Systematic review of targeted extracellular vesicles for drug delivery

Considerations on methodological and biological heterogeneity

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Targeted extracellular vesicles for drug delivery – considerations on methodological and biological heterogeneity

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ABSTRACT

The idea of using extracellular vesicles (EVs) for targeted drug delivery was first introduced in 2011 and has since then gained increasing attention as promising new candidates in the field. Targeting EVs to areas of disease can be achieved through a complex process of designing and inserting a targeting ligand to the surface of the EVs. Although this can be obtained via chemical conjugation, the most important strategy has been to transfect or modulate the EV-producing cell to endow the EVs with the desired targeting capabilities. However, since EVs are harvested from biological sources, their composition is highly heterogeneous, which makes it difficult to control the purity and quality of the resulting EV-based drug delivery vehicles. In this review, we present a detailed account of EVs in targeted drug delivery based on a systematic literature search. We discuss the potential advantages of EVs compared to synthetic lipid-based nanocarriers, and the methodological and biological limitations associated with their use as targeted drug delivery vehicles.

KEYWORDS

Extracellular vesicles; exosomes; microvesicles; drug delivery; liposomes; nanoparticles; pharmacokinetics; kinetics; treatment

1. INTRODUCTION

Extracellular vesicles (EVs) are a highly heterogeneous class of vesicles that are secreted from all types of cells considered as a mean of intercellular communication [1,2]. The discovery of EVs can be tracked to the beginning of the 1980s where Trams and colleagues published a paper [3] on enzymatic activity in secreted 'microvesicles', but it was not until 2007 that EVs received substantial attention with the discovery of small RNA content in EVs and exchange of these biomolecules between cells [4]. Following this, the biology of EVs has been implemented in virtually every aspect of cell biology and pathology, which also includes the use of EVs for drug delivery.

EVs display size and compositional heterogeneity dependent on their subcellular origin. Current classification separates EVs into four distinct classes; exosomes, microvesicles, apoptotic bodies, and large oncosomes [2]. Exosomes are generated in endosomal structures called multivesicular bodies (MVB) and are the smallest with a size range between 30 – 120 nm. Microvesicles (also called shedding microvesicles or ectosomes) are generated by outward budding of the plasma membrane and span widely in size from 200 nm to several microns [5]. Apoptotic bodies are generated by vesiculation of an entire cell undergoing apoptosis and range between 1000 – 5000 nm in size, whereas large oncosomes are between 1 – 10 µm in size [6,7]. Large oncosomes are newly discovered entities that have been observed in cancer cells and are defined by their size and lack of nuclear DNA (since they can be mistaken for cells). Apoptotic bodies and large oncosomes will not be addressed in this review due to their physicochemical characteristics making them not suitable as drug delivery vehicles [8]. The term "EVs" will be used throughout this review to highlight the smaller subset of extracellular vesicles that have a size relevant for drug delivery purposes and can be isolated with traditional exosome isolation protocols. The purpose of this review is to discuss different aspects related to EVs used for targeted drug delivery. Other therapeutic strategies utilizing EVs, such as EV-based vaccines, or the generation of EV-mimetic or bio-inspired nanotherapeutics will not be adressed here [9–13].

EV biogenesis

EV biogenesis differ according to the subset; microvesicles or exosomes. The biogenesis of microvesicles is not well understood, but some mechanisms were identified to be involved in their shedding from the plasma membrane [5]. The shedding was found to be dependent on translocation of phosphatidylserines (carried out by aminophospholipid translocases) from the inner leaflet of the plasma membrane to the exterior leaflet, followed by a contraction of the resident actin cytoskeleton to release the microvesicles [14]. A few distinct proteins have been identified in relation to this process, including ADP-Ribosylation

factor 6 (ARF6), arrestin domain-containing protein 1 (ARRDC1), and tumor susceptibility gene 101 (TSG101) (Figure 1, step 1) [15–17].

Several pathways are involved in the biogenesis of exosomes. The exosome biogenesis can, in general, be divided into four steps; a) endocytosis and formation of an early endosome, b) formation of intraluminal vesicles by inward budding into the endosomes, c) formation of a MVB or late endosome, and d) the release of exosomes from the cell (Figure 1, step 2A-D). The most studied step in this cycle is the generation of MVBs where several protein complexes have been identified as essential, including the endosomal sorting complex required for transport (ESCRT) 0 – III, ceramide and sphingosine-1-phosphate (S1P), small integral membrane protein of the lysosome/late endosome (SIMPLE), and syndecan-syntenin-alix [15,16,18–21]. The secretion of EVs to the extracellular milieu has, so far, mainly been attributed to Rab-GTPases [22–24].

After isolation of exosomes and/or microvesicles, several protein signatures are widely accepted to identify these different subopoulations of EVs. As an example, the tetraspanin protein trinity of CD9, CD63, and CD81 are widely used as universal exosomal markers along with TSG101, heat shock protein (HSP)70, and HSP90 [25]. However, since the EV research field is still in its infancy with respect to its basic biology, such characterizations are to be carefully interpreted because the correlation between EV characteristics (size and composition) and their cellular compartment of origin is poorly understood [26]. In the context of EV-based drug delivery, these protein signatures that define the homing property of the EVs can also be used as targeting ligands.

EVs are taken up by the target cells, when using them in a therapeutic setting as drug delivery vesicles. EVs are taken up by cells through almost all major uptake pathways including membrane fusion, (macro)pinocytosis and lipid raft-mediated endocytosis, phagocytosis, and receptor-mediated endocytosis. Each of these mechanisms have been identified with several different pathway inhibitors specific for each mechanism (Figure 1, step 3A-D).

Isolation of EVs

The number of methods for isolating EVs has increased drastically in the past few years, with more and more commercial reagents becoming available for this purpose. The most frequently used way to isolate EVs is by differential centrifugation followed by ultracentrifugation, and can optionally be floated on a gradient (sucrose or iodixanol) for further density-based fractionation/purification. A general ultracentrifugation protocol usually collects cell culture supernatant after 24-72 hours of conditioning, centrifuges it in steps of 300, 2000 and 10-20,000 RCF, and then precipitates the EVs by ultracentrifugation at >100,000 RCF. Other common EV isolation protocols include size exclusion chromatography-based

isolation, affinity purification, anion exchange, and volume-excluding precipitation (e.g. with polyethylene glycol, PEG) [27]. The different isolation protocols will yield different amounts of EVs and impact the size distribution [28,29]. After isolation, EVs are often characterized by size, concentration, protein concentration, protein markers, and shape. However, all types of characterization are not necessarily included in all studies [30]. Size can be estimated with dynamic light scattering (DLS), however, this analysis is mostly suitable for monodisperse nanoparticles. The most frequent and preferred method is nanoparticle tracking analysis (NTA) which measures both size and concentration by tracking individual particles [29,31,32]. Protein concentration is estimated with colorimetric assays such as BCA or Bradford, protein markers are assessed with western blot and mass spectrometry (MS), and shape is typically described using transmission electron microscopy (TEM) [27,33,34].

