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Perspective

Extracellular Vesicles for Drug Delivery and Theranostics *In Vivo*

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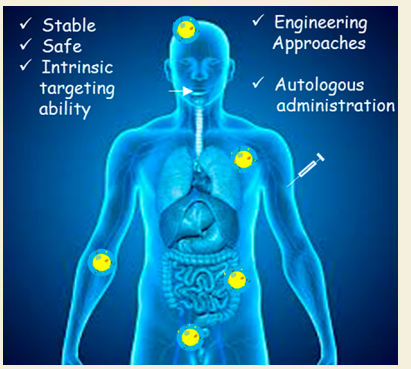
ABSTRACT: Extracellular vesicles (EVs) are lipid bilayer-enclosed nanopouches generated by all cells and are abundant in various body fluids. Depending on the parent and recipient cells, EVs exchange diverse constituents including nucleic acids, proteins, carbohydrates, and metabolites. Morphologically, EVs suffer from low zeta potentials and short circulation times, but they also offer low intrinsic immunogenicity and inherent stability. Some crucial factors for the effective clinical application of EVs include controlling immune system clearance, achieving the large-scale production of EVs with efficient quality control, and determining the dominant mechanism of the *in vivo* action of EVs. In this Perspective, we shed light on how these intriguing nano-objects are utilized in cellular imaging and drug delivery for disease therapeutics. We also discuss potential strategies for overcoming the associated limitations.



KEYWORDS: *extracellular vesicle, drug delivery, liposome, theranostics, cellular imaging, personalized medicine*

INTRODUCTION

Extracellular vesicles (EVs) were first reported by Chargaff and West in 1946 as platelet-derived procoagulant particles via the high-speed centrifugation of human plasma.[1] In the succeeding decades, vesicles from various tissues and tumor cells were also reported, but the functions of these particles were unknown. In the 1990s, EVs were identified to play a role in cell-to-cell communication, and in 2007, the seminal work of Valadi et al. on genetic material exchange via EVs sparked an emergent interest in the field that continues today.[2]

All cells secrete EVs during their normal or diseased state. EVs can be broadly classified into two categories: microvesicles and exosomes. Microvesicles are lipid bilayer-enclosed large pouches (50−1000 nm) formed via outward budding of the plasma membrane. Exosomes (40−160 nm) are endosome-derived vesicles (Figure 1). They are generated as intraluminal vesicles within endosomal compartments that fuse with the plasma membrane (PM), which is controlled by endoplasmic reticulum (ER)-late endosome membrane contact sites and GTPase switching.[3] This confounding exosome biogenesis contributes to the diversity of its constituents. This mechanism could be a means of discarding excess cellular constituents to maintain cellular homeostasis, but recent findings suggest that exosomes carry specific nucleic acids, lipids, metabolites, cytosolic and membrane proteins, and glycans from its parent cell to either a neighboring cell or a distant cell, which advocates for exosomes' role in cellular crosstalk. The cargo packing in exosomes depends on the parent cell, the cell's metabolic state, and the cell microenvironment.[4]

SOURCES AND ISOLATION STRATEGIES

Exosomes are abundant in most of our body fluids, including plasma, urine, amniotic fluid, synovial fluid, feces, sweat, breast milk, and saliva. Plant extract and animal-milk-based EV isolation has also been reported.[5] The tetraspanins CD9, CD63, CD81, syntenin, integrins, Alix, TSG101, and flotillin are enriched in exosomes and microvesicles. The isolation of exosomes is a great challenge. The major reasons are the inherent heterogeneity, the complexity of biological fluids, the presence of similar marker proteins, similarly sized microvesicles, and nanoscale contaminants.[6] Traditional isolation methods of EVs include the following: differential ultracentrifugation (commonly used to separate small EVs from larger EVs, but can lead to aggregation of EVs with high-speed ultracentrifugation), density gradient separation (usually, sucrose or iodixanol is used to improve EV purity, the yield of EV is compromised, and removing the density gradient material from the EVs is a challenge), size exclusion chromatography (capable of removing some soluble contaminating proteins from the EV solution, and the columns required for chromatography can be easily procured or made in the lab), immune-affinity-based separation (usually, polyethylene glycol (PEG) is used, and the purity of EVs is a concern since this PEG reagent will

