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RNA and Protein Delivery by Cell-Secreted and Bioengineered Extracellular Vesicles

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Extracellular vesicles (EVs) are carriers of biological signals through export and delivery of RNAs and proteins. Of increasing interest is the use of EVs as a platform for delivery of biomolecules. Preclinical studies have effectively used EVs to treat a number of diseases. Uniquely, endogenous machinery within cells can be manipulated in order to produce desirable loading of cargo within secreted EVs. In order to inform the development of such approaches, an understanding of the cellular mechanisms by which cargo is sorted to EVs is required. Here, the current knowledge of cargo sorting within EVs is reviewed. Here is given an overview of recent bioengineering approaches that leverage these advances. Methods of externally manipulating EV cargo are also discussed. Finally, a perspective on the current challenges of EVs as a drug delivery platform is offered. It is proposed that standardized bioengineering methods for therapeutic EV preparation will be required to create a well-defined clinical product.

1. Introduction

Extracellular vesicle (EV) research has exploded in the last decade, with the current literature describing their role in a swathe of biological processes and pathologies, from cancer to heart disease and neurological injury. EVs are lipid-bilayer enclosed vesicles secreted by all types of cells and released into the extracellular space. Containing a variety of lipids, proteins, and nucleic acids, EVs serve as intercellular communicators, delivering their cargo into the target cells. The term EV broadly encompasses a heterogeneous group of bio-nanoparticles which vary in size, cargo and biogenesis. While consensus has not yet emerged on the specific markers of EV biological origin, size remains the main metric for their classification, according to the International Society for Extracellular Vesicles' published Minimal Information for Studies of Extracellular Vesicles (MISEV 2018).[1]

Small extracellular vesicles (<200 nm in diameter) include the often-cited exosomes, which are produced in the endosomal compartment of the cell. Medium to large EVs include microvesicles (MVs, 100–1000 nm) and apoptotic bodies (100–5000 nm), which are formed from the outward budding of the cell membrane.[2] EVs have been shown to play major roles in intercellular communication throughout the body, in physiological processes such as maintaining the hematopoetic bone-marrow niche,[3] and pathological processes such as cancer metastasis.[4,5] Because of this, EVs are actively considered as potential biomarkers for diseases; much of this work involves utilization of the landscape of tumor-secreted EVs in order to detect cancer at earlier stages.[6] Finally, and of note to this Review, the ability to deliver bioactive cargo has made EVs of interest for therapeutics use, with as many as 79 clinical studies currently in progress (clinicaltrials.gov). Regenerative approaches in tissue repair using EVs are especially promising, with preclinical data showing efficacy in treating cardiac,[7,8] lung,[9] and liver[10] diseases.

The investigation of EVs as bioinspired drug carriers has also grown, particularly for bioactive molecules such as nucleic acids and proteins. Current drug delivery systems (DDS) are largely centered on lipid-based carriers such as liposomes and lipid nanoparticles (LNPs). These are fully synthetic, well defined membrane-bound carriers composed of phospholipids and cholesterol. Since their regulatory approval, liposomes have been used for the delivery of therapeutic agents and vaccines. However, they have nonspecific organ distribution and have caused issues with immunogenicity. On the other hand, there are certain properties of EVs which are highly desirable for DDS. The first is their ability to deliver native bioactive cargo (protein, nucleic acids), which can affect the function and phenotype of targeted cells. Perhaps the most significant advantage that EVs may hold is that the cellular machinery can be leveraged to produce specific therapeutic cargo and transfer it via EVs. This approach may eliminate much of the limitations surrounding the manufacturing, purification, and storage of the EV cargo. Such properties make EVs an exciting prospect for future DDS development. Therefore, the understanding of EV cargo-loading biology and developing bioengineering strategies which recapitulate these properties are of key interest.

Here, we propose that optimization of EVs through engineering strategies would further improve their clinical potential as

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adhm.202101557

DOI: 10.1002/adhm.202101557

carriers of therapeutic biomolecules. After elucidation of EV cargo pathways, engineering approaches can be used to create well-defined EV formulations. We first underline the rationale for EVs to serve as drug delivery systems and compare them to conventional lipid-based carriers. In an effort to understand how cellular mechanisms may be harnessed for engineering EVs, we discuss the biological basis of EV cargo. Then, we review recent bioengineering advances in EV cargo for drug delivery optimization, with focus on two major approaches: 1) Endogenous loading, in which the parent cells are genetically engineered to secrete EVs containing a desired RNA or protein of interest, and 2) exogenous loading, in which a desired cargo is incorporated into EVs after their isolation from the cells. Finally, we discuss the current challenges to using EVs as drug delivery systems and some research needs.

2. EVs versus Lipid-Based Drug Delivery Systems

It is now well established that EVs can deliver a number of functional bioactive molecules including RNAs and proteins to target cells. Unsurprisingly, their ability to carry cargo has had significant implication for the delivery of proteins or nucleic acids for therapeutic use. The number of publications centered around loading EVs with a specific cargo has risen dramatically over the last six years.[11] EVs, at first glance, solve much of the problems that current traditional based lipid-based carrier systems are facing. For example, it has been shown by many groups that EVs can cross biological barriers, including the blood-brain barrier[12,13] that has classically avoided passage of most DDS. In addition, lipid-based nanoparticles have a large clinical side-effect profile, including immune over-activation.[14] In comparison, current data suggest that EVs have low toxicity and immunogenicity, both in animal models and in human clinical trials.[15,16] However, EV-based delivery systems have their own challenges, including lack of standardized methodology and inefficient loading. As EV research is still a burgeoning field, careful examination of preceding DDS paradigms may offer valuable lessons in EV-based therapy. In this section, we briefly review the history of lipid-based drug delivery development, and compare them to native EVs for drug delivery.

2.1. Lipid-Based Drug Carriers: a Primer

Initially pioneered by Dr. Bangham at the University of Cambridge in the 1960s, lipid-based materials such as liposomes and LNPs for drug delivery have taken a strong foothold in modern medicine. Broadly defined, these carriers have lipid bilayers, and range from 20 nm to 30 μm in diameter, encapsulating an aqueous core. LNPs are a subset of synthetic particles, less than 100 nm in diameter, and are most comparable with sEVs. The lipid bilayer allows for the encapsulation of both hydrophobic and hydrophilic drugs, as well as for efficient exchange of cargo through the cell membrane. Their formulation differs mainly on two variables: 1) their size and 2) the number of bilayers (uni- or multilamellar). The ability to modulate these two parameters brings the structural complexity to liposome design. The first FDA approved drug using lipid-based drug carriers was Doxil in 1995, an anticancer drug for advanced ovarian cancer, multiple myeloma and Kaposi's sarcoma, composed of doxorubicin encapsulated in PEGylated nanoliposomes. Since then, a handful of other drugs using liposomal formulations came to the market, primarily chemotherapeutic agents.[17]

Over the course of their development, modifications to the conventional liposome formulations were primarily focused at improving their stability and half-life in circulation. When injected intravenously, liposomes have destabilizing interactions with high-density lipoproteins present in blood. Early experiments showed that modifying the cholesterol content of the liposome had significant effects on liposome stability. In a seminal study, the Gregoriadis group showed that the rigid tetracyclic ring structure of cholesterol improved liposome half-life.[18] Similarly, the incorporation of cholesterol with sphingomyelin[19] into the formulation tightened the fluid bilayer and increased cargo retention.[20] Incorporation of egg phosphatidylcholine (egg PC) and ganglioside GM1 into membranes made of sphingomyelin or 1,2-distearoyl-sn-glycero-3-phosphocholine (DPSC) also increased circulation half-life.[21] This was mechanistically described to be due to the decreased uptake by macrophages, thus increasing the clearance time from circulation.

