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Engineering and loading therapeutic extracellular vesicles for clinical translation: a data reporting frame for comparability

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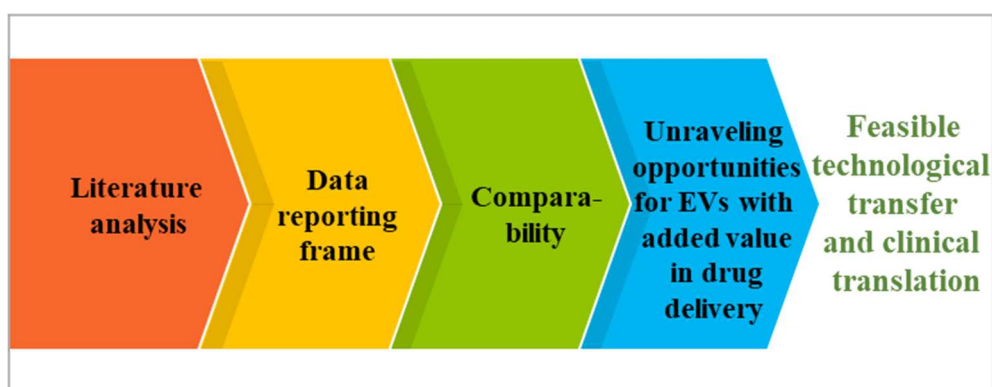
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Abstract

Extracellular vesicles (EVs) have emerged as new drug delivery systems as well as a regenerative cell-free effectors going beyond academic research to reach industrial research and development (R&D). Many proof-of-concept studies are now published describing the delivery of drugs, nanoparticles or biologics among which nucleic acids, proteins, viruses, etc. Their main interests rely on their intrinsic biocompatibility, targeting capabilities and biological activities. The possibility of loading EVs with exogenous therapeutic drug/nanoparticles or imaging tracers opens up the perspectives to extend EV therapeutic properties and enable EV tracking. Clinical translation is still hampered by the difficulty to produce and load EVs with large scale, efficient and cGMP methods. In this review, we critically discuss important notions related to EV engineering and the methods available with a particular focus on technologies fitted for clinical translation. Besides, we provide a tentative data reporting frame in order to support comparability and standardization in the field.

Key-words: extracellular vesicles, exosomes, engineering, loading, clinical translation.



Introduction

Extracellular vesicles (EVs), encompassing exosomes, microvesicles or apoptotic bodies, have emerged from academic research to enter in the pipeline of a growing number of new companies dedicated to EV bio-production, engineering and clinical translation [1]. This stems from the fact that EVs represent a paradigm shift for both the regenerative medicine and drug delivery fields. Concerning regenerative medicine, EVs offer a new alternative to cell therapy holding promises to mitigate limitations associated with the transplantation of viable cells, including the risks of uncontrolled cell replication, differentiation or vasculature occlusion [2]. Additionally, EVs benefit from leading advantages in terms of sterilization, storage and shelf-life compared to their cellular counterparts [3]. Regarding drug delivery, there is a large amount of proof-of-concept studies in the literature on EVs as carriers for drugs and biologics including nucleic acids, proteins, viruses, etc [4–6]. Indeed, EVs represent a biocompatible bio-inspired delivery system as this is their primary role in living organisms. EVs feature some advantages over already existing synthetic counterparts (such as liposome, micelles, inorganic nanoparticles, etc). For instance, EVs are expected to be less immunogenic than artificial delivery vehicles. Moreover, their phospholipid bilayer may be capable of fusing with recipient cell membrane, thus improving the uptake of the encapsulated molecule [7,8]. Additional advantages of EVs over synthetic drug delivery systems are their intrinsic biocompatibility, their natural stability in blood (systemic administration) or tissue (local/topical administration) and their intrinsic biological properties with potential inflammation/tumor targeting properties inherited from parental producer cells [9–13] (Figure 1).

Up to date, clinical translation of EVs for drug delivery is still limited. Although there are more than 20 clinical trials on EV for therapy registered at [clinical-trials.gov](https://clinicaltrials.gov), the majority of them concern native EVs for regenerative medicine. Just a few clinical trials relate to engineered EVs for drug delivery. This is the case of the NCT01294072 (sponsored by the University of Louisville, USA) to investigate plant exosomes to deliver curcumin to normal colon tissue as well as colon tumors. An additional example is the NCT03608631 (sponsored by the M.D. Anderson Cancer Center, USA) on mesenchymal stromal cells (MSC)-derived EVs engineered with a KrasG12D siRNA for the therapy of pancreatic cancer with KrasG12D mutation. The limited number of clinical trials on EVs for drug delivery stems from the difficulty to efficiently produce and load EVs with batch consistency and complying with good manufacture practice (GMP) requirements [1].

Setting-up efficient current GMP (cGMP) and scalable EV engineering strategies for high-yield and consistent drug loading remains a challenge. In this review, we critically discuss methods reported in the literature and important notions for engineering therapeutic EVs, with a particular focus on technologies prone to clinical translation. It is important to highlight that despite the many strategies currently reported in the literature, inter-study comparability to identify the best-performing approaches is hampered by the lack of information within the published studies. In this regard, we propose herein a tentative frame with key parameter entries that would be useful when comparing studies. The adoption of such a frame or equivalent may facilitate consistent quantification of drug-loading performances while allowing a more straightforward comparability across studies.