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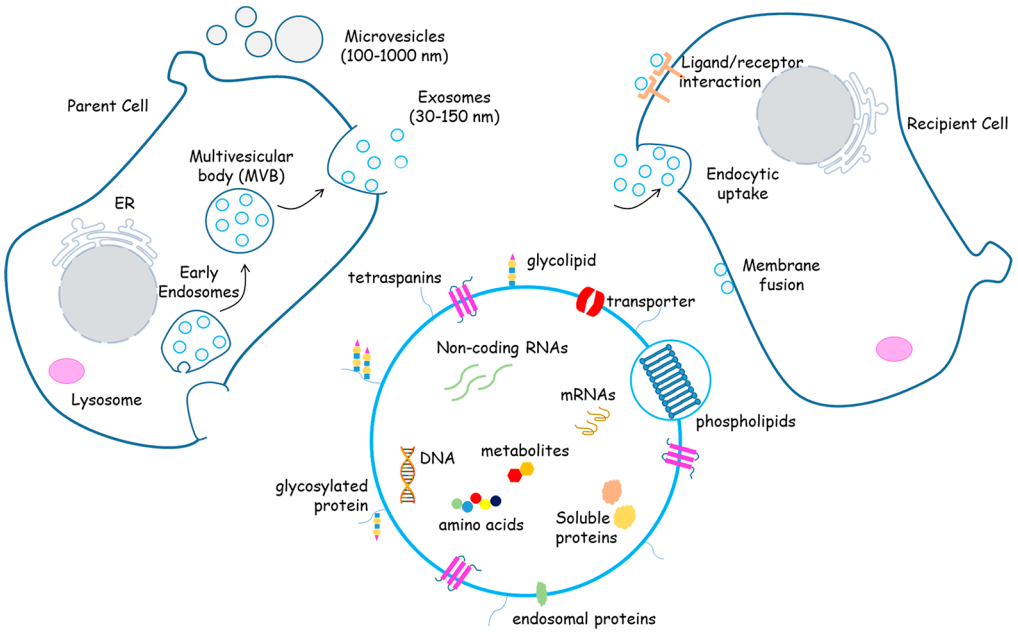

Figure 1. General graphic of EV release and uptake. Exosomes are produced via inward budding of the multivesicular body (MVB) membrane and are released by means of the fusion of MVBs with the plasma membrane. Microvesicles are formed by outward budding, followed by fission of the plasma membrane. Uptake of EVs happens through either direct EV membrane fusion with the plasma membrane (the endocytic pathway) or interactions with plasma membrane receptors. Components of an EV: a wide variety of biomolecules are present in the interior and surface of EVs, such as nucleic acids, proteins, carbohydrates, metabolites, and lipids.

recognize other nonvesicular contaminants), ultrafiltration (an initial step for removing larger EVs with sizes >300 nm; during filtration, the membrane can be easily clogged with large particles while dealing with a large sample volume, and high-pressure filtration will also damage the EV membrane and should therefore be avoided), and affinity-based EV isolation (using specific antibodies that recognize a particular subset of EVs, the yield of EVs will be substantially less, and further assessments become necessary to check for EV functionality after recovering EVs from the employed antibodies). In recent years, microfluidic chips, electrodeposition, and hydrogel-based technologies have emerged, but these are still not the common methods used for EV isolation, and they require special expertise/equipment.[7] All of the methods mentioned above have different pros and cons. So far, ultracentrifugation is the gold standard method used for isolating EVs, but aggregation-induced altered *in vivo* EV biodistribution is a threat. Chromatographic methods may remove proteins of a non-vesicular nature, but contamination due to lipoproteins and miRNA of a nonvesicular nature cannot be eliminated by density gradient centrifugation or ultracentrifugation. Therefore, we need to couple more than one method for the isolation and purification of EVs. Since we know that the purity of isolated EVs using any of these methods (or a combination) relies upon the starting material, its volume, and the media used for EV separation, the isolation strategy of the EVs should depend on a particular type of application. We must configure a combination strategy to separate the subpopulation of EVs from the entire cohort of the isolated EVs as required for a particular type of experiment.[7,8] To improve exosome yield and purity, proper standardization, guidelines, and refinements of isolation methods were defined in 2018 by minimal information for studies of extracellular vesicles (MISEV).[9] Interestingly, diseased cells secrete more EVs than normal cells; however, the physiological urge of diseased cells to shed more EVs is unknown. Reports have shown that modifying their encapsulated content could inhibit diseased cells from producing EVs.[10]

THE CONTENTS OF EVS

EVs are double-membrane-bound lipid nanoparticles. The tetraspanin proteins CD9, CD63, CD81, syntenin, integrins, Alix, TSG101, and flotillin are enriched in exosome and microvesicle membranes. Quantitative proteomics with amino acid isotope labeling−HRMS (high-resolution mass spectrometry) data were reported by Kalluri et al., where 22 proteins (including Syntenin-1, biogenesis-related proteins, GTPases, and membrane proteins, such as CD47 and ITGB1, etc.) were found to be universally enriched in all cell-type-derived exosomes.[11] Interestingly, the protein patterns of microvesicles have been found to be similar to those of their cells of origin. In contrast, exosomes are more likely to be enriched in protein patterns different from those of their parent cells. Proteins of the extracellular matrix, immune response, heparin-binding, and cell adhesion functions are abundant in exosomes. In contrast, microvesicles exhibit proteins from the endoplasmic reticulum, proteasome, and mitochondria. Exosomes and microvesicles possess different lipid contents as well. In exosomes, glycolipids and free fatty acids are plentiful, whereas ceramides and sphingomyelins are mostly found in microvesicles.[12] Glycans are an essential component of exosomes. Recent glycomics studies have indicated that exosomes are enriched with high-mannose glycans (up to nine residues) and complex-type fucosylated/sialylated di-, tri- and tetra-antennary structures of GlcNAc, whereas a few breast cancer exosomes are enriched in GalNAc over GlcNAc associated with Mucin 1 glycosylation. Different cancer exosomes also exhibit glycosaminoglycans (e.g., hyaluronan), as evident from molecular biology including confocal microscopy and single-molecule studies by atomic force microscopy (AFM).[13−15] EVs are associated with different types of DNA, including single-stranded or double-stranded