In the field of nanoparticle-based drug delivery, artificial vesicles known as liposomes have been the most extensively investigated nanoparticle type so far and most successful in the clinic in terms of FDA approvals [35,36]. EVs have just recently entered this field as biological alternatives with the hope of possessing favorable natural traits that synthetic nanoparticles including liposomes do not have. In 2011, Alvarez-Erviti et al. published a study on engineered EVs targeted to acetylcholine receptors [37], and showed mRNA knockdown in the mouse brain after intravenous injection of EVs loaded with siRNA against GAPDH and BACE1 [37]. This sparked a wave of studies investigating similar and different approaches of utilizing EVs as targeted drug delivery vehicles with the excitement and belief that EVs might be able to cross biological barriers such as the blood-brain barrier (BBB).

The aim of this systematic review is to give an overview of strategies used to generate EVs for targeted drug delivery, to discuss procedures used in the evaluation of targeting efficiency and the current clinical outline and relevance for the utility of such approaches.

2. Targeted extracellular vesicles

To systematically identify relevant studies to be used for Section 2.1, a literature search was done with the following criteria: '(exosome? OR "extracellular vesicle?" OR microvesicle?) AND ("drug delivery" OR targeting) NOT review'. First, articles were sorted based on title and abstract, and later on whether they utilized EVs for targeted drug delivery (Figure 2A). To investigate the pharmacokinetic characteristics of EVs after administration (section 2.2), the following search terms were employed '(exosome? OR microvesicle? OR "extracellular vesicle?") AND (biodistribution OR "drug delivery") NOT review'. Articles were sorted as mentioned above (Figure 2B). Studies utilizing chemical alteration of EV surfaces will not be covered in detail but will be broadly discussed in a later section (section 3).

2.1. Strategies for creating targeted extracellular vesicles

Using nanoparticles for drug delivery is a popular strategy due to the need of more precise treatments with less side effects [38]. Nanoparticles such as EVs can be engineered to deliver their cargo more specifically to the disease sites and therefore has the possibility to minimize the systemic effect [39]. Several approaches have been used to generate targeted EVs and these can roughly be divided into two main categories; passive and active [39]. We define passive targeting as a strategical choice of the EV-producing cell type and/or manipulation of EV-producing cells without introducing physical alteration of the existing transcriptome. Active targeting encompasses alteration of the EV-producing cell's transcriptome, for instance by transfecting the cells with a recombinant targeting vector. Both of these approaches use different targeting ligands and targets. Overall, the most prominent type of targeting ligands were small peptides (approximately 38%), although transmembrane proteins (34%) and antibody fragments (25%) were also frequently used (Figure 3A, Table S1). Sixteen different targets were found in the included studies and spanned from acetylcholine receptor as the most prominent to cancer cells and Neuropilin-1 and folate receptor among the least applied targets (Figure 3B, Table S1).

The passive approach

The homing of EVs towards certain tissues is dependent on the producing cells' phenotype and hence the composition of the EVs produced [25]. A prominent study from 2012 by Peinado et al. investigated the effect of melanoma-derived EVs on metastasis in the bone marrow. They treated mice with exosomes and harvested the bone marrows, and then transplanted the marrows into C57BL/6 mice. The authors found that tumor growth, recruitment of bone marrow derived cells, and tumor vascular density were enhanced compared to PBS-treated bone marrow transplanted controls, indicating that melanoma-derived EVs prepare the area for metastasis [25]. Similar studies on EV tropism to certain tissues for preparing metastatic niches have been done [40,41]. These results were further supported in a study, where it was demonstrated that exosomes facilitate degradation of matrix and invasiveness of metastasis through the tetraspanins CD151 and Tspan8 [42]. These traits have been exploited for the use of EVs in drug delivery, where EVs from certain cell types have been loaded with a therapeutic cargo and administered to cells in culture or to animals [9,25,42–49]. The interaction between the EVs and the target cells is shown by several studies to be partially dependent on a particular ligand present on the EV surface (Figure 3A, Table S1). For example, EVs isolated from U937 macrophages were shown to target endothelial cells, which was hypothesized to be mediated by the interaction of LFA-1 on macrophage-derived EVs with eCAM on endothelial cells [9]. These EVs were shown to effectively target tumor endothelial cells and deliver doxorubicin to reduce the tumor size [9]. A similar approach was utilized by another study where isolated

EVs from primary glioblastoma (GBM) cells showed that the presence of semaphorin 3A on EVs reduced the integrity of the BBB *in vivo* by targeting Neuropilin-1 on brain endothelial cells resulting in enhanced vascular permeability independent of VEGF [44]. Another, but less used, passive approach of obtaining targeted EVs is by exogenous stimulation, where EVs or the cells secreting EVs are stimulated by external factors to target a cell more specifically (Figure 3C). One example is that $CD8^+$ T cell-derived EVs isolated from allergen-induced mice could be used as drug delivery vehicles to deliver miRNA-150 to T effector cells *in vivo* [50]. They here showed that EV-based delivery of miRNA-150 could suppress contact sensitivity by modulating effector T cells [50]. Not only the cell type but also the conditions under which the cells are cultured could influence targeting capabilities. Endothelial cells cultured under hypoxic conditions or with TNF-α showed changed both the protein and RNA composition of isolated EVs compared to controls [51]. Alteration of culture conditions has not yet, to our knowledge, been used to direct EV-mediated drug delivery, but could potentially be used to further enhance certain targeting traits. One way to more precisely study the involvement of certain native EV proteins in targeting is to do knock down or knock out analyses of these proteins (if possible), or to specifically block the interaction with their respective receptor with antibodies or small molecule inhibitors.

The active approach

Since the targeting protein of interest might not be expressed in sufficient quantities or might not even be expressed in EVs, an overexpression or recombinant expression strategy might be helpful. Three different approaches within active targeting have been used; 1) insertion of a targeting epitope into a wildtype protein, 2) construction of a fusion protein, or 3) overexpression of a wildtype protein (Figure 3D, Figure 4, Table S1).

A popular choice for active targeting has been insertion of a targeting epitope into a wildtype protein. The wildtype protein Lamp2b has been used in five studies who all used the same targeting peptide (Rabies virus glycoprotein, or RVG) to target acetylcholine receptors in the brain (Figure 4A) [37,52–54]. Alvarez-Erviti et al. were the first to present this strategy where RVG-Lamp2b EVs packaged with siRNA against BACE1 were intravenously administered. They found a knockdown of BACE1 mRNA of up to 60 % in brain cell lysates from treated mice compared to controls. Similar results were achieved with the same targeting system using α-synuclein as a target [52]. Others have indicated that the glycosylation status of RVG when fused to Lamp2b dictates whether it will be cleaved prior to surface expression [54]. Thus, introducing glycosylation motifs at different positions in the N-terminus increased the expression of the fusion construct and protected the RVG from cleavage and degradation [54].

