differentiated SH-SY5Y cells. *Molecules* 24(17), 3058 (2019).
23. Morse MA, Garst J, Osada T *et al.* A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J. Transl. Med.* 3(1), 9 (2005).
24. Escudier B, Dorval T, Chaput N *et al.* Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. *J. Transl. Med.* 3(1), 10 (2005).
25. Park O, Choi ES, Yu G *et al.* Efficient delivery of tyrosinase related protein-2 (TRP2) peptides to lymph nodes using serum-derived exosomes. *Macromol. Biosci.* 18(12), e1800301 (2018).
26. Liu Y, Fu NS, Su JL, Wang XM, Li XH. Rapid enkephalin delivery using exosomes to promote neurons recovery in ischemic stroke by inhibiting neuronal p53/caspase-3. *Biomed. Res. Int.* 2019, https://doi.org/10.1155/2019/4273290 (2019).
27. Lv LH, Wan YL, Lin Y *et al.* Anticancer drugs cause release of exosomes with heat shock proteins from human hepatocellular carcinoma cells that elicit effective natural killer cell antitumor responses *in vitro*. *J. Biol. Chem.* 287(19), 15874–15885 (2012).
28. Katsuda T, Tsuchiya R, Kosaka N *et al.* Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes. *Sci. Rep.* 3, 1197 (2013).
29. Haney MJ, Klyachko NL, Zhao Y *et al.* Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J. Control. Rel.* 207, 18–30 (2015).
30. Yim N, Ryu SW, Choi K *et al.* Exosome engineering for efficient intracellular delivery of soluble proteins using optically reversible protein-protein interaction module. *Nat. Commun.* 7 (2016).

31. Malhotra H, Sheokand N, Kumar S *et al.* Exosomes: tunable nano vehicles for macromolecular delivery of transferrin and lactoferrin to specific intracellular compartment. *J. Biomed. Nanotechnol.* 12(5), 1101–1114 (2016).

32. Yuan Z, Kolluri KK, Gowers KH, Janes SM. TRAIL delivery by MSC-derived extracellular vesicles is an effective anticancer therapy. *J. Extracell. Vesicles* 6(1), 1265291 (2017).

33. Nakase I, Noguchi K, Aoki A, Takatani-Nakase T, Fujii I, Futaki S. Arginine-rich cell-penetrating peptide-modified extracellular vesicles for active macropinocytosis induction and efficient intracellular delivery. *Sci. Rep.* 7(1), 1991 (2017).

34. Meyer C, Losacco J, Stickney Z, Li LX, Marriott G, Lu BA. Pseudotyping exosomes for enhanced protein delivery in mammalian cells. *Int. J. Nanomed.* 12, 3153–3170 (2017).

35. Kanuma T, Yamamoto T, Kobiyama K *et al.* CD63-mediated antigen delivery into extracellular vesicles via dna vaccination results in robust CD8(+) T cell responses. *J. Immunol.* 198(12), 4707–4715 (2017).

36. Cho E, Nam GH, Hong Y *et al.* Comparison of exosomes and ferritin protein nanocages for the delivery of membrane protein therapeutics. *J. Control. Rel.* 279, 326–335 (2018).

37. Chen R, Huang HX, Liu H *et al.* Friend or foe? Evidence indicates endogenous exosomes can deliver functional gRNA and Cas9 protein. *Small* 15(38), e1902686 (2019).

38. Sims CM, Hanna SK, Heller DA *et al.* Redox-active nanomaterials for nanomedicine applications. *Nanoscale* 9(40), 15226–15251 (2017).

39. De Toro J, Herschlik L, Waldner C, Mongini C. Emerging roles of exosomes in normal and pathological conditions: new insights for diagnosis and therapeutic applications. *Front. Immunol.* 6, 203 (2015).

40. Théry C, Duban L, Segura E, Veron P, Lantz O, Amigorena S. Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. *Nat. Immunol.* 3(12), 1156–1162 (2002).

41. Skog J, Wurdinger T, Van Rijn S *et al.* Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell. Biol.* 10(12), 1470–U1209 (2008).

42. Corcoran C, Friel AM, Duffy MJ, Crown J, O'Driscoll L. Intracellular and extracellular microRNAs in breast cancer. *Clin. Chem.* 57(1), 18–32 (2011).