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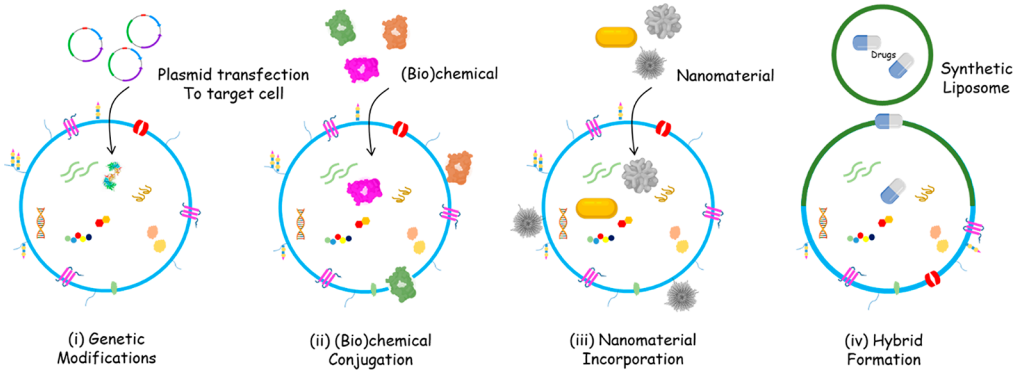

Figure 2. Four common types of EV engineering approaches. (i) Genetic pathway: a plasmid is transfected into the target cell so that the EVs carry a specific protein of interest. (ii) Biochemical pathway: conjugation, hydrophobic inclusion, or chemical bonding of functional ligands, peptides, aptamers, or small molecules to EVs to improve recognition. (iii) Nanomaterials, including magnetic metal nanoparticles, nanosheets, organic polymeric materials, loading inside EVs or conjugating on EV surface for effective targeting. (iv) Hybrid EV preparation by fusion of synthetic liposomes with natural EVs using fusogenic agents, for example, PEG, to improve the drug binding and loading efficacy of EVs.

DNA, mitochondrial DNA, and even viral DNA. During EV biogenesis, cytoplasmic (both genomic and mitochondrial) DNA are sequestered inside/on the surface of EVs.[16] Cellular stress-mediated DNA damage can expose DNA to the cytoplasm and facilitate its incorporation into intraluminal endosomal vesicles. This packaging of DNA could protect them from external invasive environments and from the immune system. Double-stranded DNA association with exosomes has been demonstrated through two different approaches: enzymatic methods and AFM by Thakur et al.[17] They reported that exosomal DNA (>2.5 kb size) represents the entire genomic DNA and, importantly, reflects the mutation of originating tumor cells. Exosomal RNAs include both coding and noncoding RNAs, as evidenced by RNA sequencing methods. Valadi et al. reported an abundance of functional "exosomal shuttle RNA" (both messenger (mRNA) and microRNA (miRNA) with little or no 18S and 28S ribosomal RNA (rRNA)) by microarray assessments from approximately 1300 genes: many of the RNA molecules were not present in the cytoplasm of the parent cell.[2] Later studies have identified exosomal long noncoding RNA (lncRNA) and circular RNA (cirRNA).[6,18] Herein, ExoCarta, an Internet exosome database (http://www.exocarta.org/), is a quite useful resource. Biochemical, cell-based, and computational strategies have revealed that RNA-binding proteins (RBPs) play a crucial role in the sorting of coding and noncoding RNAs during the active RNA-packaging process in exosomes. Proteins that have been so far identified are members of the hnRNP family (hnRNPA2B1, hnRNPC1, hnRNPG, hnRNPH1, hnRNPK, and hnRNPQ) and also include others such as YBX1, HuR, AGO2, IGF2BP1, MEX3C, ANXA2, ALIX, NCL, FUS, TDP-43, MVP, LIN28, SRP9/14, QKI, and TERT.[19]