The fusion protein strategy is the most widely used where only the desired domain of the epitope hosting protein is used instead of inserting an epitope into whole wild-type proteins. This is typically the domain that dictates its subcellular location, namely a transmembrane or membrane-tethering domain (Figure 4B, Table S1). Using this strategy, the epidermal growth factor (EGF)-like targeting peptide sequence, GE11, was fused to the transmembrane domain of PDGFR to obtain a higher tumor uptake of the injected EVs, as EGFR is upregulated in epithelial tumors [55]. The let-7-a miRNA was packaged into the resulting PDGFRTM-GE11 EVs, and the combined effects of the active targeting and therapeutic cargo yielded a functional uptake *in vitro* and tumor growth inhibition *in vivo*. Instead of using a transmembrane domain, nanobodies can be used, which are small single variable domains derived from heavy-chain antibodies [56]. A nanobody specific for EGFR was attached to a glycosylphosphatidylinositol(GPI)-linker, hereby improving the cell association of the resulting targeted EVs to EGFR-expressing cells under flow conditions *in vitro* [56]. Liang et al. used this strategy by transfecting HEK293T cells with a fusion construct of apolipoprotein A1 and the transmembrane protein CD63. Apolipoprotein A1 is a key component in high-density lipoproteins (HDL) and mediates HDL cargo uptake in liver cells via the SR-B1 receptor. They loaded the exosomes with miR-26a and demonstrated an uptake of the exosomes in HepG2 cells with a release of miR-26a, which resulted in decreased cell migration and proliferation [57]. Furthermore, two studies made use of an anti-HER2 scFv by fusing it to the C1C2-domain of lactadherin and express the construct in cells to produce HER2-targeted EVs [58,59]. However, there are still issues related to the level of expression of the scFv, since the reported ligand density of > 1000 scFvs per exosomes must be deemed unphysical when considering the surface area occupied by an individual C1C2-domain of lactadherin. Another approach is the use of a biotin acceptor peptide fused to the transmembrane domain from PDGFR to target biotinylated ligands [60].

A simpler but less popular strategy to generate targeted EVs is to overexpress a wildtype protein that has affinity for a desired target (Figure 4C). EVs harvested from rat choroidal epithelial cells Z310 overexpressing the folate receptor-α could accumulate in brain parenchyma by crossing the blood-cerebrospinal fluid barrier after intracerebroventricular injection [61]. Similarly, overexpressing MHC-II in murine melanoma cells B16F1 yielded an overexpression in the EV fraction. By intradermal injection of these MHC-II-positive EVs in tumor-bearing mice, they mediated tumor growth reduction and prolonged the survival by 20 % compared to controls [62].

Each of these active approaches have pros and cons. Inserting a targeting ligand into a wildtype protein requires it to be expressed and folded correctly with the targeting ligand highly accessible to its target. Another possible concern would be if the insertion will cause a loss of its original function, and because of the overexpression, the wildtype protein might be ‘outnumbered’ and hence the overall

function of that protein could be reduced or ablated. Fusion proteins are more flexible in design since only the membrane-anchoring part of a given EV- or surface-enriched protein is used with the targeting ligand inserted between flexible linkers ensuring high accessibility. The pitfall of fusion proteins could be a higher degree of immune activation due to more of the targeting construct being recombinant and hence considered 'foreign' by the immune system, whereas there being a less chance of the ligand inserted into a wildtype protein to be recognized. Overexpression of a wild-type protein could in theory circumvent this issue, however, it would be less flexible in ligand design since it would have to be a wildtype protein. The immunogenicity of a targeting ligand is of high relevance since treatments, targeted or not, most often require repeated dosage. For instance, repeated dose of PEGylated liposomes has long been a problem for liposome-based drug delivery due to IgM-mediated immune recognition and clearance [63]. Also in the field of non-carrier protein therapeutics the immunogenicity causes severe issues, not only that the treatment compound is cleared but also in some cases induces immune activation resulting in anaphylaxis [64,65]. Severe side-effects of EV-based treatments have, to our knowledge, not yet been reported. Toxicity of engineered EVs from HEK293T cells was not seen after repeated administration to C57BL/6 mice [66]. Repeated dose pharmacokinetics have not been investigated in the studies evaluated here, but should be investigated further in future studies to ensure a clinical potential. Several considerations on the pharmacokinetics of EVs and their biological heterogeneity in a drug delivery setting will be discussed in further details in the coming sections.

2.2. Pharmacokinetics of extracellular vesicles

When considering the use of EVs as drug delivery vehicles in a clinical setting, examination of the basic pharmacokinetics *in vivo* is required. To ensure sufficient information is gathered in such assessment, these must include a relevant choice of labelling strategy, quantification methodology, biodistribution analysis, analysis of the circulatory properties, and considerations regarding the route of administration and dosing regimen. The outcome of each of these points will impact the future clinical relevance of the proposed EV-based drug delivery strategy, since potential therapeutic efficacy is largely irrelevant if the absorption, distribution, metabolism, and excretion (ADME) profile of the strategy is unfavorable for clinical translation.

EV labelling and quantitation

One important aspect in evaluating the *in vivo* properties of EV drug delivery vehicles, is the choice of quantification parameter, i.e. the labelling strategy. Many different labelling strategies have been used to assess EV uptake and biodistribution, which has led to some rather interesting findings. The most widely used labelling entities are lipophilic dyes, with dialkylated cyanines (Di-)/PKH-dyes encompassing the vast

majority (Figure 5A, Table S2). The reason for them being the most popular is likely the ease-of-use and the fact that lipophilic dyes generally incorporate into membrane bilayers including EV membranes. An example is the use of DiI and DiD to label MSC-derived EVs to investigate the biodistribution and localization of EVs *in vivo* by optical imaging of whole mice [67]. PKH2 was used in another study to evaluate *in vitro* uptake of PC3-derived EVs and *in vivo* biodistribution [68]. Other popular labelling strategies include luciferase and fluorescent proteins. For example, EVs expressing gLucB were generated by transducing HEK293T cells with a lentivirus vector [69]. Additionally, cellular expression of CD63-EGFP or palmytoilated EGFP has been done to generate EGFP-positive EVs [53,70]. One of the included studies in this review used radiolabeling to label EVs, where ^{99m}Tc-tricarbonyl complex was inserted into the EVs [71]. Subsequently, EVs were injected intravenously into mice, and CT and SPECT imaging were performed. Another approach is the use of RNA-dyes, which was used to track EVs both in vitro and in vivo [72]. Quantification of cell association (*in vitro*) or accumulation of EVs in specific organs (*in vivo*) depends on the labelling strategy and is in most cases done with fluorescence imaging. However, one has to be aware that fluorescence imaging cannot directly be used quantitively due to different factors such as thickness of the tissue and the resulting loss of fluorescent signal, but it can be used relatively compared to healthy controls. Some studies, however, did quantitation by analyzing the effects of shuttled cargo, i.e. RNA knockdown by siRNA cargo [37,52].

