Published in final edited form as:

Adv Drug Deliv Rev. 2018 May 1; 130: 12–16. doi:10.1016/j.addr.2018.06.017.

Strategic design of extracellular vesicle drug delivery systems

James PK Armstrong¹ and Molly M Stevens^{1,*}

¹Department of Materials, Department of Bioengineering, and Institute for Biomedical Engineering, Imperial College London, London, SW7 2AZ, United Kingdom

Abstract

Extracellular vesicles (EVs), nanoscale vectors used in intercellular communication, have demonstrated great promise as natural drug delivery systems. Recent reports have detailed impressive *in vivo* results from the administration of EVs pre-loaded with therapeutic cargo, including small molecules, nanoparticles, proteins and oligonucleotides. These results have sparked intensive research interest across a huge range of disease models. There are, however, enduring limitations that have restricted widespread clinical and pharmaceutical adoption. In this perspective, we discuss these practical and biological concerns, critically compare the relative merit of EVs and synthetic drug delivery systems, and highlight the need for a more comprehensive understanding of *in vivo* transport and delivery. Within this framework, we seek to establish key areas in which EVs can gain a competitive advantage in order to provide the tangible added value required for widespread translation.

Keywords

Drug Delivery; Extracellular Vesicles; Exosomes; Microvesicles; Liposomes

Extracellular Vesicles: Next Generation Drug Delivery System?

Extracellular vesicles are potent biological vectors capable of transporting functional biomolecules between cells over large intercellular distances.^{1,2} This communication process, critical for many physiological and pathological processes,^{2–5} has been heavily investigated as a therapeutic strategy to combat disease or rejuvenate damaged tissue.^{6,7} In this regard, two distinct approaches can be classified: first, therapies that exploit native biological functions of EVs to mimic natural repair processes and second, drug delivery approaches that use EVs as vectors to deliver therapeutic entities to the site of repair.⁸ The focus on EV-mediated drug delivery systems has intensified since early animal studies by the Zhang group showed that EVs could be used as *in vivo* vectors for anti-inflammatory cargo. The first study, by Sun *et al.* in 2010, used intraperitoneal injections of curcumin-laden EVs in a lipopolysaccharide (LPS)-induced septic shock mouse model.⁹ A follow-up study, this time employing intranasal injections, demonstrated that EVs loaded with curcumin or Stat3 inhibitor could be used to treat LPS-induced brain inflammation, experimental autoimmune encephalitis and a GL26 brain tumour model.¹⁰ The same year, Alvarez-Erviti *et al.* showed

*Corresponding author: m.stevens@imperial.ac.uk.

that EVs carrying short interfering ribonucleic acid (siRNA) sequences could be delivered intravenously into wild-type mice to knockdown expression of *BACE1*, a therapeutic target in Alzheimer's disease.^{11,12} More recently, Tang *et al.*¹³ and Silva *et al.*¹⁴ reported that chemotherapeutic drugs (methotrexate, *cis*-platin) and photosensitizers (m-THPC), respectively, could be packaged into EVs by cells and used to inhibit tumour growth in murine cancer models. Indeed, a wide range of therapeutic entities (small molecules, nanoparticles, proteins, oligonucleotides) have been encapsulated into EVs using a host of different approaches (electroporation, surfactant permeabilization, sonication, hypotonic dialysis, freeze-thaw cycles, extrusion, cell-mediated packaging).^{15–17} Cargo-loaded EVs have subsequently shown promising therapeutic effects in a variety of disease models, including cancer,^{18–23} cerebral occlusion,²⁴ and neurodegenerative diseases.^{25,26}