Indeed, the major detriment to particle half-life is their sequestration in the reticuloendothelial system (RES) in the liver and spleen.[22] Tissue-resident macrophages in these tissues engulf and phagocytose nanoparticles after injection, reducing the therapeutic concentration in blood and decreasing the opportunity for liposomes to reach their targets of interest. Moreover, serum proteins bind liposomal complexes and activate the complement response, further reducing the liposome half-life. A polymer coating effectively prevents opsonization by serum proteins and macrophage clearance. Polyethylene glycol (PEG) remains the most prominent molecule used, but other polymers have also be used, including amphiphilic poly-*N*-vinylpyrrolidones,[23] poly[*N*-(2-hydroxypropyl) methacrylamide],[24] L-amino-acid-based biodegradable polymer–lipid,[25] and polyvinyl alcohol.[26] These coatings form a hydrating shield around the nanoparticle, protecting it from phagocytosis.[27] In addition, PEGylation sterically stabilize LNPs, leading to enhanced permeability and retention.[28] Doxil takes advantage of these benefits to increase the half-life to 77 h in circulation.[29,30] In comparison, pharmacokinetic studies[31,32] of EVs have estimated that their half-life is on the order of minutes, however, more studies in large animals are needed.[33] It is clear that lessons learned from LNP development can also aid the EV field. For example, PEGlyation of EVs increased their circulation time in mice.[34]

More recently, LNP formulations have been at the forefront of development of novel drugs and vaccines that deliver nucleic acid cargo. The introduction of ionizable cationic lipids has largely enabled these drugs. At low pH, the lipids are positively charged, thus enabling complexation of negatively charged oligonucleotides. Afterward, the pH can be raised to the physiological level, leading to vesicles with a neutral surface.[35,36] In the last year, the most notable examples of drugs using such methods are the massively successful mRNA-based COVID-19 vaccines by Pfizer-BioNTech and Moderna.[37,38] LNPs are also integral to the formulation of many first-in-class drugs, such as the first FDA-approved RNAi therapeutic,[39] as well as the first case report of CRISPR-Cas9 for clinical genetic editing in humans.[40] Each of

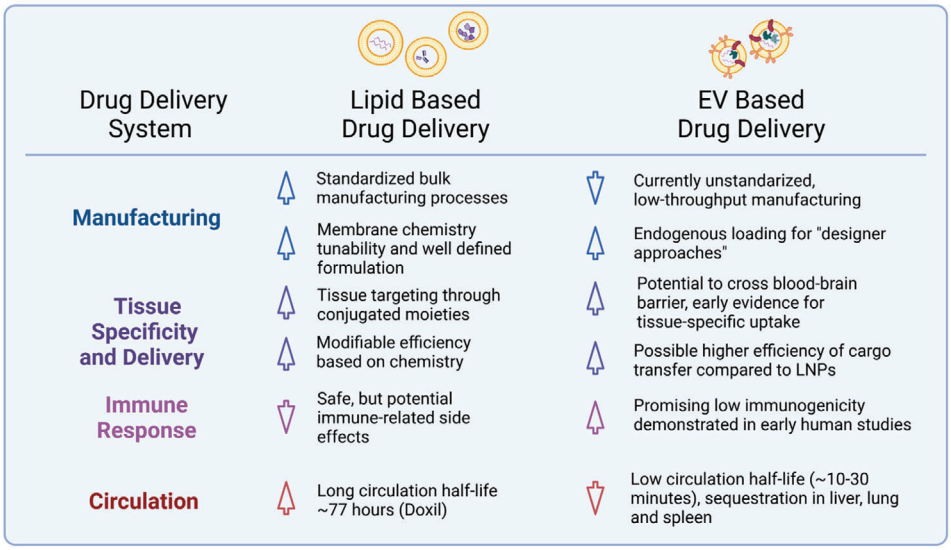

Figure 1. Comparison of lipid-based drug delivery systems and EVs. Lipid-based systems are well-defined and FDA-approved, with versatile chemistries. However, they have immunological side effects and, when compared to EVs, may lack tissue-specificity as well has cargo transfer efficiency. EV-based drug carriers could mitigate these issues, and also leverage endogenous loading mechanisms to deliver more complex cargo. Figure created with Biorender.

these applications utilizes custom LNP formulations, speaking to the versatility of available chemistries.[34] Overall, LNPs and liposomes represent a mature method for drug delivery that is FDA-approved and widely adopted. Still, LNPs face a number of limitations that could potentially be addressed by using EVs as a drug delivery system (**Figure 1**).

2.2. Unique Characteristics of EVs as Drug Delivery Systems

The rationale for EV-based drug delivery has three pillars. Whereas immune-related hypersensitivity reactions to LNPs and liposomes has been well described,[41] EVs are widely considered to have low immunogenic potential.

In general, intravenous injections of EV has been safe in the majority of small animal studies conducted to characterize EV toxicity profiles.[42] For example, intravenous injection of bovine milk EVs into mice showed no systemic effects.[43] Similarly, repeated long-term injection of HEK293 EVs in mice over 22 days produced virtually no deviation in body weight, serum cytokines, and blood cell composition.[15] While these early studies are encouraging, an open question remains whether repeated dosing in humans may cause immunological responses. In a preprint, the Witwer group repeatedly dosed macaque monkeys with EVs.[44] They found that EV circulation times decreased with repeated administration, perhaps due to heightened immune response. In addition, there are applications in which an immunological response may be desirable, such as in EV-based vaccines.[45] Various types of EVs have been shown to modulate immune responses such as macrophage polarization,[46,47] T-cell suppression,[48] but also to display pro-inflammatory effects.[49]

Still, human trials are now proceeding, and a number of first-in-human clinical trials evaluating safety have been completed. These include EVs sourced from a variety of cell types, including dendritic cells,[50] autologous tumor cells,[51] and umbilical cord stem cells,[16] further supporting the safety of intravenous injection of EVs. Although such studies must be done for each EV type, they are reassuring. Of note, one of the studies utilized EVs exogenously loaded with the chemotherapy drug methotrexate through UV-irradiation.[51] The most frequently reported adverse events included dizziness and vomiting (these are also the effects of the chemotherapeutic agent). There were no reports of whole-organ toxicities or autoimmune reactions. However, there were transient differences in blood cell composition and cytokine levels, although all these parameters remained within normal ranges. Nevertheless, this was one of the first studies to demonstrate that modified EVs can be used clinically.

Another argument for the use of EVs is their ability to cross biological barriers, both at the tissue level and the cellular level. At the cellular level, EVs are efficiently uptaken by cells through endocytosis, and several processes have been reported, including clathrin-dependent and independent pathways.[2] At present, a de-facto EV receptor has not been identified. A number of studies have compared the uptake of drug-laden EVs to conventional liposomal formulations and found that EVs have enhanced cellular uptake.[52–54] In a study by the Vader's group, a sensitive CRISPR-Cas9 based reporter system was utilized, in which the delivery of a small guide RNA (sgRNA) to a target cell enabled Cas9 genetic editing and subsequent activation of GFP expression. When the authors compared sgRNA delivery via EVs to that of Dlin-MC3-DMA-LNP, used in the commercial product Onpattro, EVs were much more efficient in activating the reporter.[55] These studies provide evidence that EVs may outperform LNPs in cargo delivery, although more studies of EVs compared to liposomal controls are needed.[56]

At the tissue level, EVs have been shown to deliver signals to many hard-to-reach organs. Specifically, they exhibit the ability to cross the blood brain barrier, an obstacle which current synthetic particles struggle with. In a seminal study, Alvarez-Erviti from Matthew Wood's group engineered dendritic cell EVs to express

a neuron-specific rabies virus glycoprotein peptide (RVG), which binds the acetylcholine receptor present in neurons.[12] This was done through endogenous loading, transfecting the engineered constructs into the cells before EV isolation. Downstream, isolated EVs were further loaded with small-interfering RNA (siRNA) using electroporation. Intravenously injected EVs showed efficient knockdown of mRNA and protein in murine brains, thus demonstrating functional effect. In a separate study, a very similar approach was utilized, this time co-transfecting the cells with both siRNA and the RVG construct.[57] Engineered EVs were able to significantly reduce opioid receptor mu mRNA and protein levels in the mouse brain. Together, these studies suggest two possibilities. First, the blood-brain-barrier may be sufficiently breached using the more physiological EVs when compared to their synthetic counterparts, though the mechanisms are still unknown. Second, it showed that the biodistribution of EVs may be controlled using bioengineering methods. This is exciting as there is evidence that EVs may have distinct organ and cell-type specific targets. In one study, genetically modified mice with cardiac-specific EV luciferase expression were used to track the biodistribution of cardiac EVs in the healthy whole animal. Cardiac EVs localized in the thymus, testis, and kidneys.[58] Using EVs from a specific cell source or further modifying these tropisms may enable tissue-specific drug delivery.