PKH- and Di-dyes are thought to only label double lipid-layered membranes, however, studies have shown that these types of lipophilic dyes are retained in other (and more abundant) plasma species when used to label plasma-EVs [39,73,74]. When applying size-exclusion chromatography, non-EV containing SEC-fractions were shown to retain lipophilic dyes which could be transferred to cells (if the EV sample prior the SEC-purifation was used for the uptake study), hereby mimicking cellular uptake of EVs [75,76]. The same study also found high abundance of lipoprotein particles (LDL,VLDL, IDL, and chylomicron remnants, not HDL) were eluded in the same fraction as the EVs [73,77], and that these particles could also host the lipophilic dyes and thus contribute to transfer of dye to cells [75] via lipoprotein-mediated uptake. In line with these observations, the purity of EV isolates was shown to highly impact non-EV associated dye retention [78]. The study found that commercial precipitation kits and traditional ultracentrifugation protocols yielded impure samples compared to density-gradient isolated EVs. After floating isolated EVs, which were stained with lipophilic dyes, on a density gradient it was shown that a larger proportion of fluorescence was present in fractions of higher densities than EVs [78]. Methods such as confocal microscopy and flow cytometry also limit tracking, as they cannot quantify the proportion of the total EV population inside a cell [74]. The fluorescence signal from the flourophores also highly depend on the environment such as pH, and thus, it might be difficult to quantitively compare the signal in different

milieus. This means that by using a lipid-anchored flourophore you lose track of how much is taken up by the different kind of EVs and other protein complexes. In addition to concerns related to co-staining of non-EV species in apparently purified EV samples, the desorption of lipophilic fluorophores from EVs to lipoproteins during the uptake experiment might also take place and affect the interpretation [76]. Also, the formation of lipophilic dye-based EV-like particles during the staining could pose challenges to the outcome and interpretation of EV uptake and biodistribution studies that rely on the fluorescent readout from lipophilic dyes [74,79]. A way to circumvent excess/free dye to generate particles or find harbor in non-lipid bilayer particles, could be to stain EV-producing cells with lipophilic dyes and isolate the supposedly stained EVs [80]. However, this does not alter the issue of desorption of the lipophilic dyes from EVs to lipoproteins when injected i.v.

Some studies "prove" active EV uptake by incubation of cells with EVs added at 4°C to halt endocytosis or by blocking a specific endocytosis pathway with a small molecule inhibitor [81–83]. However, none of these methods show a preference for EV-mediated uptake, since different lipoprotein particles that have also been shown to retain lipophilic dyes also utilize these pathways. To minimize the confusion of whether the stained EVs or the non-EV-stained particles are giving rise to the fluorescent signal from the cells/tissue, proper internal controls should be included, or more specific experimental approaches should be used. Although merely a small step in the right direction, Takov et al. show a great example of internal controls by using non-EV protein and/or lipid-enriched fractions from SEC to estimate unspecific labelling [75]. However, the authors also show the retention of large lipoprotein particles within the EV fractions, hence not accounting for unspecific labeling within these fractions. Otherwise, genetic engineering of cells to express a fluorescently labelled protein that is enriched in EVs could be a better alternative (although one might select for a sub-population of EVs) to avoid staining of non-EV species, or a more complex model using Cre-loxP, a recombinase system used to control gene expression, could visualize functional uptake *in vitro* and *in vivo* [84].

The abovementioned issues indicate that isolation procedures and, hence, purity of isolated EV samples and the subsequent labeling strategy displays great impact on the interpretation of downstream EV-cell dynamics. When interpreting results obtained using lipophilic dyes to label EVs, one must consider: 1) the source and medium from where the EVs are isolated, 2) the isolation protocol and subsequent sample purity, and 3) the controls used in the experiments.

Administration route and dosing

When using EVs as a drug delivery vehicles it is also important to consider the administration route. The most widely used administration route is intravenous injection, which was used in approximately 90% of

the identified studies (Figure 5B). Intraperitoneal, subcutaneous, and intranasal injections were also used to a lesser extent. A study by Wiklander et al. compared the biodistribution by intravenous, intraperitoneal, and subcutaneous administration and found that the distribution is dependent on the administration route [53]. For example, intravenous injection increased the distribution in the liver and spleen and decreased the distribution in pancreas and intestines compared to subcutaneous and intraperitoneal injection [53]. Hence, it is important to consider the route of administration when targeting a specific disease-site or to minimize potential side effects. The total dose of injected EVs was mostly reported as mass amount of protein and varied from 1.25 µg to 200 µg per animal. To specify this, the most abundant dose was less than 10 µg, although a dose of 11-50 µg and >50 µg accounted for 33% and 20% of the studies, respectively (Figure 5C, Table S2). From a volumetric perspective, Eugene Sverdlov calculated the theoretical amount of 1 µg of small EVs (what he refers to as exosomes) to be approximately 2×10^9 particles based on an average mass of protein from an averagely-sized cell. Using our study cohort, this conversion corresponds to injected EV amount between 2.5×10^9 (1.25 µg) and 4×10^{11} (200 µg) with a median of 3.5×10^{10} (17.5 µg) [85]. Five studies reported the injected dose as particle amount, which varied between 4×10^6 and 3×10^{11} [47,53,86–88] and one study adjusted for amount of radioactivity injected (^{99m}Tc, measured in MBq) [71]. The differences in injected dose span widely, where in some cases the interstudy dose difference reaches a factor of x100 even when using the same host animals. The justification (for example by titration of EV doses) of the amount of EVs injected is absent in most studies. For example, accelerated blood clearance is often observed after the second administration of PEGylated liposomes, however, this can be reduced by increasing the initial dose (lipid dose of up to 5 µmol/kg) [63,89]. In contrast, conventional liposomes without PEG coating were shown to induce the phenomenon at the same high lipid doses, whereas it was reduced at lower lipid doses [63]. This phenomenon might also apply for repeated injection of EVs and thus the dose needs justification by a complete titration with lower and upper thresholds (or as a compromise, at least two doses). Furthermore, characterization of accelerated clearance due to repeated EV injection needs to be investigated as a function of the dose.