Opportunities for EVs in drug delivery

Envisioning EVs as a drug delivery system for industrial transfer and clinical translation requires to surpass the current drug delivery agents, mainly synthetic vectors like

nanoparticles, liposomes, dendrimers, etc. Compared to these synthetic vectors, EVs suffer from lower loading capabilities and more difficult batch consistency [1]. Besides, higher process complexity has a direct impact on regulatory issues and also in the cost per dose. The main interest of EVs compared to synthetic vectors, which is expected to overcome their drawbacks, is their intrinsic targeting [14] and therapeutic properties [15] as biotherapies (**Figure 1**). Therefore, some of the most competitive use of EVs as drug delivery vectors are probably related to the pro-regenerative and targeting properties of MSC-EVs to deliver drugs to enhance or enlarge their own therapeutic action. EVs from dendritic cell to deliver immune modulating proteins or molecules in oncology also seem a valuable strategy. Alternatively, the delivery of membrane proteins, in particular if directly produced by the parental cell, and the delivery of drugs via low-cost and high-performant strategies based on EVs from widely available cells (*e.g.* red blood cells) may also be promising. As a general rule, particular attention should be paid to the choice of the loading technologies to ensure that it is well adapted to the cargo to enhance feasibility, performance and translational relevance. This will be the focus of the next section.

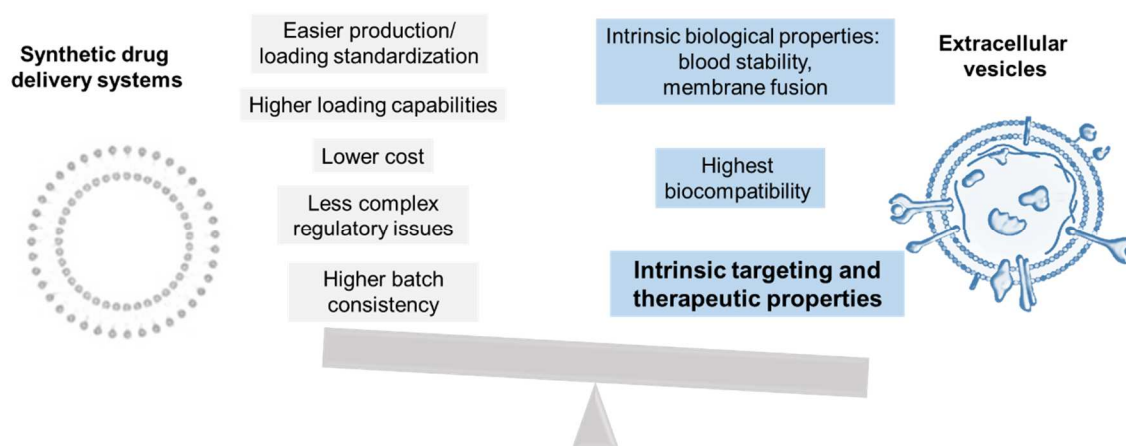


Figure 1: Advantages of synthetic drug delivery systems and extracellular vesicles considering intrinsic properties, manufacturing and regulatory aspects.

Searching for straightforward, high performant, scalable and/or cGMP EV engineering technologies

When considering EVs as a drug delivery system, EVs can be simplified as a hydrophilic core (lumen), encapsulated in a protein rich lipo/amphiphilic layer (membrane). Therefore, EVs can encapsulate both hydrophilic, amphiphilic and lipophilic molecules. In order to do so, three different strategies may be used: pre-production, per-production and post-production loading. These approaches will be further detailed in this section, indicating their appropriateness as a function of the physico-chemical features of the cargo. Of note, we focus on strategies that we consider straightforward, high performant, scalable and/or compatible with cGMP requirements for a more translational relevance. When applicable, considerations based on well-established metrics in the drug delivery field were highlighted. In particular, performance parameters such as the Encapsulation Efficiency (EE) and Loading Capacity (LC) were commented. We detail these parameters and their relevance in **Box 1**.

Box 1: Metric parameters from (or adapted from) the drug delivery field that are of interest comparing the performance EV engineering strategies.

Encapsulation Efficiency (EE)

$$\text{Encapsulation efficiency} = \frac{\text{Amount of the cargo in EVs}}{\text{Amount of the cargo used (total input)}} \times 100 \quad \text{Eq.1}$$

EE is defined as the percentage of drug that is encapsulated in the vector at the end of the process. As this parameter provides information about the amount of drug that is not encapsulated during the process (drug loss), it is an important economic indicator of loading performance. For instance, typical liposomal formulation has an EE of 5-15% with hydrophilic drugs [16] and 70-80% with lipophilic ones. Of note, this parameter may be

particularly critical for very costly cargoes (biologics), but much less relevant for easy to source drugs (small molecules).