43. Li J, Sherman-Baust CA, Tsai-Turton M, Bristow RE, Roden RB, Morin PJ. Claudin-containing exosomes in the peripheral circulation of women with ovarian cancer. *BMC Cancer* 9, 244 (2009).

44. Nilsson J, Skog J, Nordstrand A *et al.* Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. *Br. J. Cancer* 100(10), 1603–1607 (2009).

45. Ohno S, Takanashi M, Sudo K *et al.* Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells. *Mol. Ther.* 21(1), 185–191 (2013).

46. Momen-Heravi F, Bala S, Bukong T, Szabo G. Exosome-mediated delivery of functionally active miRNA-155 inhibitor to macrophages. *Nanomedicine* 10(7), 1517–1527 (2014).

47. Zhang D, Lee H, Zhu Z, Minhas JK, Jin Y. Enrichment of selective miRNAs in exosomes and delivery of exosomal miRNAs *in vitro* and *in vivo*. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 312(1), L110–L121 (2017).

48. Wang B, Zhang AQ, Wang HD *et al.* miR-26a limits muscle wasting and cardiac fibrosis through exosome-mediated microRNA transfer in chronic didney disease. *Theranostics* 9(7), 1864–1877 (2019).

49. O'brien K, Lowry MC, Corcoran C *et al.* miR-134 in extracellular vesicles reduces triple-negative breast cancer aggression and increases drug sensitivity. *Oncotarget* 6(32), 32774–32789 (2015).

50. Lou GH, Song XL, Yang F *et al.* Exosomes derived from miR-122-modified adipose tissue-derived MSCs increase chemosensitivity of hepatocellular carcinoma. *J. Hematol. Oncol.* 8 (2015).

51. Katakowski M, Buller B, Zheng XG *et al.* Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. *Cancer Lett.* 335(1), 201–204 (2013).

52. Gong CA, Tian J, Wang Z *et al.* Functional exosome-mediated co-delivery of doxorubicin and hydrophobically modified microRNA 159 for triple-negative breast cancer therapy. *J. Nanobiotechnol.* 17(1), https://doi.org/10.1186/s12951-019-0526-7 (2019).

53. Jeong Y, Yu YOJ, You AEY, Rhee WO, Kim EOA. Exosome-mediated microRNA-497 delivery for anti-cancer therapy in a microfluidic 3D lung cancer model. *Lab Chip* 20(3), 548–557 (2020).

54. Alvarez-Erviti L, Seow YQ, Yin HF, Betts C, Lakhal S, Wood MJA. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat. Biotechnol.* 29(4), 341–U179 (2011).

55. Didiot MC, Hall LM, Coles AH *et al.* Exosome-mediated delivery of hydrophobically modified siRNA for Huntingtin mRNA silencing. *Mol. Ther.* 24(10), 1836–1847 (2016).

56. Lamichhane TN, Jeyaram A, Patel DB *et al.* Oncogene knockdown via active loading of small RNAs into extracellular vesicles by sonication. *Cell. Mol. Bioeng.* 9(3), 315–324 (2016).

57. Wahlgren J, De LKT, Brisslert M *et al.* Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes. *Nucleic Acids Res.* 40(17), e130 (2012).

58. Bellavia D, Raimondo S, Calabrese G *et al.* Interleukin 3- receptor targeted exosomes inhibit *in vitro* and *in vivo* chronic myelogenous leukemia cell growth. *Theranostics* 7(5), 1333–1345 (2017).

59. Zhao L, Gu C, Gan Y, Shao L, Chen H, Zhu H. Exosome-mediated siRNA delivery to suppress postoperative breast cancer metastasis. *J. Control Rel.* 318, 1–15 (2020).

60. Stranford DM, Leonard JN. Delivery of biomolecules via extracellular vesicles: a budding therapeutic strategy. *Adv. Genet.* 98, 155–175 (2017).

•• **Elucidates emerging strategies for EV engineering and how different approaches to EV production, purification and design may impact the efficacy of EV-based therapeutics.**

61. Kanada M, Bachmann MH, Hardy JW *et al.* Differential fates of biomolecules delivered to target cells via extracellular vesicles. *Proc. Natl Acad. Sci. USA* 112(12), E1433–1442 (2015).