Biodistribution and circulation time

EV biodistribution *in vivo* has been examined in different mouse strains with EVs from various cell sources (Table S2). No two setups are identical with variations mostly being in cellular source of EVs, EV dose administered, animal model, labelling strategy, and time point of analysis. The distribution of the targeted EVs are most prominent in the liver followed by spleen and lungs (Figure 5D). Accumulation in intestine, brain, bladder, pancreas, bone marrow, and kidney were demonstrated in a lesser extend with the brain accumulation likely being dependent on the targeting potential endowed to the EVs rather than illustrating

a passive accumulation potential. Wiklander et al. demonstrated that administering EVs intravenously increased the accumulation in the liver and spleen while decreasing the accumulation in pancreas and intestines compared to intraperitoneal- and subcutaneous administration [53]. However, the organ distribution resembles that of other lipid nanoparticles (such as liposomes) with a main accumulation in liver and spleen, and to a lesser extent lung (Figure 5D) [90]. When EVs were either actively or passively targeted to specific disease sites, accumulation was also prominent in the respective target organs [37,53]. EV circulatory properties have only been recorded in five of the included studies, reporting a half-life between 2 – 20 minutes (Figure 5E). This half-life is substantially lower than that recorded for liposomes that can have a half-life of up to several days [91]. However, the liposomes used for drug delivery are often PEGylated, thereby increasing the circulation time. One problem about PEGylation is, however, that an anti-PEG-IgM response occurs about week after the first injection, meaning that the liposomes is cleared faster after second or third injection due to the accelerated blood clearance-effect [92]. EVs used for drug delivery are mostly not PEGylated, which means that EVs might have a chance to sustain their circulatory properties, e.g. circulation time after several injections. In order to obtain a proper targeting of organs/tissue beyond the liver and spleen a long circulation time is typically a requirement. Thus, the fairly short circulation times reported for EVs seem to pose a challenge to the efficiency of EV-targeting.

Possible unwanted effects from recombinant protein tags on EV pharmacokinetics

Most of the recombinant constructs used for EV targeting include some sort of tag to verify insertion of the targeting construct into EV membranes and for purification purposes. However, it has long been known that such tags, e.g. the hexahistidine tag (His-tag), might induce several unwanted effects on the recombinant protein construct. Studies have reported that the His-tag might alter protein conformation and solubility, which might ultimately result in a different functional behavior of the protein[93,94]. Furthermore, the position of the His-tag (N- or C-terminal) was shown to affect the binding affinity of a single-chain variable fragment (scFv), where C-terminal His-tag was shown to reduce its affinity to its target by around 40 % compared to N-terminal tagged and no-tag control[95]. Due to the convenience for purification purposes, protein tags have also been extensively applied in vaccine research. A study by Khan et al. showed that small conformational changes of a malaria vaccine antigen induced by the His-tag resulted in altered immune response compared to non-His-tagged antigens[96]. These ‘adverse effects’ of His-tags indicate that tagged proteins for EV targeting purposes should be carefully considered. When using His-tags for purification purposes it is suggested that it is important to remove the tag prior to working with the protein and to demonstrate that this is removed [93]. This can be done by inserting enzymatic cleavage

sequences to remove affinity tags after purification (strategies reviewed here [97]). If these criteria are met the use of His-tags might serve as a good method for isolation/purification purposes.

2.3. Determining specificity – the heterogenic dogma

There is little doubt that heterogeneity in isolation and characterization protocols of EV studies has an impact on functional outcomes, but biological heterogeneity within the isolated EV population might also contribute to this. Determining how well EVs are suited for targeted drug delivery purposes is an even more difficult task than with homogenous synthetic nanoparticles such as liposomes, and thus some extraordinary precautions should be considered when interpreting data from EVs used as targeted drug delivery vehicles. In this section, we will address some parameters that could be critical for the experimental outcome.

Research groups working with synthetic drug delivery vehicles usually strive to keep the size and composition of a batch of carriers within a narrow range. Since EV research is still in its infancy, the lack of basic biological knowledge limits the methodological approaches to isolate homogenous EV populations optimal for targeted drug delivery [39]. Often EV separation and isolation methods are based on physical properties such as size and density, for example by size-exclusion chromatography or ultracentrifugation. With the EVs spanning from 30 – 2000 nm in size, it is nearly impossible to achieve a homogenous EV population with respect to physical parameters. Even if size or density-based separation could yield physically homogenous EVs, the biological composition of the EV membranes (proteins and lipids) is also very heterogeneous and thus adds another layer of unforeseeable factors in relation to targeted drug delivery [73]. As an example, the EV database ExoCarta.org has registered more than 6000 human proteins from EV samples from less than 280 studies, mainly identified with mass spectrometry [98]. According to the UniProt database, the total annotated human proteome encompasses around 20,000 proteins. This means that more than 25 % of *all* previously detected proteins have been found in the modest amount of EV studies included in the ExoCarta database. This could illustrate that regardless of the cargo used or the targeting ligand applied, the diverse proteins present could account for some or most of the function or targeting seen. The diversity of proteins present could also vary from isolation to isolation, depending on the ratio of EV subpopulations (if such exist) that are extracted. One question arises from this notion: With the heterogeneous EVs containing an array of different proteins, will there always be some types of EVs being taken up by any type of cell at any given time? A typical EV proteomic study approximately identifies between 400 and 2000 different proteins from a given cell line *in vitro* [99–101], and one even found 4937 distinct proteins in EVs from porcine MSCs [102]. Using trypsin-based shotgun mass spectrometry for protein identification most likely results in an underestimation of the actual number of proteins present

due to some proteins being less prone to trypsin cleavage [103]. Most of the proteins associated with EVs are membrane-bound proteins, which means that if an average of 1000 different proteins (in varying concentrations) are present in EVs from one cell type most of these proteins will be accessible for interaction with just as many (or more) receptors on surrounding cells. Amongst all these ligands inherently present on EVs, how much will the targeting pattern change by introducing a targeting ligand to the EV surface in high abundance? And will the introduced targeting ligand be expressed in higher concentrations on EVs than the most abundant of the inherent proteins present in the sample? Considering the methodological limitations in EV isolation and characterization, the amount and diversity of proteins found is likely dependent on the isolation protocol [104]. A recent study by *Jeppesen et al.* investigated EV composition by high-resolution density fractionation and direct immune-affinity capture [105]. By dissecting the many different fractions, the authors showed that DNA and Ago-proteins (miRNA effector proteins) are not associated with small EVs, which was thus far thought to be the case. This indicates that the biological heterogeneity observed in EV samples could, at least partially, be explained by a general lack of applying methods with suitable resolution to properly dissect EV composition.

There are pros and cons when using EVs in targeted drug delivery. However, it cannot be ignored that several studies have reported biological traits that synthetic particles either cannot achieve or have yet to achieve. EVs are very complex entities and much is still not known about these biological particles. This complexity is perhaps what makes the EVs so interesting in a drug delivery perspective, however, it is very important to investigate the unknown and to consider the methods used in the different studies. It has to be acknowledged that the effects seen most likely are not entirely based on the EVs, due to the complexity ranging from the isolation methods to labelling, which should definitely be considered in the conclusions.