Loading Capacity (LC)

$$\text{Loading capacity} = \frac{\text{Amount of the cargo in EVs}}{\text{Given amount of EVs (and producer cells)}} \quad \text{Eq.2}$$

LC is commonly the weight of drug loaded per unit weight of the vector. Typical liposomal formulation has a LC of 0.5-10 wt/wt % with hydrophilic drugs and about 2-30 wt/wt % [17] with lipophilic ones. The exact calculation of LC is difficult for EVs because (i) they cannot be simply dried like synthetic vectors; (ii) the presence of salt in the conservation media of EVs would mislead this calculation; (iii) the estimation of EV weight from their size distribution would not be a valuable strategy due to the fact that the high variability in size distributions leads to a high variability in volume/weight estimation (proportional to r^3). Considering technical difficulties, this parameter can be adapted taking into account the amount of cargo in EVs for a given number of EVs. This proxy remains limited because the exact number of EVs in a sample is difficult to estimate accurately due to analytical limitations (lack of specificity by nanoparticle tracking analysis (NTA) and lack of sensibility for small EVs by NTA and particularly by flow cytometry). While considering drug loading per EV amount, it would be also of interest to integrate the information of the number of EV per producing cell, in order to estimate the drug loaded per EV producer cell ratio. This would represent an interesting factor for industrial translation. Indeed, EVs being costly to produce, it is of a particular interest to limit the amount of EVs and producer cells needed to deliver a therapeutic dose for a patient in order to limit the cost of the treatment.

Pre-production loading methods

Loading parental cells with the cargo of interest before EV production is one of the strategies that can be envisioned for engineering EVs. Indeed, many compounds that are able to cross the cell membrane (*i.e.* lipophilic and small amphiphilic drugs) may be released by the cells via EVs in a *spontaneous cell entry / EV exit basis* [14,18,19]. Alternatively, molecules overexpressed by engineered cells may also exit the cells via EVs in an *induced cell expression / spontaneous EV exit basis*. EE for this approach is relatively limited for the former strategy, but it may be increased with an efficient EV production method in a small volume of media. For both strategies, LC may be boosted, especially if the molecule of interest is also present at high concentration before and during the EV production and if the cargo can have a tropism for enriched exit via EVs.

The main interest of the spontaneous cell entry / EV exit strategy is the fact that it is straightforward. In particular, it can be of interest for cargoes that are spontaneously directed to endosomal compartments. This is the case of theranostic iron oxide nanoparticles and carbon nanotubes, as demonstrated by our group [20–22]. Our team published proof-of-concept studies showing that parental cells incubated with iron oxide nanoparticles or carbon nanotubes produced EVs loaded with these nanoparticle cargoes in their lumen. It was also evidenced that EVs loaded with nanoparticles were able to deliver the encapsulated theranostic cargo to recipient cells in both *in vitro* and *in vivo* experiments [18]. It was also possible to co-load these EVs with drugs like doxorubicin (lipophilic), t-PA (hydrophilic), TPCS2a (an amphiphilic photosensitizer) [19] or mTHPC (a rather lipophilic photosensitizer) [18]. EE and LC were not calculated in these studies. However, a poor loading performance can be deduced from the reported concentration of iron oxide nanoparticles loaded in EVs (in a volume basis) that was estimated to be 1.5% v/v [23]. Altogether, the method of spontaneous entry / exit is relatively interesting due to the straightforward implementation. However, EE and LC are usually relatively limited and highly dependent on the chosen cargo.

Such a spontaneous strategy may be of interest for loading low cost, relatively lipophilic molecules or nanoparticles.

Concerning the induced cell expression / spontaneous EV exit strategy, many teams published proof-of-concept studies indicating that the overexpression of proteins, mRNAs or miRNAs [24–26] was a valuable approach for producing EVs featuring them. For this strategy, EE is not a relevant parameter considering that there is not an incubation step with a cargo input (the cargo is generated by the producer cell itself). LC can be considered a useful parameter. However, the LC is expected to be low. For instance, the physiological miRNA loading rate in EVs was described to be about 1 miRNA copy per 100 EVs (0.00825 ± 0.02 miRNA molecules/EVs for abundant ones [27]). In a related approach, Maguire et al [28] reported the isolation of natural adeno associated virus (AAV) loaded EVs, called vexosomes. AAV encapsulation into EVs enabled them to escape antibody mediated neutralization and provided a near 10 fold improvement in their ability to transfect cells compared to classical AAVs [29–31]. These vexosomes most probably comprise EVs encapsulating an AAV vector but also aggregates of EV and AAV [28]. In addition, EV encapsulation significantly reduces the AAV titer in a production compared to non-encapsulated AAVs.