Challenges of EV-Based Drug Delivery

Amidst these undoubtedly exciting findings, it is worthwhile taking a step back to consider the precise motivation behind using EVs to deliver drugs, in particular, determining the real benefits that may be gained over synthetic vectors, such as liposomes or nanoparticles (Table 1). By their very nature, synthetic vectors can be designed and loaded using various flexible strategies and produced in the large-scale quantity that is required for therapeutic application. In contrast, the biogenesis of EVs is a natural process, and while there are many strategies for incorporating cargo (pre-secretion cell engineering or post-purification vesicle loading), particularly harsh reaction conditions can adversely affect EVs or their parent cells.¹⁵ More pertinently, vast quantities of cells are needed to generate enough EVs for *in vitro* assays and *in vivo* animal models. Scaling up these quantities for clinical treatment in human patients poses a major challenge for the field.²⁷ Alongside these practical concerns, there are several important biological factors that must be considered when using EVs for *in vivo* drug delivery. For instance, does the mode of interaction between the EV and the cell correspond with the underlying mechanism of the delivered therapeutic? It has been suggested that EVs can interact with cells in several different ways: they may bind with receptors on the cell surface to induce signalling cascades, fuse with the cytoplasmic membrane to release intraluminal contents into the cytoplasm, be internalized *via* endocytosis, or remain docked on the surface of the cell.²⁸ While these mechanisms are poorly understood, what is clear is that the mode of interaction will affect the efficacy of the delivered therapeutic. Our comparatively greater knowledge of the interaction between synthetic vectors and cells has allowed us to design smart strategies to mediate cell binding, internalization and endosomal escape,²⁹ and it remains to be seen whether such approaches are applicable for EV-based drug delivery. It is also important to remember that EVs are responsible for a wide range of biological processes, which presents two potential concerns. It is possible that intercellular communication of endogenous EVs could be disrupted by the presence of large numbers of exogenous EVs. Moreover, an unknown or poorly-understood mechanism could lead to unwanted side effects, such as off-target signalling from proteins on the vesicle surface, or the co-delivery of species present in the lumen, such as oncogenes,³⁰ viral miRNAs,³¹ or prion particles.³² In addition, many widely-used protocols for purifying EVs fail to eliminate co-eluted particles or soluble factors,³³ which could also present biological side effects. It is imperative that robust purification protocols³⁴ and safety

profiling is applied to minimize these confounding factors, for both *in vitro* and *in vivo* studies. Yet even when using highly-purified populations of drug-loaded EVs, it can be a challenge to conclusively define the active substance, non-active components and mode of action; all key factors required for pharmacological classification.³⁵

Exploiting the Advantages of EVs for Drug Delivery

It is clear that the biological origin and complexity of EVs, which makes them such attractive therapeutic candidates, is also responsible for many of the challenges facing vesicle-mediated drug delivery. Caution should therefore be taken when designing an EV-based therapy, and it is essential that researchers ask the million-dollar question: *do EVs offer any specific benefits over synthetic vectors for delivering drug X to target Y?* When viewed in this context, the biological origin and complexity of EVs can present specific advantages at various stages of the drug delivery workflow (drug loading, *in vivo* stability, and targeting). For example, the biogenesis of EVs provides unique opportunities for the cellular production and endogenous loading of therapeutic factors. In this scenario, therapeutic drugs, oligonucleotides and nanoparticles can be delivered to a cell and subsequently re-packaged into secreted vesicles.^{12–14,24,25} Exploiting cells to fabricate, load and release drug-laden vesicles simplifies the loading process, provides a basis for site-specific cargo loading (*e.g.* the lumen or vesicle membrane), and may also allow higher uptake efficiency for species that are not easily loaded into pre-formed systems. *In vivo*, certain EVs are also thought to benefit from innate mechanisms that increase their physicochemical stability. Whereas liposomes typically elicit complement activation that triggers degradation and clearance, these processes are reduced in antigen-presenting cell derived EVs that express the membrane-bound complement regulators CD55 and CD59.³⁶ This characteristic could offer real benefits for drug-loaded EVs that require higher circulation times, or those that are exposed to harsh, inflammatory environments.

There have also been reports that EVs can bypass certain biological barriers to access challenging target sites for drug delivery. For instance, some biodistribution studies suggest that untargeted, systemically-administered EVs can accumulate at tumour sites.³⁷ One theory is that EVs may benefit from the enhanced permeability and retention (EPR) effect, in which nanoscale entities are purported to preferentially access leaky vasculature formed by expanding tumour tissue. However, it is highly contentious whether the EPR effect bears clinical relevance,³⁸ or whether EVs would benefit any more than synthetic vectors of the same dimensions. The small size of EVs do undoubtedly contribute to the observations made by Headland *et al.*, who reported that neutrophil-derived vesicles can penetrate deep into dense cartilage tissue.³⁹ Interestingly, macrophage-derived EVs were shown not to penetrate cartilage to the same extent. This discrepancy indicates a biological or biophysical influence, or as the authors speculate, a directed chemotactic response, which could potentially be harnessed in the EV-mediated delivery of osteoarthritic drugs. Another highly desirable tissue target for drug delivery is the brain, indeed, Zhuang *et al.*¹⁰ and Haney *et al.*²⁶ have elegantly demonstrated neuroprotective effects in murine models using EVs loaded with curcumin and catalase, respectively. These therapies used intranasal delivery, however, a more desirable administration route would involve the transversing of systemically-delivered EVs across the blood-brain barrier (BBB).⁴⁰ Recent reports have indicated that EVs may

have intrinsic mechanisms for bypassing the BBB,^{12,25,41} which if harnessed, could provide new opportunities for drug delivery to the brain. Finally, it has been reported that certain EVs can transfer contents to different cell types with different efficiency,⁴² and that the biodistribution of EVs is dependent upon the parent cell.³⁷ Understanding and harnessing the mechanisms underpinning these cell-selective interactions may allow the innate targeting of drug-loaded EVs to regions of therapeutic interest, such as tumours or wound sites.