3. Comparison to liposomal golden standards

Liposomes and EVs

One main question that the future research into EV-based drug delivery must answer is whether the functionality and therapeutic efficacy of the EV strategy is superior or comparable to what can be obtained for synthetic nanocarriers. Recently, we pointed out that the current choice of liposomal controls in EV-based drug delivery studies were poor, because the chosen liposomal formulations were rarely clinically relevant [106]. This means that the superiority of EVs compared to liposomes (if present) is likely exaggerated. Choosing clinically used formulations of liposomes may illustrate possible differences between the two strategies much better and provide a valid foundation on which to build assumptions regarding EV's superiority. For example, inclusion of PEG molecules on the liposome surface will increase

the circulation time of the system, which may be beneficial for therapeutic efficacy. EVs are in general known to circulate poorly when injected intravenously. However, since anti-PEG responses are often observed already by the second dose of PEGylated liposomes [107], it may be that the performance of EVs with respect to plasma stability will improve the therapeutic efficacy compared to their synthetic counterparts. This illustrates that too little information is available at the moment to deem one strategy better than the other, but with improved controls we will approach the answer to this question in the near future. In addition, the two fields could be inspired by each other and include important elements from the respective counterpart to improve the functionality and therapeutic efficacy.

Chemical alteration of EV surfaces

The use of EVs as drug delivery vehicles often requires an alteration of the EV surface to target a specific disease site. As discussed earlier, the surface of particles can be altered by expressing a specific ligand towards a certain receptor or, as opposed to biological incorporation of the ligand, this expression can be performed by the use of click chemistry. Click chemistry is defined as chemical reactions that are easy to perform, insensitive to water and oxygen, have a high yield, and must not require chromatographic purification [108]. Click chemistry can consist of cycloaddition reactions, nucleophilic ring-opening reactions, and carbonyl chemistry. However, the most popular strategy is coupling of azides to alkynes by Cu(I)-catalysis [109]. Smyth et al. used this click chemistry approach to conjugate an azide-fluor-545 to the surface of EVs. The membrane proteins were modified with alkyne groups and subsequently the azide-fluor-545 by click chemistry reaction catalyzed by Cu(I). They found that the surface alteration did not change the size or cell uptake of the EVs [110]. Hence, it seems like this click chemistry reaction is safe to use without compromising the morphology or function of the EVs.

Nanoparticles used for drug delivery purposes often have a relatively short half-life when administered systemically. As in the case of liposomes, addition of PEG to the surface of these nanoparticles might serve as a good approach. This strategy was also used to modify EVs to improve the circulation time and to enhance specific uptake in a tumor. EGa1 nanobodies were conjugated to PEG-phospholipid micelles and post-inserted into Neuro2A-derived EVs to target EGFR. The PEGylation of EVs decreased the EV-cell interactions *in vitro*, hence, shielding the EV resulting in increased circulation time *in vivo* [111]. As previously mentioned, however, one downside of PEGylation is that it can induce an anti-PEG IgM response [107], resulting in accelerated clearance from the circulation.

The idea of using EVs as drug delivery vehicles originate from the perspective of EVs being more biologically relavant compared to other nanoparticles as they are naturally occuring in the human body. Alteration of the EV surface by click chemistry or PEGylation could compromise the natural traits of

these particles, which is one of the main advantages. Changing the surface of the EVs - thereby, compromising the aforementioned traits - might not make them more suitable to drug delivery than liposomes, which are more homogenous in size and composition, and generally more stable in proportion to storing of/delivering the cargo.

4. Clinical perspective: a long way to go

To date, no approved, EV-based treatment exist, however, some clinical trials have been completed. A search on clinicaltrials.gov using "exosomes OR extracellular vesicles, phase I-IV" gave 36 hits. A phase II clinical trial from 2016 (NCT01159288) investigated the use of IFNγ matured DC-derived EVs to target inoperable non-small cell lung cancer. They aimed to observe 50 % of the patients with progression-free survival after 4 months, however, this was only found for 32 % of the patients, and additionally, one patient developed grade three hepatotoxicity. Even though they did not meet their primary endpoint, they found enhanced NK cell functions. [112] The effect of MSC-derived EVs on graft-versus-host disease was investigated in a clinical study from 2014, where they found that MSC-derived EVs reduced the symptoms [113]. Several other clinical trials are about to be executed. A phase I/II study starting in April 2019 (NCT03384433) aims to investigate whether the disability of patients with acute ischemic stroke can be improved by treatment with MSC-derived EVs loaded with miR-124. Another phase I study loaded MSC-derived EVs with KrasG12D siRNA to study dose and side effects in patients with pancreatic cancer (NCT03608631).

EV drug delivery systems are still a relatively new thing and has some challenges compared to e.g. liposomes, which are already in the clinic. Codiak Biosciences is working on platforms to generate targeted EVs and are planning a few phase I/II studies to be executed in 2020 (http://www.codiakbio.com/therapeutics/), however, as we have outlined throughout this review it still seems as there is a long way to go. Since EVs are complex entities from biological sources, the road to the clinic is paved with different obstacles than for synthetic nanoparticles. The first step in need of consideration is from which specimen to harvest the EVs; from plasma or cell cultures, and should it be autograft or is allograft (or even generic cell cultures) sufficient? Next challenge is which targeting strategy to use and the scalability and reproducibility of EV production needed for therapeutic application. Apart from this, EVs are still required to go through rigorous characterization similarly to synthetic nanoparticles used in the clinic; toxicity, targeting efficiency, drug loading capacity, biodistribution and more.

