Extracellular Nanovesicles: From Intercellular Messengers to Efficient Drug Delivery Systems

Sachin Kumar Deshmukh, Mohammad Aslam Khan, Seema Singh, and Ajay Pratap Singh

ABSTRACT: Nanosized extracellular vesicles (nEV) are released by all the eukaryotic cells into the extracellular spaces. They serve as crucial mediators of intercellular communication, and their presence has been detected in a variety of body fluids. nEV carry nucleic acids, lipids, proteins, and metabolites from the donor cells and transfer them to the recipient cells in the vicinity or distant locations to cause changes in their biological phenotypes. This very property of nEV makes them a suitable carrier of the drugs for therapeutic applications. The use of nEV as a drug delivery system offers several advantages over synthetic nanoparticles, including biocompatibility, natural targeting ability, and long-term safety. Further, nEV can be isolated from various biological sources, quickly loaded with the drug of choice, and modified to further enhance their utility as targeted drug delivery vehicles. Here we review these aspects of nEV and discuss the parameters that should be kept in mind while choosing the nEV source, drug loading method, and surface modification strategies. We also discuss the challenges associated with the nEV-based drug delivery platforms that must be overcome before realizing their full potential in clinical applications.

1. INTRODUCTION
Extracellular vesicles (EV) are lipid bilayer membrane particles released by the eukaryotic cells into the extracellular space. EV can originate either via the endocytic pathway or through membrane budding. The term “exosomes” is given to those EV that form via the endocytic route, while “microparticles” are released via direct membrane budding. Exosomes and microparticles typically range in size from 30 to 100 nm and 100–1000 nm, respectively. The size distribution of exosomes and microparticles is not very strict and can vary depending upon the physiological condition. Similarly, surface marker protein expression is also not very strict. Therefore, the International Society for Extracellular Vesicles (ISEV) recommends that the use of these terms should be avoided unless there is direct evidence of their biogenesis.

The presence of EV has been reported in most body fluids, including urine, saliva, and blood. Although initially thought to serve as mere trash bags released by the cells to get rid of unwanted biomaterial, EV are now well-established as intercellular communicators playing significant functional roles in cellular biology. EV act both in paracrine and autocrine fashion and exert their activity in nearby or distant places. The biological activity of EV is attributed to their surface composition and the cargo that they carry, which includes lipids, nucleic acids, proteins, and metabolites. The composition and physicochemical properties of EV are influenced by the donor cell type and can change under pathophysiological conditions leading to their altered biological functions. Because of these properties and the fact that they are widely present in body fluids, their clinical utility in biomarker development is being explored extensively. Further, because of their natural function as bioinformation carriers, their potential utility in drug delivery has also caught significant attention. The efficient delivery of the drug to the target site has remained a challenging task. The efficacy of most therapeutic drugs is limited due to the lack of targeted delivery and rapid systemic clearance. As a result, patients cannot benefit optimally and suffer from unwanted short- or long-term toxicity. To overcome these limitations, synthetic delivery systems such as metal nanoparticles, liposomes, and carbon nanotubes have been developed; however, their stability, biocompatibility, and long-term safety remain a clinical concern. In that regard, nanosized EV (nEV) have provided a lot of hope as an emerging drug delivery system. nEV can be isolated from a variety of sources and have several desirable characteristics. Further, they have the exceptional ability to interact with the recipient cells and have a selective homing
capability due to their unique membrane composition. In the following sections, we discuss additional aspects of nEV and salient progress in their development as efficient drug delivery systems.

2. SOURCES OF EXTRACELLULAR NANOVESICLES

Although nearly all types of cells can produce nEV, not all cell-derived nEV are suitable for use in human therapeutic applications. nEV for slated use as drug carriers should be available in abundance and have appropriate surface protein composition to not induce adverse immune reaction when delivered. Human tumor cells, red blood cells, dendritic cells, mesenchymal stem cells, bovine milk, and plant juices or extracts have been used as sources for the isolation of nEV for drug loading and delivery purposes2,5−7 (Figure 1). Advantages and disadvantages of these sources are discussed below:

2.1. Tumor Cells. Tumor cells are an appealing source for the isolation of nEV for drug delivery purposes, as their derived nEV carry tumor-targeting surface proteins. Ovarian cancer cells-derived nEV loaded with cisplatin are shown to inhibit the tumor growth in the mice model with ovarian cancer.8 However, their use has been discouraged, as tumor cell-derived nEV are shown to potentiate malignant behavior and cause immune suppression due to tumor-promoting cargo and surface protein composition. Importantly, tumor cell-derived nEV can inhibit T cell effector function, since they carry PD-L1 on their surface that binds to PD-1 expressed on the T cells.