Future Direction: Realising Tangible Benefits

The ultimate target of any drug delivery system is to realise real clinical application and tangible patient benefit. In order to achieve this goal, we need a robust understanding of how administered EVs will be transported *in vivo*, reach the intended tissue target and deliver their therapeutic cargo. The simplest therapies involve local administration of EVs to the target tissue, for instance, in intranasal delivery to brain tissue^{10,26} or direct injection into subcutaneous tumours.⁴³ Systemic administration, on the other hand, presents a complex interplay of fluid dynamics, biological barriers and immune clearance. For liposomes, we have a relatively comprehensive understanding of how subtle changes in size, charge and flexibility dictate *in vivo* circulation, barrier crossing, and margination in the bloodstream.⁴⁴ Applying these principles to EVs (naturally nanoscale, flexible and anionic), we expect to see poor margination, entrapment in the red blood cell core and clearance by the mononuclear phagocyte system. However, as discussed, the biochemical complexity of EVs present additional considerations beyond this liposome analogy.^{36,37,42} Ultimately, although there is strong preclinical evidence that systemically administered EVs are able to reach therapeutic tissue targets (*e.g.* the brain^{11,12} or cartilage tissue³⁹), it is imperative that we fully understand the *in vivo* transport mechanisms in order to develop clinical therapies that can more effectively evade clearance and target tissues or tumours. In doing so, care must be taken not to overinterpret results from preclinical models in the context of clinical translation. For example, the feature size of relevant biological barriers, such as the fenestrae in the liver sinusoidal wall, varies significantly between small animals and humans.⁴⁴ Similarly, while the transport of labelled EVs between contralateral mammary glands can be rightly considered as “long-distance” communication in a mouse model,⁴⁵ such results should not be readily used to infer transport properties in larger organisms, such as humans.

In conclusion, if we can overcome the challenges of EV-based drug delivery and develop a more comprehensive mechanistic framework of transport in human patients, then we will be much better placed to develop clinical therapies that can fully harness the specific benefits of EVs. Even then, it is important to remember that EVs are unlikely to provide a universal nanomedicine solution. The specific benefits of using EVs are dependent upon the precise details of the therapy: the chemical nature of the drug, the mode of delivery, the target tissue and the mechanism of action. These characteristics will heavily influence factors such as loading efficiency, cellular uptake, administration route and potential side effects. Thus, for each system, the advantages of using drug-loaded EVs must be carefully weighed against the limitations, and also against the pros and cons of competing systems. It is not in doubt that EVs are able to deliver drugs in an *in vivo* setting, but ultimately, for pharmaceutical companies and clinicians to fully embrace EV-mediated drug delivery, there must be

tangible added value. For instance, the “academic measures” of increased circulation or targeting are only likely to disrupt the *status quo* if they lead to real pharmaceutical benefits, such as decreased cost, higher therapeutic index or reduced number of treatment cycles. 46,47 Nevertheless, it is clear that emergence of EVs have opened up a host of exciting and unexplored opportunities for drug delivery research. To fully realise the potential of EV-mediated drug delivery, we believe that the field must look to intelligently designed strategies that actively exploit the biological characteristics of EVs. While far from exhaustive, the criteria discussed in this article (cargo loading, *in vivo* stability or site-specific targeting) highlight some of the most promising areas for achieving the competitive advantage over synthetic systems that is needed to establish EVs as clinically-relevant drug delivery vectors.

Funding Sources

JPKA was funded by Arthritis Research U.K. Foundation (21138). MMS acknowledges support by the grant from the UK Regenerative Medicine Platform “Acellular Approaches for Therapeutic Delivery” (MR/K026682/1), the “State of the Art Biomaterials Development and Characterization of the Cell-Biomaterial Interface” (MR/L012677/1) grant from the MRC, the ERC Seventh Framework Programme Consolidator grant “Naturale CG” (616417), the Wellcome Trust Senior Investigator Award (098411/Z/12/Z) and GlaxoSmithKline through the Imperial College London Engineered Medicines Laboratory Project.

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Figure 1. Specific advantages of EVs as drug delivery vectors.

(1) EV biogenesis can be hijacked for drug loading, which can be particularly suitable for loading biological therapeutics, such as proteins or oligonucleotides. (2) There has been some evidence that EVs possess enhanced *in vivo* stability and circulation compared to synthetic vectors, such as liposomes. (3) There have also been reports that EVs can bypass the blood-brain barrier, which offers opportunities for systemic delivery of drugs to the brain. (4) It has also been shown that certain EVs exhibit cell-dependent cargo delivery and that the biodistribution of systemically-delivered EVs can depend upon the parent cell. These innate processes provide a biological platform for targeted delivery of therapeutics to specific tissues or tumours.