62. Lamichhane TN, Raiker RS, Jay SM. Exogenous DNA loading into extracellular vesicles via electroporation is size-dependent and enables limited gene delivery. *Mol. Pharm.* 12(10), 3650–3657 (2015).

63. Shenoda BB, Ajit SK. Modulation of immune responses by exosomes derived from antigen-presenting cells. *Clin. Med. Insights Pathol.* 9(Suppl. 1), 1–8 (2016).

64. Cheng L, Zhang K, Wu S, Cui M, Xu T. Focus on mesenchymal stem cell-derived exosomes: opportunities and challenges in cell-free therapy. *Stem Cells Int.* 2017, 6305295 (2017).

65. Familtseva A, Jeremic N, Tyagi SC. Exosomes: cell-created drug delivery systems. *Mol. Cell. Biochem.* 459(1–2), 1–6 (2019).

66. Margolis L, Sadovsky Y. The biology of extracellular vesicles: the known unknowns. *PLoS Biol.* 17(7), e3000363 (2019).

67. Zhang Y, Liu Y, Liu H, Tang WH. Exosomes: biogenesis, biologic function and clinical potential. *Cell Biosci.* 9, 19 (2019).

• **Goes into depth on the complexity of exosome biology, how this class of EVs can be leveraged for therapy and current paths toward translation to clinic.**

68. Zhang ZG, Buller B, Chopp M. Exosomes – beyond stem cells for restorative therapy in stroke and neurological injury. *Nat. Rev. Neurol.* 15(4), 193–203 (2019).

69. Kawikova I, Askenase PW. Diagnostic and therapeutic potentials of exosomes in CNS diseases. *Brain Res.* 1617, 63–71 (2015).

70. Zipkin M. Exosome redux. *Nat. Biotechnol.* 37(12), 1395–1400 (2019).

•• **Provides overview on the current state of exosome therapeutics including cost considerations and a summary of therapeutic products in development as of December 2019.**

71. Riazifar M, Pone EJ, Lotvall J, Zhao W. Stem cell extracellular vesicles: extended messages of regeneration. *Annu. Rev. Pharmacol. Toxicol.* 57, 125–154 (2017).

72. Ofir-Birin Y, Heidenreich M, Regev-Rudzki N. Pathogen-derived extracellular vesicles coordinate social behaviour and host manipulation. *Semin. Cell Dev. Biol.* 67, 83–90 (2017).

73. Whisner CM, Athena Aktipis C. The role of the microbiome in cancer initiation and progression: how microbes and cancer cells utilize excess energy and promote one another's growth. *Curr. Nutr. Rep.* 8(1), 42–51 (2019).

74. Carvalho AL, Fonseca S, Miquel-Clopes A *et al.* Bioengineering commensal bacteria-derived outer membrane vesicles for delivery of biologics to the gastrointestinal and respiratory tract. *J. Extracell. Vesicles* 8(1), 1632100 (2019).

75. Liu Y, Smid EJ, Abee T, Notebaart RA. Delivery of genome editing tools by bacterial extracellular vesicles. *Microb. Biotechnol.* 12(1), 71–73 (2019).

76. Usman WM, Pham TC, Kwok YY *et al.* Efficient RNA drug delivery using red blood cell extracellular vesicles. *Nat. Commun.* 9(1), 2359 (2018).

• **A first of its kind that demonstrates the novel use of red blood cells to generate exosomes containing the CRISPR-Cas9 genome editing system and illustrating the potential for these exosomes to act as vectors for delivering functional genome editing biomolecules to cells.**

77. Kuo WP, Tigges JC, Toxavidis V, Ghiran I. Red blood cells: a source of extracellular vesicles. *Methods Mol. Biol.* 1660, 15–22 (2017).

78. Oliveira GP Jr, Zigon E, Rogers G *et al.* Detection of extracellular vesicle RNA using molecular beacons. *iScience* 23(1), 100782 (2020).

79. Simak J, Gelderman MP. Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers. *Transfus. Med. Rev.* 20(1), 1–26 (2006).