EVS VERSUS LIPOSOMES

Nanocarriers for drug delivery are a promising approach for improving the efficacy of drugs by altering the pharmacodynamics and kinetics.[20] This strategy also reduces unwanted side effects, such as toxicity and immunogenicity.[21] The last few decades have seen an enormous development of nanomaterial-based delivery systems with specific organ-targeting and stimuli-responsive drug delivery applications in various disease conditions. In this regard, lipid nanoparticles (LNPs) have emerged as the most favorable vehicle for drugs and vaccines.[22] Nonetheless, clinical applications of LNPs suffer from substantial drawbacks due to low bioavailability, toxicity and immunogenicity, and rapid clearance by the mononuclear phagocyte system (MPS), negatively impacting the translation of these formulations.[23] To alleviate this drawback, researchers favor EVs as viable alternatives to LNPs due to their structural similarity. Because of their biological origin, EVs are endowed with several advantages compared to LNPs. EVs are inherently nontoxic and nonimmunogenic. The biological origin of EVs entails the metabolism and excretion of the carrier in the human body, causing no side effects.[24] The drug delivery efficacy is found to be greater than that of synthetic counterparts due to a favorable curvature.[25] Further, EVs promote angiogenesis, provide cytoprotection, and reduce apoptosis.[26] Unlike synthetic nanomaterials, EVs can also traverse through different biological barriers such as the blood−brain barrier (BBB). Therefore, EV-based drug delivery platforms have shown enormous promise in drug delivery applications.

Another advantage of using EVs is the versatility of cargo loading in the vector. Various molecular and macromolecular cargo, including drugs, proteins, mRNAs, and lipids, can be loaded in the matrix, extending the circulation half-life and helping the delivery to distant cells.[27−30] The assimilation of imaging modality in this macromolecular conglomerate further enhances EV functionality. Collectively, this formulation is termed "theranostics". In this Perspective, we provide recent advances in drug delivery and theranostics via EVs. We limit our discussion to *in vivo* systems and aim to make the reader aware of the untapped potential of this field.

EV-BASED DRUG DELIVERY: ENGINEERING APPROACHES

EVs are likely to serve as exciting drug delivery vectors because of their natural origin, inherent targeting ability, nonimmunogenicity, protein and nucleic acid content, and intrinsic therapeutic properties that could create new opportunities in the field of drug delivery. EVs on their own, EVs isolated from genetically modified cells, EVs conjugated with nanomaterials, and EV hybrids with synthetic liposomes are all emerging new vehicles for efficient drug delivery (Figure 2). Due to inherent bio-molecular stuffing, EVs lack drug binding abilities, unlike liposomes. Therefore, EV−nanomaterial conjugates and EV−liposome hybrids are increasingly used for better drug loading and subsequent delivery.[5,31,32]

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JACS Au 2024, 4, 318−327

EVs are generated from cells as heterogeneous mixtures of vesicles, and they take part in proximal and distal cross-talk between cells. For *in vivo* drug delivery, EVs have an intrinsic short half-life, which affects the effective delivery of EVs at the site of injury. Intravenously administered EVs in our bodies have circulation times higher than those of systemically injected EVs. After EV circulation, the EVs are removed by different organs. Macrophages are known to play an important role in sequestering EVs from the bloodstream. Therefore, to increase the EV circulation time, EVs must avoid eliminating organs, such as the liver, lungs, kidneys, and immune cells, after they enter the systemic circulation. One way to improve their half-life is by engineering EVs to block recognition by macrophages after EV administration. Direct injection of EVs specific to a target organ could be helpful to prevent immune system clearance, but more investigations are necessary to find an optimal assessment.[33] In general, the tissue-specific targeting efficiency of EVs could be enhanced by using chemical functionalization; the EV-PEGylation strategy is one such method.[34]

EVs are also aggregation-prone for *in vivo* applications since they have low zeta potential values. Aggregation of EVs has an adverse effect on delivery. In the case of prior administration, aggregation reduces the effective number of EVs that reach the target site. For post-delivery aggregation, it will affect the EV circulation time. To prevent this, trehalose is used in the EV solution to lower the attractive forces between EVs, or EV surface proteins can be engineered or blocked using protein inhibitors. EV-based hydrogels could also be a creative solution.[33] It is to be noted that the long-term adverse effects of increasing EV circulation time *in vivo* are currently unknown, and therefore, more studies are necessary.

DRUG LOADING IN EVS

Approaches for drug loading to EVs mainly include: (i) physical methods (sonication, electroporation, freeze−thaw, and extrusion), (ii) chemical methods (saponin-assisted permeation and transfection; here, saponin being a weak detergent helps to permeabilize the EV membrane to encapsulate cargos into the EVs), and (iii) biological methods (incubation and viral transduction; the latter process is quite complex, as it involves alterations to the EV structure, and functionality may be reduced). It is worth noting that EV loading with cargos should be non-invasive, and crucial parameters need to be optimized for efficient loading, such as the forces involved during physical interactions, the duration of loading operation, and the cell type-specific changes in EV concentration. By proper selection or modification of the cells from which EVs are collected, various platforms for EV loadings and conjugating targeting molecules are developed, for example, natural EVs, which are native or obtained from genetically modified cells; and EVs postmodified with drugs, small interfering RNAs (siRNAs), or surface ligand moieties to increase target specificity and EV-inspired lipid vesicles where the lipid molecules are obtained from natural EV sources.[35]

Due to unique bioactive components, EV-based nanodrug formulations could permit different biodistributions for free drugs or currently marketed liposomal formulations. It is now accepted that the bioactive constituents in EVs hold potential as biopharmaceuticals. However, the clinical translation of EVs is challenging since it has not been properly elucidated until now which part of the EV (lipid/miRNA/protein/glycan) primarily contributes to its recognition of the target cell and its therapeutic potency.[33] After careful evaluation, the biomanufacturing processes for EVs should be adjusted from the existing protocols of biologics, liposomes, and cell-based treatments since EVs require additional controls and measures due to their intrinsic size and compositional complexity, for example, EV dimensions and surface charge, protein markers, drug loading efficiency evaluation, and additional chromatography techniques to purify the EVs from contaminants.