An interesting approach for the induced cell expression / spontaneous EV exit strategy involves the modification of the cargo of interest with a biomolecule enriched in EVs (membrane protein, RNA sequence, protein cargo). This may be performed by modifying the protein sequence of interest to add a membrane targeting sequence (*e.g.* myristoylation domain, PIP3 binding, prenylation domain) [32] or by creating synthetic fusion proteins with natural EV targeted proteins such as lamp2b, PDGFR [24], C1-C2 domains of lactadherin [33], PTGFRN, BASP-1 [34] or tetraspanins. This method was used to produce EVs with neuronal targeting by fusing the lamp2b exosomal protein to the neuron-targeting RVG peptide [35]. The fusion construct approach is used by Codiak bioscience (USA) to express a

therapeutic protein on the surface or in the lumen of EVs by fusing it to PTGFRN or BASP-1, respectively [34]. In a related approach, Cilloa company (France) has described a polypeptide sequence enriched in EVs and uses it to create fusion constructs with proteins of interest [36]. The chimeric DNA is introduced in producer cells that will express the fusion construct and enrich it in the released EVs. As far as we know, LC have not been reported. However, LC is expected to be higher than for the previous approaches. It is important to consider that fusing a peptide to an already expressed EV membrane protein could impact the functions of both the membrane protein and the fused peptide and jeopardize their targeting properties. For example, it was demonstrated that peptides displayed on the exosome surface can be degraded during exosome biogenesis by endosomal proteases. Peptides fused on the N terminus of lamp2b are vulnerable to acid-dependent proteolysis due to their localization in the lumen of endosomes. Interestingly, a strategy of glycosylation could protect the peptide from degradation, enhance Lamp2b fusion expression in cells and released EVs, and augment targeted delivery of exosomes to neuroblastoma cells [37].

As a general remark, it is important to highlight that the cargo of interest may have an impact on the parental cell, in a positive (*i.e.* increase EV production [38]) or negative way (limit EV production, denature its composition, etc). As the cargo itself may change the physiology of producer cells, it may impact as well the constitution of released EVs in terms of the other present biomolecules.

Per-production EV (or EV-like vesicle) loading

EVs can be loaded during their production, encapsulating the cargo during their formation. This approach is usually coupled to EV production using physical methods that implies plasma membrane “destruction-reformation”. For instance, Toledano *et al.* report drug loading of EV-like vesicles (nanoghost) produced from MSC membranes [39]. For that, cells

were emptied from their cytosolic content following a hypotonic shock (although the exact mechanism and its efficiency is not clear) and sonicated to fractionate their membrane in small vesicles. Vesicles were then extruded by 0.4 μm polycarbonate membranes in presence of the cargo (tumor-necrosis-factor related apoptosis inducing ligand, TRAIL) to obtain TRAIL-loaded nano-sized vesicles [39]. A similar method was reported to produce EVs from platelets by sonication in presence of polymeric nanoparticles as a cargo [40]. Serial extrusion through filters with decreasing pore size of macrophages in the presence of different chemotherapeutics like doxorubicin and carboplatin (without cytosol removal) was also reported to allow the production of loaded EVs [41]. Overall, disruption/reformation techniques applied to cells in the presence of the cargo to form cargo-loaded EVs may be cGMP and scalable method. It may lead to interesting EE and LC for lipophilic compounds. However, membrane disruption during the process may induce a potential loss of membrane asymmetry and inner physiological cargo of the EVs (miRNA, soluble proteins), impairing the intrinsic protective, targeting and delivery properties of native EVs.

Post-production loading methods

Compounds with medium hydrophilic / lipophilic properties and amphiphilic compounds are interesting for their ability to spontaneously integrate EV membrane by simple incubation [42]. However, the same properties also give them the ability to exit the EV membrane to get back in the surrounding media (hydrophilic solvent). Therefore, compounds with medium hydrophilic / lipophilic properties are expected not to be stably loaded in EVs. In these conditions, the processes of cargo entry in / exit from EVs may reach a dynamic equilibrium with a relatively good EE and LC in particular settings, *e.g.* with very concentrated EVs in a small amount of hydrophilic solvent (where much of the drug would be in the EVs). However, there may be a sudden shift in this dynamic equilibrium towards the exit process with drug redistribution upon high volume dilution. The relative proportion in membrane and solvent

depending on the octanol-water partition coefficient (K_{ow}) of the drug of interest. Considerations on the post-production loading of cargoes featuring lipophilic or amphiphilic properties are outlined in **Box 2** while hydrophilic cargoes are highlighted in **Box 3**.

Box 2: Focus on particularities of lipophilic cargoes.

Lipophilic and amphiphilic compounds

Lipophilic compounds are not very difficult to load, which means that the simple incubation method may enable a moderate EE. For instance, Gomari et al. [43] reported an EE of 13% for doxorubicin. In theory, the incorporated lipid compound may represent a large fraction of the EV membrane. In case it represents 50% of the membrane mass, it would lead to an approximate maximum of 10% LC. For instance, Gong et al reported that their experiments enabled to reach up to 16% mg of doxorubicin per mg of protein [42]. However, such a loading may have an impact on EV membrane properties considering the membrane “dilution” effect. Incorporation of lipophilic compounds in EVs or amphiphilic ones may be biased by their ability to form lipid aggregates or micelles, respectively. These 1-300 nm structures [44] are relatively stable in an hydrophilic environment, which limits their ability to fuse with the EV membrane, although micelles have a tendency to coalesce with EVs [45]. Coalescence may not be total and may lead to some “contamination” of the EV product by the remaining product micelles. Micelles are usually quite difficult to separate from EVs [26,45], even if their size range is smaller than EVs.

Box 3: Focus on particularities of hydrophilic cargoes of medium and high molecular weight.