2.2. Red Blood Cells. Human red blood cells (RBCs) belonging to the “O” group are being explored as a source of nEV for therapeutic purposes. RBCs can be obtained in large quantities from the blood bank. Moreover, to avoid any unwanted immunogenic reaction due to cell contamination, RBCs can be isolated from the same patient. nEV secretion from RBCs can be increased by treating them with calcium ionophore, phorbol-12-myristate-13-acetate, or lysosphosphatidic acid. The addition of ascorbic acid to the stored murine RBCs has been shown to increase post-transfusion recovery and decrease nEV formation and release.9

2.3. Dendritic Cells. Dendritic cells (DCs) are the most potent antigen-presenting cells of the immune system. Immature dendritic cells (imDCs)-derived nEV (imDC-nEV) have been explored for drug delivery and can be generated from the peripheral blood mononuclear cells (PBMCs) of the healthy donors. imDC-nEV display low levels of surface biomarkers such as MHC-I, MHC-II, CD86, and CD40, potentially reducing the immune reaction and protecting them from systemic clearance. It is suggested that nEV of different size (large vs small) released from imDCs may have different roles in T helper cell response. While larger-sized nEV trigger Th2 cytokine secretion, smaller EV induce the secretion of Th1 cytokines suggesting their differences in cargo contents and MHC distribution on the nEV surface.10

2.4. Mesenchymal Stem Cells. Mesenchymal stem cells (MSCs) have emerged as an excellent choice, since they can be efficiently isolated from different tissues such as umbilical cords, bone marrow, and adipose tissues. MSCs can also be expanded in vitro and thus serve as an abundant source of nEV. nEV derived from MSCs does not produce any adverse effect, since they are nonimmunogenic.6 However, in some cases, MSC-derived nEV are shown to enhance the proliferation of recipient cells.11 Concerns are also raised that MSC-nEV can also promote tumor vascularization. In contrast, some reports suggest antitumorigenic effects of MSC-derived nEV. Clearly, more research is needed to understand these differential actions of MSC-derived nEV, as they are continued to be explored as drug delivery systems.

2.5. Milk. Bovine milk has emerged as a cost-effective, readily available, and abundant source of nEV. Milk-derived nEV are resistant to digestive juices and exhibit cross-species tolerance, and they are therefore attractive carriers of oral delivery drugs. These nEV are equally efficient in loading both hydrophobic and hydrophilic drugs. A study conducted in mice showed that fluorescently labeled bovine milk nEV injected into mice were stable in circulation for up to 6 d.7 However, extensive studies are warranted to rule out potential toxicity or prion contamination with milk nEV as a drug vehicle.

2.6. Plant. Extracts from edible plants can yield a good quantity of nEV, and plant-derived nEV do not pose any safety issues in oral formulations. They are stable at a wide range of pH and can also serve as natural therapeutic agents in several pathological conditions. Interestingly, some plant-derived nEV exhibit specificity toward certain types of recipient cells. For example, nEV derived from grapes are specifically taken up by the intestinal stem cells and inhibit dextran sulfate sodium-induced colitis in mice.12

3. METHODS FOR DRUG ENCAPSULATION

The drug can be encapsulated into the nEV through a passive or active approach. Passive drug loading utilizes natural mechanisms, while in active loading, external physical (sonication, extrusion, freeze–thaw cycle, and electroporation) or chemical methods (use of saponin and click chemistry) are employed. Active cargo loading causes temporary disruption or
stretching of the nEV membrane to allow the drug’s import and is considerably more efficient than passive loading.  

3.1. Passive Loading. Passive loading is achieved by incubating the drug, either with the nEV or the donor cell (Figure 2A). On the one hand, the direct diffusion of the drug into nEV is a slow process facilitated through the hydrophobic interaction between the lipid layer of nEV and the drug. On the other hand, when the drug is incubated with the donor cell, it can be actively taken up using endogenous cellular mechanisms and then packaged into nEV and released into the culture medium. For therapeutic miRNA or peptide packaging into nEV, donor cells can be transfected with a plasmid overexpressing the therapeutic entity. The donor cells then express the therapeutic, package it into nEV, and release it into the culture medium.

![Figure 2. Passive loading of the drug into nEV. (A) The drug and nEV mixture is kept at room temperature to allow drug loading through the diffusion process. Alternatively, donor cells are incubated with the drug at 37 °C, where they take up the drug, package it into nEV, and release them in the culture medium. (B) For therapeutic miRNA or peptide packaging into nEV, donor cells can be transfected with a plasmid overexpressing the therapeutic entity. The donor cells then express the therapeutic, package it into nEV, and release it into the culture medium.](https://dx.doi.org/10.1021/acsomega.0c05539)

Several procedures have been explored for active drug loading as described below.