AUTOLOGOUS ADMINISTRATION OF EVS

The use of EVs in personalized medicine could also be of potential interest, where either patient cells would be cultured or blood samples would be collected for isolating EVs, followed by readministration of the EVs to the patient's body. Autologous EVs, usually readily available, could be particularly important for certain applications such as maintaining genetic compatibility via EV-mediated transfer to mitochondria. Mitochondria-rich extracellular vesicles from autologous stem cell-derived cardiomyocytes restore the energetics of ischemic myocardium.[36,37] This strategy offers superior target specificity and nonimmunogenicity of EVs as well. In a similar direction, blood- or plasma-derived EVs from cancer patients could be used in an autologous transplantation manner for delivering therapeutics to tumor tissues, mesenchymal stem cell (MSC)-derived EVs for regenerative medicine, and dendritic cell EVs for vaccine delivery for various purposes.[38] Several phase I trials are underway in EV-based personalized drug delivery applications.[39] However, this strategy has several limitations, particularly for patients with acute diseases, such as infections and cardiovascular issues. Herein, autologous EV administration could lead to undesired outcomes. Importantly, nanoscale contaminants present in biological EV isolation could impose potential risks in the immune response. There is also ambiguity in evaluating the EV dosing, the frequency of repeat dosing to improve homeostasis, the duration in which benefits can be obtained, any infection at the injection site, and the short-term and long-term side effects of EVs.[40] The future of this strategy would depend on critical comparative assessments of autologous EVs and well-established nonautologous EVs in terms of the need for a particular application and safety issues.

EVS FROM NATURAL SOURCES

Another exciting avenue in EV delivery could be using natural fruit-, vegetable-, and animal-milk-based sources. Harmless microbe EVs (e.g., bacteria and fungi) could be another option. These are scalable, amenable, nontoxic, and relatively cheaper choices compared to liposomes. Plant-derived EVs possess intrinsic antitumor activity and are also stable across pH and temperature ranges, suggesting their ability to deliver drugs in an acidic tumor microenvironment. Microbe-derived EVs efficiently permit genetic and other biomolecules across kingdoms to be efficient vaccine carriers in the future. Milk-derived EVs could be particularly useful for increasing the bioavailability of any EV-packaged drug for oral administration since they would be easily absorbed from the gastrointestinal tract in humans without any immunogenic effect.[41] These abundant and organic EV sources could be helpful in cost-effective, efficient drug delivery if we streamline the EV isolation and purification strategies on a case-by-case basis (batch-to-batch critical quality check).

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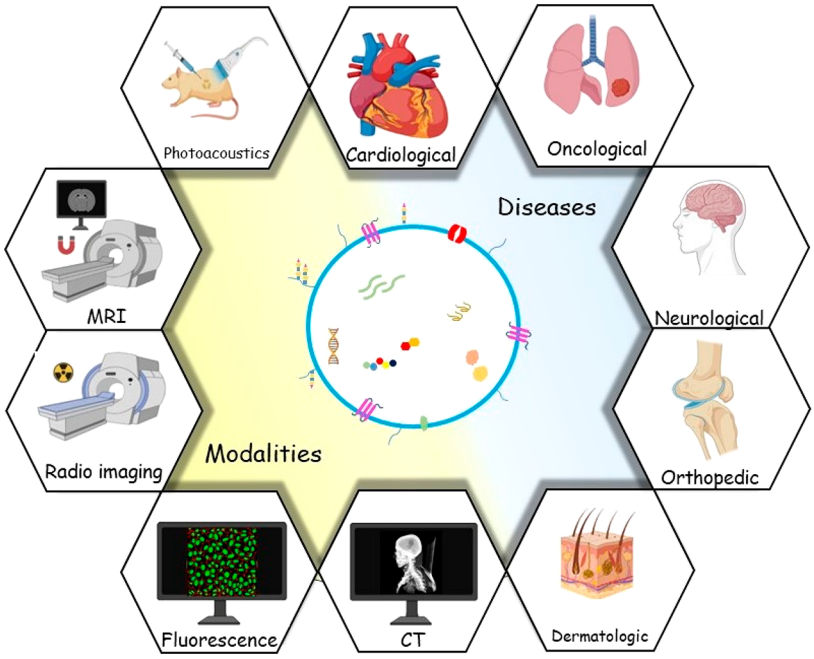

Figure 3. Imaging strategies for *in vivo* tracking of EVs. Various approaches have been developed to elucidate EV trafficking and specific targeting in different human disease models.