80. Yamashita T, Takahashi Y, Takakura Y. Possibility of exosome-based therapeutics and challenges in production of exosomes eligible for therapeutic application. *Biol. Pharm. Bull.* 41(6), 835–842 (2018).

81. Food and Drug Administration (FDA). Public safety notification on exosome products (2020). www.fda.gov/vaccines-blood-biologics/safety-availability-biologics/public-safety-notification-exosome-products

82. Greening DW, Simpson RJ. Understanding extracellular vesicle diversity – current status. *Expert Rev. Proteomics* 15(11), 887–910 (2018).

83. Vagner T, Chin A, Mariscal J, Bannykh S, Engman DM, Di Vizio D. Protein composition reflects extracellular vesicle heterogeneity. *Proteomics* 19(8), e1800167 (2019).

84. Wang S, Khan A, Huang R *et al.* Recent advances in single extracellular vesicle detection methods. *Biosens. Bioelectron.* 154, 112056 (2020).

85. Murphy DE, De Jong OG, Brouwer M *et al.* Extracellular vesicle-based therapeutics: natural versus engineered targeting and trafficking. *Exp. Mol. Med.* 51(3), 1–12 (2019).

86. Andaloussi SE, Mager I, Breakefield XO, Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat. Rev. Drug Discov.* 12(5), 347–357 (2013).

87. Van Der Pol E, Boing AN, Gool EL, Nieuwland R. Recent developments in the nomenclature, presence, isolation, detection and clinical impact of extracellular vesicles. *J. Thromb. Haemost.* 14(1), 48–56 (2016).

88. Witwer KW, Buzas EI, Bemis LT *et al.* Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J. Extracell. Vesicles* 2, doi:10.3402/jev.v2i0.20360 (2013).

89. Willms E, Johansson HJ, Mager I *et al.* Cells release subpopulations of exosomes with distinct molecular and biological properties. *Sci. Rep.* 6, 22519 (2016).

90. Kalra H, Adda CG, Liem M *et al.* Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma. *Proteomics* 13(22), 3354–3364 (2013).

91. Tauro BJ, Greening DW, Mathias RA *et al.* Comparison of ultracentrifugation, density gradient separation and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. *Methods* 56(2), 293–304 (2012).

92. Andreu Z, Yanez-Mo M. Tetraspanins in extracellular vesicle formation and function. *Front. Immunol.* 5, doi:10.3389/fimmu.2014.00442 (2014).

93. Kowal J, Arras G, Colombo M *et al.* Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc. Natl Acad. Sci. USA* 113(8), E968–977 (2016).

94. Jeyaram A, Jay SM. Preservation and storage stability of extracellular vesicles for therapeutic applications. *AAPS J.* 20(1), doi:10.1208/s12248-017-0160-y (2018).

95. Lőrincz ÁM, Timár CI, Marosvári KA *et al.* Effect of storage on physical and functional properties of extracellular vesicles derived from neutrophilic granulocytes. 3(1), 25465 (2014).

96. Valkonen S, Van Der Pol E, Boing A *et al.* Biological reference materials for extracellular vesicle studies. *Eur. J. Pharm. Sci.* 98, 4–16 (2017).

97. Geeurickx E, Tulkens J, Dhondt B *et al.* The generation and use of recombinant extracellular vesicles as biological reference material. *Nat. Commun.* 10, https://doi.org/10.1038/s41467-019-11182-0 (2019).

•• **This article describes a cutting-edge technology for the preparation of an EV reference material. The authors propose the use of recombinant EVs (rEVs) as a biological reference material source for quality control, data normalization, method development and calibration of EV measurement methods.**

98. Bouwstra JA, Gooris GS, Bras W, Talsma H. Small-angle x-ray-scattering – possibilities and limitations in characterization of vesicles. *Chem. Phys. Lipids* 64(1–3), 83–98 (1993).

99. Dubochet J. Cryo-EM-the first thirty years. *J. Microsc.* 245(3), 221–224 (2012).

100. Erdbrügger U, Lannigan J. Analytical challenges of extracellular vesicle detection: a comparison of different techniques. *Cytom. Part A* 89a(2), 123–134 (2016).

101. Hammouda B. *Probing Nanoscale Structures – The SANS Toolbox*. National Institute of Standards and Technology, MD, USA (2008).