Finally, bioengineering of parent cells to produce therapeutic EVs is unique to EVs as a drug carrier modality. Genetic modification of parent cells which exploit the cellular machinery for EV cargo sorting may be used to produce massive quantities of biologic agents (nucleic acids, proteins). This is of particular interest in the current drug approval landscape, as biologics apprise nearly 30% of all drugs approved by the FDA in 2015–2018, and that number is continuing to rise.[59,60] Biologics have complex manufacturing processes, are difficult to store and package,[61] and stability in circulation, due to the presence of degrading enzymes in serum. Thus, efficient loading of biologics into the relatively stable, membrane-bound EVs may well offer a solution to these problems. In order to better engineer EVs, a better understanding of EV biology and sorting depending on the origin or cargo of EVs is required. In the next section, we will review our current mechanistic understanding of these processes and highlight the most recent bioengineering strategies which employ these advances.

3. EV Cargo and Sorting

Here, we focus on the sorting of specific cargo into EVs and their delivery into specific tissue compartments, as the basis for developing EV bioengineering delivery strategies. The biogenesis of EVs is complex and varies with their size. Various methods have been developed for the isolation of different EV populations, and such practices are continually evolving, as described in several excellent reviews.[2,62,63] However, an overwhelming amount of the current literature focuses on sEVs, whereas the molecular mechanisms of cargo loading to other EV subclasses are less understood and should be the focus of further study.

We have classified the current body of work into those for RNAs and those for proteins. **Figure 2** summarizes our current understanding of these mechanisms, which are covered in detail below.

3.1. EV Associated RNAs

The hypothesis that EVs deliver extracellular RNA (exRNAs) to the recipient cells to mediate signaling first came to the spotlight when a number of pioneering studies in the last two decades demonstrated the potential for EV-associated RNAs for extracellular communication. In 2006, a report showed the horizontal transfer of RNA between EVs and target cells.[64] Similarly, Valadi et al. demonstrated that exRNAs are translation competent using an in vitro translation assay,[65] begging the question of whether or not the transferred RNA can result in functional protein in the target cell. Skog et al. demonstrated that the incorporation of a RNA encoding *Gluc* in microvesicles conveyed luciferase activity in recipient cells.[66] These discoveries inspired numerous attempts at developing an understanding of the types and species of RNAs contained within EVs. Through further experimentation, including unbiased transcriptomics approaches, it has emerged that nearly all types of RNAs including noncoding (ncRNA) RNAs such as small nuclear RNAs, microRNAs (miRNAs/miRs), rRNAs, lncRNAs, mitochondrial RNAs, circular RNAs,[67,68] and transfer RNAs are present in EVs.[69,70] Emphasis has been placed on characterizing the role of RNAs with a known function (e.g., miRNAs, mRNAs). Less understood is the way that fragments of RNAs (tRNAs) or other ncRNAs mediate intercellular communication.

The transcriptome of EVs reflect their cell type of origin but also contain specifically enriched RNA species.[71–73] Moreover, the abundance of certain RNA biotypes differs between the types of EVs, with selective loading of separate species into sEVs and microvesicles. In a comprehensive study, Wei et al. used serial size-filtration combined with RNA sequencing of tumor-derived conditioned media to ascertain differences in MV, sEVs, and free ribonucleoprotein RNA content. They showed that small and fragmented RNAs comprise ≈64 and 93% of all exRNA, respectively, and that miRNA comprises <10% of all RNAs. In agreement with other studies,[74] they found that sEVs are enriched in miRNAs compared to other EV subclasses.[75] These miRNAs are of particular interest as studies suggest a significant difference in the miRNA composition of EVs versus their parent cells.[76,77] Indeed, an increasing number of EV studies now evaluate the miRNA cargo of vesicles. Several mechanisms for RNA sorting into EVs have been proposed (**Table 1**), in two main categories: RNA-binding proteins (RBPs), and EV biogenesis-related membrane proteins.

3.1.1. RNA Binding Proteins

RBPs couple to RNAs and are sorted concurrently into EVs. In a notable study, heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) was found to control microRNA sorting into sEVs, as well as microRNA transcript processing.[78,79] Using microarray analysis and subsequent motif detection, Villarroya-Beltri et al. described a specific GGAG/UGCA "EXO-motif" which is common in EV-derived miRNAs but not in cell-specific miRNAs. Pull-down of an EV-RNA (miR-198) and mass-spectrometry showed that hnRNPA2B1 recognizes this motif. Moreover, t SUMOylation of hnRNPA2B1 was required for miRNA binding, indicating an important role in post-translational modifications

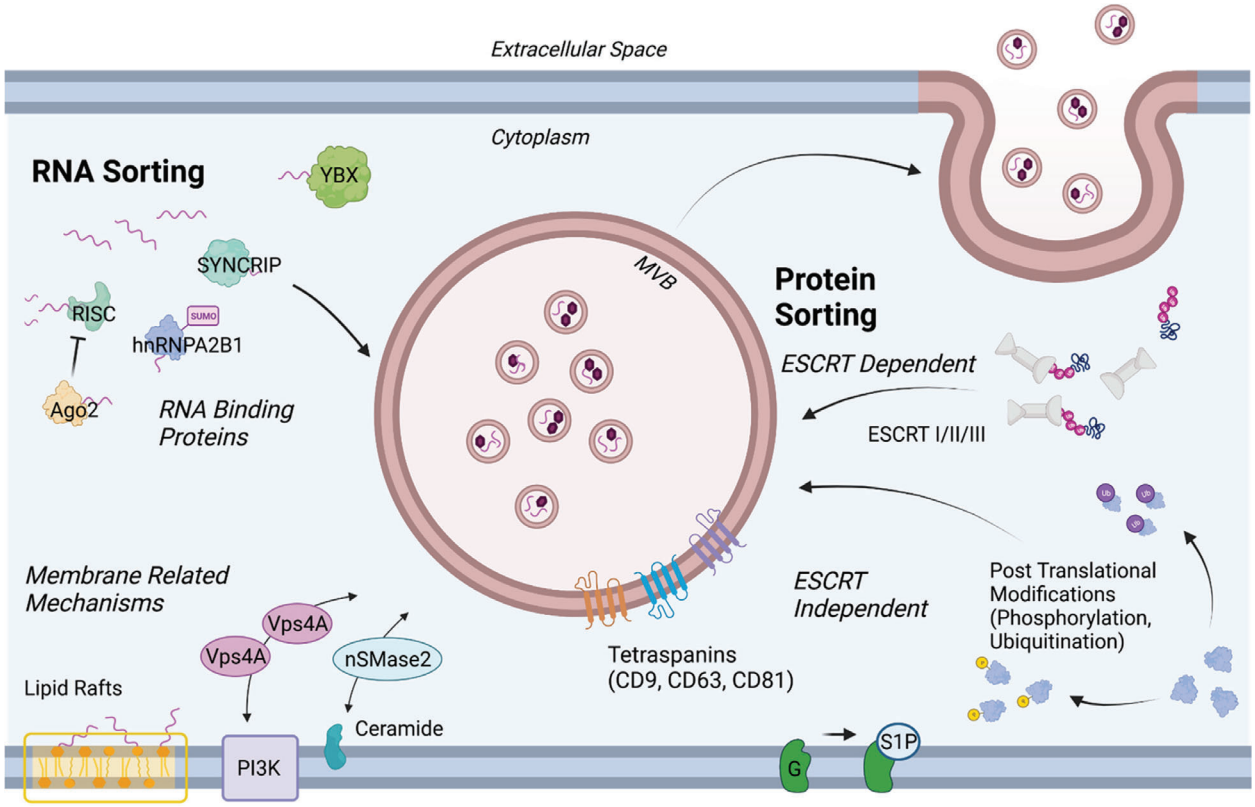

Figure 2. Overview of RNA and protein sorting to EVs. RNA sorting largely involves RNA binding proteins that recognize the specific RNA motifs. These motifs preferentially target multivesicular bodies (MVB) during EV biogenesis. Proteins can be sorted through biological pathways that include post-translational modifications, and the Endosomal Sorting Complex Required for Transport (ESCRT) machinery. Other mechanisms, such as membrane related lipid rafts, are less well understood, but are known to contribute to EV cargo. Figure created with Biorender.