3.2.1. Sonication. Sonication is the act of harnessing the energy of sound waves, mostly of ultrasonic frequencies (>20 kHz), to agitate particles in a solution. Light sonication can transiently disrupt the membrane to release and take up the material such as drugs from the suspension media. For optimal loading, the cycling frequency and the sonication duration are adjusted depending upon the type of drug being loaded. We recently reported the maximum loading of honokiol into nEV at six cycles of sonication with 30 s pulse/30 s pause without compromising the integrity and desirable polydispersity index and size. Another study that loaded PTX into nEV through sonication also made a similar observation. In some cases, sonication can inadvertently attach the drug to the outer surface of nEV along with inside encapsulation. The drug anchored to the outer surface is released quickly, followed by a slow release of the drug-loaded inside nEV.

3.2.2. Extrusion. The drug and nEV are mixed and loaded into a lipid syringe extruder with 100–400 nm porous membranes and pushed through forcefully. The process is performed under a controlled temperature. During the process, the exosome membrane is transiently disrupted, enabling the drug uptake. Severe mechanical force, however, can permanently disrupt nEV membrane and affect drug loading efficiency. There is also a risk that other membrane properties get altered as well in an undesirable manner. On the one hand, an earlier study conducted with cultured breast cancer cell-derived nEV suggested that the exhaustive extrusion does have unwanted consequences on the integrity and zeta potential of nEV. On the other hand, milder extrusion of nEV derived from RAW264.7 macrophages produced optimal catalase loading without compromising the integrity. However, it could be argued that the sources of nEV and loading entities were different in both the studies, and thus, more investigations are needed.

3.2.3. Freeze and Thaw Cycles. This process is straightforward and does not require much technical skill or specialized instruments. Here, nEV and drugs are incubated at room temperature, and then the mixture is quickly frozen at –80 °C or in liquid nitrogen. Subsequently, the mixture is thawed again at room temperature, and the process is repeated for efficient loading. The ratio of drug and nEV, the incubation time, and the number of freeze–thaw cycles can vary and need to be optimized for different drug-nEV-type combinations. The freeze–thaw method has also been used for membrane fusion between phospholipid-based liposomes and nEV to create exosome-mimetic nanoparticles. The production of exosome-mimetic particles is less cumbersome and scalable for its use in preclinical or clinical settings. Besides, the process of fusion is more controllable. On the negative side, repetitive freeze and thaw cycles can induce nEV aggregation and increase the polydispersity index with a broad size distribution of the drug-loaded nEV that ultimately affects drug uptake efficiency. It is also suggested that the drug-loading yield of the freeze–thaw method is lower than that resulting from sonication or extrusion methods.

3.2.4. Electroporation. Conventionally, the electroporation technique is used to introduce DNA, RNA, and drugs into the cells, but lately, it has also been exploited to load miRNA/siRNA inside the nEV. siRNA or miRNA are charged and larger in size than some hydrophobic drugs such as PTX and, therefore, cannot passively diffuse inside the nEV. The electric
current generated in the electroporation method creates spontaneous pores into the lipid bilayer membrane of the nEV facilitating the inward movement of the siRNA/miRNA or drug. Once the loading process is complete, the EV membrane integrity is quickly recovered. Since the exposure times are in the millisecond range in the electroporation method, a very minimal rise in temperature occurs (1 °C rise per pulse) that avoids any thermal damage to the nEV. It is reported that the size of nEV increases during electroporation in proportion to the applied electric fields. Saponin treatment also creates membrane pores and allows drug loading into nEV. In the "click chemistry" reaction, drugs are anchored to the external membrane surfaces of the nEV.

3.2.5. Saponin Treatment. Saponin is a surfactant molecule that forms complexes with membrane cholesterol. As a result, upon incubation with nEV, it generates pores into the EV membrane, increasing permeability. Saponin treatment is shown to be a practical approach for drug encapsulation into the nEV compared to other techniques such as incubation and electroporation. The saponin method results in high loading efficiency without having a significant impact on the nEV size. However, some concerns are raised for this method, including the fear of in vivo hemolytic activity and saponin-induced pore formation in the recipient cell membrane. Therefore, using a minimum concentration of saponin and their complete removal from the solution is crucial before delivering the nEV.