102. Hong Q, Sheetz M, Elson E. Single-particle tracking: analysis of diffusion and flow in 2-dimensional systems. *Biophys. J.* 60(4), 910–921 (1991).

103. Kiselev MA, Zemlyanaya EV, Aswal VK, Neubert RHH. What can we learn about the lipid vesicle structure from the small-angle neutron scattering experiment? *Eur. Biophys. J. Biophy.* 35(6), 477–493 (2006).

104. Øgendal L. *Light Scattering Demystified Theory and Practice.* University of Copenhagen, København, Denmark (2017).

105. Song Y, Zhang J, Li D. Microfluidic and nanofluidic resistive pulse sensing: a review. *Micromachines* 8(7), doi:10.3390/mi8070204 (2017).

106. Varga Z, Yuana Y, Grootemaat AE *et al.* Towards traceable size determination of extracellular vesicles. *J. Extracell. Vesicles* 3, doi:10.3402/jev.v3.23298 (2014).

107. Conde-Vancells J, Rodriguez-Suarez E, Embade N *et al.* Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. *J. Proteome Res.* 7(12), 5157–5166 (2008).

108. Rikkert LG, Nieuwland R, Terstappen LWMM, Coumans FaW. Quality of extracellular vesicle images by transmission electron microscopy is operator and protocol dependent. *J. Extracell. Vesicles* 8(1), https://doi.org/10.1080/20013078.2018.1555419 (2019).

109. Defante AP, Vreeland WN, Benkstein KD, Ripple DC. Using image attributes to assure accurate particle size and count using nanoparticle tracking analysis. *J. Pharm. Sci.* 107(5), 1383–1391 (2018).

110. Wyatt Technology Corporation. US6774994B1 (2003). https://patents.google.com/patent/US6774994B1/en

111. Medrano R, Laguna MTR, Saiz E, Tarazona MP. Analysis of copolymers of styrene and methyl methacrylate using size exclusion chromatography with multiple detection. *Phys. Chem. Chem. Phys.* 5(1), 151–157 (2003).

112. AN1617: quantifying quality attributes of AAV gene therapy vectors by SEC-UV-MALS-dRI (2020). www.biopharminternational.com/quantifying-quality-attributes-aav-gene-therapy-vectors-sec-uv-mals-dri

113. Hindson CM, Chevillet JR, Briggs HA *et al.* Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat. Methods* 10(10), 1003–1005 2013).

114. Karimi N, Cvjetkovic A, Jang SC *et al.* Detailed analysis of the plasma extracellular vesicle proteome after separation from lipoproteins. *Cell. Mol. Life Sci.* 75(15), 2873–2886 (2018).

115. The ACS Symposium Series. *State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 1: Monoclonal Antibody Therapeutics: Structure, Function and Regulatory Space.* Schiel JE, Davis DL, Borisov OV. (Eds). American Chemical Society, DC, USA (2014).

116. The ACS Symposium Series. *State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 2. Biopharmaceutical Characterization: the NISTmAb Case Study.* Schiel JE, Davis DL, Borisov OV. (Eds). American Chemical Society, DC, USA (2015).

117. The ACS Symposium Series. *State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 3. Defining the Next Generation of Analytical and Biophysical Techniques.* Schiel JE, Davis DL, Borisov OV. (Eds). American Chemical Society, DC, USA (2015).

118. Carter B. Adeno-associated virus and adeno-associated virus vectors for gene delivery. In: *Gene Therapy*. Smyth Templeton N, Lasic DD (Eds). Marcel Dekker, NY, USA, 41–59 (2000).

119. Mittereder N, March KL, Trapnell BC. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J. Virol.* 70(11), 7498–7509 (1996).

120. Manufacturing USA. NIIMBL gene therapy roadmap 2019 (2020). https://niimbl.force.com/s/gene-therapy-roadmap

121. Nelson BC, Minelli C, Doak SH, Roesslein M. Emerging standards and analytical science for nanoenabled medical products. *Annu. Rev. Anal. Chem.* 13(1), 431–452 (2020).

122. Tickner JA, Urquhart AJ, Stephenson SA, Richard DJ, O'Byrne KJ. Functions and therapeutic roles of exosomes in cancer. *Front. Oncol.* 4, 127 (2014).