Table 1. Mechanisms for RNA sorting into EVs.

Target	Mechanism	Reference
miR-198	Sumoylated hnRNPA2B1	[78–80]
miR-3470a, miR-194-2-3p	SYNCRIP	[81,82]
miR-100, miR-320a	Ago2	[83,84]
miR-233, miR-133	YBX-1	[85]
miR-193	MVP	[86]
miR-122	La protein, HuR	[87–89]
miR-16, miR-146a, miR-210	nSMase2	[90–92]
miR-27b-3p, miR-92a-3p, miR-150	Vps4A	[93,94]
Synthetic RNAs	Lipid rafts	[95–97]

in EV-RNA packaging. Using X-ray crystallography, Wu et al. confirmed the specific recognition of the AG core motif by hnRNPA2B1 RNA binding motif.[80] Another report showed that hnRNPA2B1 inhibited rather than enriched the EV export of miR-503.[98] In this study, authors note that miR-503 does not contain EXO-motifs. Together, these studies suggest that hnRNPA2B1 plays multiple roles in EV-RNA packaging, which may or may not be sequence binding-dependent.

Another RNA-binding protein which has been described to mediate miRNA sorting into EVs is SYNCRIP, also known as hnRNPQ or NSAP1.[81] Santangelo et al. quantified the miRNA cargo of hepatocyte-derived EVs and found that Syncrip co-precipitated with biotinylated miR-3470a and miR-194-2-3p using mass spectrometry analysis. shRNA knockdown of SYNCRIP reduced miRNA quantity in EVs. Using sequence comparison, the RNA motif GGCU was required for SYNCRIP binding. Mutation of this sequence reduced miRNA yield in SYNCRIP pull-down, while insertion of this sequence into a nontarget miRNA resulted in exosomal loading. Furthermore, SYNCRIP and hnRNPA2B1 RNA binding were independent of each other, displaying sequence-specific loading of miRNAs into EVs. The role of SYNCRIP in miRNA-sorting was further explored by the same group that identified an N-terminal RNA domain (NURR) in SYNCRIP that recognizes the GG motif.[82] Mutation of the exo-motif or removal of the NURR domain led to the same decrease in binding affinity measured by biolayer interferometry, and these results were confirmed in a cellular environment.

Argonaute 2 (Ago2) is an RNA-binding protein integral to the RNA-induced silencing of complex (RISC) which binds miRNA and their target mRNA and facilitates the degradation of the mRNA. Ago2 has been shown to be a the primary microRNA carrier in human serum, independent of EVs.[83] Other parts of the miRNA processing machinery, such as Dicer and Exportin, have been described in tumor EVs.[99] However, there is also evidence for the opposite, with many proteomic studies failing to detect Ago2. It is difficult to separate naked Ago2 -miRNA

complexes from EVs without additional purification methods such as density-gradient centrifugation, which leads to contaminating complexes in preparations of EVs via differential ultracentrifugation, the most commonly used isolation practice.[100,101] Despite this difficulty, an elegant study by the Weaver group highlighted the KRAS-MEK axis in controlling Ago2 sorting into tumor EVs. Activation of the pathway resulted in the phosphorylation of Ago2 on serine 387 reducing miRNA export into EVs, suggesting that Ago2 is indeed part of EV cargo. These findings are further backed by studies that show that mutant-KRAS alters the miRNA profile of tumor EVs.[102]

Y-Box Binding (YBX) protein is another well-described RNA-binding protein which mediates RNA sorting into extracellular vesicles. YBX-1 has been shown to mediate packaging of miR-223 and miR-133. Using a YBX-null cell line and thermostable group II intron reverse transcriptase sequencing(TGIRT-seq), further work by Shurtleff et al. determined a broad role for YBX in the sorting of not only miRNAs, but also tRNAs and Y RNAs.[85]

Other RNA binding proteins involved in RNA sorting to EVs include Major Vault Protein (MVP), which has been implicated in the export of tumor suppressor miR-193 in colon cancer cell EVs.[86] Knockdown of the commonly used exosomal marker Alix in human liver stem-like cells resulted in decreased export and transfer of miRNAs through secreted EVs.[103] The RBP lupus La protein, through a combination of biochemical and genetic experiments, was shown to be responsible for the sorting of miR-122 in breast cancer cell lines.[87] The authors further identified a UUUG motif in miR-122 which La protein binds to, in concordance with earlier studies.[88] Another protein which regulates the export of miR-122 is ELAV-like protein 1/HuR. In a thorough study, Mukurhjee and colleagues showed that HuR ubiquitylation is a novel mechanism for targeting its bound miRNA for EV export.[89] Indeed, post-translational modification of RBPs seem to play an important role in EV loading, including sumoylation, phosphorylation, and uridylation in addition to ubiquitylation. For comprehensive review of the current knowledge of RBPs in extracellular vesicles, please refer to Fabbiano et al.[104]

3.1.2. Membrane-Related Sorting Mechanisms

Membrane proteins related to EV biogenesis have also been implicated in RNA sorting to EVs. Neural Sphingomyelinase 2 (nSMase2) is an enzyme which catabolizes ceramide biosynthesis but also has well-established roles in EV synthesis.[90] For this reason, it is difficult to pinpoint the exact function of nSMase2 on RNA loading, as the inhibition of nSMAse2 will result in a decrease of miRNA secretion either due to the decreased loading, or to the decreased EV number. Nonetheless, it is a known that nSMase2 is essential for miRNA secretion from cells. Overexpression of nSMase2 caused an increase in miR-16 and miR-146a in EVs secreted by COS-7 cells.[91] Knockdown of nSMase2 in 4T1 cancer cells resulted in a loss of miR-210 in EVs.[92]

Vacuolar protein sorted associated protein 4 (Vps4A) has also been shown to be important in the loading of miRNAs in liver cancer. Wei et al. demonstrated that hepatocellular carcinoma cells overexpressing Vps4A had higher levels of oncogenic miRNAs miR-92a-3p and miR-27b-3p in their EVs. In addition, Vps4A overexpression caused higher levels of tumor suppressor miRNAs within the cells. Pathway analysis of differentially expressed miRNAs identified the PI3K pathway as a potential target, and indeed the activity of this pathway was reduced in Vps4A cells.[93] Knockdown of Vps4A has been shown to have the opposite effect, decreasing miR-92a and miR-150 levels.[94] Together, these data demonstrate a role of Vps4A in EV packaging.