EXOSOME THERAPY

Several examples of EV-based drug delivery applications have matured to *in vivo* settings. A recent report by Su et al. used colitis mouse models to study systematic bone loss linked to inflammatory bowel diseases (IBDs). They investigated the bone development capacity, the bone marrow MSC differentiation nature, and the intricate molecular-level mechanisms. They employed Golgi glycoprotein-1-modified EVs through intravenous administration to target bone marrow MSCs in order to rescue osteoblastic bone development.[42] The study suffers from a few shortcomings; for example, to study subtypes of IBD, more animal models are required. Additionally, the bone metabolism of patients needs to be studied with serum samples, and the role of the immune system in IBDs also needs to be addressed. In another recent study, Yang et al. demonstrated the mitochondrial delivery of circRNA mSCAR (steatohepatitis-associated circRNA ATP5B regulator), which could alleviate sepsis by modulating macrophage activation. Sepsis is directly linked to unrestrained inflammation and can be instigated by the dysregulation of the macrophage (M1) polarization. To improve the incorporation of circRNA mSCAR into the mitochondria, an EV−circRNA mSCAR conjugate was electroporated using poly-D-lysine-graft-triphenylphosphine. An *in vivo* mouse model confirmed the delivery of circRNA mSCAR via modified EVs in the macrophages and facilitated the M1 macrophage polarization to M2, resulting in the attenuation of inflammation and improved mortality. The circRNA mSCAR linear form that was co-transcribed from the mitochondrial DNA and the plasmid used may have important roles that should be studied further.[43] In another paper on liver fibrosis, CD44-targeting hyaluronan-coated milk-derived EVs encapsulated with a traditional Chinese medicine, Forsythiaside A, were employed. *In vivo* results on zebrafish larvae demonstrated that the constructed EV conjugate could improve the morphology and function of the liver, where it could inhibit NLRP3-linked pyroptosis (a type of sterile inflammation).[44] These recent studies demonstrate the potential of EVs in delivery and therapeutics under different pathological conditions. More than 150 clinical trials are registered at ClinicalTrials.gov and deal with EV therapies for numerous diseases. One-fifth of these trials used MSC cell EVs. These trials tested cell-free EV therapies as an alternative to stem cell therapy since EVs are more potent in growth factors than adult stem cells, which brings greater opportunity for faster repair, tissue regeneration, and reprogramming of target cells. EVs are small and stable, and they pose a minimum risk of immune response and tumor development; additionally, storage can be optimized to some extent. Importantly, no ethical issues are associated with EV-based therapies, unlike in stem cell therapy, where there are chances of tumor formation after engraftment, possibilities of migration to the injured site, viability and ethical issues, unavailability of allogeneic (genetically different and immunologically unsupported) stem cells (donated), and chances of transmission of infection. Nonetheless, stem cell therapy has well-established Food and Drug Administration (FDA) guidelines, and more than 7000 studies have been conducted already.[45]

Although there are some important benefits of using EV therapy, many challenges still remain in the field. It is difficult to upscale the manufacturing of EVs due to their biological heterogeneity, and no good manufacturing practice (GMP) is in place. EVs are still not approved by the FDA for treatment or diagnosis of any disease, and EVs can only be used for FDA biologic licenses and clinical applications. In 2020, the FDA published a consumer alert detailing the risks and unproven claims of uncontrolled EV therapies. Therefore, the use of EVs for therapeutic purposes is still in its infancy; we must address biogenesis, mass production, *in vivo* biodistribution, long-term safety, and therapeutic efficacy issues in great detail to move the use of EV therapies further along.

EV-BASED THERANOSTIC APPLICATIONS

EVs hold great potential in theranostic imaging for different human diseases. Popular imaging modalities, such as fluo-