3.2.6. Click Chemistry. The copper-catalyzed azide–alkyne cycloaddition (click chemistry) method is used to directly attach the molecules to the surface of nEV through covalent bonds. The click chemistry method has fast reaction times, better control over the conjugation site, and compatibility in aqueous buffers. nEV are cross-linked with alkyne groups with the help of carbodiimide-mediated coupling. A fluorescent molecule, azide-fluor 545, was efficiently attached to the surface of 4T1 cells derived nEV by cross-linking with the carbosyl group of 4-pentyenoic acid. Amine groups present on the nEV membrane facilitated this cross-linking. Importantly, conjugation of anazide-fluor 545 with nEV did not affect the size and uptake by the recipient cells. Further, the azide group bearing nEV were conjugated with targeting peptide using copper-free click chemistry to improve their delivery to the cancer cells.

4. MODIFICATION OF EXTRACELLULAR NANOVESSICLES FOR TARGETED DELIVERY

Like other drugs and drug carriers, nEV are also prone to systemic clearance via the liver and spleen, as demonstrated by intravenous injection in mice. The complement system, a significant part of the innate immune system, and immune cells, including macrophages, participate in the clearance of the circulating nEV as well. The fate of nEV in circulation is governed by their surface composition, as they may display specific antigen proteins on their surfaces. To address these limitations, surface modifications of nEV are crucial. Surface modification can also improve targeted drug delivery leading to optimal treatment response while minimizing the drug-originated undesired off-target systemic toxic effects. Various surface modification strategies have been explored, as discussed below.
4.2.1. Pegylation. Nanovesicles.  

the size of plasmid vectors, which relies on the donor cell type, transfection reagent, and xenografts and delivered let-7a miRNA cargo. However, a is achieving optimal and consistent transfection effi

intravenously into the mice.20 PEG-coated nEV remained neuroblastoma cells were coated with PEG and administered in the circulation. In a study, nEV derived from mouse the surface of nEV and decreases the recognition of nEV as a increased bioavailability. Pegylation forms a hydration layer on increased circulation time of nanomaterials in the blood, leading to increased bioavailability. Pegylation, is the most widely used approach to prolong the nanomaterials with poly(ethylene glycol) (PEG), termed as pegylation, is the most widely used approach to prolong the membrane domain of platelet-derived growth factor receptor (PDGF-R) fused to αv integrin-specific iRGD peptide. nEV shed by these engineered imDCs expressed this peptide on their surface. These nEV were later loaded with doxorubicin (DOX) and intravenously (i.v.) administered in mice. nEV efficiently delivered DOX to αv integrin-positive breast cancer cells and suppressed their growth without causing any noticeable toxicity.18 In another study, HEK293 cells were forced to express the trans-

Figure 4. Nanosized extracellular vesicles are being evaluated as a carrier of chemical drugs and therapeutic RNA, miRNA, proteins, and peptides for the treatment of various ailments, including inflammatory diseases, neurodegenerative disorders, and cancer.

4.2.2. pH-Responsive Modification of Extracellular Nanovesicles. 4.2.1. Pegylation. Surface modification of nanomaterials with poly(ethylene glycol) (PEG), termed as pegylation, is the most widely used approach to prolong the circulation time of nanomaterials in the blood, leading to increased bioavailability. Pegylation forms a hydration layer on the surface of nEV and decreases the recognition of nEV as a foreign material, thus preventing the clearance of the nEV from the circulation. In a study, nEV derived from mouse neuroblastoma cells were coated with PEG and administered intravenously into the mice.20 PEG-coated nEV remained detectable in circulation for more than 60 min, while nonpegylated nEV were cleared rapidly within 10 min.20 In another study, RAW264.7 macrophage-derived nEV were fused with pegylated liposomes to achieve a PEG coating, which resulted in their increased bioavailability.21

4.2.2. pH-Responsive Modification. Tumor cells reside in an acidic tumor microenvironment (TME), which can be exploited for targeted drug delivery by introducing pH-sensitive functional groups on the nEV surface. Lee et al. demonstrated the advantage of pH-responsive nEV created by incorporating 3-(diethylamino)propylamine (HDEA) onto the surface of mouse macrophage RAW 264.7 macrophages-derived nEV. Further, HDEA-conjugated nEV were loaded with the antitumor drug DOX. These surface-modified nEV efficiently responded to low pH and actively bound to CD44 receptors on HCT-116 tumor cells and inhibited their growth in vitro and in vivo.22 In another study, DOX was conjugated with bovine milk-derived nEVs by a pH-labile imine bond, which dissociated under an acidic microenvironment. The modified nEV showed controlled DOX release under acidic conditions in vitro and reduced squamous cell carcinomas growth in mice.23