Finally, a less-described model for EV sorting using lipid microdomains, called lipid rafts, has been proposed by the Janas group.[105] Lipid rafts are areas enriched in cholesterol and sphingolipids which form discrete domains. The model is based on the observation that nucleic acids specifically bind phospholipids rafts, and is certainly plausible given that the sphingolipids and ceramides which constitute lipid rafts are also enriched in EVs.[96,97,95,106] At this point, it is not well understood whether or not this phenomenon plays a large role in sorting of RNAs to EVs in vivo.[107]

3.1.3. Additional Considerations of EV-RNA

The hypothesis that EVs deliver functional RNAs is still debated.[108] Many studies highlight RNA transfer in the context of a number of confounding variables in experimental design. For example, researchers often use overexpression systems to modulate EV cargo, potentially altering other aspects of EV biology and other cargo. Furthermore, delivery of specific RNA species is concomitant with the delivery of EV-associated proteins which may also modulate gene expression. Additionally, proof of exRNA uptake is not proof of function, as exRNA needs to undergo endosomal escape in order to reach the cytoplasm of the cell, where it can then be transcribed. Limiting the expression the RNA of interest in recipient cells may be a clever approach to overcome some of these problems, and accurately track RNA function post-transfer.[109]

Another argument against EV-delivered RNAs, specifically miRNAs, are stoichiometric studies, which show less than one copy of a specific miRNA per 100 vesicles, raising questions about the number of EVs necessary to deliver an adequate level of miRNA into a recipient cell to induce gene silencing (with an estimated 3000–5000 copies of miRNA per cell).[75,110–112] Modulation of gene expression via an EV delivered miRNA would require either a large amount of EVs delivered at once, or consistent EV delivery over time, or extreme specificity for cell-type uptake. Novel tools to study EV-cargo uptake will help in this manner, including novel split luciferase-based systems.[113] Vader's group, again using their CRISPR/Cas9 based reporter for EV transfer (CROSS-FIRE), showed that reporter activity was less than 1% in EV-transfer experiments,[114] which the authors attributed to sgRNA stoichiometry: one sgRNA molecule per 450 000 EVs. Overall, these results suggest that a more in-depth understanding of EV RNA trafficking and uptake is required to properly optimize RNA delivery via EVs.

3.2. EV Associated Protein Sorting

Proteins are the second class of EV cargo of main focus in the literature. It is clear how the delivery of a protein into the cytoplasm or membrane of a target cell can affect biological function. The

Table 2. Mechanisms for Protein Sorting into EVs.

Mechanism	Reference
ESCRT	[120–122]
Ubiquitylation	[123–126]
Ubiquintin Like Proteins (UBL)	[127]
SUMO1-4, NEDD8, ISG15	[128]
ATGs	[129]
UBL3	[130,131]
SUMOylation	[132,133]
Sphingosine 1-phosphate(S1P)	[134]
Tetraspanins	
CD63	[135]
CD9, CD82	[136]
Phosphorylation	[137–139]

first in-depth characterization of EV protein came from Théry et al in 1999, utilizing peptide mapping to identify the presence of heat shock proteins, annexin II, and major histocompatibility complexes (MHCs) in EVs.[115] Subsequent proteomic studies of EVs have since mapped the proteome of vesicles secreted by multiple cell types, and detailed databases can be found at Vesiclepedia and ExoCarta.[70,116] Over 32 000 unique proteins among 1254 EV studies have been identified. Like EV RNA cargo, the protein cargo of EVs vary with the parent cell state.[117–119] EV proteins can be broadly separated into those present on the vesicle membrane or those solubilized within the membrane enclosure (**Table 2**).

Common EV membrane proteins include those associated with biogenesis (CD9, CD81, CD63), while the soluble cargo often includes heat shock proteins and parts of the endosomal sorting machinery (TSG101, Alix). Because sEVs are generated from the endosomal compartment, their cargo may be more divergent from that of their parent cell. Targeting proteins to the endosomal compartment may therefore enable altering EV cargo and should be better understood in order to improve engineered EVs. Biological mechanisms of protein sorting to the endosomal compartment are separated between those that depend and do not depend on the Endosomal Sorting Complex Required for Transport machinery (ESCRT).

3.2.1. ESCRT-Dependent Sorting

Proteins which are marked for the endosomal pathway eventually coalescence in the late-stage endosome as intraluminal vesicles (ILVs) inside multivesicular bodies (MVBs).[140] From there, MVBs either fuse with lysosomes for degradation, or with the cell membrane to be released as EVs.[141] Thus, there is a degree of overlap between protein degradation pathways and EV secretion. For example, membrane proteins are often sorted to EVs due to a common shared pathway between receptor recycling and EV packaging. Integral to the endosomal sorting pathway is ESCRT, which is composed of ≈20 proteins that comprise 4 distinct complexes. These complexes associate with other proteins, including markers specific for sEVs, Alix, TSG101, and VPS4. Syndecan proteoglycans and their cytoplasmic adaptor syntenin were reported to also interact with Alix through LYPX(n)L motifs, supporting the formation of ILVs.

In the canonical pathway, ESCRT complexes shuttle ubiquitinylated proteins to the endosome. The early ESCRT complexes, ESCRT-0, -I, and -II, initiate recognition and binding of ubiquitinated proteins. ESCRT-0 recruits ESCRT-1 through the interaction of HRS domains, which binds both ubiquitinated protein and the ESCRT-1 subunit TSG-101.[120,142] ESCRT-1 encapsulates cargo and passes it on to ESCRT-II. ESCRT-II coordinates the assembly of ESCRT-III.[121,122] ESCRT-III is a transient complex which recruits deubiquintinases for ubiquitin recycling and plays a role in MVB membrane budding. A prime example of this canonical pathway is found in epidermal growth factor receptors (EGFRs) and other transmembrane receptors present on the plasma membrane. Cell surface receptors monoubiquitinated at their intracellular regions are sorted to the early endosome. Indeed, the role of EGFR in EVs is well established in the cancer literature. EGFR-laden sEVs secreted by SGC7901 cells promoted hepatotropic metastasis in mice,[143] and larger microvesicles containing mutant EGFRvIII secreted by glioblastoma increased oncogenic potential in vitro.[66]

3.2.2. Ubiquitylation and Ubiquitin-Like Proteins (UBLs)

Due to ESCRT, ubiquitylation of proteins is a primary PTM by which proteins may be secreted through EVs. Ubiquitylation is catalyzed by a series of ligases (E1, E2, and E3) which recruit activate, conjugate and ligate ubiquitin, respectively. Ubiquitin is a small 8.5kDa protein with β-grasp fold structure that contains seven lysine residues at position 6, 11, 27, 29, 33, 48, and 63 in its sequence, and is ligated to substrate protein lysine residues.[123,124] Ubiquitylation of these residues forms polymeric ubiquitin structures, which have distinct conformations and differential effects on proteins. Current evidence suggests that proteins with K29 and K48 chains are targeted toward the endosome. Ubiquitin itself can also be the subject of PTMs, either through phosphorylation or acetylation.

UBLs are proteins with close conformational homology to ubiquitin, and some of them have relatively no sequence homology.[127] The most characterized proteins of this class include small ubiquitin-like modifiers (SUMO1-4), neuronal precursor cell-expressed proteins, developmental downregulated 8 (NEDD8), interferon-stimulated gene 15 (ISG15), and autophagy related proteins (ATGs). Of these, NEDD8 is the closest to ubiquitin and similarly forms chain structures at residues 22 and 48.[128]

In 2004 and 2005, two separate reports confirmed the presence of ubiquitinated proteins in EVs.[125,126] Since then, specific proteins enriched in EVs have been identified. For example, in EVs secreted by myeloid-derived suppressor cells, ≈10% of the EV lysate is ubiquinated. Intriguingly, this analysis identified several ubiquitinated histones, which may play a role in mediating inflammation.[144] In infectious disease of the lung, *Mycobacterium tuberculosis*-infected macrophages produce EVs which stimulated Toll-like receptors (TLRs) and mediated inflammation by conferring pathogen-associated molecular patterns (PAMPs), both in vitro and in vivo. Intranasal delivery of these EVs into mice increased neutrophil and macrophage recruitment in the lung.[145] In a follow-up study by the same group, inhibition of

ubiquitination in stimulated RAW264.7 macrophages prevented secretion of mycobacterial proteins in EVs.