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rescence imaging, magnetic resonance imaging (MRI), positron emission tomography/single photon emission computed tomography (PET/SPECT), and photoacoustics, are routinely used in clinical settings for disease diagnosis (Figure 3). To fabricate EVs with theranostic capabilities, one has to incorporate a therapeutic drug module and an imaging module into the EVs. Further, modified EVs are prone to home in RES organs, such as the liver and spleen, rather than in the desired disease sites. To alleviate this issue, surface modifications with targeting agents are used. Several examples of external MRI contrast agents are incorporated in the EV formulation. For example, curcumin- and superparamagnetic iron oxide nanoparticle (SPION)-loaded EVs with neuropilin-1 peptide surface modifications have been developed and used to cross the blood−brain barrier for a successful theranostics application in glioma. The EV formulation helped the magnetic resonance imaging, along with the therapeutic potential of curcumin.[46] EVs from blood cells have been modified with superparamagnetic nanoparticles for imaging and transferrin for organ targeting. The modified exosomes were shown to possess a better *in vivo* therapeutic efficacy.[47] In one study, Interleukin 3 receptor-targeted EVs were loaded with near-infrared (NIR) fluorophores and Imatinib or siRNA for imaging and treatment of chronic myelogenous leukemia.[48] In another work, EVs derived from genetically modified human embryonic kidney cell line 293 (HEK293) cells were used to deliver miRNA to EGFR-expressing breast cancer.[49] Embryonic stem cell-derived and c(RGDyK)-functionalized EVs have been loaded with paclitaxel (PTX) for the treatment of glioblastoma.[50] Fluorescence imaging was concurrently used for the localization of therapeutic EVs. Vanadium carbide quantum dots have also been loaded to an RGD-peptide-modified exosome vector as photothermal agents.[51] The incorporated nanomaterial possessed photoacoustics and MRI capabilities as well as a photothermal therapy modality that enabled efficient *in vivo* theranostics. Liu et al. invented a designer EV for the desired drug release profile in the tumor site. In this innovative design, exosomes derived from macrophages were attached to gold nanorods for the photo-controlled release profile of entrapped drugs from the construct. Folate groups were attached to the EV surface for tissue targeting, and fluorophores were used for *in vivo* imaging, showing the versatility of the construct.[52] In another study, EVs derived from M1 macrophages were attached with antibodies of CD47 and SiRPα through an imine linker.[53] The modified EVs showed excellent therapeutic efficacy, along with *in vivo* imaging capabilities. In another interesting strategy, a hybrid vesicle derived from genetically engineered EVs and thermosensitive liposomes was fabricated. This newly developed nanoconstruct efficiently delivered docetaxel (DTX) in cancer tissue, and fluorescence imaging was performed.[54]

Radio imaging is the most preferred imaging technique for cancer in the clinic. In this regard, several exosome-inspired designs have emerged. For instance, Exosomes have been labeled with ^{131}I, which allowed for *in vivo* tracking and clearance studies using PET imaging.[55] Subsequently, this same group demonstrated single photon emission computed tomography (SPECT) imaging using a clinically approved ^{111}In-oxine-labeled exosome in a breast cancer murine model. In another strategy, HER2-targeted exosomes were labeled with ^{99m}Tc to impart *in vivo* SPECT imaging capabilities. This method was instrumental in determining the exosome's biodistribution and tumor homing abilities.[56]

Recently, EVs have gained significant attention as theranostic devices for neurological disorders, such as traumatic brain injuries, malignant brain tumors, peripheral nerve injuries, Alzheimer's disease, and Parkinson's disease, as they pass through the blood−brain barrier (BBB). Offren et al. pioneered the development of EVs derived from mesenchymal stem cells labeled with gold nanoparticles.[57,58] The inherent X-ray CT contrast of gold allows one to track the homing patterns of EVs in various neurological disorders. The gold nanoparticle surface was modified with brain-targeting EVs to allow for neurological imaging.[59] In another study, blood-derived EVs were loaded with dopamine; the brain-targeting ability of the exosomes were used to deliver dopamine to a Parkison's disease-affected brain, and fluorescence imaging of the affected area was performed.[60] The targeting capabilities of EVs could be improved by attaching c(RGDyK) peptides to the surface, which target the ischemic brain, and the disease could be imaged by visualizing the curcumin loaded in the exosome via fluorescence imaging.[61] In another study, hydrophobically modified small interfering RNAs (hsiRNAs) were incorporated in EVs for the treatment of Huntington's disease with concurrent fluorescence imaging.[62] Further, EVs have been used to deliver anti-inflammatory drugs, curcumin, and JSI124 (a signal transducer and activator of the transcription 3 (Stat3) inhibitor) to microglia cells via an intranasal route for the treatment of brain inflammation.[63]

OUTLOOK: PROSPECTS AND LIMITATIONS

Due to their biocompatibility, physiochemical stability, and nonimmunogenicity, EVs are promising biological products in drug delivery and theranostic applications. They offer an opportunity for personalized medicines. EVs also have tremendous potential in regenerative medicine; unlike stem-cell-based therapy, EV therapy is cell-free. EVs can be extracted from donated human MSCs. However, before proceeding further, we should critically assess the following points.

First, for EV-based clinical applications, it is vital to uphold the highest standard with good practice regulations in EV production and quality control, which must comply with regional accreditation. Second, the safety, storage, toxicity, and immunogenicity of EVs must be examined closely in early phase clinical trials for donors and recipients. Having knowledge of the pharmacokinetic behavior and biodistribution profile of EVs should also be required. Finally, the efficiency and long-term adversative effects of autologous EV administration should be obtained from later-phase clinical trials. Academia−industry collaboration is the key to accelerating preclinical progress and fruitful clinical translation.