Hydrophilic compounds of medium and high molecular weight are probably the most difficultly loaded molecules due to their relative inability to cross the intact EV membrane.

EV loading methods usually involve membrane disruption (slicing, extrusion, sonication, etc [46]) or permeabilization (saponin, electroporation, etc), and may lead to the loss of some EV properties. More importantly, the maximal theoretical LC is usually very limited, mostly due to the compound solubility. As an example, 5-Fluorouracil is a hydrophilic molecule that has a maximal solubility of about 1g/L in water, with a therapeutic dose of about 0.8g/day. In these conditions, if no crystallization takes place inside EVs, a performant loading method would only load about 4.2×10^{-19} g/EV (for ideal 100 nm EVs), *i.e.* a 0.08% LC. This means that about 1.9×10^{18} EVs would be needed to deliver a therapeutic dose daily. Reaching a moderate EE for hydrophilic drugs of medium and high molecular weight is also a particular challenge. Even a performant loading method would probably only lead to a maximal theoretical 1-5% EE. Indeed, the maximal EE if loading is only based on a passive transport into EVs in this system is limited by the maximal percentage of the volume represented by intra-vesicular fraction of EVs, *i.e.* occupying 1% of the volume with a suspension of 100 nm EVs requires a concentration of about 2×10^{13} EVs/ml, 5% requiring a concentration of 10^{14} /ml. However, working at highly concentrated EV suspensions [17] may lead to purification difficulties and EV aggregation during the process. Keeping these theoretical calculations in mind, moderate loading hydrophilic drugs of medium and high molecular weight may be achieved if the compound is highly soluble, particularly potent, or combined with a method to achieve a substantial cargo content increase in EVs *e.g.* via drug crystallization in the EV lumen (pH gradient method used in liposomes [47]), drug solubility increase [48], cargo (in case of nanoparticles) coating on the EV membrane [49]). Furthermore, the high cost of EV production should favor optimization of LC instead of EE.

Examples of spontaneous post-production loading with lipophilic curcumin [50], or doxorubicin [41] have been reported. Although not useful for clinical applications, examples of EV loading with lipophilic dyes such as PKH67 and PKH26 or 1,1-dioctadecyl-3,3,3,3-

tetramethylindotricarbocyanine iodide (DiR) confirms that hydrophobic interactions successfully allow loading [51,52]. However, EV staining/loading with lipophilic molecules should be performed avoiding dye/drug excess to avoid contamination with micelles or lipid aggregates (**Box 2**). Whatever the compounds used, loading is most often biased by the presence of contaminants that may even be made of the compound itself (micelles of lipophilic compounds [26]). EV preparations are usually not perfectly pure and are co-purified with protein aggregates [53], most of the time coming from the cell culture media (human serum albumin, platelet lysate, etc). Most compounds are known to be able to bind to proteins in a non-specific way [54], and probably also with protein co-purified with EVs.

Consequently, drug encapsulation quantification in EVs has to be made with caution to avoid biases by the non-encapsulated drug. Purification, *e.g.* with density gradients, is a major step to remove non-encapsulated drug, protein-bound drug, and drug aggregate [55]. The notion of dynamic equilibrium has also to be taken in consideration when preparing samples for analysis, if sample preparation and purification is not fast enough (the time scale highly depends on drug K_{ow}) or made in a too large volume of solvent, it may lead to drug exit from the EVs.

In addition to this spontaneous strategy of post-production loading, EVs may be also engineered by inducing membrane destabilization. Destruction-reformation or permeabilization with saponin at room temperature, freeze-thaw cycles or sonication and extrusion [56] were benchmarked compared to simple incubation to encapsulate catalase (high molecular weight macromolecule). Sonication and extrusion methods are reported to be the most efficient approaches. However, the highest EE reported is probably limited to near 3-4%, including potential precipitation (no controls are reported to avoid this bias). A study reports the use of high-energy sonication to load MSC-derived nanoghosts with pDNA [57]. Of note, high energy applied may lead to heating (protein denaturation) and drug

denaturation. For instance, to avoid naked pDNA fragmentation, polyethylenimine was used compressing the DNA and protecting it from degradation [57].

Box 4: Focus on nucleic acids

Nucleic acid loading is of a high interest for both academic and industrial sector, with specific constraints compared to small molecules. Most nucleic acids of interest have no intrinsic ability to cross membranes, have a high molecular weight and are relatively large compared to classical small molecules. They may therefore rather be considered as nanomaterials, siRNA being for example a hydrophilic, 13 kDa, 7,5x2x2 nm object. Therefore, one must keep in mind that entry of these molecules inside a vesicle is not at all a thermodynamically favored process, and that simple aggregation with the vesicle or with surrounding proteins is a much more likely to happen process. Another critical aspect is that the nucleic acids should not be degraded by the loading method in order to remain functional.

Chemical-based transfection via commercial and commonly used transfection agents like lipofectamine (that contain positively charged lipids) may be used to load siRNA in EVs with limited efficacy [58]. Attention should be paid to the risk of lipofectamine micelle co-purification with EVs as a contaminant.