In a similar way, many viruses take advantage of ubiquitylation in order to export viral proteins. Human T-cell Lymphoma Virus (HTLV) Gag protein is ubiquitinated by Nedd4.1 ubiquitin ligase.[146] Subsequent recruitment of TSG101 ensures trafficking to MVBs. Human immunodeficiency virus, another retrovirus, requires ubiquitin in ESCRT-dependent viral release.[147] In latent infection of the Epstein-Barr virus(EBV), latent membrane protein 2A (LMP2A) alters B cell development and contributes to pathogenesis of disease. LMP2A ubiquitination was found to be mediated by plasma membrane cholesterol and reduced secretion in EVs.[148] EBV protein LMP1 was also found to be localized in EVs.

Recently, the Tsuchida group identified UBL3 as a post-translational modification that is required for protein sorting into EVs. The authors identified UBL3 using bioinformatics analysis and confirmed its activity as a PTM factor. In contrast to classical ubiquitylation, UBL3 creates disulfide bonds with its target. UBL3 was found to localize with CD63, marking the endosomal compartment. Proteomics analysis identified 1241 proteins as interacting with UBL3, with 29% annotated as related to EVs. Genetic knockout of UBL3 in mice resulted in an astounding 60% reduction in total EV protein, indicating a general role of UBL3 in EV sorting. Among other proteins, tubulin and Ras sorting to EVs were increased by UBL3 modification.[130,131] Importantly, the overexpression of UBL3-tagged GFP in parent cells resulted in sorting of GFP to their vesicles, whereas simple overexpression of GFP did not. These results indicate that cargo not normally present in EVs may be sorted to them via bioengineering techniques.

SUMOylation has also been reported as a PTM factor enhancing sorting to EVs. Extracellular α-Synuclein, a protein implicated in Parkinson's disease, is secreted in EVs in a SUMOylated form. Kunadt et al. from the Schneider group comprehensively demonstrated that the sorting of α-Synuclein into extracellular vesicles of mouse neruroblastoma cells is dependent on SUMOylation.[132] They present evidence that SUMO-2 interacts with phosphoinositol-3-phosphate, which have been previously demonstrated to recruit the ESCRT complex.[149] Moreover, SUMO-1 has been implicated in the cellular response to α-Synuclein in neurodegenerative disease.[133]

3.2.3. ESCRT Independent Sorting

ESCRT is not required for MVB biogenesis, as evidenced by studies in cells lacking functional ESCRT-0, -I, -II,-III.[150] Therefore, other mechanisms of EV sorting Ceramide generation by nSMase 2, covered previously, have been reported as required for ESCRT-independent ILV biogenesis.[90] In a key study reported by Kajimoto et al., sphingosine 1-phosphate(S1P) coupling to inhibitory G protein was shown to mediate exosomal MVB maturation, and the treatment of cells with S1P increased intraluminal cargo. Depletion of S1P using siRNA significantly limited the EV cargo but not the total number of EVs. This observation suggests that S1P is necessary for cargo sorting into ILVs but is not necessarily required for ILV formation.[134] Furthermore, tetraspanin membrane proteins CD9, CD63, and CD82 have also been shown to mediate sorting of proteins to EVs. Melanocytes export premelanosome protein (PMEL) through melanosomes in a process identical to EV biogenesis. The Raposo Group demonstrated that the loading of PMEL into MVB is affected by CD63, and that PMEL sequestration and export were significantly decreased by CD63 knockdown.[135,151] Likewise, the export of β-catenin, the Wnt pathway effector, in EVs is decreased when CD9 and CD82 are silenced.[136] In that work, Chairoungdua showed that that CD82 and CD9 down-regulate the cellular levels of β-catenin through increased EV export, without exploring the effects of β-catenin enriched EVs on target cell function.

3.2.4. Phosphorylation

Proteins are processed by post-translational modifications (PTM) including phosphorylation, acetylation and glycosylation, among others. These additions to specific amino acid residues markedly increase the level of complexity in the protein cargo.[131,152–154] Phosphorylation of proteins has been discussed in the sorting of RNA binding proteins to EVs.[84,102,155] Indeed, phosphorylation is increasingly recognized as a signal for trafficking to EVs, but only recently has the phosphoproteomic landscape of EVs started to be explored.[156] Chen et al. and Rontogianni et al. both show the use of the EV phosphoproteome as a biomarker in breast cancer.[157] At present, there is some evidence for the role of phosphorylation for the loading of EVs. Impairing EGFR phosphorylation prevents its ubiquitination and subsequent endosomal sorting.[137] The Fas ligand, a key protein triggering apoptosis in the immune system, has been shown to require phosphorylation by the Src-family tyrosine kinase Fgr for its internalization into MVBs. In Alzheimer's disease, the tau protein secreted in extracellular vesicles has been shown to be selectively phosphorylated.[138,139] Other PTMs currently being explored in the context of sorting to EVs include glycosylation,[158–160] citrullination, deamination,[161] and acetylation.[162] However, these approaches are limited in part by the ability to conduct unbiased surveys of PTM landscapes.

4. Engineering of Extracellular Vesicles for Drug Delivery

Despite exciting preclinical evidence, a common challenge to all EV based therapies is to determine the dosing required to achieve therapeutic action, largely due to the intrinsic heterogeneity in EVs. For example, the properties of mesenchymal stem cell derived EVs vary based on the original cell seeding density, passage number, and even the frequency of EV collection.[163] Therefore, in order to standardize EV characteristics and bioactivity, it may be desirable to specifically engineer EVs with cargo of interest to a target stoichiometry.

In this section, we explore such engineering platforms designed to bolster EVs as a drug-delivery system through manipulation of cargo content. Following our delineation of EV loading into endogenous and exogenous loading categories (**Figure 3**), we first describe the exogenous loading methods, which occur after EV isolation. Next, we describe the most recent approaches for endogenous loading, taking abreast the mechanisms of cargo-loading we have covered thus far.

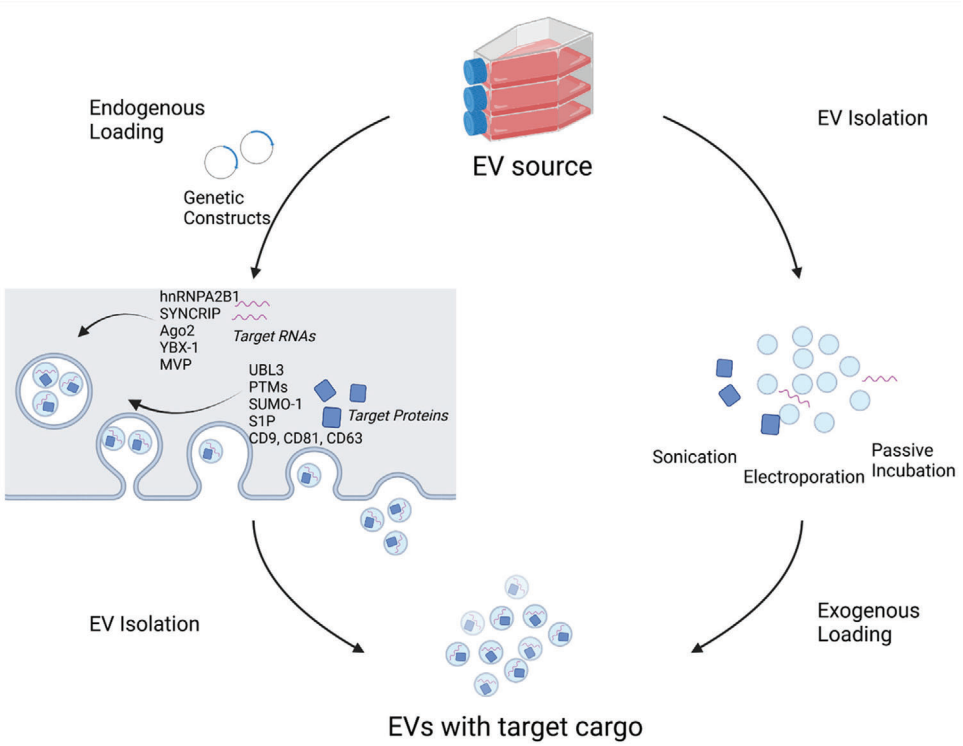

Figure 3. Overview of EV bioengineering. EVs can either be endogenously loaded using engineered genetic constructs or exogenously loaded post-isolation using a number of membrane disruption methods such as electroporation. Endogenous loading mechanisms may take advantage of cellular machinery to load EVs. Figure created with Biorender.