Table 1
Comparison between extracellular vesicles and liposomes

	Extracellular Vesicles	Liposomes
Production Methods	<ul style="list-style-type: none"> Naturally and continuously secreted by all living cells into culture medium and body fluids.² Purification (<i>e.g.</i> centrifugation, filtration, size exclusion) is required to remove cells and soluble factors.⁴⁸ 	<ul style="list-style-type: none"> Usually formed by bulk mixing or thin film hydration.⁴⁹ Further processing steps (<i>e.g.</i> homogenization, sonication, extrusion, freeze-thaw cycles) are usually performed to reduce the size and lamellarity.⁴⁹
Size Range	<ul style="list-style-type: none"> Exosomes $\approx$ 30 - 100 nm.⁵⁰ Microvesicles $\approx$ 100 - 1000 nm.⁵⁰ Apoptotic bodies $\approx$ 500 - 2000 nm.⁵⁰ 	<ul style="list-style-type: none"> Small unilamellar vesicles $\approx$ 30 - 100 nm.⁵¹ Large unilamellar vesicles $\approx$ 100 - 500 nm.⁵¹ Giant unilamellar vesicles $\approx$ 1 - 200 μm.^{51,52}
Charge	<ul style="list-style-type: none"> Naturally anionic but can be surface modified.⁵⁰ 	<ul style="list-style-type: none"> Tunable - anionic, cationic or neutral.⁴⁹
Loading Mechanisms	<ul style="list-style-type: none"> Endogenous loading: drugs are introduced into cells and subsequently packaged and secreted in EVs.⁵⁰ Exogenous loading: actively loading the drug into purified EVs using sonication, electroporation, <i>etc.</i>...^{16,50} 	<ul style="list-style-type: none"> Passive loading: introducing the drug to the lipid mixture during liposome formation.⁵³ Remote loading: using pH or ion gradients to transport the drug across the liposome membrane.⁵³
Circulation and Clearance	<ul style="list-style-type: none"> Distribution half-life $\approx$ 1 - 20 mins.⁵⁴⁻⁵⁶ Elimination half-life $\approx$ 1 - 6 hr.⁵⁴⁻⁵⁶ Clearance is mediated by opsonization and the mononuclear phagocyte system, with the majority of EVs taken up into the liver and spleen.⁴⁴ EVs derived from antigen-presenting cells can reduce complement activation and inflammatory toxicity.³⁶ Circulation time can be significantly increased by coating the vesicle surface with poly(ethylene glycol) (PEG).⁵⁷ 	<ul style="list-style-type: none"> Distribution half-life without PEG $\approx$ 3 - 30 mins.⁵⁸⁻⁶¹ Elimination half-life without PEG $\approx$ 3 - 25 hr.⁵⁸⁻⁶¹ Circulation time and clearance mechanism depends on the liposome characteristics (size, charge, flexibility).⁴⁴ Small liposomes (< 80 nm) are cleared by the liver.⁴⁴ Large liposomes (> 250 nm) are cleared by the spleen.⁴⁴ Circulation time can be increased by designing liposome formulations with intermediate size (80 - 250 nm) and a neutral or poly(ethylene glycol)-coated surface.⁴⁴
Selected Clinical Examples	<p>There are currently no established clinical therapies employing EVs as drug delivery systems. However, examples of EV therapies tested clinically include:</p> <ul style="list-style-type: none"> EVs derived from pulsed dendritic cells used to promote immune cell response in cancer immunotherapy.⁶²⁻⁶⁴ EVs derived from mesenchymal stem cells used to reduce the symptoms of graft-versus-host disease.⁶⁵ EVs isolated from ascites fluid, in combination with granulocyte macrophage colony stimulating factor, 	<p>Several formulations already on the market,⁶⁷ including:</p> <ul style="list-style-type: none"> AmBisome[®], Amphotec[®], Abelcet[®] (Amphotericin B). Doxil[®], Myocet[®], Lipodox[®] (Doxorubicin). DaunoXome[®] (Daunorubicin). Visudyne[®] (Verteporphin). DepoDur[®] (Morphine sulfate).

	Extracellular Vesicles	Liposomes
	used to stimulate the activity of T-cells in cancer patients. ⁶⁶	<ul style="list-style-type: none">• DepoCyt® (Cytosine, arabinoside).• Diprivan® (Propofol).• Estrasorb (Estrogen).• Marqibo (Vincristine).