Electroporation is one of the most efficient, scalable and GMP-compatible methods available. Electroporation is based on applying an electrical field to cells, bacteria or vesicles in the presence of the cargo of interest. The electrical pulse creates pores into the lipid bilayer membrane to elicit EV loading. Electroporation is considered as an efficient method to load large macromolecules like siRNA into the lumen of the exosomes [35,59]. In a previous report, electroporation was used to load siRNAs into EVs to knockdown a therapeutic target in Alzheimer's disease [60]. However, Kooijmans *et al.* reported that this result was probably an artefact [61]. In fact, the process of electroporation induced metal ions release from the

metal electrodes. These ions were able to induce the formation of siRNA aggregates that were co-purified with EVs. Kooijmans *et al.* indicated that in the presence of a chelator (EDTA), metal ions no more induced siRNA aggregation and co-purification with EVs, drastically dropping EE from near 20% to <0.05%. This very detailed study has shed light on a bias that is still not taken into consideration by most studies published up to date. Altogether, although electroporation is a pretty straightforward, scalable and cGMP method, careful characterization of the real EE must be performed. Beside electroporation, other active methods using sonication or heat shock have been proposed, but should be used with caution in order to preserve nucleic acid as well as EV functionalities [62,63].

Another method inspired from the liposome field is to generate a pH gradient across EV membranes in order to enhance EV loading of negatively charged cargo such as miRNA, siRNA, and single-stranded DNA [64]. The interior of HEK293T-derived EVs was rendered acidic (pH 2.5) by first dehydrating the EVs in 70% ethanol, then rehydrating them in an acidic citrate buffer and dialyzed them with a neutral buffer. This method enabled reuse of unloaded nucleic acid cargo in subsequent loading operations, resulting in an increase of RNA loading rate from about 1% to 6,5%. Importantly the protein per EV level decreased following EV acidification and dialysis suggesting modification of protein content of some EV subsets or contaminants. Nevertheless the cell uptake and functional delivery of pro- and anti-inflammatory miRNA by the modified EVs were demonstrated.

Another approach to load EVs is to induce EV fusion with drug-loaded liposomes or nanocarriers potentially modified on their surface to harbor PEGylated lipids or other membrane compounds. Such strategy is valuable to tune the composition of the membrane and of the lumen of the resulting hybrid drug vector. To do so, Sato *et al.* [65] used several freeze-thaw cycles and our group proposed the use of a PEG-mediated fusion [55]. For

instance, we reported an EE of 90% for the hydrophobic drug mTHPC after the fusion of EVs with mTHPC liposomes. A Swiss based start-up company (Anjarium bioscience) proposed the use of pH sensitive cationic lipids liposomes (that are cationic in acidic environment and neutral at neutral pH) to be fused with EVs in acidic environment by electrostatic interaction. On a later step, the lipids shifted to a neutral charge (*i.e.* not toxic) by changing the pH to neutral one. Another team proposed a similar method to load cas9 protein in EVs [66]. The technique relies on the use of undisclosed positively charged nanoparticle aggregated with the cas9 protein, and subsequently incubated with naturally negatively charged EVs.

These methods are relatively complex, needing in particular an efficient production and loading of liposomes. In a related approach, micelles (*i.e.* without luminal hydrophilic cargo) composed of lipids and amphiphilic molecules have been reported to be incorporated into EV membranes by co-incubation. A proof-of-concept study has shown prolonged half-life when using PEGylated lipids [45]. This method is relatively straightforward and simple, *i.e.* possible to transfer in cGMP, but only allows modification of the EV membrane and not of its luminal cargo.

Click chemistry was used to graft molecules on the EV surface in proof-of-concept studies. Although the chemical functionalization may change or disrupt the EV biological property, this method allows the covalent binding of a potentially large variety of molecules [67,68]. Briefly, an alkyne group is attached to the EV membrane proteins, it is then linked to an azide-functionalized compound of interest in presence of copper. The complexity of the method, with a moderate grafting efficiency, may limit its implementation at the industrial scale.

A schematic overview of pre-production, per-production and post-production loading strategies is provided in **Figure 2**. It also emphasizes the preferential location of hydrophilic

compounds in the lumen of EVs, amphiphilic compounds on EV membrane as well as the location of lipid compounds on the membrane or the lumen of EVs (particular case of lipid aggregates [69]).