5. Conclusion/Perspectives

To date, several studies have explored the use of EVs as targeted drug delivery vehicles with success. However, the lack of knowledge of fundamental EV biology and methodology to isolate and analyze these pose substantial challenges. We have described the strategies used in EV targeting, discussed pitfalls in the interpretation of EV pharmacokinetics, and compared the EVs to synthetic liposomes. One critical factor is the EV isolation strategy, which has to be considered both with respect to the source of EVs, which EV isolation method (and therefore sample purity), and proper controls to ensure that the demonstrated effects are a result of the EVs and not contaminants such as lipoproteins. Lipophilic dyes are the most widely used labeling strategy, however, this strategy is also known to label non-EVs, which highlights the importance of sample purity and the pitfalls in interpreting EV uptake and function. The dose of EV injection in drug delivery studies is usually not justified. Hence a titration of upper and lower limits or, as a compromise, at least two different doses should be tested. Our understanding of EVs is limited by the methodology available to study them, and thus better ways to isolate and characterize EVs are needed before these can be used reliably in a therapeutic setting. When comparing EVs to the liposomal golden standards one cannot deem one strategy better than the other. However, it only makes sense to use the EVs due to their natural traits and these will be compromised by e.g. surface chemistry to improve circulation time. One keyword relates to most problems presented in this review; heterogeneity. When working with EVs it is highly important to consider their heterogeneity, also when it comes to drug delivery. Fundamental knowledge on EV biology is scarce and hence more basic research of what EVs in fact are is needed before considering them as candidates for targeted drug delivery in the clinic. The heterogeneity observed in EV isolates, or our lack of knowledge hereof, severely limits the current potential of EVs as targeted drug delivery vehicles. We therefore urge researchers working in the field of EVs to focus more on fundamental EV biology to help evaluate whether these have a future within the field of drug delivery.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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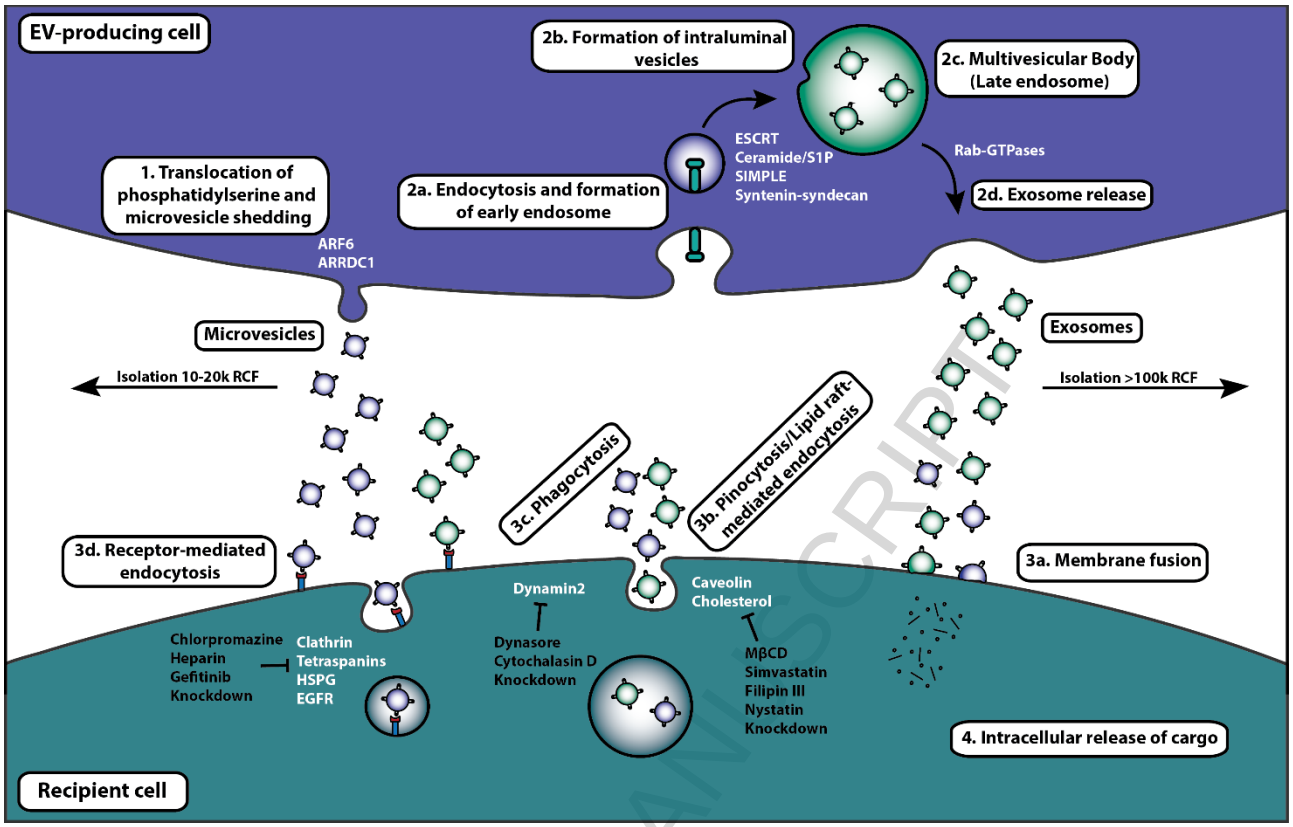

***Figure 1**: Overview of EV biogenesis and uptake. 1) Microvesicle biogenesis. 2A-D) Steps of exosome biogenesis. 3A-D) Different routes of EV uptake. 4) Release of EV cargo within recipient cell. Left arrow indicates isolation of microvesicles, right arrow exosome isolation.*

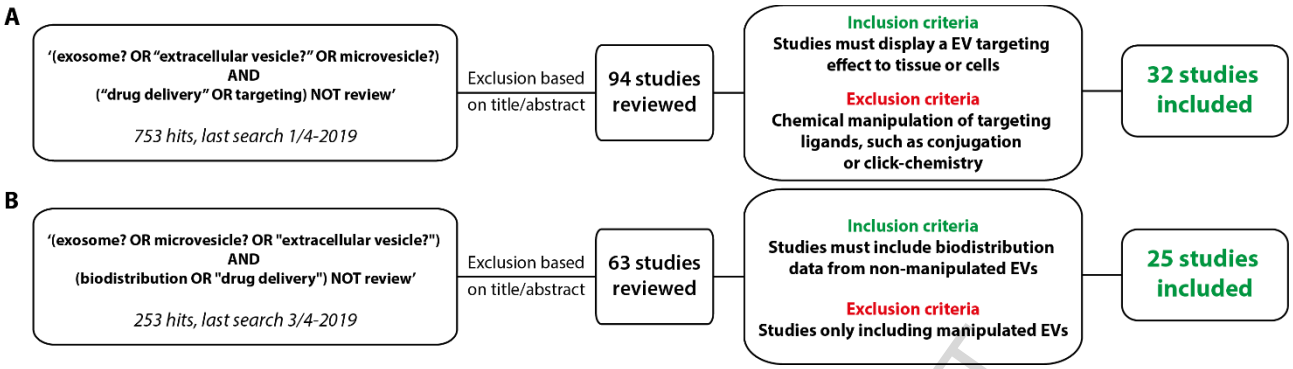

***Figure 2**: A) PubMed search and systematic review to find studies using targeted EVs for drug delivery. B) PubMed search and systematic review to find studies displaying EV biodistribution data.*