4.1. Exogenous Loading

EVs can be loaded exogenously, after isolation from target cells. These loading strategies have the advantage of starting from a clearly defined substrate and modifying their cargo externally. Additionally, these methods must be used in lieu of endogenous loading when the ideal cargo is a small molecule drug, which cells lack the machinery to manufacture. However, there is also a tradeoff: the methods outlined below may also damage the integrity of EVs, perhaps altering their pharmacokinetic properties or bioactivity.[164]

The earliest and simplest strategy to load EVs is through passive incubation with the cargo. For example, EVs co-incubated with curcumin and then administrated through the nasal cavity prevented neural inflammation in a mouse model of experimental encephalomyelitis.[165] Doxorubucin passively loaded into EVs also significantly increased survival in murine models of cancer.[166] An alternative passive loading approach is to incubate cells with the target molecule, causes them to uptake it and then secrete it inside EVs. In this way, Millard et al. passively loaded EVs with photodynamic drug, meta-tetra(hydroxyphenyl)chlorine (mTHPC), and tested them against liposomal mTHPC in an 3D in vitro model of a tumor microenvironment, finding that the EVs performed significantly better. A similar approach was used by Pascucci et al. to load the chemotherapeutic paclitaxel into EVs, showing in vitro efficacy.[167]

Passive loading, while effective, may still fall short of other active loading methods in terms of efficiency.[54] Ubiquitously, active loading strategies disrupt EV plasma membrane structure in order to allow cargo to enter. The most popular method currently used is electroporation. In one study, the authors showed that electroporated EVs could deliver miRNA-155 mimic to the liver, in vitro and in vivo.[168] However, they did not show functional knockdown of the mRNAs which miR-155 target. In another study using electroporation to load EVs with short interfering RNA specific to oncogenic KRAS, Kamerkar et al. demonstrated the therapeutic ability of these EVs in multiple models of pancreatic ductal carcinoma in mice.[169] Moreover, they found that the anti-tumor ability of EVs were much higher than that of liposomes loaded with the same siRNA. This research formed the foundation for a phase 1 clinical trial investigating the use of these engineered EVs in pancreatic cancer patients. (clinicaltrials.gov, NCT03608631) The functional difference between these two studies suggests that rigorous optimization of EV source and dosing may be required for each application and type of therapeutic cargo.

Small molecules can also be delivered via electroporation. Tian et al. isolated EVs from immature murine dendritic cells and then electroporated them with doxorubicin. After recovery, washing and re-isolation, the EVs could inhibit tumor burden in mice inoculated with breast cancer cells.[170] Using a similar approach, Schindler et al. loaded HEK293T EVs with doxorubicin and performed cellular uptake studies using doxorubicin's

inherent fluorescence. When compared to on-the-market liposomal formulations of doxorubicin, they found that loaded EVs performed substantially better, perhaps showing the superiority of EVs as a drug-delivery vehicle. However, the authors tested HEK293T to HEK293T transfer, and did not exclude a cell-type specific effect of EV uptake. Other methods for EV plasma membrane disruption to allow cargo loading include sonication[171] and freeze-thaw cycling.[172]

4.2. Endogenous Loading

Endogenous loading is the use of genetic overexpression in parent cells to produce EVs with a desired cargo. One of the first reports was put forth by Mizrak et al., in which HEK293-T cells were transfected with a construct overexpressing cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT). EVs containing these proteins and mRNAs were isolated and injected into mouse tumors in vivo. Subsequent conversion of a prodrug by these two enzymes into an active chemotherapeutic agent halted tumor progression.[173]

Further engineering of EV cargo has been accomplished by leveraging novel technologies such as optogenetics. The Choi group reported the EXPLOR method (exosomes for protein loading via optically reversible protein-protein interactions).[174] This approach achieved protein cargo loading in two steps. First, they introduced a construct expressing the photoreceptor cryptochrome 2 (CRY2) fused to a cargo protein of interest in EV-producing cells. Next, they exploited a CRY-interacting protein conjugated to CD9, a tetraspanin specific to sEVs. Upon stimulation of the cells with blue light, transient complexing of the CRY-2 fused cargo protein and CD9 occurred, leading to the sorting of the cargo and secretion in EVs. Of note, the authors compared the loading capacity of their method with other commercially available methods, such as XPACK (System Biosciences), and found that EXPLOR outperformed them. Finally, in an in vivo model, they demonstrated the feasibility of their platform by demonstrating functional delivery of EV-targeted Cre recombinase delivered to floxxed reporter cells in a mouse model.

We have already covered how the novel mechanism of UBL3 can be manipulated to sort cargos into EVs.[130] Similarly, Sterzenbach leveraged the ESCRT sorting pathway to load biologically active proteins into EVs.[175] The authors hypothesized that because the late domain (L-domain) proteins are used for the recruitment of ESCRT, and because L-domain containing protein Ndfip1 recognizes WW domains, the addition of a WW domain could target proteins into EVs. Indeed, addition of a WW-tag to Cre recombinase resulted in functional Cre transfer via EVs. By thorough characterization, they demonstrated that WW-Cre was present inside EV lumens and that sorting was dependent on Ndfip1, as they had hypothesized. Injection of WW-Cre EVs into a mouse brain further demonstrated their in vivo activity, when the delivered Cre induced tdTomato expression in multiple brain regions.

EV subtypes other than sEVs and exosomes have also been used for endogenous loading. Arrestin domain containing protein-mediated microvesicles (ARMMs) bud directly at the plasma membrane rather than through the endosomal pathway. ARMMs require arrestin domain containing protein 1 (ARRDC1) for biogenesis. The overexpression of ARRDC1 increases the production of ARMMs in cells, a simple method for bolstering production. [176] Like sEVs and exosomes, transfer of cargo via ARMMs has also been described, showing transfer of NOTCH receptors.[177] These candidates make ARMMs an appealing choice for cargo delivery. In a recent study, Quan Lu's group has demonstrated ARMMs as drug delivery vehicles, and characterized the packaging and bioactivity of proteins, RNAs, and CRISPR-Cas9 complexes.

To generate ARMM-directed protein, the authors overexpressed a construct that contained ARRDC1 fused to the n-terminus of the tumor suppressor protein p53. Then, they isolated EVs from these cells, and detected only the ARRDC1-p53 but not native p53 in their cargo. ARMMs loaded with p53 demonstrated both in vitro and in vivo activity of p53. To load RNAs, the authors leveraged a dual-construct system. One construct overexpressed ARRDC1 fused with transactivator of transcription (Tat) protein, which strongly binds transactivating response (TAR) elements in RNA. The second construct expressed a target mRNA with TAR elements. EVs generated from these cells demonstrated the delivery of p53 mRNA in vitro by upregulated transcription of p53 target genes Mdm2 and p21. As the proof-of-concept, the authors loaded the Cas9 protein and associated small guide RNA into ARMMs. This time, rather than using direct ARRDC1 fusion, the authors leveraged the fact that ARRDC1 also binds WW-domains. A WW-tag successfully directed Cas9 into ARMMs, and subsequent incubation with target cells resulted in successful gene editing.

Lastly, a study by Kojima et al combined a number of different genetic manipulations to the parent cell in order to increase key properties of EVs.[178] Termed the "EXOtic" platform, the authors optimized five properties of EVs into one hyper-functionalized product. First, they overexpressed three genes (STEAP3, SDC4, NadB), in order to vastly increase EV production by parent cells. Next, mRNA packaging was achieved by using a dual construct system. The ribosomal protein L7Ae, which binds C/D box RNA structures, was conjugated to CD63 and overexpressed. C/D box motifs were then conjugated to mRNAs of interest. Finally, connexin 43 was also overexpressed to help cytosolic delivery of EV cargo. To show therapeutic use of these "designer" EVs, the authors injected EXOtic EVs containing catalase mRNA to the brain in Parkinsonian mice to attenuate neuroinflammation. These studies demonstrate how genetic manipulation can be used to combinatorially modify EVs by adding the properties of interest.