It is currently ambiguous how one defines a "control" EV in clinical settings. For example, in one study during cardiovascular treatment, after myocardial infarction, EVs isolated from induced pluripotent stem cell-derived cardiomyocytes (iCMs) and mesenchymal stem cells (MSCs) were applied, and it was found that both types of EVs improved cardiac functions. It was, therefore, difficult to recognize the differences and similarities between the two EV types without having a proper EV as a control.[64] EV content, especially microRNAs, is highly dependent on the parent cell metabolic state and the microenvironment. Therefore, choosing a proper control EV becomes very challenging. In this case, a liposome made out of EV-extracted lipid could be used as a control, as the pharmacokinetic and dynamic properties of the liposomes would be comparable with the therapeutic EVs. This step is crucial in identifying which bioactive molecules (proteins, nucleic acids, glycans, and

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miRNAs) are responsible for a particular therapeutic intervention.

Since cardiovascular disease and strokes remain an alarming epidemic, EV therapy could be a new ray of hope. Emerging evidence suggests that EVs have tremendous potential in treating heart diseases. It is known that the human heart has physical and cellular differences between the two sexes, and miRNA expression also varies; therefore, it is important to assess how EV therapy is related to these biological sex differences. This will also elucidate EV clinical application pathways for other relevant diseases.[33]

From a basic research perspective, much information still needs to be discovered. Although EV biogenesis is an active area of research, the following points need to be perused in great detail in the future. The exact mechanisms involved in EV biogenesis, secretion, and fusion have not yet been fully revealed. Whether cargo incorporation in EVs is selective or random needs to be elucidated; this critical information will pave the way for controlling the cargo/drug incorporation into EVs with purpose. The detailed recognition mechanism of EVs with the target cell and whether it is primarily through EV surface glycans have also not been enlightened so far. A mechanobiological perspective of cells and EVs could also be valuable to address the intrinsic targeting ability of natural EVs.[65] Previous reports have demonstrated that EVs can cross the BBB, but the knowledge we currently have is inadequate for modulating EV surface markers for efficient targeting to exploit this valuable route of therapeutics.[66] Furthermore, for the visualization of EVs with sizes <200 nm, fluorescence microscopy is not a great choice due to its inadequate resolution. Therefore, sophisticated technology needs to be developed to study EVs at the single particle level; atomic force microscopy (AFM) is one such option.[13] For studying the cellular uptake of EVs, fluorescence microscopy and flow cytometry are popularly used. However, labeling the EVs with a fluorescent dye and storing them overnight could lead to diminished fluorescence intensity without impacting EV uptake. Therefore, label-free techniques should be developed, and in situ techniques would be a plus. AFM coupled with nano-IR spectroscopy could be a viable solution.[67] Although the drug loading ability of natural EVs is limited, we still need to explore the possibility of loading more than one drug for EV-based combination therapy (immuno-, chemo-, and radiotherapy) in the future. Engineering approaches are great ways to enhance EV drug loading capabilities. EV hybrids created with synthetic liposomes or soluble proteins could be very useful.[32]

Also, the abundant plant- and milk-based sources of EVs need to be critically evaluated as delivery vectors, especially for oral routes. In order to increase EV production in industrial settings, different molecules can be used as stimuli, for example, for the hormone dopamine treatment to cells. Since cells can modulate the composition of EVs in response to exogenous stress and stimuli, it is essential to critically assess the content of EV production with/without stimuli. The cell culture conditions are also very important. For example, fetal bovine serum (FBS) is routinely used in cell culture. This must be made EV-free before it can be added to the cell cultures dedicated to EV isolation; otherwise, the FBS EVs would be mixed with the desired EV population. It is to be noted that with EV-depleted FBS, the growth of the cells is usually less; therefore, the content of the isolated EVs could also be different. Thus, more control experiments are required. Moreover, irrespective of the source of EVs, immunogenicity studies are indispensable for realizing any clinical translation. Since liposome-based drug delivery and imaging have existed for 20 years, the EV field should take direct inspiration from it in most aspects of large-scale production, drug loading, and delivery. Comparing the aspects of synthetic liposomes and natural EVs would be valuable to thoroughly judge the full potential and unavoidable limitations of EVs.

Considering the potential of EVs as a biologics and a multibillion dollar pharmaceutical market, we should strongly envision many successful EV therapies for cancer and cardiovascular, neurodegenerative, and respiratory diseases. Additionally, when large-scale EV production with good manufacturing practices is ensured and well-established FDA guidelines are critically followed in the future, more EV therapies will be available. The potential environmental impact of using EVs on a large scale should also be carefully considered, as this is an increasingly important consideration in the development of new technologies and therapies. It is well-accepted in the field that manufacturing choices can directly impact the environment and the well-being of our planet. One must consider the use of batch versus continuous manufacturing processes (the latter has a lower environmental impact in terms of process mass intensity, a metric used to evaluate the efficiency of a manufacturing process), the life cycle assessment of the biologics, and most importantly, the water-related impact of energy that is directly linked to the carbon dioxide that is emitted due to mass biologics production.

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CRediT: **Tatini Rakshit** conceptualization, funding acquisition, project administration, supervision, visualization, writing-original draft, writing-review & editing; **Suchetan Pal** conceptualization, funding acquisition, project administration, visualization, writing-original draft, writing-review & editing.

Notes

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