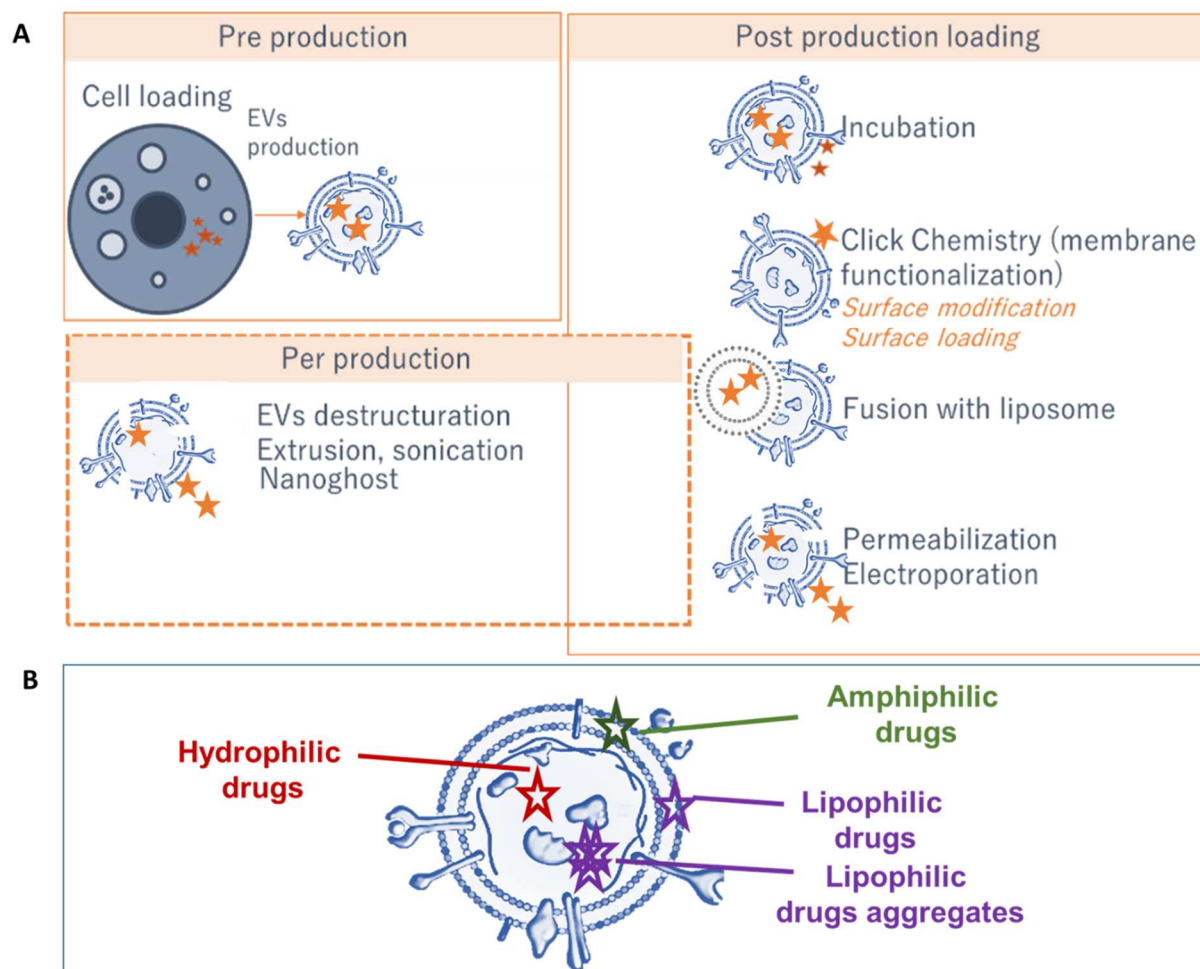


Figure 2: Schematic representation of EV engineering strategies based on pre-production, per-production or post-production loading (A). Schematic representation of the EV localization of hydrophilic, lipophilic and amphiphilic cargoes (B).

GMP compatibility and scalability

A method of choice has to, as much as possible, use techniques and material/machines that have already been approved and used in the industry. Indeed, each material used during the process has to be qualified, quality controlled, and considered as safe for human health (or

demonstrated not to be in the final product). It is also the case for machines that have to be qualified and controlled following the FDA Code of Federal Regulations Title 21 [70] in the US and are usually dedicated to the clean room use, therefore limiting the possibility to use very costly/unique machines. Most often, methods are developed in R&D labs before being transferred with the same material in clean room settings. Increasing the method complexity is highly related to an increased number of controls, time of clean room occupation, technician time, all of them being important drivers of total cost. Scalability is also a key aspect that can sometimes counter-balance the relative complexity of a process. It has to be kept in mind that a small scale production and loading of EVs performed for a proof of concept clinical study will be much easier to scale up for future use if performed with an already “scale-up ready” method, instead of developing a whole new method, demonstrate its equivalence and re-transfer it in GMP settings. We recently published a review on the GMP mass production of EVs with a focus on these aspects [71].

A tentative data reporting frame for comparability in the development of performant strategies

Different EV engineering strategies were commented in the previous section with the attempt to highlight their advantages, disadvantages and performance, focusing on the most appropriate cargoes for each approach. Concerning performance assessment, metrics based on EE and LC were discussed when data were available. However, this information is often missing in the literature, which hampers the systematic assessment and comparability investigation in order to point out performant strategies for EV loading. Considering this, we suggest herein a tentative frame for EV loading studies. Besides including EE and LC metrics widely adopted in the drug delivery field, this frame comprises entries related to the EV

production process itself as well as the characterization methods, which are of interest in order to provide a more complete view of the EV production and loading processes as a whole.