4.3. Other Approaches to Engineered EVs

Other types of engineered EVs have been developed, not just for cargo purposes. EVs have been modified with surface epitopes in an attempt to modify their tissue distribution.[179,180] For example, expression of the WLSEAGPVVTVRALRGTGSW peptide on EV surfaces targeted EVs to myocardium, [181] and may be useful for repair after myocardial infarction. Combining EVs with synthetic lipids into so called EV hybrids, is also an area of interest. Piffoux et al. utilized PEG to catalyze fusion between EVs and liposomes and demonstrated encapsulation of EV cargo in their hybrid system.[182] Similarly, Sato et al. created a number of different hybrid formulations utilizing a variety of cationic lipids and

showed that their ability to deliver cargo in vitro was superior to that of unmodified EVs.[183]

Recently, a novel method for engineering EVs was pioneered by Votteler et al., who screened synthetic proteins with elements encoding for the following functions: contained membrane binding, self-assembly, and ESCRT recruitment. The result was a synthetic, hybrid biological carrier termed an "enveloped protein nanocage," or EPN. [184] When the synthetic construct is expressed inside cells, cellular machinery cause the EPNs to be loaded with functional cargo and secreted into EVs. This study is a prime example of how, on the basis of understanding EV biology, bioengineering approaches can be leveraged to improve EV function.

5. Conclusion and Outlook

EV research has tremendously expanded in the last decade. As native carriers of biomolecules, EVs are promising candidates for drug delivery, especially for cargos that can be synthesized using cell's own machinery. As we have reviewed, simple exogenous and endogenous loading methods are now evolving into more targeted approaches utilizing natural mechanisms of cargo loading. For RNAs, the addition of certain motifs outside of the coding sequence may be useful for RBP binding and incorporation into EVs. For proteins, the expression of fusion proteins or domains which link the cargo of interest to an EV specific marker (e.g., CD81, CD63), may increase loading. The addition of lysine residues, which are the targets of ubiquitination, may also be a strategy to push protein toward the ESCRT machinery. Moreover, multifactorial cellular engineering now allows the use of independent genetic constructs bolstering separate aspects of EV production (i.e., EXOtic). As the molecular mechanisms regulating EV secretion become more clear, more avenues of opportunity will open for EV bioengineering.

We believe that a large contributor to the obstacles that EVs face for clinical translation is the current lack of standardization. Moving forward, standards for assessing the cargo loading will be essential to EV bioengineering. The creation of such standards will ultimately be the purview of field experts, and is currently a problem that a number of consortia are trying to address. [185] We posit here some of the relevant questions.

The first question is that of stoichiometry: how can we show that the loading of RNAs and proteins is effective? To assess loading of proteins, it may be useful to use a nonendogenous protein, such as GFP or a luciferase, to benchmark the amount of cargo that has been loaded. For RNAs, this approach may be significantly more difficult. Given their relative paucity, high-resolution quantification of mRNA or miRNA copy numbers are needed for EV isolate. Advances in fluorescence in situ hybridization (FISH) techniques may allow the use of fluorescence-based quantification systems, as reviewed by de Voogt.[186] Similarly, new droplet based digital qPCR enables high-resolution of absolute RNA copy number.[187]

The next question is that of function. How can we show that the loaded cargo is having its desired effect? For protein delivery, the function is probably most easily measured either by the activity of the downstream molecular pathways or transcriptional targets. Dose dependence experiments, in terms of both the cargo loaded as well as the amount of EVs used in treatment will be crucial to prove bioactivity. The same framework should be applied to the functional RNA transfer, though it may depend on both the type and abundance of the RNAs in question. For miRNAs, the use of luciferase reporter systems in target cells is a common approach to assess functional miRNA transfer. Similarly, delivery of an siRNA may be tested by the expression levels of the target protein in the cell. However, for mRNA species, the problem is compounded by the fact that functional transfer of mRNA and encoded protein is virtually indistinguishable in experimental settings (this is a common problem of Cre-lox EV reporter systems). A potential solution here would be to limit mRNA expression in the target cell, such that any protein expression after EV transfer would be due to transcription of the EV cargo itself. Moreover, ablation of this effect using a translation inhibitor such as cyclohexamide could convincingly show that the mRNA, and not the protein, is being transferred. Finally, functional studies should be conducted both in vitro and in vivo, ideally utilizing LNP-loaded cargo as a positive control.

The questions of stoichiometry and function are inherently tied to EVs' potential as a drug carrier. While it may be argued that EVs have potential to deliver complex signals consisting of multiple different types of cargo, this capability may complicate the regulatory approval. Without a clearly defined function of the EV products, clinical dosing may be difficult to determine.[188] On the other hand, abundant loading of a specific biomolecule such as a miRNA or protein in stoichiometric excess would decrease batch-to-batch variability of EV effects, as well as help define the therapeutic mode of action. An ISEV position paper[189] suggested that if the mode of action of a drug can be attributed to the specific cargo alone, then the EVs would be considered excipients. If so, only the safety profile of the EVs would be required, perhaps paving the way to regulatory approval.

An aspect of EV bioengineering that we have not covered in this review is their production. To increase capacity, new methods such as adapting cell monolayers to 3D or suspension culture, [190] or nanoelectroporation are being explored.[191] However, the absolute numbers of EVs needed to treat each patient will again depend on stoichiometry and function.

Considerations of cell source and EV isolation will also be important for regulatory approval of EV therapies. For example, EVs may be subject to different regulatory requirements if they are endogenously loaded (i.e., genetically modified), or exogenously loaded.[189] As all components of a drug must be well-defined, it is necessary to demonstrate low batch-to-batch variation. To do this, EVs isolated from cell culture supernatant will likely need to be standardized in terms of culture time, cell density, oxygen content, and media formulation, as all of these factors affect EV quality.

In terms of the cell sources, MSCs are a strong candidate, as their safety and efficacy has already been shown in the clinic. However, it is unlikely that this is a one-size-fits-all scenario, as EVs from different cells may significantly differ in terms of their effectiveness. Therefore, standardized protocols for the production, isolation, and storage of EVs from cell sources of interest must be established. In most cases, EVs are isolated by ultracentrifugation, which is time consuming and not compatible with large-scale production; instead, recent methods such as tangential flow filtration may be used.[192,193] Another important requirement for all EVs is the compliance with good manufacturing

practice.[42] Many of the production problems are being solved,[194] and with the continued interest in the EV field, we remain optimistic for the future translation of EV therapies such as those targeting the heart.[195]

In conclusion, the ability to define the desired EV cargo would be advantageous to both designing more effective EV therapeutics and obtaining regulatory approval. Therefore, continued investigation into EV biogenesis and cargo will be critical for advancing bioengineering approaches to generate EVs with a specific cargo. The present preclinical proof-of-concept studies are highly promising.[12,51,169,179] Moving forward, interactions between biologists, bioengineers, and nanomedicine experts are likely to further accelerate progress toward engineered EV drug carriers.

Acknowledgements

The authors are grateful for funding support by NIH (grants UH3 EB025765, P41 EB027062, and 3R01 HL076485).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

bioengineering, drug delivery systems, extracellular vesicles, lipid nanoparticles

Received: July 30, 2021
Revised: September 28, 2021
Published online: November 11, 2021

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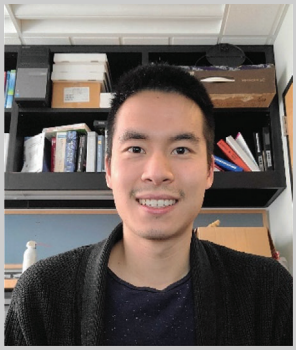

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