4.2.3. Glycan Modification. Endogenous surface molecules such as glycans have been implicated in mediating nEV targeting and absorption through charge-based interaction or pattern recognition, or both. Royo et al. removed sialic acid from the surfaces of nEV derived from mice lung cells by neuraminidase treatment.24 The removal of sialic acid reduced the negative charge of nEV, which enhanced their interaction with lung cells and resulted in an increased accumulation in the lungs after i.v. administration in mice.24 In another study, bovine-serum-derived nEVs were surface-modified with α-D-mannose.25 After an intradermal administration in mice, a higher number of mannose-modified nEV were observed in the lymph nodes. These studies provide a rationale to explore glycan modification on the nEV surface as a strategy for the selective delivery of the drug to the intended sites.

5. CLINICAL TRIALS

Having observed overwhelming success in multiple preclinical studies, several clinical trials have been launched to analyze the feasibility, safety, and efficacy of nEV as a drug delivery system. nEV derived from human sources as well as milk and plants are in clinical trials (www.ClinicalTrials.gov). KRAS mutation is a common occurrence in pancreatic ductal adenocarcinoma (PDAC) and is associated with continued tumor growth and poorer patient survival. A phase I clinical trial study (NCT03608631), sponsored by the M.D. Anderson Cancer Center, is currently ongoing to test the efficacy of nEV derived from MSCs loaded with small interference RNA (siRNA) against KrasG12D in metastatic PDAC patients carrying this mutation. An earlier study in a murine hepatocarcinoma ascites model examined the effectiveness of nEV derived from the mouse hepatocarcinoma tumor cell line H22 for methotrexate (MTX) delivery and showed significant inhibition of tumor growth without typical side effects.7 These findings built the foundation of a clinical trial (NCT02657460) that is currently ongoing to test the efficacy of autologous
tumor cell-derived MTX-loaded nEV for the treatment of malignant pleural effusion in patients with advanced lung cancer. Curcumin has been shown to inhibit colon carcinogenesis growth; however, its hydrophobic nature limits its clinical translation as a drug. A phase I clinical trial (NCT01294072) is currently evaluating the ability of plant-derived nEV to effectively deliver curcumin to colon tumors. Grape-derived nEV are shown to trigger the process of tissue remodeling in a situation of pathological damage. On the basis of these findings, a phase I clinical trial (NCT01668849) is currently underway to analyze the effect of grape-derived nEV for the inhibition of oral mucositis associated with the chemoradiation treatment of head and neck cancer.

6. CONCLUSION AND FUTURE PERSPECTIVE

Nature-derived nEV are emerging as next-generation drug delivery systems for the treatment of a variety of diseases due to their strong biocompatibility (Figure 4). Formulations using nEV from different biological sources are being tested for efficacy in preclinical models and, more recently, in clinical trials. There are, however, some limitations associated with the use of nEV as well. For example, obtaining exosomes from an autologous source in the desired amount may often be difficult. Moreover, autologous nEV, such as those derived from the patient’s tumor cells, may pose concerns about immunosuppression and chemoresistance due to their inherent bioactivity. Furthermore, the safety and efficacy of autologous nEV cannot be evaluated in clinical trials due to the limited availability of these nEV. Efficient drug loading into the nEV can also be an issue that could be influenced by the quality and purity of nEV. Therefore, finding an acceptable source of nEV and developing an isolation method that provides pure and intact nEV at a large scale is highly significant. Future investigations must focus on producing large amounts of well-characterized nEV with a high loading capacity from reliable allogeneic sources. Efforts should also be focused on developing ways to tailor nEV to enhance their homing capabilities and achieve the desired intracellular fate of the encapsulated drug.

It is anticipated that future years will witness significant progress in the field of EV and the uses of EV in disease diagnostics and therapeutics. Advances in biology, chemistry, and nanotechnology are consistently happening that will help in bypassing the current obstacles. Newer disease models are being developed that could more accurately provide preclinical data on the efficacy and safety of nEV drug delivery systems. The ongoing clinical trials exploring nEV as a drug carrier in cancer patients are expected to announce top-line results. With more systematic in vivo studies and continuous improvement in nEV isolation, drug loading, and engineering, it will be possible to bring these attractive drug delivery vehicles to clinical practice in the not-too-distant future.

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Notes

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**ACKNOWLEDGMENTS**

The authors acknowledge the funding support from the National Institute of Health/National Cancer Institute [CA224306 and CA185490 to A.P.S. and CA204801 and CA231925 to S.S.] and the University of South Alabama Mitchell Cancer Institute.

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https://doi.org/10.1021/acs omega.0c05539

ACS Omega 2021, 6, 1773–1779