As indicated above, the main opportunities of EV for drug delivery are related to their own potency and intrinsic capabilities to improve the drug biodistribution, cell targeting as well as cargo internalization and delivery in addition to the modulation of the microenvironment that EVs can foster. For instance, our group [14] reported that tumoral selectivity of mTHPC photosensitizer drastically increased when delivered in MSC EVs in comparison to the free drug and to the liposomal formulation Foslip (mean ratio of the drug in tumors/organs of 40 for EVs-mTHPC versus 1.5 for the free drug and 5.5 for the liposomes-mTHPC). The apoptosis level was 5-fold higher in the laser-exposed group treated with EVs-mTHPC in comparison to the Foslip and free drug at equivalent dose. Besides, in comparison to the other groups, EVs-mTHPC promoted pro-inflammatory immune environment with inflammatory macrophages and cytotoxic T cell infiltration that is mostly promoted by mTHPC vectorization and photodynamic therapy with MSC-derived EVs [14]. Therefore, we consider that the metrics for comparability should also integrate parameters related to EV capabilities (for instance target specificity) and potency in comparison to the free drug and, if relevant/possible, a synthetic delivery system. These entries were also introduced in Table 1. The adoption of such a tentative frame for data reporting would be valuable to standardize the information provided, facilitate comparability and access the added-value of EVs for drug delivery. It would also provide a framework to compare genuine EVs (with pre- or post-production loading preserving EV integrity) to EV-like vesicles loaded by a destruction-reformation method or synthesized to mimic some EV features. The production of EV-like vesicles might be more easily scalable and the regulatory quality requirements simpler than for genuine EVs if the starting components are well defined. However, for vesicles formed by extrusion of cell membranes, the complexity/heterogeneity of the final product might be

equivalent to the native EVs and thus difficult to control. So far, there is no direct comparison of targeting and therapeutic activities of EV-like vesicles and genuine EVs from the same cell source. Native EVs are expected to recapitulate intrinsic biological activities (immunomodulation, fusion capability, enhanced targeting and delivery...) that might not be conserved in EV-like vesicles, although drug loading could be higher in the latter. Hence this reinforces the need to benchmark both extrinsic and intrinsic capabilities of EVs and EV-like vesicles as well as their clinical translation potential.

Table 1: Tentative frame for data reporting including EV production, loading, isolation, counting, characterization as well as metrics for cargo loading and EV intrinsic properties.

Strategy Parameter	Pre production	Per production	Post production
EV Production	Cell source (cell type, cell line or primary cell, ...)		
	Number of producing cells		
			Number of EVs as input
	Production protocol (starvation, chemical or physical stimulation, ...)		
	Medium used		
EV Loading	Physico-chemical properties of the cargo		
	Initial amount of the cargo (mol for drugs) // concentration & volume		
	Protocol		
	Medium used		
	Number of EVs		
EV isolation	Protocol for EV isolation after loading (ultracentrifugation, tangential flow filtration, ...)		Protocol for EV isolation before loading and after loading (ultracentrifugation, tangential flow filtration, ..)
EV counting and characterization (size and markers)	Protocol and data for EV counting and characterization after loading (nanoparticles tracking analysis, Zetaview, ..)		Protocol and data for EV counting and characterization before loading and after loading (nanoparticles tracking analysis, Zetaview, ..)
	Number of EVs /mL and total EV amount		
EV loading metrics	Encapsulation Efficiency (EE)		
	Loading Capacity (LC)		

EV intrinsic capabilities metrics	Test used (target cell internalisation, biodistribution, potency, ...)
	Response ratio EV/free cargo (Response ratio EV/synthetic vector if possible/relevant)

Concluding remarks and perspectives

This paper provides a critical overview of the strength and pitfalls of EVs use in drug delivery. It focuses on the choice of EV engineering technologies to render feasible industrial transfer and clinical translation. In brief, a method of choice should rely on strategies and equipment already approved and used in the pharmaceutical industry. Indeed, each material used during the process has to be qualified, quality controlled, and considered safe for human health (or demonstrated to be eliminated in process to comply with upper limits in the final product). Increasing the method complexity is highly related to an increased number of controls, time of clean room occupation, technician time, all of them being important drivers of total cost. Scalability is also a key aspect that can sometimes counter-balance the relative complexity of a process. It has to be kept in mind that a small scale production and loading of EVs performed for a proof-of-concept clinical study will be easier to scale up for future use if performed with an already “scale-up ready” method, instead of developing a whole new method, demonstrate its equivalence and re-transfer it in GMP settings. The feasibility of the industrial transfer and clinical translation of an EV engineering technology will highly depend on its loading capabilities, which in turn will depend on the cargo properties and the choice of the most appropriate loading strategy adapted to it.

A large palette of potential methods may be appropriate for each cargo. However, decision on the best choice is obscured by the lack of relevant data on currently available methods reported in the literature and the adoption of a universal metrics. In view of this fact, a tentative frame for data reporting is proposed in this paper. Besides including well-established metrics from the drug delivery field, it comprises additional information entries to allow global view of the EV production and loading processes as a whole, as well as the intrinsic capabilities of EV as vectors. We foresee that the adoption of such a tentative frame for data reporting would support comparability and standardization

in the field while facilitating decision choice for the development of high-performant manufacturing process more amenable to industrial transfer and clinical translation.

Declaration of interest

Florence Gazeau, Amanda K. A. Silva, Max Piffoux and Claire Wilhelm are co-founders of the spin-off Evora Biosciences. Amanda K. A. Silva, Jeanne Volatron, Max Piffoux and Claire Wilhelm are co-founders of the spin-off EverZom.

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