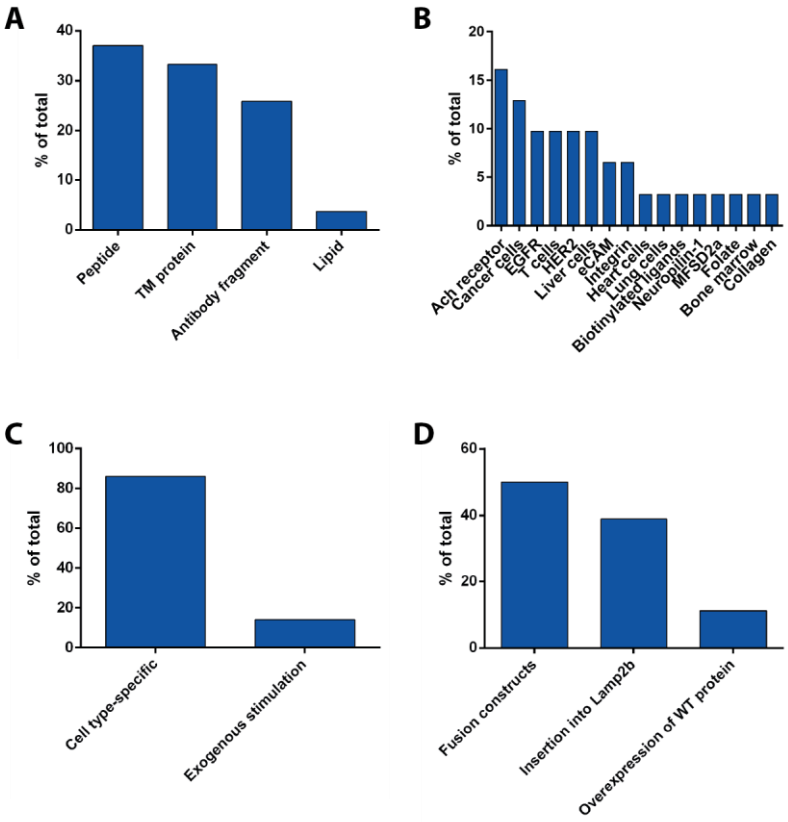

***Figure 3**: Data from systematic review of EV drug delivery studies. A) Quantification of studies using different types of targeting ligands; small peptides, transmembrane proteins, antibody fragments, and lipids. B) Different targets utilized in the studies included. C) Percentage of studies categorized by type of passive targeting. D) Quantification of studies using the different active targeting strategies.*

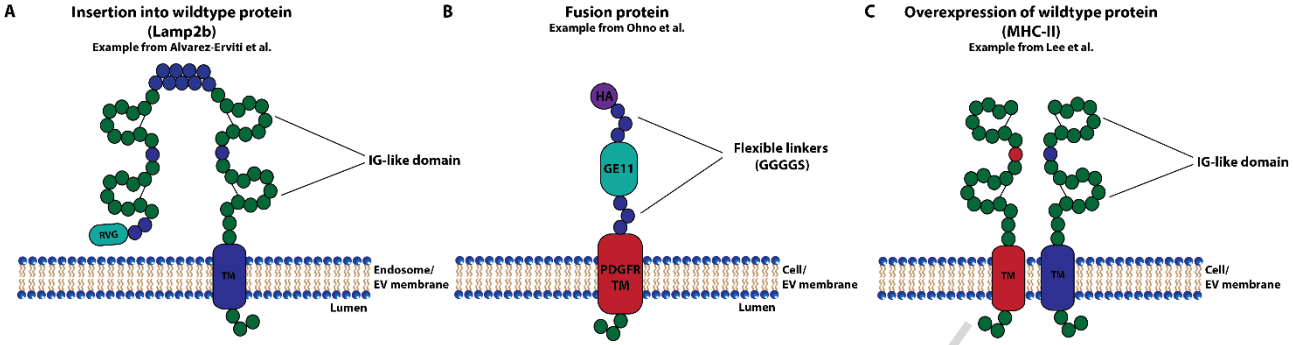

***Figure 4**: Examples of the different active targeting approaches used by the studies included. A) Insertion of a targeting peptide into the N-terminal part of wildtype Lamp2b. B) Example of attaching a targeting peptide to a transmembrane domain via flexible glycine-serine linkers. C) Overexpression of non-edited wildtype proteins.*

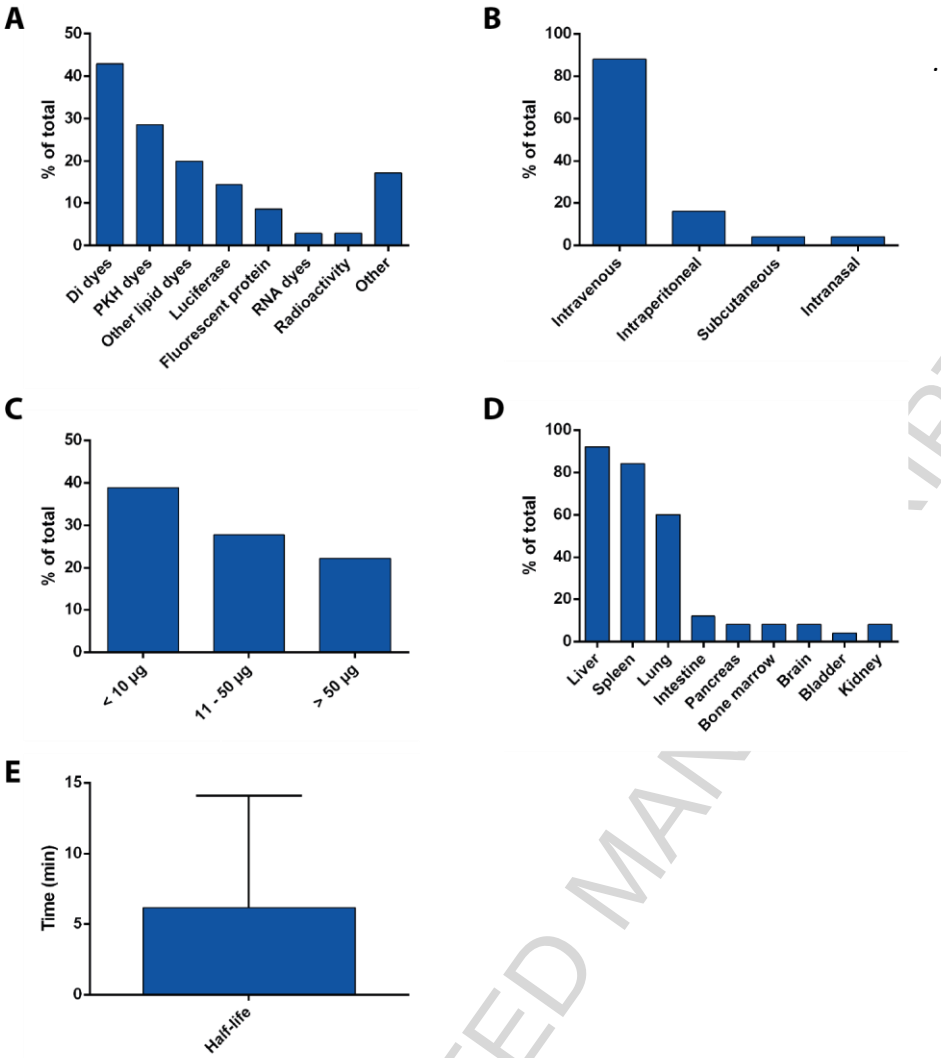

***Figure 5**: Data from systematic review of EV biodistribution studies. A) Percentage of studies using the different EV labeling strategies. B) Study distribution categorized based on route of administration. C) Study distribution categorized based on dose expressed in µg. D) Study distribution based on which organs EVs mostly accumulated in. E) Average half life of EVs in circulation expressed as mean + SD.*